Glucocorticoid Receptor Interaction with 14-3-3 and Raf-1, a Proposed Mechanism for Cross-talk of Two Signal Transduction Pathways*

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Christina Widén, Johanna Zilliacus, Jan-Åke Gustafsson, and Ann-Charlotte Wikström‡

From the Department of Medical Nutrition, Karolinska Institutet, NOVUM, S-141 86 Huddinge, Sweden

The glucocorticoid receptor (GR) functions as a ligand-dependent transcription factor. In the present study we describe a specific immunoaffinity chromatographic purification of GR from liver cytosol from adrenalectomized rats that may be used to identify hitherto unknown cytosolic GR interacting proteins. We have identified the ubiquitously expressed 14-3-3 as well as Raf-1, a downstream effector of Ras, as GR co-purifying proteins. In our semi-quantitative analysis liganded/activated GR showed the strongest interaction with 14-3-3 and Raf-1, but 14-3-3 was also found to co-purify with GR in a nonliganded/nonactivated state. By extensive salt washes we were also able to demonstrate that the glucocorticoid induced interaction between GR, 14-3-3, and Raf-1, respectively, is remarkably stable and withstood 2.4 M salt. The interaction between GR and 14-3-3 was also verified by 14-3-3 co-immunoprecipitation studies. Our observations that GR and Raf-1 are found within the same protein complex (‘‘receptosome’’) in the cytoplasm of rat liver cells could provide a mechanistic explanation for glucocorticoid effects on the Raf-1-Ras signaling pathway.

The glucocorticoid receptor (GR) belongs to a family of nuclear receptors that function as ligand-dependent transcription factors. In the cytoplasm, the nonliganded GR forms a multiprotein complex in which, among other proteins, a dimer of a heat shock protein (hsp) 90, as well as monomeric hsp70 and FKBP52 are known to be included. The proteins in this multiprotein complex play a role in the function of GR. For example the dimer of hsp90 keeps the GR inactive and in an optimal conformation for ligand binding (1). Upon ligand binding, GR is activated (i.e. GR dissociates from the hsp complex), the nuclear localization signal within the ligand binding domain of GR is unmasked, and the receptor is able to translocate into the nucleus and regulate gene transcription (for a review see Bamberg et al. (2)). In various experimental systems GR has been reported to associate with proteins involved in other signaling pathways such as NF-κB (3, 4), STAT3 (5), and STAT5 (6), suggesting that the GR-multiprotein complex in vivo could contain a larger number of proteins. One such protein is the 14-3-3 protein. In a yeast two-hybrid screening, 14-3-3η has been shown to be a GR-binding protein using the ligand-binding domain of GR as a bait (7). In the same study Wakui et al. (7) also showed, by glutathione S-transferase pull-down experiments, that 14-3-3η interacted with liganded GR, whereas the addition of molybdate inhibited the GR-14-3-3 interaction, indicating that hsp90 prevented GR-14-3-3η interaction. Molybdate is known to stabilize the GR-hsp90 interaction (8). Proteins of the 14-3-3-family are highly conserved and widely distributed, and they have also been found to associate with proteins involved in cellular signaling, for example Cdc25, IRS-1, and Raf-1 (9). Members of the 14-3-3 protein family seem to have a variety of functions; for example they affect the cell cycle by binding to Cdc25 and thereby function as a nuclear export signal (10). Furthermore, the 14-3-3 interaction with IRS-1 seems to interrupt the association between the insulin receptor and IRS-1 (11), whereas the 14-3-3 interaction with Raf-1 has been shown to be a prerequisite for plasma membrane recruitment and activation of Ras (12). 14-3-3 can exist as a dimer in vivo and thereby function as an adaptor protein (i.e. bind two different proteins at the same time). For example the proteins Bcr and Raf-1 have been shown to be connected through a dimer of 14-3-3 (13).

Because the methods used to investigate GR-14-3-3 interaction have relied on the interaction of overexpressed proteins, we found it of interest to investigate whether GR interacts with 14-3-3 under conditions where GR and its associated partners are present at concentrations comparable to the in vivo situation. In the present study we describe a specific immunoaffinity chromatographic purification of GR in liver cytosol from adrenalectomized rats in which 14-3-3 was found to co-purify with GR. 14-3-3 co-purified with GR in several functional states: nonliganded/nonactivated, liganded/nonactivated (i.e. in the presence of molybdate) and liganded/activated. Liganded/activated GR showed the strongest 14-3-3 interaction in this semi-quantitative analysis. The 14-3-3 interaction with nonliganded GR suggested that 14-3-3 and GR could associate when GR is complexed to hsp90. However, a stringent washing procedure involving stepwise salt gradient washes from 50 mM to 2.4 M NaCl indicated that 14-3-3 interaction predominantly occurred with liganded/activated GR.

Because glucocorticoids are involved in regulation of glucose homeostasis and also affect cellular growth rates, it was of interest to study whether we could observe possible 14-3-3-mediated GR interactions relevant for these processes. We found that Raf-1 was co-immunopurified with GR, whereas we were not able to demonstrate the presence of IRS-1 in the GR-associated complex. Our observations that GR and Raf-1 are found within the same protein complex in the cytoplasm of

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‡ To whom correspondence should be addressed. Tel.: 46-8-58-58-37-30; Fax: 46-8-711-66-59; E-mail: lotta.wikstrom@mednut.ki.se.

The abbreviations used are: GR, glucocorticoid receptor; hsp, heat shock proteins; NMIIgG, normal mouse IgG; EPG, EDTA phosphate buffer glycerol; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.
rat liver cells provide a mechanistic explanation for glucocorticoid inhibitory effects on the Raf-1-Ras signaling pathway reported by Croxall et al. (14). Glucocorticoids could therefore exert rapid effects on Ras-Raf signaling pathways via GR, which interacts directly or possibly indirectly via 14-3-3 with Raf-1 in the cytoplasm.

**EXPERIMENTAL PROCEDURES**

**Cytosol Preparation and Immunooaffinity Chromatographic Purification of Rat GR**—Rat liver cytosol was prepared from four 6–8-week-old adrenalectomized female rats (M&B, Rey, Denmark). The livers were perfused with a Tris buffer (1 mM EDTA, 20 mM phosphate buffer, pH 7.4, 10% glycerol, 50 mM NaCl, 2 mM dithiothreitol) and homogenized in EPG buffer (with 0.5 μg/ml leupeptin, 1 μg/ml aprotinin (Roche Diagnostics Scandinavia AB, Stockholm, Sweden), 0.7 μg/ml pepstatin, and 200 μg/ml phenylmethylsulfonyl fluoride (Sigma) added) and ultracentrifuged for 30 min at 210,000 × g at 4 °C. To obtain GR in different functional states the cytosol was prepared as follows: (a) in case of nonactivated GR, 20 mM Na2MoO4 was added to the EPG buffer in all steps; (b) in case of liganded GR, triamcinolone acetonide (20 μm) was added to the cytosol for 1 h at 4 °C; and (c) in case of activated GR, the cytosol was diluted 1:2 in EPG buffer and heat-activated at 25 °C for 30 min. The cytosol was filtered and then, at 4 °C, run through (a) a column containing 1 ml 4B-Sepharose 4B, (b) a column with 1 ml of normal mouse IgG (NMIgG) antibodies coupled to CNBr-activated Sepharose 4B (referred to as the NMIgG column), and (c) a column with 1 ml of the GR-specific mouse monoclonal antibody 250 (originally called mAb 7) (15) coupled to CNBr-activated Sepharose 4B (referred to as the 250 column). (The chromatography columns were from Bio-Rad and Sepharose 4B, and CNBr-activated Sepharose 4B was from Amersham Pharmacia Biotech.) After washing with 15 ml of EPG buffer with 50 mM NaCl, 15 ml of EPG buffer with 150 mM NaCl (salt wash), and 15 ml of EPG buffer with 50 mM NaCl, respectively, four column volumes of the corresponding peptide D-I2-3 (synthesized by NeoSystem, Strasbourg, France) (16) at a concentration of 1.4 mg/ml in EPG buffer was used for elution of GR and GR-associated proteins at 4 °C. Because the elution efficiency was dependent on the functional state of GR, peptide elution was not sufficient to elute liganded/activated GR, which required 2.5 mM NaSCN in EPG buffer for complete elution. The eluted proteins were precipitated with 0.02% sodium deoxycholate for 15 min and 20% trichloroacetic acid at 4 °C overnight. After centrifugation at 3000 × g for 30 min and washing with ice-cold acetone the proteins were resuspended in sample dilution buffer.

**SDS-PAGE, Western Blot, and Immunostaining**—The proteins were run on SDS-PAGE (7.2% or 9% polyacrylamide gel) under reducing conditions and blotted to polyvinylidene difluoride membrane (Bio-Rad). The membrane was stained with Coomassie Brilliant Blue R 250 (Fluka, Stockholm, Sweden), cut to obtain one lane per strip, and run on SDS-PAGE (7.2% or 9% polyacrylamide gel) under reducing conditions. The proteins were then probed with agarose-coupled normal rabbit IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA), respectively, in PBS and 1% defatted dry milk for 2 h at room temperature. After washing in washing buffer (PBS + 0.5% Tween 20), the polyvinylidene difluoride strips were incubated with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad and Amersham Pharmacia Biotech) diluted in PBS and 1% defatted dry milk for 1 h. The strips were again washed in washing buffer and incubated with SuperSignal Substrate or SuperSignal WestFemto Maximum Sensitivity Substrate (Pierce) for 5 min. The signals were detected according to the protocol enclosed from Pierce.

**Co-immunoprecipitation Using Agarose-coupled Antibody Directed against 14-3-3**—Liver cytosols were prepared as described above and 10 mM Tris, pH 7.8, 0.1% Nonidet P-40, 0.15 μM NaCl, 1 mM EDTA, and flavin adenine dinucleotide (Flavin, Stockholm, Sweden), cut to obtain one lane per strip, and run on SDS-PAGE (7.2% or 9% polyacrylamide gel) under reducing conditions. The proteins were then probed with agarose-coupled normal rabbit IgG (Santa Cruz). The preabsorbed cytosol (500 μg of the total amount of protein) was diluted to a total volume of 100 μl in 10 mM Tris, pH 7.8, 0.1% Nonidet P-40, 0.15 μM NaCl, 1 mM EDTA, and a slurry of 20 μl 14-3-3 agarose-coupled beads was added to it. The beads were gently agitated overnight and then washed three times in PBS with 0.02% sodium deoxycholate and the agarose beads, which were boiled before analysis by SDS-PAGE and Western blot. As a negative control, agarose-coupled normal rabbit antibodies from Santa Cruz were used. All steps were carried out at 4 °C.

**Salt Gradient Analysis of GR and GR Co-purifying Proteins**—Liver cytosols from adrenalectomized rats were prepared to contain either nonliganded/nonactivated or liganded/activated GR as described above, and the cytosols were run, in parallel, through three subsequent columns; Sepharose, NMIgG, and 250 columns, respectively. A stepwise NaCl gradient elution of the 250 columns was performed using EPG buffer with the increasing salt concentrations: 30 mM + 20 ml of 50 mM, 20 ml of 300 mM, 20 ml of 600 mM, 20 ml of 1.2 M, and 20 ml of 2.4 M. To each fraction, a 20-ml 50 mM wash followed by a 5-ml peptide elution and a 10-ml NaSCN elution was performed. The salt eluted fractions (apart from the first 30 ml of the first washing with 50 mM) as well as the peptide and NaSCN eluted fractions were precipitated as described above and analyzed by SDS-PAGE and Western blotting.

**RESULTS**

**Cytosol Preparation and Immunooaffinity Chromatographic Purification of Rat GR**—To investigate cytosolic GR-interacting proteins we have established a GR immunooaffinity chromatographic purification method where GR was purified from cytosol prepared from livers of adrenalectomized rats. To avoid nonspecific binding, the cytosol was prepurified, first on a Sepharose column followed by a column containing NMIgG immobilized on a Sepharose matrix. The prepurified cytosol was subsequently applied to a column containing the anti-GR monoclonal antibody (mAb7 also denoted 250) (15) coupled to a Sepharose matrix. A peptide corresponding to the identified epitope for mAb 250 (16) was used for elution of GR. The elution is mild and specific and allowed GR-interacting proteins to co-purify with the receptor. After the peptide elution, the column was eluted with NaSCN. Liganded/activated GR required NaSCN for complete elution. Peptide-eluted and NaSCN-eluted GR and its co-purifying proteins were separated using SDS-PAGE followed by blotting to polyvinylidene difluorde and Coomassie staining (Fig. 1, A and C). The pattern of GR and GR co-purifying proteins was analyzed with GR in different functional states: (a) nonliganded/nonactivated, (b) liganded/nonactivated, and (c) liganded/activated. To investigate the specificity of the GR immunooaffinity chromatographic purification, prepurified cytosol was also run through a normal mouse IgG column and eluted with peptide followed by NaSCN, as a negative control (Fig. 1, A, lane 4, and C, lane 3). GR was detected using immunostaining with the mAb 250 (Fig. 1B). Several co-purifying proteins were observed in Fig. 1 (A, lanes 1–3, and C, lanes 1 and 2).

14-3-3 Co-purifies with GR from Rat Liver Cytosol—14-3-3 protein has been reported to bind to GR in a yeast two-hybrid screening, by using the ligand binding domain of GR as a bait (7). In the same study it was shown that the 14-3-3 protein interacted with liganded GR, whereas molybdate inhibited this interaction as judged from a glutathione S-transferase pull-down assay. This indicates that, in vitro, hsp90 prevents 14-3-3 binding to GR. To further investigate possible 14-3-3 protein interaction with GR in a system using endogenous protein levels, immunooaffinity chromatographic purification of GR was performed in a semi-quantitative manner (see “Experimental Procedures” and Fig. 2). Western blot analysis showed that 14-3-3 co-purified with GR in all functional states, even when molybdate was present in the cytosol. Although not formally quantitative, the Western blot signal intensity of a GR co-purified protein as compared with the GR signal intensity gives an indication of the amount of proteins co-purified with GR in the various states. In light of this, we noted that liganded/activated GR gave rise to a stronger 14-3-3 signal than the nonliganded/ nonactivated GR (Fig. 2, compare A and C). This suggests that 14-3-3 and GR associate even when GR is bound to hsp90, although GR devoid of hsp90 showed a higher degree of binding to 14-3-3. This is somewhat in contrast to what has previously been described with methods using overexpressed protein levels (7). The presence of hsp90 was used as an indicator of the functional state of GR in the various eluates; accordingly, the presence of hsp90 in Fig. 2A indicates that GR...
in this experiment was in a nonactivated state, whereas the eluate in Fig. 2C is devoid of hsp90 and thus indicates that GR was activated. We also noted a relative increase of hsp70, as compared with GR, co-immunopurified with activated GR in parallel to the increase of GR-interacting 14-3-3 (Fig. 2, compare A and C).

The 14-3-3-interacting Protein Raf-1, but Not IRS-1, Co-purifies with GR—We further investigated whether the previously reported 14-3-3 interacting proteins Raf-1 and IRS-1 were to be found among the GR co-purifying proteins. The presence of these two proteins in rat liver cytosol was first determined by Western blotting (data not shown) using commercially available polyclonal antibodies. Western blotting analysis of GR interacting proteins purified as presented in Fig. 1 showed that Raf-1 co-purified with GR in the liganded/activated state (Fig. 3B), whereas a Raf-1 signal was not de-
Concentrations of NaCl from 50 mM to 2.4 M salt was performed. Washing—To examine the stability of the GR-antibody interactions obtained when using GR antibodies. An agarose-coupled polyclonal antibody directed against 14-3-3 was used for precipitation. A slight background immunoprecipitation of Raf-1 was seen when using 20 μl of agarose-coupled NMigG. The positive control (+) is 5% (25 μg of total protein) of input (500 μg of total protein).

**Fig. 3. Raf-1 co-purifies with GR.** Western blotting analysis of nonliganded/nonactivated and liganded/activated GR and its copurified proteins from liver cytosol from adrenalectomized rats. Antibodies directed against GR, 14-3-3, Raf-1, and IRS-1 were used for analysis. A, peptide elution of GR purified in a nonliganded/nonactivated state. B, NaSCN elution of GR purified in a liganded/activated state. C, negative control; GR purified and peptide eluted in a nonliganded/nonactivated state on a normal mouse IgG column. Occasionally a weak Raf-1 signal was detected in the nonliganded/nonactivated fraction (not shown). Molecular mass markers are shown in kilodaltons.

**GR and Raf-1 Co-immunoprecipitate with 14-3-3—**By using specific rabbit antibodies to 14-3-3, we performed another set of co-immunoprecipitation studies to further corroborate the findings obtained when using GR antibodies. An agarose-coupled polyclonal antibody directed against 14-3-3 was used for precipitation of 14-3-3 in rat liver cytosol treated to obtain GR in two different functional states: nonliganded/nonactivated and liganded/activated. The 14-3-3 co-immunoprecipitates were analyzed on SDS-PAGE followed by Western blotting. Both nonliganded/nonactivated and liganded/activated GR co-immunoprecipitated with 14-3-3 (Fig. 4). The precipitates were also analyzed for the presence of Raf-1 using immunostaining. Raf-1 was found in the 14-3-3 immunoprecipitate. Raf-1 co-immunoprecipitated with 14-3-3 to the same extent regardless of molybdate or glucocorticoid addition to the cytosol (Fig. 4). A slight background immunoprecipitation of Raf-1 was seen when using 20 μl of agarose-coupled NMigG as negative control (Fig. 4).

**GR Interaction with Raf-1 and 14-3-3 Withstands High Salt Washing—**To examine the stability of the GR-antibody interaction and also to investigate the strength of GR interaction with 14-3-3 and Raf-1, a stepwise salt elution with increasing concentrations of NaCl from 50 mM to 2.4 M salt was performed. Liver cytosols from adrenalectomized rats were prepared to contain either nonliganded/nonactivated or liganded/activated GR, and the cytosols were run, in parallel, through three subsequent columns: Sepharose, NMigG, and 250 columns, respectively. A stepwise salt gradient elution of the 250 columns was performed using EPG buffer with increasing salt concentrations: 50 mM, 300 mM, 600 mM, 1.2 M, and 2.4 M, followed by a salt washing out using 50 mM NaCl. Thereafter, a peptide elution followed by a NaSCN elution was performed. The salt eluted fractions as well as the peptide and NaSCN eluted fractions were precipitated and analyzed on SDS-PAGE and by Western blot. The immunoaffinity chromatographic purification of GR was found to be very stable, despite the extensive washing with increasing salt concentrations the column still retained GR that could be eluted using the epitope corresponding peptide or NaSCN (Fig. 5). Both nonliganded/nonactivated GR (Fig. 5A) and liganded/activated GR (Fig. 5B) were found to form stable bonds to the 250 column, and both of these two functional GR states withstood the salt washings. Some elution of nonliganded/nonactivated GR occurred from the 250 column at 50 mM, 300 mM, 600 mM, and 1.2 M salt washing (Fig. 5A), whereas the liganded/activated GR eluted at 300 and 600 mM NaCl (Fig. 5B). This indicates that the liganded/activated GR binds with a higher affinity to the mAb 250 than the nonliganded/nonactivated GR.

The salt washing fractions were also analyzed with regard to 14-3-3 and Raf-1 to investigate the strength of their binding to GR. Surprisingly, after the stepwise salt gradient, Raf-1 and 14-3-3 were still associated with GR, in its liganded/activated state, and both 14-3-3 and Raf-1 co-eluted with GR when eluting with the peptide as well as with the following GR elution using NaSCN (Fig. 5B). The interaction between nonliganded/nonactivated GR and 14-3-3 was much weaker, and a first 30-ml salt washing with 50 mM NaCl seemed to be sufficient to abolish 14-3-3 interaction with the nonliganded/nonactivated GR (Fig. 5A). In the case of Raf-1 and its association with nonliganded/nonactivated GR, we obtained varying results, showing on the one hand that the interaction was almost as strong as with the liganded/activated GR (Fig. 5B) and on the other hand that the association was as weak as for 14-3-3 and nonliganded/nonactivated GR (not shown). The reason for this variability is further addressed under “Discussion.”
A mechanistic option for rapid effects of glucocorticoids could be of relevance in GR interactions with other signaling pathways. Glucocorticoid-induced transcriptional events but may well be the glucocorticoid receptor has not been obvious in relation to factor I and thereby activated in the absence of estrogen (20). Kinases upon stimulation by for example insulin-like growth which may be phosphorylated by mitogen-activated protein

Rapid effects have been demonstrated with estrogen receptor activation has been documented for estrogen receptor, progesterin receptor, and androgen receptor (for a review see Ref. 20). Rapid effects have been demonstrated with estrogen receptor α, which may be phosphorylated by mitogen-activated protein kinases upon stimulation by for example insulin-like growth factor I and thereby activated in the absence of estrogen (20).

The relevance of glucocorticoid-induced phosphorylation of the glucocorticoid receptor has not been obvious in relation to glucocorticoid-induced transcriptional events but may well be of relevance in GR interactions with other signaling pathways. A mechanistic option for rapid effects of glucocorticoids could be direct protein-protein interactions. Such interactions have been demonstrated to occur for GR with among others STAT3 (5) and STAT5 (6) and has been implied for NF-κB and GR (3, 4). Some of these interactions may be dependent on phosphorylation events.

In the cytoplasm, nonliganded/nonactivated GR has previously been demonstrated to exist in a multiprotein complex (see Ref. 21 and references therein). Several of the receptors in the nuclear receptor family including GR have been demonstrated to interact with cofactors (i.e. coactivators and corepressors present in nuclear extracts or detected in in vitro based systems probing for protein-protein interaction). Based on findings in this study we would like to propose a multiprotein complex, a receptosome, in which GR participates, containing more proteins than previously suggested and existing not only in the cell nucleus but also in the cytoplasm harboring a number of putatively functionally relevant proteins, some of which may be of importance for rapid glucocorticoid effects. Such a receptosome may be a subset of a larger “signalsosome,” a term often used to describe multiprotein complexes consisting of proteins participating in signal transduction (Refs. 22 and 23; for a review see Ref. 24).

We have developed a purification method for GR allowing the receptor to be purified in various functional states and also allowing detection of co-purifying proteins. By running parallel experiments with equal fractions of the same cytosol containing nonliganded/nonactivated or liganded/activated GR on equal-sized immunoaffinity columns, we have been able to perform semiquantitative comparisons of GR co-purifying proteins. As seen in Fig. 1, a number of proteins co-purify with GR and most of them are specific in the sense that they are not observed when substituting the anti-GR antibody for normal mouse immunoglobulins. We are in the process of identifying the GR-co-purifying proteins and have found that it is possible to corroborate the previously demonstrated interaction of GR with 14-3-3 in this system. Because the GR/14-3-3 interaction has previously been demonstrated using yeast-two-hybrid and glutathione S-transferase pull-down experiments, our findings confirm the validity of these experimental approaches in this particular case. Furthermore, the unexpected finding of Raf-1 in the GR multiprotein complex indicates the usefulness of our immunoaffinity-based technique in native systems to discover yet other currently unknown GR-protein interaction.

It is at this point unclear whether GR interacts directly with Raf-1 or whether this interaction is mediated via 14-3-3. Studies of the effect of 14-3-3 on Raf-1 are so far not conclusive as 14-3-3 has been reported to either have no effect on Raf (25) or to activate Raf (26, 27) in in vitro experiments. A more recent study implies that 14-3-3 is bound to Raf-1 in the cytosol and that this binding is necessary for shuttling of Raf-1 to the cell membrane and for activation by Ras. This study also shows that 14-3-3 dissociates completely from Raf-1 at the plasma membrane (12). Because we have found several co-purifying proteins for liganded/activated GR in cytosolic extracts, any other protein present in the extract may actually mediate the GR-Raf-1 interaction. It could for instance be noted in Fig. 2 that hsp70 seems to be enriched in the liganded/activated fraction. Raf-1 is also known to complex with hsp90 (28), but because we found that Raf-1 co-purified with GR in the liganded/activated state (i.e. when GR is dissociated from hsp90), we exclude that, in our experiments, the GR-Raf-1 interaction is mediated via hsp90.

To be able to distinguish nonliganded/nonactivated from liganded/activated receptor, we used adrenalectomized rats to

![Image](140x589 to 464x729)
eliminate endogenous ligand and also included 20 mM molybdate to maintain the receptor in the nonactivated state (8). However, we sometimes noted the presence of 14-3-3 and Raf-1 in the nonliganded/nonactivated GR co-purifying protein population albeit at lower levels. This may indicate that a low affinity interaction of GR, 14-3-3 and Raf-1 could occur regardless of ligand and GR activation state but that the conformational change of GR most likely induced by ligand binding as well as the concomitant detachment from hsp90 leads to an increased recruitment of 14-3-3 and Raf-1 to GR. Another possibility is that the experimental procedure involving dilution and washing with 150 mM NaCl leads to activation of a fraction of GR at low temperature and in the absence of ligand and that we observe presence of 14-3-3 and Raf-1 in the nonliganded/nonactivated GR fraction as a consequence of a unintentional GR activation.

To further study the interaction between 14-3-3 and GR, we also used 14-3-3-antibodies to immunoprecipitate 14-3-3 and looked for co-precipitating proteins. The antibody we used recognized all 14-3-3 isoforms including the 14-3-3-7. We were able to co-immunoprecipitate a small amount of both GR and Raf-1 to a similar extent regardless of GR ligandation and activation state. This verifies the interaction but also probably reflects that only a fraction of 14-3-3, being an abundant protein with several isoforms, interacts with GR and that the activation state of GR is difficult to control under the experimental conditions of this particular co-immunoprecipitation study.

The observation of 14-3-3 complexed with liganded/activated GR may indicate the need for GR to be continuously chaperoned in all functional states. The functional relevance of GR-Raf-1 interaction could be of special importance in regulation of cellular growth and differentiation. Glucocorticoids have been reported to affect cellular growth events in a cell- and context-dependent fashion; for example, glucocorticoids lead to induction of apoptosis in certain T lymphocyte populations and to growth arrest in G1 in lymphocytes (reviewed in Ref. 29), and the expression of IRS-1 either in the GR co-purifying fraction or in the 14-3-3 immunoprecipitate. This may reflect a mutually exclusive binding of GR and IRS-1 to 14-3-3 or, in the case of lack of IRS-1 co-immunoprecipitation by 14-3-3 antibodies, experimental conditions different from those used by Ogihara and co-workers (11).

It is tempting to speculate that cytosolic GR may exist together with other specific proteins in a tightly held together multiprotein complex, a receptosome, forming the biochemical basis for cross-talk between GR and other signaling pathways, one example of which is Ras/Raf-1. The presence of the 14-3-3 protein may facilitate GR interactions with other proteins. Because the GR-protein interactions studied in this investigation appeared to be significantly strengthened following ligand activation of GR, it is quite conceivable that introduction of glucocorticoids into this system might trigger changes in GR-protein interaction leading to rapid nongenomic effects. Ongoing studies are aimed at identifying other possible proteins participating in the formation of the GR containing receptosome.

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REFERENCES

1. Nemoto, T., Ohara-Nemoto, Y., Denis, M., and Gustafsson, J. Å. (1990) Biochemistry 29, 1880–1886
2. Bamberger, C. M., Schulte, H. M., and Chrousos, G. P. (1996) Endocr. Rev. 17, 245–261
3. Caldenhoven, E., Liden, J., Wissink, S., Van de Stolpe, A., Raaijmakers, J., Koenderman, L., Okret, S., Gustafsson, J. Å., and Van der Saag, P. T. (1995) Mol. Endocrinol. 9, 401–412
4. Ray, A., and Prefontaine, K. E. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 752–756
5. Zhang, Z., Jones, S., Haggard, J. S., Fuentes, N. L., and Fuller, G. M. (1997) J. Biol. Chem. 272, 30607–30610
6. Stocklin, E., Wissler, M., Gouilleux, F., and Groner, B. (1996) Nature 383, 726–728
7. Wakai, H., Wright, A. P., Gustafsson, J., and Zilbius, J. (1997) J. Biol. Chem. 272, 8158–8166
8. Leach, K. L., Dahmer, M. K., Hammond, N. D., Sando, J. J., and Pratt, W. B. (1979) J. Biol. Chem. 254, 11884–11890
9. Fu, H., Subramanian, R. R., and Masters, S. C. (2000) Annu. Rev. Pharmacol. Toxicol. 40, 617–647
10. Lopez-Girona, A., Furnari, B., Mondesert, O., and Russell, P. (1999) Nature 397, 172–175
11. Ogihara, T., Isobe, T., Ichimura, T., Tasaka, M., Funak, M., Sakoda, H., Ozishi, Y., Inukai, K., Anai, M., Fukushama, Y., Kikuchi, M., Yazaki, Y., Oka, Y., and Anaso, T. (1997) J. Biol. Chem. 272, 25267–25274
12. Roy, S., McPherson, R. A., Apolloni, A., Yan, J., Lann, A., Clyde-Smith, J., and Hancock, J. F. (1996) Mol. Cell. Biol. 18, 3947–3955
13. Braselmann, S., and McCormick, F. (1995) EMBO J. 14, 4839–4848
14. Croxatt, J. D., Choudhury, Q., and Flower, R. J. (2000) Br. J. Pharmacol. 130, 299–304
15. Okret, S., Wikström, A. C., Wrangle, O., Andersson, B., and Gustafsson, J. Å. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1609–1613
16. McEwan, I. J., Almlof, T., Wikström, A. C., Dahlman-Wright, K., Wright, A. P., and Gustafsson, J. Å. (1994) J. Biol. Chem. 269, 25629–25636
17. Akner, G., Mosberg, K., Sundqvist, K. G., Gustafsson, J. Å., and Wikström, A. C. (1992) Eur. J. Cell Biol. 58, 356–361
18. Merry, W. H., Caplan, R. H., Wickus, G. G., Reynertson, R. H., Kiskan, W. A., Cobgill, T. H., and Landercasper, J. (1994) Surgery 116, 1095–1100
19. Reichardt, H. M., Kaestner, K. H., Tuckermann, J., Kretz, O., Weisso, B., Bock, R., Gass, P., Schmid, W., Herrlich, P., Angel, P., and Schütz, G. (1998) Cell 93, 531–541
20. Cenni, B., and Picard, D. (1999) Trends Endocrinol. Metab. 10, 41–46
21. Pratt, W. B., and Toft, D. O. (1997) J. Biol. Chem. 272, 1854–1862
22. Van Aelst, L., Barr, M., Marcus, S., Polverino, A., and Wigler, M. (1993) Science 260, 1659–1661
23. Moodie, S. A., Willumsen, B. M., Weber, M. J., and Wolfman, A. (1993) Science 260, 1659–1661
24. Burack, W. R., and Shaw, A. S. (2000) Curr. Opin. Cell Biol. 12, 211–216
25. Suen, K. L., Bustello, X. R., and Barbacid, M. (1995) Oncogene 11, 825–831
26. Frati, W. J., Muslin, A. J., Kikuchi, A., Martin, J. A., MacNeil, A. M., Gross, R. W., and Williams, L. T. (1994) Nature 371, 612–614
27. Irie, K., Gotoh, Y., Yashar, B. M., Errede, B., Nishida, E., and Matsumoto, K. (1994) Science 265, 1710–1712
28. Stancato, L. F., Chow, Y. H., Hutchinson, K. A., Perdew, G. H., Jove, R., and Pratt, W. B. (1993) J. Biol. Chem. 268, 21711–21716
29. King, K. L., and Cidlowski, J. A. (1998) Annu. Rev. Physiol. 60, 601–617
30. Evans-Storms, R. B., and Cidlowski, J. A. (2000) Endocrinology 141, 1431–1443
31. Campbell, S. L., Khorasvari-Far, R., Rossman, K. L., Clark, G. J., and Der, C. J. (1998) Oncogene 17, 1385–1413