The glucocorticoid receptor (GR) is a ligand-dependent transcription factor that is able to modulate gene activity by binding to its response element, interacting with other transcription factors, and contacting several accessory proteins such as coactivators. Here we show that GRIP120, one of the factors we have identified to interact with the glucocorticoid receptor, is identical to the heterogeneous nuclear ribonucleoprotein U (hnRNP U), a nuclear matrix protein binding to RNA as well as to scaffold attachment regions. GR-hnRNP U complexes were identified by blotting and coimmunoprecipitation. The subnuclear distribution of GR and hnRNP U was characterized by indirect immunofluorescent labeling and confocal laser microscopy demonstrating a colocalization of both proteins. Using a nuclear transport-deficient deletion of hnRNP U, nuclear translocation was seen to be dependent on GR and dexamethasone. Transient transfections were used to identify possible interaction domains. Overexpressed hnRNP U interfered with glucocorticoid induction, and the COOH-terminal domains of both proteins were sufficient in mediating the transcriptional interference. A possible functional role for this GR binding-protein in addition to its binding to the nuclear matrix, to RNA, and to scaffold attachment regions is discussed.

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methylsulfonyl fluoride, 5 μM aprotinin, 5 μM of leupeptin, 0.2% Nonidet P-40 under mild agitation at 4 °C. After centrifugation (750 × g) for 3 min, the supernatant was incubated with 70 μl of protein A solution that had been mixed with the respective antisera (α-GR antibody kindly provided by Elisabeth Stocklin, Tumor Biology Center, Freiburg, Germany; control antibody, anti-α-TRITC goat antibody kindly provided by Renkawitz-Pohl, Universitat Marburg, Germany) for 1.5 h under mild agitation at 4 °C. The solution was centrifuged for 3 min (750 × g), and the supernatant was removed carefully. After washing, the pellet was resuspended in sample buffer and applied to SDS-PAGE.

Electrophoresis and Electroblotting—Crude nuclear protein extracts, bacterial protein extracts, and chromatographically purified protein fractions were precipitated as described (34) and separated by SDS-PAGE (35).

Electroblotting was carried out for 2 h in a semidry blotting system with a constant current of 1 mA/cm² gel using a ternary buffer system (buffer 1: 200 mM Tris, pH 10.4; buffer 2: 20 mM Tris, pH 10.4; buffer 3: 40 mM capric acid, pH 7.6; all buffers contain 20% methanol).

Immunoblotting and Cross-linked Far South-Western Assay—After electroblotting the polyvinylidene difluoride membrane (Millipore, Germany) was blocked overnight with PBS containing 0.05% Tween (PBS-T) and 5% milk powder (Carnation). The membranes were incubated with the respective antibody for 1.5 h at room temperature and with the secondary, peroxidase-coupled antibody for 1 h. The immune complexes were visualized by enhanced chemiluminescence according to the manufacturer’s protocol (Amersham). The cross-linked far South-Western analysis was performed as described (29).

Protein Purification—Crude nuclear protein extracts were separated on different columns, and buffers and conditions were as described recently (29) except for heparin-Sepharose, where a linear gradient between 0 and 1 M salt was applied. Positive fractions were identified by electroblotting the polyvinylidene difluoride membrane (Millipore, Germany) which was blocked overnight with PBS containing 0.05% Tween (PBS-T) and 5% milk powder (Carnation). The membranes were incubated with the respective antibody for 1.5 h at room temperature and with the secondary, peroxidase-coupled antibody for 1 h. The immune complexes were visualized by enhanced chemiluminescence according to the manufacturer’s protocol (Amersham). The cross-linked far South-Western analysis was performed as described (29).

Indirect Immunofluorescence—Cells were fixed in 3.7% paraformaldehyde, pH 7.4, washed with PBS, and permeabilized with 0.5% Nonidet P-40. The cells were incubated for 30 min with a blocking solution containing 10% glycerol, 1% bovine serum albumin, and 100 mM glycolic acid. Antibody incubations were done in incubation buffer (0.5% bovine serum albumin, 0.05% Tween 20 in PBS) for 60 min at room temperature and reversed phase HPLC separation and microsequencing were performed as described (36).

RESULTS

**GRIP120 Is Identical to hnRNP U**—To identify and characterize possible interaction partners of the GR, we analyzed HeLa nuclear proteins for their ability to interact with the DNA-bound GR in vitro. To delimit such a search to those proteins interacting with a functional hormone-responsive unit, we used a probe consisting of baculovirus-expressed, activated human GR bound to a [-32P]labeled DNA oligonucleotide with two adjacent GRE sequences. Before incubation with the blotted nuclear extract the probe was UV cross-linked in the presence of hormone (29). As shown in Fig. 1, four prominent bands were detected (lane 1), whereas there was no signal in bacterial extracts (data not shown). These GRIPs were designated in accordance with their molecular masses: GRIP170, GRIP120, and GRIP95. Except for the band at the molecular mass position of 115 kDa, which shows direct DNA binding, we have shown previously that the binding was specific for GR cross-linked to a double GRE, suggesting that the proteins identified are putative interaction partners of GR (29). Here, we describe the identification and characterization of GRIP120.

The purification started with an anion exchange chromatography of 2 g of HeLa nuclear extract, and the resulting fractions were electrophoresed, generating BromH III and EcoRI sites, and was inserted in a pBSK + vector. This construct was used to express full-length hnRNP U as well as deletions in the expression vector pAB linker (43) which was combined with a nuclear localization signal (NLS) from the T-antigen of SV40 virus (5'-GCCCAAGAGAAGAAGGTG-GGAG-3') at the Bgl II/Sal I-digested pAB linker and named pAB-NLS. The purified GRIP120 from pBSK + -hnRNP U fusion-generating BamH I and EcoRI sites, was ligated into a pBSK + vector. This construct was used to express full-length hnRNP U as well as deletions in the expression vector pAB linker (43) which was combined with a nuclear localization signal (NLS) from the T-antigen of SV40 virus (5'-GCCCAAGAGAAGAAGGTG-GGAG-3') at the Bgl II/Sal I-digested pAB linker and named pAB-NLS. The purified GRIP120 from pBSK + -hnRNP U fusion-generating BamH I and EcoRI sites, was ligated into a pBSK + vector. This construct was used to express full-length hnRNP U as well as deletions in the expression vector pAB linker (43) which was combined with a nuclear localization signal (NLS) from the T-antigen of SV40 virus (5'-GCCCAAGAGAAGAAGGTG-GGAG-3') at the Bgl II/Sal I-digested pAB linker and named pAB-NLS.
small amount of GRIP95. GRIP170 was detected in fractions containing 350–450 mM salt, and the residual amounts of the 115-kDa protein were found in fractions containing 375–525 mM salt. Further purification was achieved by heparin-Sepharose, where GRIP120 eluted in a range of 725–980 mM salt (Fig. 1, lane 5). Finally, GRIP120 was concentrated by anion exchange chromatography (Fig. 1, lane 5), resulting in a strong enrichment of GRIP120 which could be visualized with Coomassie staining (Fig. 1, lane 6). The purification steps for GRIP120 enrichment are summarized in Fig. 1, scheme of the purification protocol.

**Nuclear Complexes Contain GR and HnRNP U—**HnRNP U had been identified previously in the context of two different activities. First, this protein had been demonstrated to be a component of the nuclear hnRNA particles (7). Second, it had been shown to be identical to a protein attached to the nuclear scaffold (SAP A) and binding to several scaffold attachment regions from vertebrates (8). Here we wanted to analyze whether the third function we have seen in vitro, binding to GR, could be confirmed further by communoprecipitation of GR and hhnRNP U. Therefore, we immunoprecipitated proteins from HeLa nuclear extract with an antibody against GR or with an antibody specific for hhnRNP U. As shown in Fig. 2A the GR-specific antibody coprecipitates hhnRNP U; an immunoblot probed with the anti-hhnRNP U antibody clearly detects hhnRNP U in the precipitate (Fig. 2A, lane 2) migrating at a position identical to that of the 120-kDa band detected in the anti-hhnRNP U precipitate (Fig. 2A, lane 3) as well as in the untreated nuclear extract (Fig. 2A, lane 1). A control antibody, used to detect unspecific interactions with antibodies in general, or using only the protein A-Sepharose matrix (Fig. 2A, lanes 4 and 5) led to no specific hhnRNP U enrichment in the precipitate. A second band with a molecular mass of about 100 kDa seen in both specific precipitates might be the result of protein degradation during incubation or caused by an unknown protein modification.

To substantiate further the occurrence of GR-hhnRNP U complexes we carried out the reverse experiment, by testing whether GR could be coprecipitated by an hhnRNP U-specific antibody. Analysis of the precipitates by GR-specific immunoblotting resulted in a clearly detectable band in the hhnRNP U-specific precipitate (Fig. 2B, lane 3). This band comigrates

**Table I**

| Peptide sequence | HnRNP U residues |
|------------------|------------------|
| SSGPTSFLAVTVPPGARQ | 187–205          |
| RPREDHGYGYFETYLRNK | 229–246          |
| PYPFIPBETYTFFQVNPLEDRVR | 446–467 |
| YNLGQNTIMDK | 506–517 |
| MMVAGFK | 518–524 |
| RNFILDGTVNSAAQRKKG | 556–573 |

**Fig. 1. Enrichment of GRIP120.** Panel A, a 120-μg nuclear extract of dexamethasone-induced HeLa cells (lane 1, NE) and GRIP120-positive fractions of each purification step were separated by SDS-PAGE and blotted on a polyvinylidene difluoride membrane. Proteins were probed with the 32P-labeled GR-double GRE complex for the presence of GRIPs. Bands were visualized by autoradiography. Positive fractions (in μg of total protein) are shown after fractionation on Q-Sepharose (lane 2, QS, 80), Mono S (lane 3, MS, 80), heparin-Sepharose (lane 4, HS, 46), and Mono Q (lane 5, MQ, 38). Finally, 80 μg of the concentrated fraction was separated by SDS-PAGE and stained with Coomassie Blue (lane 6, CM). Panel B, scheme of the purification protocol.
with GR, detected in the GR-specific precipitate (Fig. 2B, lane 2) and is also seen in unfractionated nuclear extract. In addition, the GR antibody identifies several degradation bands, most prominently the already published 45-kDa degradation product of GR (44) and a protein of about 190 kDa, probably caused by cross-reactivity of the polyclonal serum (note that this protein is only bound in denatured Western blot, whereas the native protein is not precipitated by the same antibody; see lane 2). The precipitates with the control antibody and using no antibody did not generate any GR-specific signal (Fig. 2B, lanes 4 and 5). Thus, nuclear complexes having GR and hnRNP U molecules in common support the idea of an in vivo association.

Nuclear Colocalization of GR and hnRNP U—In addition to the biochemical evidence for the existence of GR-hnRNP U complexes we wanted to characterize the subnuclear location of hnRNP U and of GR. Therefore, we analyzed the distribution of both endogenous proteins within HeLa cell nuclei, without any overexpression, by indirect immunofluorescence microscopy. As seen in Fig. 3, immunolocalization of GR confirmed previous results (45) on the focal clustering within the nucleus. Using the laser confocal microscope we could dissect stained nuclei into several adjacent optical layers, three of which are shown for a single nucleus (Fig. 3). We detected roughly about 30–70 stained GR clusters or speckles distributed evenly within the three-dimensional nuclei. Immunolocalization of hnRNP U revealed two types of patterns (Fig. 3). HnRNP U is found to be concentrated in larger clusters. The merged images of both antibodies reveal that the GR speckles and the large clusters of hnRNP U colocalize in all optical sections analyzed (Fig. 3, d–f). In addition, there was an almost even distribution of granular patterns with the majority of granules much smaller compared with the GR speckles. In addition to this pattern, for control, the distribution of a different antigen (fibrillarin) shows clearly a different pattern and no colocalization with GR (Fig. 3j). Fibrillarin is found in the nucleolus and in regions adjacent to the nucleoli (46).

To substantiate further the significance of the colocalization we applied several statistical methods to the confocal data sets. We wanted to determine whether signals distributed randomly within a nucleus would result in colocalization with the GR signals as well, or whether the measured colocalization of GR and hnRNP U could only be observed if both antigens would be indeed different components of identical focal clusters. Therefore, we used a model-based segmentation algorithm (see “Experimental Procedures”) and determined the distances between individual GR signals and the hnRNP U signal next to it. We found that about half of the determined distances were within less than 250 nm (Fig. 4), with none of the distances longer than 500 nm. In contrast, the distances between individual GR signals and the nearest signal from a random, simulated pattern generated by a Monte Carlo simulation (see “Experimental Procedures”) were significantly longer (Fig. 4); the calculated distances reached up to 2,000 nm. Thus, we conclude that the colocalization seen between GR and hnRNP U is significant and not the result of an overlap between randomly distributed hnRNP U signals and the GR speckles.

Functional Interaction Is Mediated by the COOH-terminal Domains of GR and of HnRNP U—Because the NLS of hnRNP U has been identified on the NH2-terminal region (7) we tested whether deletion of the NLS resulted in a cytoplasmic location of hnRNP U. This was indeed the case (data not shown), and therefore we could make use of the nuclear cotranslocation assay to study the GR-hnRNP U interaction. We generated c-Myc-tagged hnRNP U deletions that were overexpressed in COS-1 cells together with hGR and tested the influence of...
hormone on the nuclear translocation of the c-Myc signal. The hnRNPU deletion (hnRNPU406–806), in the presence of dexamethasone, resulted in predominantly stained nuclei (Fig. 5A), whereas in the absence of hormone, staining was seen more evenly in the cytoplasm as well as the nucleus (Fig. 5B). This reflects the hormone-dependent distribution of GR, which in many cases has been shown to be enriched in the nuclei after hormone induction (15). Using a more severe deletion of hnRNPU (hnRNPU685–806) the hormone-dependent nuclear location was lost, and the cytoplasm was stained preferentially with c-Myc antibody irrespective of the presence of hormone (Fig. 5C and D).

To delineate both the hnRNPU and the GR regions involved in the interaction we used a transient expression assay. In case of a functional interaction between GR and hnRNPU, we expected that overexpression of hnRNPU could interfere with the GR/GRE-mediated transcriptional induction of a reporter gene. Therefore, we transfected a tkCAT reporter gene fused to a double GRE and determined the glucocorticoid induction of CAT activity in the absence or presence of a hGR expression vector in mouse L cells. For all of these transfections we used identical amounts of reporter and of total DNA. The hormone induction seen was about 3-fold in the absence of the hGR expression plasmid (i.e. activity of the endogenous GR) and about 8 fold in its presence (Fig. 6). Cotransfection of the hnRNPU expression plasmid coding for the full-length protein resulted in the inhibition of induction without interfering with the basal level of transcription. Similar effects were obtained with the progesterone receptor instead of GR when coexpressed with hnRNPU (data not shown) or when using the natural occurring murine mammary tumor virus promoter instead of the synthetic double GRE fused to the tk promoter.

The glucocorticoid induction mediated by both the endogenous receptor as well as by the expressed hGR was impaired by overexpressed hnRNPU (Fig. 6). Using the indicated hnRNPU deletions in this reporter assay only the hnRNPU406–806 and 517–806 regions were efficient in interfering with the GR-mediated induction (Fig. 6). Again, this interference was seen on the endogenous GR as well as on the expressed hGR. Further deletion of hnRNPU to the region 685–806 showed no GR interference, suggesting that the important region is located between amino acids 517 and 685. This agrees well with the data from the nuclear cotranslocation assay as described above.

Identification of the GR region responsible for the hnRNPU effect was done with different GR deletions. Both GR mutants GR1–515 and GR1–550 showed gene activation in the absence of hormone on double GREs, which was not influenced by the overexpression of hnRNPU (Fig. 7A). In contrast, the GR mutant GR418–777 mediated hormone-dependent gene activation, which was severely inhibited by full-length hnRNPU as well as by hnRNPU406–806 (Fig. 7A). Because this GR fragment contained two different domains, the DNA binding domain in addition to the hormone binding domain, we fused just the GR hormone binding domain to the DNA binding domain of Gal4 (42). This Gal-GR488–777 fusion was tested with the UAS-tkCAT reporter gene allowing the binding of the Gal-GR fusion. As expected, addition of hormone induced the CAT activity about 18 fold (Fig. 7B). Overexpression of hnRNPU406–806 abolished even this strong glucocorticoid induction without affecting the basal level gene activity.

Therefore, the transcriptional interference seen by overexpression of hnRNPU is independent of the DNA binding domain of GR and requires both of the COOH-terminal regions: amino acids 488–777 of GR and 517–806 of hnRNPU.

**DISCUSSION**

Molecules associated with the nuclear matrix appear to act at all stages of gene expression. Here we have shown that the GR is complexed with hnRNPU, a matrix protein for which binding to RNA and to scaffold attachment regions had been described previously (7, 8). Although a functional interaction of nuclear hormone receptors with the nuclear matrix had been demonstrated, this is the first time that a matrix-associated protein interacting with the GR has been identified.

**Localization of GR and HnRNPU**—The GR distribution and localization in eukaryotic cell has been found to be clustered. This was seen with the help of antibodies (45, 47) as well as with a GFP-GR fusion protein (48).

Several reports demonstrated the GR association with the nuclear matrix (25, 26). Tang and DeFranco (49) show a dramatic increase in GR binding to the nuclear matrix in ATP-depleted cells, and they found the DNA binding domain of GR involved in this interaction. This is in agreement with another
study that demonstrated that the DNA binding domain as well as the COOH-terminal domain of GR are required for matrix interaction (45). Our data demonstrate that the COOH terminus of GR is sufficient for GR-hnRNP U interaction, but in fact we do not know whether hnRNP U is the only matrix protein that binds to GR.

hnRNP U is an abundant nuclear protein of the hnRNP protein family, members of which bind pre-mRNA and nuclear mRNA and play an important role in processing and transport of mRNA (50). In addition, hnRNP U is known to bind to scaffold attachment region sequences (8, 11). Here, we analyzed the subnuclear distribution of hnRNP U and found a granular staining after indirect immunofluorescence labeling. In addition to this distribution the hnRNP U signals showed a clustering similar to the GR speckles. Double staining detected the colocalization of GR and hnRNP U within these speckles, whereas outside of the speckles hnRNP U did not colabel with GR antibodies. In vitro binding, immunoprecipitation, and functional assays argue for the association of GR and hnRNP U. For the following reasons we do not know whether the interaction seen is dependent on hormone. The GR is especially difficult to be analyzed for hormone-dependent effects because in vivo, hormone mediates at least two different functions, which are nuclear translocation and protein interaction with other proteins (14). Therefore, our results on the cotranslocation of hnRNP U cannot distinguish between these two possibilities. The in vitro analysis of hormone-dependent effects is almost impossible because GR is activated in vitro even in the absence of hormone (51).

Possible Functions of the GR-HnRNP U Complex—A matrix association of transcription factors has been seen in other cases as well. The nuclear proteins PML, Oct-1, and YY 1 represent examples of matrix-associated DNA binding factors (52–55), but a matrix protein responsible for interaction with the respective transcription factor has not yet been identified. In contrast to the receptor-binding factor 1, which generates high affinity binding sites for the progesterone receptor on avian genomic DNA (56, 57), hnRNP U binds to the nuclear hormone receptor. In vitro binding of GR and hnRNP U is specific and direct. HnRNP U binding to DNA is not possible for DNA fragments smaller than 350 base pairs. Whether a direct interaction is found in vivo cannot be concluded. An indirect interaction mediated by other nuclear proteins, such as hnRNPs or small nuclear RNPs, might be possible as well.

The nuclear matrix has been demonstrated to be involved in many nuclear activities such as chromatin acetylation, gene transcription, and RNA processing (58). Therefore, several functions for the association of GR with the matrix protein hnRNP U could be envisaged, three of which will be discussed below.

First, the RNA binding capacity of hnRNP U (50), in addition to GR and matrix binding, may suggest a possible effect on RNA processing or stability. Indeed, glucocorticoids have been known to stabilize specific mRNAs (59–61). If hnRNP U would play a role in such a stabilization, binding to GR might take part in the mechanism of glucocorticoid-induced RNA stability.

Second, GR clustering with hnRNP U could reflect a storage of inactive GR molecules. This latter possibility was suggested by the finding that RNA polymerase II was not colocalized with the GR clusters (45). In addition, our transfection experiments show a hnRNP U-mediated inhibition of glucocorticoid induction. This may be taken as evidence for an inactivation of GR, although other interpretations are possible as well, such as that overexpression of hnRNP U generates an artificial change of the GR to hnRNP U ratios, thereby changing a positive effect

FIG. 6. Overexpression of hnRNP U inhibits glucocorticoid induction. Cotransfection of the tkCAT reporter gene fused to a double GRE with expression vectors coding for hGR (GR+) and for the different deletions of hnRNP U into mouse Ltk− cells. The fold activation of CAT activity is calculated in relation to the activity seen in the absence of ligand and in the presence of empty expression plasmids. For hnRNP U the positions of the NLS) and of the RNA binding domain (RGG) are indicated in addition to the region required for the interference with glucocorticoid induction (shaded box). The low induction values seen in the absence of hGR expression vector (GR−) are caused by the endogenous GR of the Ltk− cells.

2 F. O. Fackelmayer, unpublished data.
with small nuclear ribonucleoprotein particles and splicing factors. In fact, these speckles are probably identical to the GR speckles analyzed by us, since we could immunolocalize splicing factors to the GR-hnRNP U speckles. These data may suggest a possible colocalization of active RNApolymerase II, hnRNP U, and GR.

Several models of transcription predict that the RNApolymerase II and other essential factors for transcription are fixed to the nuclear matrix and therefore, the template moves along this multiprotein complex (63). The finding of hyperphosphorylated, active RNApolymerase II associated with the nuclear matrix (62) and our results on functional interaction between GR and hnRNP U may support this hypothesis. The multiple functions of hnRNP U invite to speculate on its possible role of an integrator providing local concentrations (factory) of active genes, of the regulatory transcription factors such as GR, and of the RNA binding and processing machinery.

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FIG. 7. The COOH-terminal domain of GR is required for hnRNP U-mediated interference. Expression vectors coding for the constitutive GR deletions (GR1–515 and GR1–550) were tested in mouse Ltk– cells in the absence of dexamethasone (Dex–), whereas GR mutants 418–777 and GalGR488–777 were regulatable and tested in the presence (+) of dexamethasone. Fold activation was determined from the CAT activities as described in the legend to Fig. 6. Panel A, expression vectors coding for GR deletions containing the GR-DBD were cotransfected with expression vectors coding for hnRNP U1–806 or 406–806 and with the double GREtkCAT reporter gene. Panel B, to test the influence of the GR-DBD the expression vector coding for Gal-GR488–777 was cotransfected with the UAStkCAT reporter.

3 S. Schneider, unpublished data.
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