A Nuclear Action of the Eukaryotic Cochaperone RAP46 in Downregulation of Glucocorticoid Receptor Activity

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Abstract. RAP46 is a eukaryotic cochaperone that associates with several proteins, including the heat shock protein hsp70/hsc70 and the glucocorticoid receptor (GR). Here we show a downregulation of GR-mediated transactivation by RAP46 via a mechanism independent of a cytoplasmic action of this cochaperone. We demonstrate a specific cytoplasmic–nuclear recruitment of RAP46 by the liganded GR that results in inhibition of the transactivation function of the receptor. A repeated sequence motif $[EEX_4]_8$ at the NH_2 terminus of RAP46 or BAG-1L, a larger isoform of RAP46, is responsible for this downregulation of GR activity. BAG-1, a shorter isoform with only a duplication of the $[EEX_4]$ sequence, does not inhibit GR activity. The $[EEX_4]_8$ motif, when linked to an otherwise unrelated protein, abrogated the inhibitory action of endogenous RAP46 on GR-mediated transactivation. The nuclear effects of RAP46 and BAG-1L are specific since GR-mediated inhibition of AP-1 activity was not affected. These studies identify the $[EEX_4]_8$ sequence as a signature motif for inhibition of GR-mediated transactivation and demonstrate a specific nuclear action of a eukaryotic cochaperone in the regulation of GR activity.

Key words: glucocorticoid receptor • mineralocorticoid receptor • transactivation • BAG-1 isoforms • cochaperone

Glucocorticoids function by binding to the glucocorticoid receptor (GR)[1], which is localized in the cytoplasm of target cells in a complex with the heat shock proteins hsp90, p60/Hop, hsp70, hsp40, and other chaperone molecules (for review see Pratt and Dittmar, 1998). These proteins, particularly hsp90, hsp70, and p60, keep the receptor in a form that is capable of binding ligand (Dittmar and Pratt, 1997).

Molecular chaperones of the hsp70/hsc70 family play important role in folding, translocation, and degradation of proteins in eukaryotic cells due to their ability to bind and stabilize nonnative protein conformation (Hartl, 1996; Rassow et al., 1997). They recognize extended hydrophobic segments in substrates and bind them with their COOH-terminal peptide domain while their NH_2-terminal ATPase domain is used for regulating the binding process (Rassow et al., 1997). This occurs through cycles of ATP binding and hydrolysis whereby denatured proteins are alternatively bound and released to effect protein folding. Substrates interact transiently with the ATP bound form of hsp70, but this binding is stabilized when ATP is hydrolyzed (for review see Bukau and Horwich, 1998).

Members of the hsp40 and p60/Hop (hsp70/hsp90 organizing protein) families modulate the protein folding activity of hsp70 through interaction with the COOH-terminal domain of this protein (Gebauer et al., 1997, 1998). In this reaction, Hop plays a particularly interesting role and establishes a physical link between hsp70 and hsp90 (Johnson et al., 1998). A protein termed Hip (hsc70 interacting protein) has been identified as a chaperone cofactor interacting with the ATPase domain of hsc70 after the initial activation of the ATPase activity by hsp40. Hip stabilizes the ADP bound form of hsc70 and prolongs the interaction of this protein with various target substrates (Höhfeld et al., 1995; Frydman and Höhfeld, 1997).

Recently, another member in the family of chaperones and cochaperones has been identified and termed Hap46, BAG-1, or RAP46. These different names originate from the functions associated with this protein. For example, Hap46 is derived from its function as an hsp70/hsc70 associating protein (Gebauer et al., 1997, 1998; Höhfeld and Jentsch, 1997; Takayama et al., 1997; Zeiner et al., 1997), BAG-1 as a Bcl-2 associated athanogene 1 (Takayama et al., 1995), and RAP46 as a steroid hormone receptor associating protein (Zeiner and Gehring, 1995). Hap46/RAP46/BAG-1 interacts with the NH_2-terminal ATP bind-

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1. *Abbreviations used in this paper:* GFP, green fluorescence protein; GR, glucocorticoid receptor; HA, hemagglutinin influenza; HBD, hormone binding domain; hsp, heat shock protein; MMTV, mouse mammary tumor virus; MR, mineralocorticoid receptor.

ing domain of hsp70/hsc70 (Takayama et al., 1997; Stuart et al., 1998) and competes with Hop, as well as Hip, for binding to this molecular chaperone (Höhfeld and Jentsch, 1997; Gebauer et al., 1998). RAP46/BAG-1 interacts with several other proteins through its COOH terminus, the same region it uses to bind hsp70/hsc70 (Bardelli et al., 1996; Wang et al., 1996; Takayama et al., 1997). Therefore, it has been postulated that protein–protein interactions involving this cochaperone must be mediated by hsp70/hsc70 (Zeiner et al., 1997).

As a protein that associates with the antiapoptotic protein Bcl-2, overexpression of BAG-1 prolongs the survival of fibroblasts challenged with apoptotic signals (Takayama et al., 1995, 1997). It also interacts with hepatocyte and PDGF receptors to enhance their antiapoptotic function (Bardelli et al., 1996), while RAP46 interacts with steroid receptors to inhibit apoptosis induced by some members of this family of ligand binding transcription factors (Kullmann et al., 1998; Liu et al., 1998).

The action of RAP46 on steroid hormone action is complicated by the fact that different isoforms of this protein exist (Froesch et al., 1998). In addition to RAP46, also known as BAG-1M, a larger isoform (BAG-1L) and a smaller isoform (BAG-1) have been described. BAG-1L arises through translation initiation at a noncannonical CTG codon upstream and in-frame with the usual ATG used for the production of RAP46, whereas BAG-1 is a product of an in-frame translation at a downstream ATG site (Froesch et al., 1998; Packham et al., 1997). BAG-1L strongly enhances transactivation by the androgen receptor, but BAG-1 and RAP46 do not drastically affect the action of this receptor. On the other hand, BAG-1 inhibits the transactivation activity of the retinoic acid receptor heterodimers (RAR/RXR), but not the homodimers (RXR/RXR; Liu et al., 1998). As the different isoforms of RAP46 still contain their domain of interaction with hsp70/hsc70, their effect on the action of the steroid receptor is thought to be partially controlled by their cochaperone activity. The effects of the various RAP46 isoforms on GR and retinoic acid receptor activity so far have not been analyzed.

In this communication, we have analyzed the effect of RAP46 and its isoforms on GR action. We have shown in confocal immunofluorescence experiments colocalization of RAP46 with the nontransformed GR in the cytoplasm. However, this interaction does not affect the hormone binding properties of the receptor. In the presence of ligand, RAP46 is specifically recruited from the cytoplasm into the nucleus by the activated GR. There, it uses its first 70 NH_2-terminal amino acids containing the sequence $[EEX_4]_8$ to inhibit DNA binding and, as a result transactivation, but without compromising the ability of the receptor to repress the activity of the transcription factor AP-1. BAG-1L, the larger isoform of RAP46, which also contains the sequence motif $[EEX_4]_8$, is constitutively nuclear. It inhibited transactivation without a major effect on the transrepression function of the receptor. Mutants of RAP46 that are similar to BAG-1 with truncations of the repeat motif do not affect GR activity. These results identify a signature motif on a eukaryotic cochaperone for inhibition of the transactivation function of the GR and demonstrate that the primary site of action of this protein is the nuclear compartment of cells.

Materials and Methods

Cell Culture

Simian kidney COS-7 cells and human mammary MCF-7 cells were cultured in DME supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in an atmosphere of 5% CO_2. For experiments involving the use of the human mineralocorticoid receptor (MR), the cells were kept in DME containing 3% charcoal treated FCS to remove residual steroids.

Plasmid Constructs

The expression vector containing the wild-type human GR and MR have been described by Giguère et al. (1986) and Arriza et al. (1987). The recombinants GMM, GGM, and GGΔ have been described by Arriza (1991). The plasmid pcDNA3RAP46 was constructed by insertion of the coding sequence of human RAP46 (Zeiner and Gehring, 1995) into the plasmid pcDNA3. The expression plasmid pT2 contains sequences encoding two copies of the hemagglutinin influenza (HA)-tag 5′-AA GCT TCC ACC ATG ATC TTT TAC CCA TAC GAT GTT CCT GAC TAT GCG GGC TAT CCC TAT GAC GTC CCG GAC TAT GCA GGA TCT ACT CGA GAG GAT CCG GTA CCT ATC TAG A-3′ cloned between the HindIII and XbaI sites of the plasmid pcDNA3 (Invitrogen Corp.). The constructs pT2RAP46 and pT2RAP46ΔC47 were constructed by inserting blunt-ended BamHI/XbaI fragments from pcDNA3RAP46 and pcDNA3RAP46ΔC47 (lacking the last 47 COOH-terminal amino acids) into XhoI and XbaI sites of pT2 after a fill-in reaction with DNA polymerase I (Klenow fragment). The constructs pT2RAP46Δ40 and pT2RAP46Δ70, containing deletion mutants of the first 40 and 70 NH_2-terminal amino acids of RAP46, were constructed by PCR amplification with XhoI and BamHI ends and insertion into the corresponding sites of pT2. The construct p46N69GFP, encoding the first 69 NH_2-terminal amino acids of RAP46 fused to the NH_2 terminus of the green fluorescence protein (GFP) sequence, was obtained by subcloning a HaeIII/PvuII fragment from pT2RAP46 into the blunt-ended HindIII site of pcDNA3.1hGFP. The pcDNA3.1hGFP construct contains sequences encoding the humanized GFP (CLONTECH Laboratories, Inc.) between the HindIII and XbaI sites of pcDNA3.1 (Invitrogen Corp.). The reporter plasmid pGL3MMTV encodes the firefly luciferase gene under the control of the mouse mammary tumor virus (MMTV) long terminal repeat region (−241 to +137), cloned as a BamHI/BglII fragment from the plasmid pHCwt CAT (Kaspar et al., 1993). The reporter plasmid −517/+63 Coll-Luc already has been described by Heck et al. (1997) and Schneikert et al. (1996). The control plasmid for transfection pTKRenilla Luc was obtained from Promega and the expression vectors GR–GFP and GFP–MR have been described by Carey et al. (1996) and Fejes-Tóth et al. (1998). The GFP plasmids GRΔ491-515GFP and GR1-515GFP were constructed by exchanging the GR sequence in the construct GR–GFP (Carey et al., 1996) with the GRΔ491-515 and GR1-515 sequences (Hollenberg et al., 1987).

Transient Transfections

100,000 MCF-7 cells were transiently transfected by the calcium phosphate coprecipitation procedure in 3.4-cm-diam plates with 2 μg reporter plasmid, 0.1 μg pTKRenilla Luc, and 2 μg expression vector. After 5 h of incubation with DNA and 2 min shock with 10% glycerol in PBS, the cells were further incubated for 36 h. Thereafter, they were harvested and cellular extracts were prepared for luciferase assay as described by Schneikert et al. (1996). Renilla luciferase assay was performed with the dual luciferase reporter assay system from Promega, according to the manufacturer's instructions. COS-7 cells were transfected by electroporation as previously described by Klocker et al. (1992) with 16.5 μg DNA containing 2 μg reporter plasmid, 0.5 μg pTKRenilla Luc, 7 μg wild-type or mutant RAP46, and 7 μg receptor expression vector.

Whole Cell Hormone Binding Assay

COS-7 cells were transiently transfected with either empty expression vectors or expression vectors encoding GR, RAP46, and RAP46Δ70. After 36 h incubation in DME containing 3% charcoal treated FCS, the cells were trypsinized and resuspended in the same medium. 200,000 cells in 100 μl medium were incubated for 90 min at room temperature with different concentrations (0–100 nM) of [^{3}H]dexamethasone (81.0 Ci/mmol;

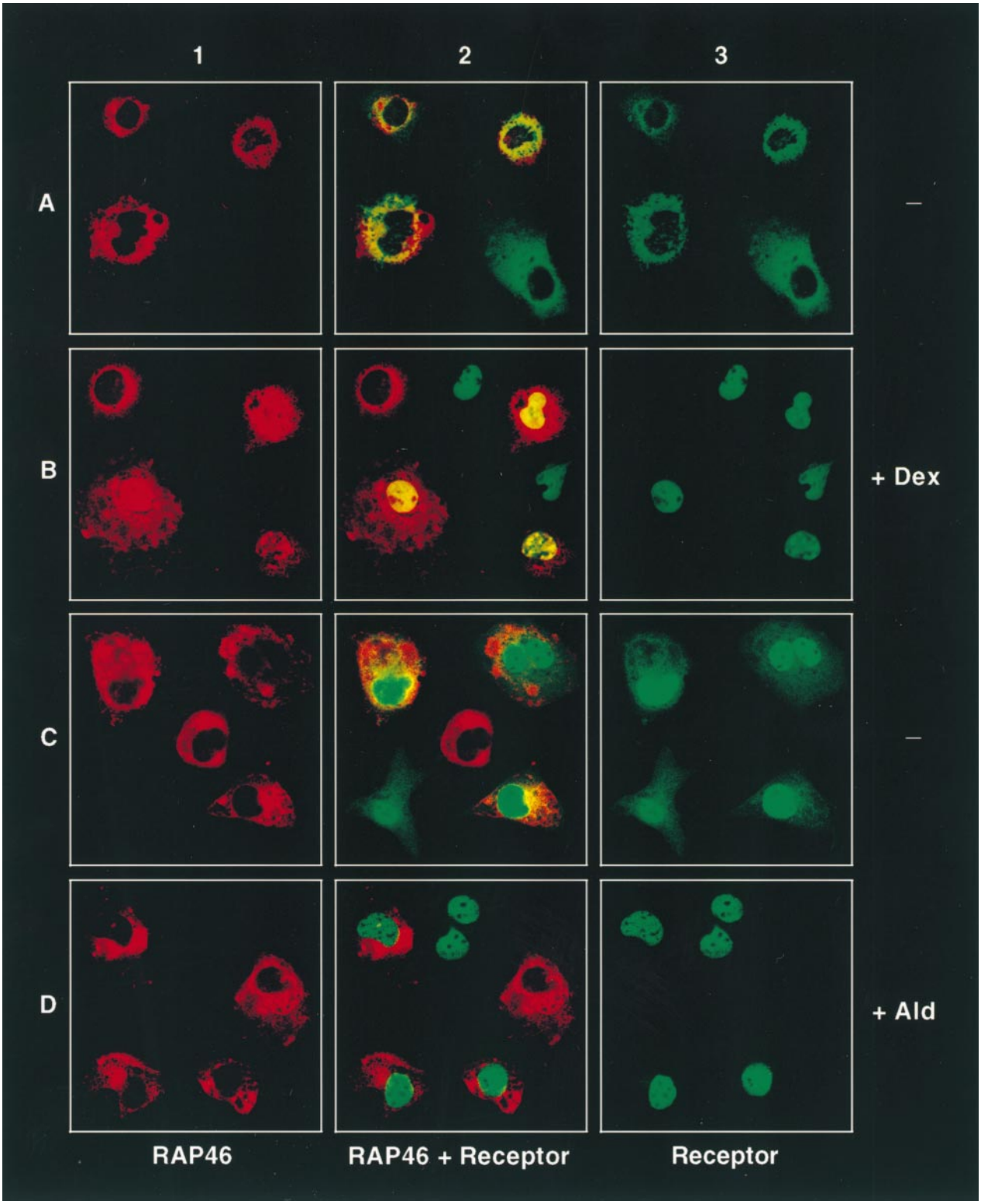


Figure 1. Confocal immunofluorescence analysis of intracellular localization of GR, MR, and RAP46. COS-7 cells were transiently transfected with expression vectors encoding either a GR–GFP (A and B) or GFP–MR (C and D) together with a plasmid expressing HA-tagged RAP46 (A–D). 36 h after transfection the cells were treated for 1 h with vehicle (0.1% ethanol) alone (−) or vehicle containing either 0.1 μM dexamethasone (+ Dex) or 0.1 μM aldosterone (+ Ald) before harvesting, processing, and visualization with a laser confocal microscope. The green fluorescence arises from the GFP-tagged receptors, whereas the red fluorescence comes from staining of RAP46 with the anti-HA mAb 12CA5 (Boehringer Mannheim Corp.), followed by an anti-mouse antibody labeled with rhodamine. The yellow to orange colors indicate areas of colocalization of the two proteins.

Nycomed Amersham Inc.) in the absence and presence of a 1,000-fold M excess of unlabeled dexamethasone. After incubation, the cells were washed three times with 1 ml PBS and recovered by centrifugation (3,800 *g* for 3 min). The cell pellet was then lysed in 5 ml scintillation fluid and the radioactivity determined by liquid scintillation counting.

Immunofluorescence

Immunofluorescence experiments were performed as previously described by Herscovics et al. (1994) and the photographs were taken with an LSM 410 invert Zeiss confocal microscope.

Electrophoretic Mobility Shift Assays and Immunoblots

Electrophoretic mobility shift and immunoblots assays were performed as described previously by Gast et al. (1995).

Results

Association and Recruitment of RAP46

RAP46 is a protein initially isolated by virtue of its association with the GR in an interaction-screening assay (Zeiner and Gehring, 1995). Since then, association of RAP46 and the GR has been shown in vitro in a glutathione S–transferase pull-down assay (Kullmann et al., 1998). To determine whether these two proteins associate in vivo, we performed immunofluorescence experiments with RAP46 and the GR in the absence and presence of hormone. To demonstrate the specificity of these interactions, control experiments were carried out with the MR, a structural and functional homologue of the GR (Arriza et al., 1987).

When expressed in COS-7 cells, most of the RAP46 protein resided in the cytoplasm as determined by laser confocal microscopy. Antibodies that recognize COOH-terminal epitopes or the HA-tag on this protein clearly showed cytoplasmic localization of RAP46 (Fig. 1, A 1 and C 1; and results not shown). In certain regions of the cytoplasm, RAP46 colocalized with the unliganded GR or MR (Fig. 1, see the yellow color in A 2 and C 2). In the absence of hormone, the MR was identified in the cytoplasm, as well as in the nucleus (Fig. 1, C 3), in agreement with the results of Fejes-Tóth et al. (1998). In the presence of ligand when the GR and MR are translocated from cytoplasm into the nucleus, RAP46 was transported with the GR (Fig. 1, see yellow staining in the nucleus of B 2), but not with the MR (Fig. 1, D 2), showing a specific in vivo association of GR and RAP46. Note that cells containing only RAP46, but not the GR, do not translocate into the nucleus in the presence of dexamethasone (Fig. 1, compare A 1 with B 1).

Effect of RAP46 on Ligand Binding and Transactivation by the GR

Through its association with the GR and MR in the cytoplasm, RAP46 may alter the ligand binding properties of these receptors in line with its cochaperone activity (Stuart et al., 1998). We therefore analyzed the hormone binding properties of these receptors in the presence of RAP46 in whole cells and in cytosol preparations. These studies revealed only a slight change in the ligand binding activity of the receptors in the presence of RAP46. For example, Scatchard plot analysis showed an insignificant change in

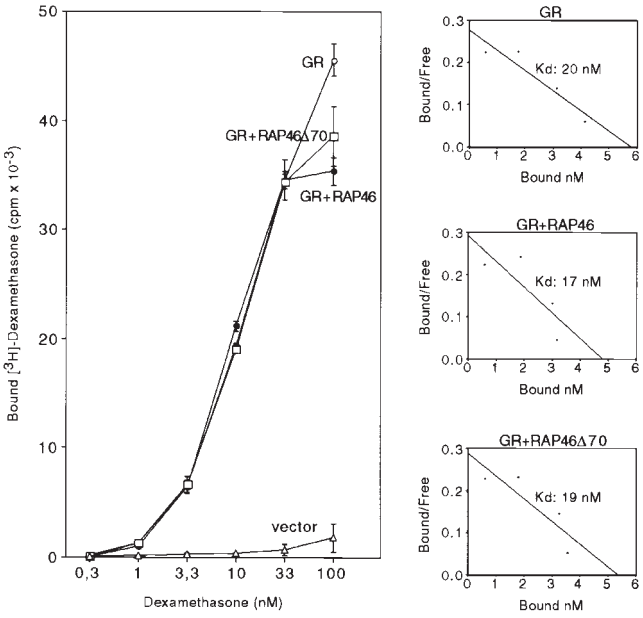


Figure 2. The effects of RAP46 and RAP46Δ70 on dexamethasone binding properties of the GR. 200,000 COS-7 cells were transiently transfected with an empty expression vector or expression vectors encoding GR, RAP46, and RAP46Δ70. Thereafter, the cells were incubated with the indicated amounts of [^{3}H]dexamethasone in the presence or absence of 1,000-fold competitor unlabeled dexamethasone and the bound radioactivity was determined. This was done by subtracting the amount of radioactivity incorporated in the presence of competitor from the radioactivity incorporated in the absence of the competitor. Left, GR-bound [^{3}H]dexamethasone is plotted as a function of hormone concentration in cells transfected with only the GR expression vector (open circles), the GR and RAP46 (filled circles), the GR and RAP46Δ70 (open squares), or with an empty expression vector (open triangles). Presented are the mean values and error bars of two different experiments. In each experiment, the individual measurements were performed in triplicate. Right, The Scatchard plots of the experiments indicating the K_d of GR for dexamethasone in the presence or absence of RAP46 and RAP46Δ70.

the dissociation constant (K_d) of the receptor for dexamethasone from 20 to 17 nM in the presence of RAP46 (Fig. 2, right). A slight (20%) reduction in the maximum hormone binding capacity (B_{max}) of the receptor was also detected (Fig. 2, left). This downregulation of the B_{max} is minimal compared with our previous report of a strong RAP46-mediated inhibition of transactivation by the GR (Kullmann et al., 1998). Thus, RAP46 must exert its negative regulatory function at other stages in the action of the GR.

In the liganded state, when both the GR and MR are in the nucleus, RAP46 downregulated the transactivation function of only the GR, but not that of the MR (Fig. 3, A and B; note that GGG and MMM refer to GR and MR). This repression was independent of whether dexamethasone, cortisol, or aldosterone was the activating ligand (Fig. 3 B). The lack of RAP46 effect on transactivation by the MR is possibly due to the different cellular localizations of these two proteins in the presence of ligand (Fig. 1, D 2; note nuclear and cytoplasmic localization of MR

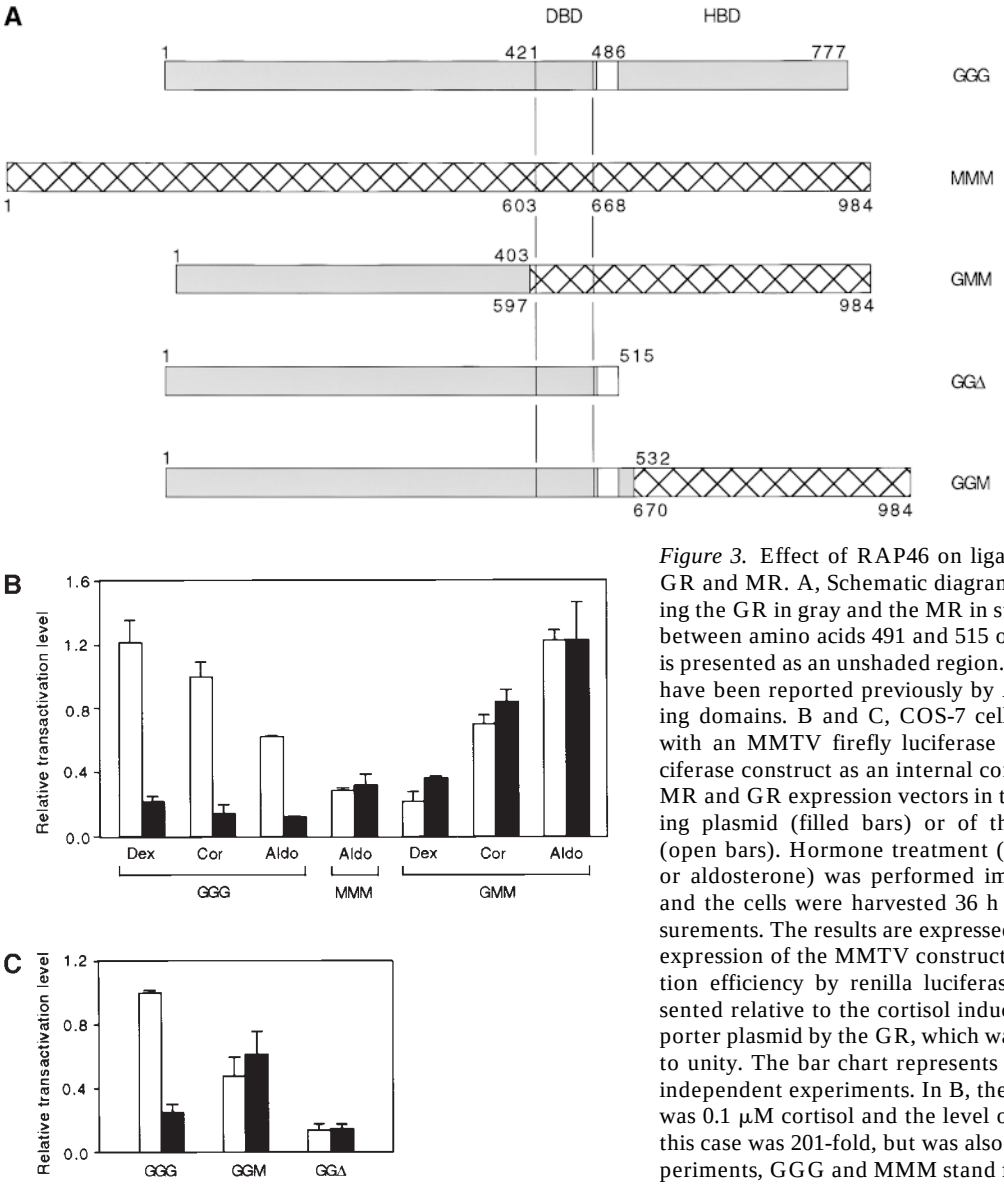
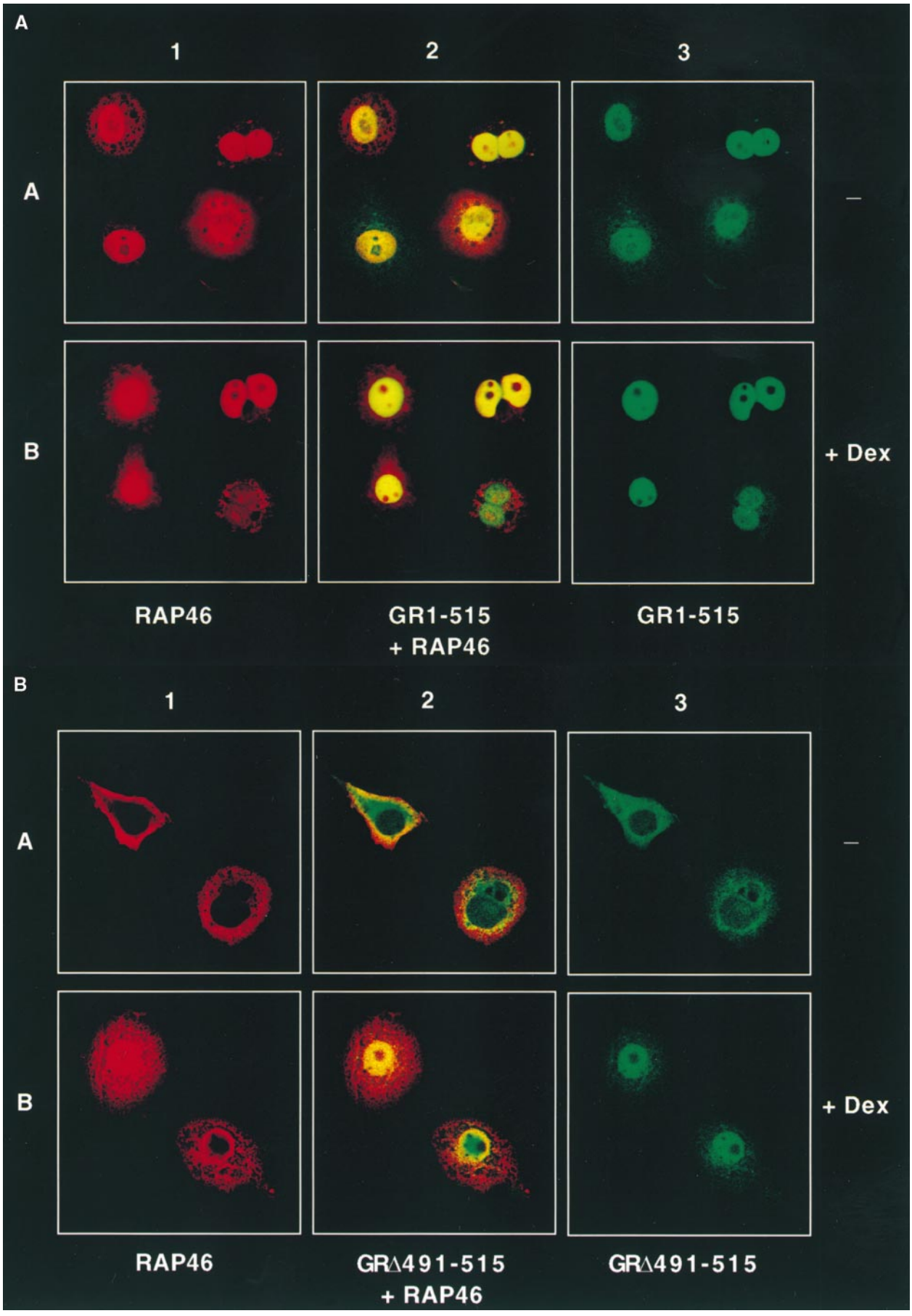


Figure 3. Effect of RAP46 on ligand dependent transactivation by GR and MR. A, Schematic diagram of the GR–MR chimeras showing the GR in gray and the MR in stripes. The RAP46 binding site in between amino acids 491 and 515 of the GR (Kullmann et al., 1998) is presented as an unshaded region. The receptor chimeras described have been reported previously by Arriza (1991). DBD, DNA binding domains. B and C, COS-7 cells were transiently cotransfected with an MMTV firefly luciferase indicator gene and a renilla luciferase construct as an internal control, together with the indicated MR and GR expression vectors in the presence of a RAP46 expressing plasmid (filled bars) or of the corresponding control vector (open bars). Hormone treatment (0.1 μM dexamethasone, cortisol, or aldosterone) was performed immediately after the transfection and the cells were harvested 36 h later for luciferase activity measurements. The results are expressed as the level of hormone-induced expression of the MMTV construct after correcting for the transfection efficiency by renilla luciferase measurements. This was presented relative to the cortisol induced expression of the MMTV reporter plasmid by the GR, which was 213-fold, but was arbitrarily set to unity. The bar chart represents the mean value (± SD) of three independent experiments. In B, the hormone used after transfection was 0.1 μM cortisol and the level of induction of MMTV activity in this case was 201-fold, but was also set arbitrarily to 1.0. In these experiments, GGG and MMM stand for the GR and MR.

and RAP46). We therefore repeated the transfection experiments with the MR and the RAP46 isoform BAG-1L, which is constitutively localized in the nucleus (Froesch et al., 1998; our unpublished results). In this study, transactivation by the MR was not repressed by BAG-1L, although the response of the GR was inhibited (results not shown; see Fig. 7). This indicates a fundamental difference in the action of RAP46 towards the MR and the GR.

GR–MR chimeric constructs were therefore used to identify the regions of the GR necessary for repression by RAP46 (Fig. 3 A). Two chimeric GR–MR constructs (GMM and GGM), as well as a truncated GR construct (GGΔ), were used in these analyses. Transactivation by the chimeric MR receptor (GMM) containing the NH_2-terminal sequence of the GR (Arriza, 1991) in the presence of either aldosterone, dexamethasone, or cortisol was not repressed by RAP46 (Fig. 3 B). This may be due to the absence of the hinge region (amino acids 491–515) of the GR that we previously identified as an interaction domain for RAP46 and demonstrated its necessity for RAP46-mediated inhibition of transactivation by the GR (Kullmann et al., 1998). The presence of this domain in the chimeric construct GGM still did not allow RAP46 to repress the transactivation function of this mutant receptor (Fig. 3 C), demonstrating the need for the presence of the hormone binding domain (HBD) of the GR. This finding was further confirmed by the use of the truncated receptor (GGΔ), which contains the interaction domain, but lacks the HBD of the GR. In cotransfection experiments, this mutant GR constitutively transactivated a glucocorticoid responsive gene construct, but its activity was not repressed by RAP46 (Fig. 3 C). Further control experiments with chimeric receptors MGG and MMG were performed. Transactivation by MGG containing both the hinge region and HBD of the GR was repressed by RAP46, but not transactivation by MMG containing only the HBD of the GR, but not the hinge region of the GR (results not shown). These results, together with our previous findings on the hinge region (Kullmann et al., 1998), demonstrate that this region, together with the HBD of the GR, are


A
1
2
3
A
–
B
+ Dex
RAP46
GR1-515
+ RAP46
GR1-515
B
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2
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A
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B
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RAP46
GRΔ491-515
+ RAP46
GRΔ491-515

both required for RAP46-mediated inhibition of transactivation by the GR.

The Role of the HBD of the Glucocorticoid Receptor in Repression by RAP46

To further determine the role of the HBD in the repression of the transactivating function of the GR by RAP46, we asked whether this region is involved in the GR-mediated recruitment of RAP46 into the nucleus. In laser confocal immunofluorescence experiments, we showed that the GGΔ mutant receptor lacking the HBD is permanently localized in the nucleus in the absence and presence of hormone, as already reported (Hollenberg et al., 1987). However, in >75% of the cells, RAP46 was colocalized with the receptor in the nucleus (Fig. 4 A). Thus, the inability of RAP46 to repress transactivation by GGΔ is not due to the preclusion of this protein from the nucleus. Rather, it must be due to the absence of an important function in the HBD required for the negative action of RAP46.

To find out how RAP46 is recruited into the nucleus by the GR, we used the mutant GR with a deletion of the hinge region (amino acids 491–515) in the confocal immunofluorescence experiments. In this study, both RAP46 and the mutant receptor were localized in the cytoplasm in the absence of hormone. But, in the presence of dexamethasone, the ability of the GR to recruit RAP46 was severely compromised (Fig. 4 B). Only ~50% of the cells containing the mutant receptor and RAP46 showed colocalization of the two proteins in the nucleus (Fig. 4 B). In the remaining cases, RAP46 was localized in the cytoplasm while the liganded receptor was translocated into the nucleus (Fig. 4 B). This demonstrates that the hinge region of the GR is indeed involved in the transport of RAP46 into the nucleus, although this process could be aided by other regions of the receptor. These regions are most likely contained in the HBD since its deletion, as shown above, slightly decreased nuclear transport of RAP46.

Nuclear Action of RAP46

As RAP46 does not drastically alter the hormone binding properties of the GR in the cytoplasm, and it is translocated into the nucleus by the GR, its major site of action must be in the nucleus. Previously, we have shown that it inhibits the DNA binding activity of the receptor (Kullmann et al., 1998), but the mechanism involved is not known. We therefore deleted the NH_2-terminal sequences of this protein encompassing the sequence motif $[EEX_4]_8$ (see underlined sequences in Fig. 5 A) to determine the effect of the truncated proteins on DNA binding by the GR and GR-mediated transactivation at the MMTV promoter.

While RAP46 inhibited DNA binding by the GR, mutants with truncation of the first 40 or 70 NH_2-terminal amino acids, eliminating five or all eight of the repeat-motif, partially or completely abolished this negative effect of RAP46 on DNA binding, as well as transactivation by the GR (Fig. 5 B). Control immunoblot experiments with extracts of the transfected cells showed that the GR and RAP46 constructs were adequately expressed in this experiment (Fig. 5 B). Note that the apparent complete inhibition of DNA binding by the GR in Fig. 5 B is due to the very short autoradiographic exposure time of the gel. Longer exposures showed that DNA binding was drastically repressed, but not completely abolished (results not shown).

The two RAP46 NH_2-terminal mutants behaved as the wild-type RAP46 in several experiments. This was demonstrated by RAP46Δ70, which slightly decreased the maximum hormone binding capacity of the GR without significantly affecting the K_d for dexamethasone, as we have reported already (Fig. 2, compare K_d = 20 nM with 19 nM). In the presence of ligand, this mutant, as in the case of the wild-type RAP46, was also recruited into the nucleus by the GR (Fig. 5 C, compare A 1 with B 2). However, unlike the wild-type RAP46, it did not downregulate DNA binding by the GR, nor did it repress GR-mediated transactivation at the MMTV promoter (Fig. 5 B). Thus, COOH-terminal sequences of RAP46 are required by the GR for the recruitment of this protein into the nucleus.

To determine the sequences at the COOH terminus necessary for the recruitment, we used a mutant RAP46 lacking the last 47 amino acids known to bind hsp70/hsc70 in immunofluorescence experiments (Froesch et al., 1998). This mutant, unlike the NH_2-terminal deletion construct, was not transported into the nucleus (Fig. 5 D), indicating that the extreme COOH-terminal sequence of RAP46 is required to get this protein into the nucleus. Interestingly, deletion of this COOH-terminal sequence also abolished the repressive action of RAP46 on DNA binding by the GR (results not shown). This demonstrates the necessity for nuclear transport of RAP46 for its negative regulatory activity. These results, together with our findings from the experiments with the NH_2-terminal deletion mutant RAP46Δ70 (Fig. 5 C), demonstrate that although nuclear transport of RAP46 is necessary, it is not sufficient for inhibition of DNA binding by the GR. This function requires the NH_2 terminus of RAP46.

To determine the contribution of the NH_2 terminus of RAP46 to downregulation of GR activity, we fused the first 69 amino acids to GFP and asked whether such a construct was sufficient in repressing GR activity. In immunofluorescence experiments, this fusion protein was found both in the cytoplasm and the nucleus (results not shown).

Figure 4. Confocal immunofluorescence analysis of intracellular localization of mutant GR and RAP46. COS-7 cells were transiently transfected with expression vectors encoding either a GR1-515-GFP (A) or GRΔ491-515-GFP (B), together with a plasmid expressing HA-tagged RAP46. 36 h after transfection, the cells were treated for 1 h with vehicle (0.1% ethanol) alone (−) or vehicle containing 0.1 μM dexamethasone (+ Dex) before harvesting, processing, and visualization with a laser confocal microscope. The green fluorescence arises from the GFP-tagged receptors, whereas the red fluorescence comes from staining of RAP46 with the anti-HA mAb 12CA5 (Boehringer Mannheim Corp.), followed by an anti-mouse antibody labeled with rhodamine. The yellow to orange colors indicate areas of colocalization of the two proteins.

In transactivation studies carried out in GR- and RAP46-positive human mammary tumor MCF-7 cells (Cato et al., 1986; Yang et al., 1998), overexpression of RAP46 inhibited transactivation by the GR, as we had previously reported (Kullmann et al., 1998; Fig. 5 B). However, overexpression of GFP alone had no effect on GR response, whereas the N69GFP construct increased transactivation by the endogenous GR (Fig. 6 A). This shows a dominant–negative effect of the N69GFP protein on the repressive action of endogenous RAP46 in GR-mediated transactivation. This dominant–negative effect was further demonstrated in DNA binding studies involving the GR. In this assay, the N69GFP fusion protein, but not GFP alone, abolished the repressive action of RAP46 on DNA binding by the GR (Fig. 6 B, compare lanes 2–4 with lanes 5–7). These studies confirm that RAP46 uses its NH_2-terminal sequence for the repression of GR activity, possibly through interaction with other factors since its negative effect could be competed by an excess of the N69 sequence fused to GFP.

RAP46 Isoforms (Transactivation Versus Transrepression)

To further determine the significance of the $[EEX_4]_8$ motif in the negative action of RAP46, we examined the effect of the RAP46 isoform BAG-1L (which also carries this sequence motif) on DNA binding by the GR and transactivation by this receptor at the MMTV promoter. In these experiments, both RAP46 and BAG-1L negatively regulated the DNA binding and transactivation functions of the GR to the same extent (Fig. 7). BAG-1, the isoform of RAP46 that lacks a considerable portion of the repeat motif, had no effect, as we have already shown for its closely related homologue RAP46Δ40 (unpublished results; see also Fig. 5 B).

Since the nuclear action of the GR involves both positive and negative regulation of gene expression (Beato et al., 1995; Cato and Wade, 1996; McEwan et al., 1997), we examined the effect of RAP46 and BAG-1L on the negative regulatory activity of the GR. This was achieved by measuring the ability of the GR to repress the expression of a human collagenase I gene construct induced by the phorbol ester 12-O-tetradecanoyl phorbol 13-acetate (TPA). This study showed that transrepression was unaffected by RAP46, whereas BAG-1L only slightly (30%) abrogated this function of the GR (Fig. 7). These effects remained unchanged, even when different amounts of the two constructs were transfected (results not shown). These findings show that the transactivating and transrepressing functions of the GR are regulated differently by RAP46. They further demonstrate that the major nuclear action of

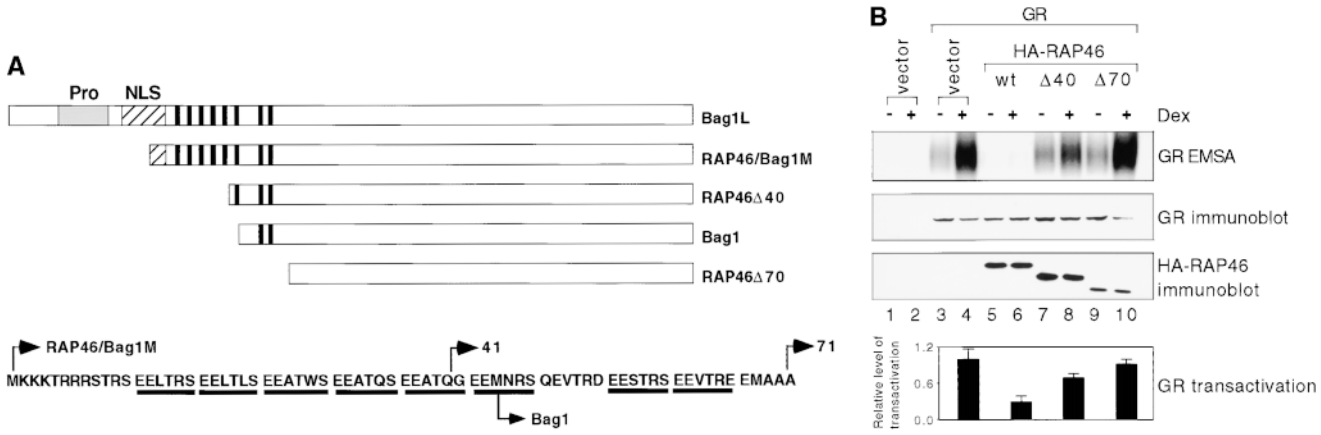

Figure 5. Regions of RAP46 required for nuclear transport and inhibition of GR activity. A, Schematic representation of different isoforms and deletion mutants of RAP46. Indicated are the isoforms BAG-1L, RAP46, and BAG-1, as well as the deletion mutants RAP46Δ40 and RAP46Δ70. The black vertical bars indicate the positions of the motif $[EEX_4]$. The gray and hatched areas represent the location of a proline rich region (Pro) and of a putative nuclear localization signal (NLS). The sequence of the first 71 NH_2-terminal amino acids of RAP46 is also shown with the arrows pointing to the first amino acid in RAP46, RAP46Δ40, BAG-1, and RAP46Δ70. Each $[EEX_4]$ motif is underlined. B, Lack of effect of a NH_2-terminal deletion mutant of RAP46 on DNA binding by the GR and transactivation by the receptor at the MMTV promoter. COS-7 cells were transiently transfected with a control vector (lanes 1 and 2) or an expression vector for GR (lanes 3–10). In addition, they were cotransfected with either a control vector (lanes 1–4) or a plasmid encoding the wild-type HA-tagged RAP46 (HA-RAP46 wt, lanes 5 and 6) or deletion mutants lacking either the first 40 amino acids (HA-RAP46Δ40, lanes 7 and 8) or the first 70 amino acids (HA-RAP46Δ70, lanes 9 and 10). After treatment with vehicle (0.1% ethanol) alone (− Dex) or with 0.1 μM dexamethasone (+ Dex) for 36 h, whole cell extracts were prepared for EMSA and for GR or RAP46 immunoblot analysis. The cells were also transfected with the MMTV firefly luciferase indicator construct and an internal control encoding the renilla luciferase. Hormone treatment was performed with 0.1 μM dexamethasone. The results are expressed as dexamethasone-induced activity of the MMTV indicator gene presented relative to the induced level in the absence of RAP46 that was set at unity. The bar chart represents the mean value (± SD) of three independent experiments. C and D, Confocal immunofluorescence analysis of intracellular localization of GR and HA-RAP46Δ70 or HA-RAP46ΔC47 constructs. COS-7 cells were transiently transfected with expression vectors encoding a GR–GFP and HA-tagged RAP46Δ70 (C) or GR–GFP and HA-tagged RAP46ΔC47 (D). 36 h after transfection, the cells were treated for 1 h with vehicle (0.1% ethanol) alone (−) or vehicle containing 0.1 μM dexamethasone (+ Dex) before harvesting, processing, and visualization with a laser confocal microscope. The green fluorescence arises from the GFP-tagged receptor, whereas the red fluorescence comes from staining of RAP46Δ70 and RAP46ΔC47 with the anti-HA mAb 12CA5, followed by an anti-mouse antibody labeled with rhodamine. The yellow to orange colors indicate areas of colocalization of the two proteins.

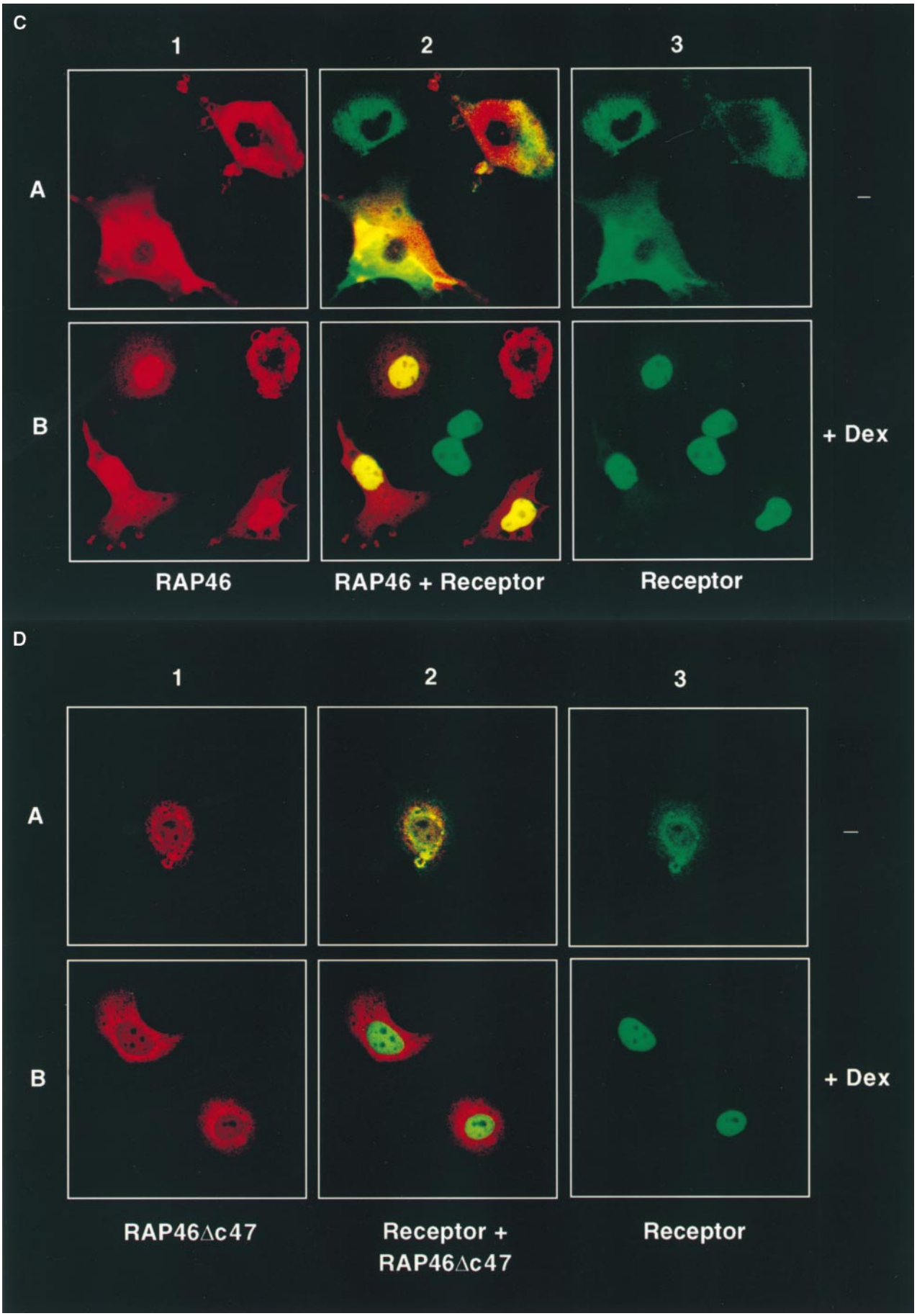

Figure 5 (continued).

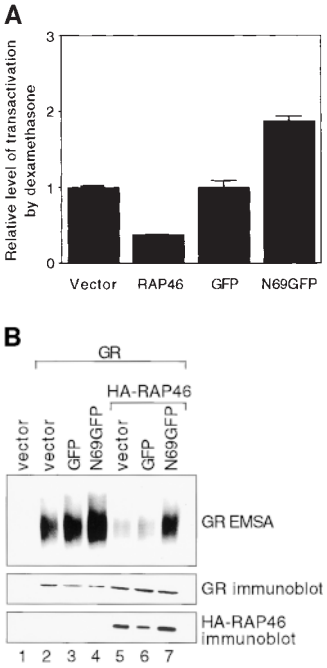

Figure 6. Dominant–negative effect of the first 69 NH_2-terminal amino acids of RAP46 on transactivation and DNA binding by the GR. A, GR-mediated enhancement of transactivation by the first 69 NH_2-terminal amino acids of RAP46 fused to GFP. MCF-7 cells were transiently transfected with the MMTV firefly luciferase indicator gene and the internal control pTKRenilla-Luc construct, as well as plasmids encoding either RAP46, GFP, or N69GFP. Dexamethasone treatment (0.1 μM) was performed immediately after the transfection. Shown are results of the dexamethasone-induced MMTV activity expressed relative to unity, which is the value given to the hormone induced activity of the indicator plasmid in the presence of the empty expression vector. The bar charts represent the mean value (± SEM) of two independent experiments. B, The N69GFP fusion overcomes the inhibitory effect of RAP46 on DNA binding by the GR. Shown are the EMSA and immunoblots with extracts of COS-7 cells transiently transfected with either a control vector (lane 1) or a plasmid encoding GR (lanes 2–7). In addition, the cells were transfected with constructs encoding HA-RAP46 (lanes 5–7), the empty expression vector pcDNA3 (lanes 1, 2, and 5), GFP (lanes 3 and 6), or the N69GFP fusion (lanes 4 and 7). After transfection, the cells were treated with 0.1 μM dexamethasone for 36 h and cellular extracts were prepared for the EMSA and immunoblot assay.

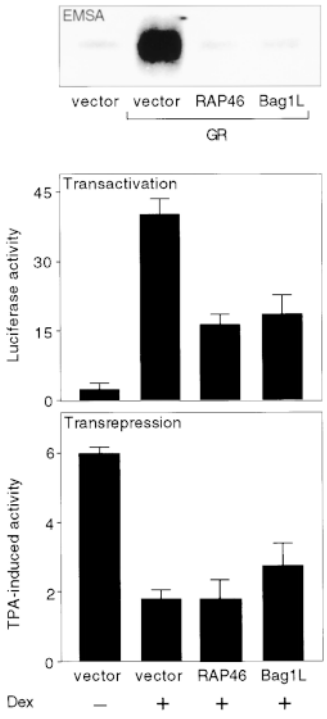

Figure 7. Effect of RAP46 and BAG-1L on DNA-binding, transactivation, and transrepression by the GR. COS-7 cells were transiently transfected with either a control vector, a plasmid encoding the GR, RAP46, or BAG-1L. After transfection, the cells were treated with 0.1 μM dexamethasone for 36 h and whole cell extracts were prepared for EMSA. In addition, the effect of RAP46 and BAG-1L on transactivation at the MMTV promoter and GR-mediated repression at the human collagenase I promoter were analyzed. MCF-7 cells were transiently transfected with a control vector or a plasmid encoding RAP46 or BAG-1L, in addition to the MMTV firefly luciferase indicator or collagenase luciferase constructs and a plasmid encoding the renilla luciferase as an internal control. The activity of the collagenase promoter was induced with 80 ng/ml TPA and the cells were treated immediately after transfection with vehicle alone (0.1% ethanol; −) or 0.1 μM dexamethasone (+) for 36 h. The bar chart shows the normalized luciferase activity (firefly/renilla) of the MMTV and collagenase gene constructs. The results represent the mean value (± SD) of three independent experiments.

RAP46, and its isoform BAG-1L, on GR activity is the repression of the transactivation function of this receptor.

Discussion

hsp70 and hsp90 provide the GR with the proper conformation for ligand binding and, therefore, play an important role in the ligand-mediated activation of this receptor (Hutchinson et al., 1994; Dittmar and Pratt, 1997; Dittmar et al., 1998). In this study we have shown that RAP46, a protein that binds hsp70 to inhibit its ability to refold denatured proteins (Gebauer et al., 1997, 1998), downregulates transactivation by the GR. This negative regulation does not involve inhibition of the ability of the receptor to bind hormone. In hormone binding studies, RAP46 did not alter the K_d of the receptor for its ligand. We therefore concluded that the negative regulation of GR activity by RAP46 must occur through other mechanisms.

We demonstrated by immunofluorescence experiments that in the presence of ligand, RAP46 is recruited from the cytoplasm into the nucleus by the GR, but not by the MR. As RAP46 binds hsp70/hsc70, the nuclear transfer could occur via this heat shock protein, especially since it is reported to be transported into the nucleus by both the GR and MR (Diehl and Schmidt, 1993; Srinivasan et al., 1994; Bruner et al., 1997). However, if RAP46 were to be transported via its interaction with hsp70/hsc70, it would be expected to be recruited into the nucleus by both receptors. Since only the GR, but not the MR, recruited RAP46 into the nucleus, it is very likely that the nuclear transport is mediated by a specific association of RAP46 with the GR.

Previously, we have shown in glutathione S–transferase pull-down experiments that the hinge region of the GR (amino acids 491–515) is necessary for the association of RAP46 with the GR. In those experiments, we showed that deletion of this region abolished RAP46-mediated downregulation of transactivation by the GR (Kullmann et al., 1998). In the present study, we have shown that deletion of the hinge region partially decreased the ability of RAP46 to be transported into the nucleus by the GR. This means that the hinge region has at least two functions. First, it contributes to nuclear recruitment of RAP46 and second, it also plays a role in inhibition of GR-mediated transactivation. As the hinge region of the GR differs considerably in sequence from that of the MR, it could explain why RAP46 is not translocated into the nucleus by the MR and why transactivation by this receptor is not repressed by RAP46.

In our study to find out which region of RAP46 is required for recruitment into the nucleus, we used a mutant that lacks the last 47 COOH-terminal amino acids known

to bind hsp70/hsc70 (Froesch et al., 1998). This protein was not translocated into the nucleus by the GR, implicating the hsp70/hsc70 binding site in the nuclear transport of RAP46. Thus, binding of hsp70/hsc70 to RAP46 may be involved in the nuclear transport and the heat shock protein must interact differently with the GR and MR to account for the differences observed in the recruitment of RAP46 into the nucleus by these two receptors. Alternatively, hsp70/hsc70 may not be necessary at all, but the same sequence used for binding the heat shock protein may be involved in a direct interaction with the GR. This would mean that RAP46 must compete with hsp70/hsc70 for binding to the GR. Our experiments so far do not distinguish between these possibilities.

We have shown that, although nuclear recruitment of RAP46 is necessary, it is not sufficient for negative regulation of GR activity. A RAP46 protein lacking the first 70 amino acid residues was transported into the nucleus by the GR, but nevertheless failed to inhibit transactivation by the receptor. This indicates that the inhibitory function of RAP46 requires its NH_2-terminal sequences. This region of RAP46 is possibly involved in interaction with other proteins, since a fusion protein containing this segment exerted a strong dominant–negative function and relieved the downregulatory action of RAP46 on transactivation by the GR. The first NH_2-terminal 70 amino acids of RAP46 contain a sequence motif $[EEX_4]_8$ that may contribute to the downregulation of transactivation by the GR since BAG-1L which contains this motif, but not BAG-1, also repressed GR-mediated transactivation. BAG-1, on the other hand contains two copies, rather than the eight copies of the $[EEX_4]$ motif. Thus, whatever the function of this sequence, several copies, rather than a duplication, are necessary for the manifestation of its repressive function. The $[EEX_4]_8$ sequence is highly enriched in threonine and serine residues (27% of the total sequence). Our preliminary in vivo labeling studies showed that RAP46 is a phosphoprotein, with almost all its phosphorylated residues localized at the $[EEX_4]_8$ sequence in the first NH_2-terminal 70 amino acid residues (Schneikert, J., and A.C.B. Cato, unpublished). This finding is rather striking and highly suggestive of a role of phosphorylation in the action of the NH_2 terminus of RAP46.

Inhibition of GR-mediated transactivation by the corepressor calreticulin has been shown to be dependent on inhibition of DNA binding brought about by the interaction of this protein with sequences in the DNA binding domain of the receptor (Burns et al., 1994). Distinct sequences in the DNA binding domain of the GR are not recognized by RAP46 for the inhibition of transactivation by the GR. The site of interaction of RAP46 on the GR is the hinge region (Kullmann et al., 1998; present results). However, the HBD of the GR also contributes to the negative action of RAP46. From our studies, we conclude that the negative action of RAP46 is effected through a prior nuclear transport of this protein via a process that requires the COOH-terminal region (the hinge region and, to some extent, the HBD) of the GR, as well as the last 47 COOH-terminal amino acids of RAP46. In the nucleus, RAP46 inhibits DNA binding by the receptor through a mechanism that is not quite clear at the moment. The fact that the HBD of the GR binds hsp70/hsc70, which is also recognized by RAP46, and all three proteins are found in the nucleus, argues in favor of at least a tertiary protein complex in the negative regulatory action of RAP46. Other proteins may be part of this complex as well, especially protein binding to the NH_2-terminal region of RAP46.

We have demonstrated that the inhibitory action of RAP46 is specific for those actions of the GR requiring DNA binding. Thus, transactivation, which is dependent on DNA binding by the GR is repressed, but not GR-mediated repression of the activity of the transcription factor AP-1, which does not require DNA binding by the receptor (Heck et al., 1994). We cannot, however, rule out the possibility that the use of TPA for the activation of AP-1 in our experiments disrupts the RAP46 complex necessary for inhibiting GR action.

The biological significance of the negative regulatory action of RAP46 are manifold. We previously have shown that RAP46-mediated inhibition of transactivation by the GR correlates with inhibition of glucocorticoid induced apoptosis (Kullmann et al., 1998). We also observed that T cells that are resistant to glucocorticoid-induced apoptosis express relatively high levels of RAP46 (Kullmann et al., 1998). Conversely, conditions that downregulate the endogenous levels of RAP46, such as exposure of thymoma cells to the immunosuppressant rapamycin, enhanced the transactivation potential of the GR and reduced GR-mediated apoptosis (Kullmann et al., 1998). In the present study we have shown, by the use of a dominant–negative mutant construct, that the transactivation function of the GR in the human mammary carcinoma MCF-7 cells is negatively regulated by endogenous RAP46. As neoplastic cells generally express relatively high levels of RAP46 and BAG-1L (Takayama et al., 1998; Yang et al., 1998), our results on the inhibition of GR-mediated transactivation by these proteins may provide mechanistic explanations for the reported cases of glucocorticoid resistance in neoplastic transformation. This point would have to be taken into consideration in studies on glucocorticoid resistance in disorders such as acute lymphocytic and nonlymphocytic leukemia (Crabtree et al., 1981; Wells et al., 1981).

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