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A Role for HDJ-2/HSDJ in Correcting Subnuclear Trafficking, Transactivation, and Transrepression Defects of a Glucocorticoid Receptor Zinc Finger Mutant

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All steroid receptors possess a bipartite nuclear localization signal sequence (NLS) that localizes within the second zinc finger of their DNA-binding domain. Fine-structure mapping of the rat glucocorticoid receptor (rGR) NLS identified a composite signal composed of three distinct proto-NLSs that function effectively when present in unique pairs. At least one of the rGR proto-NLSs appears to influence receptor trafficking within the nucleus, as revealed by a unique nuclear staining pattern of receptors possessing a point mutation (i.e., arginine at position 496; R496), at proto-NLS, pNLS-2. Specifically, carboxyl-terminal-truncated rGRs possessing various point mutations at R496 localized within a limited number of large foci in nuclei of transiently transfected Cos-1 cells. R496 mutations did not affect subnuclear targeting when present in full-length rGR, reflecting a protective effect of the receptor's ligand-binding domain that can be exerted in cis and in trans. The effects of rGR R496 mutations on subnuclear targeting were not autonomous because we also observed a coincident localization of hsp70, the 70-kDa heat shock protein, within nuclear foci that include R496 mutant receptors. The elimination of R496 mistargeting by overexpression of an hsp70 partner (i.e., the DnaJ homologue, HDJ-2/HSDJ) suggests that the hsp70/DnaJ chaperone system is mobilized to specific sites within the nucleus in response to inappropriate targeting or folding of specific mutant receptors. HDJ-2/HSDJ overexpression also corrects defective transactivation and transrepression activity of R496 mutant GRs. Thus, molecular chaperones, such as members of the hsp70 and DnaJ families, may survey the nucleus for misfolded proteins and actively participate in their refolding into biologically active conformational states.

INTRODUCTION

Newly synthesized proteins emerging from the ribosome must ultimately be targeted to appropriate subcellular compartments. The selectivity of this process is dictated in part by unique protein-encoded signal sequences that serve as recognition elements for specific organelle-targeting receptors (Schatz and Dobberstein, 1996). The precise mechanisms of organelle translocation that ensue after recognition of cognate signal sequences are diverse, because membrane-embedded, cytoskeletal, and nuclear translocation machineries vary in complexity. For example, although import into endoplasmic reticulum (Deshaies *et al.*, 1991; Wickner, 1994) and mitochondria (Lill and Neupert, 1996) occurs through multiprotein complexes possessing up to 10 individual subunits, proteins destined for import into the nucleus must pass through a 100-MDa nuclear pore complex (NPC) that is com-

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posed of more than 100 proteins (Forbes, 1992; Rout and Wente, 1994).

Proteins pass through mitochondrial and endoplasmic reticulum translocation machines in a partially unfolded state (Schatz and Dobberstein, 1996) that is stabilized by molecular chaperones such as members of the 70-kDa heat shock protein (i.e., hsp70) family (Höhfeld and Hartl, 1994). Global protein unfolding is not associated with the passage of proteins through the NPC, although cytoplasmic hsp70 has been found to be required for nuclear import of some (Imamoto *et al.*, 1992; Shi and Thomas, 1992) but not all karyophiles (Yang and DeFranco, 1994). hsp70 may function during nuclear import to facilitate interactions between translocating substrates and soluble nuclear import factors or structural components of the NPC (Shulga *et al.*, 1996).

Nuclear localization signal sequences (NLSs), first identified within the simian virus large tumor antigen (Kalderon *et al.*, 1984), have been defined for many proteins and are generally composed of a few short noncontiguous stretches of basic amino acids (Dingwall and Laskey, 1991). Although the precise arrangements of basic amino acids within identified NLSs may vary, a common mechanism of nuclear import is used by NLSs with basic amino acid character (Michaud and Goldfarb, 1991). Although various proteins have been shown to bind NLS sequences in vitro (Adam *et al.*, 1989; Silver *et al.*, 1989), recent functional assays have unequivocally identified the 54–56-kDa α subunit of karyopherin/importin as a bona fide NLS receptor (Görlich *et al.*, 1994; Moroianu *et al.*, 1995). An additional 97-kDa β subunit of karyopherin/importin, in complex with the NLS substrate and α subunit, is sufficient for docking of NLS proteins to the NPC in an in vitro nuclear transport system (Görlich *et al.*, 1995; Moroianu *et al.*, 1995).

After the appropriate interactions of NLS-protein/NLS-receptor complexes with specific NPC proteins (i.e., nucleoporins; Radu *et al.*, 1995; Rexach and Blobel, 1995), NLS proteins must engage components of the NPC that make up the translocation machine to complete the nuclear import process. The soluble GTP-binding protein Ran/TC4 (Melchior *et al.*, 1993; Moore and Blobel, 1993), is used in this process to aid in the delivery and/or release of NLS proteins to various nucleoporins that are encountered during passage through the interior 50 nm of the NPC (Rexach and Blobel, 1995). Once NLS proteins are released from the NPC, they are presumably free to proceed to various subnuclear compartments. Although in some cases, specific signal sequences have been identified that target proteins to specific subnuclear compartments (Li and Bingham, 1991; Leonhardt *et al.*, 1992; Hedley *et al.*, 1995), relatively little is known about the mechanisms of directed protein and RNA trafficking within the nucleus.

The glucocorticoid receptor (GR), a member of the nuclear receptor superfamily of transcription factors (Mangelsdorf *et al.*, 1995), has provided a useful model for the analysis of regulated nuclear import. GRs are retained within the cytoplasm through their association with various members of heat shock and immunophilin families of proteins (Pratt, 1993; Smith and Toft, 1993; Yang and DeFranco, 1996). The interactions of these cytoplasmic anchors with the GR carboxyl-terminal ligand-binding domain (LBD) (Pratt *et al.*, 1988; Howard *et al.*, 1990; Dalman *et al.*, 1991) serves to shield the GR NLS, which becomes exposed after ligand binding and the release of receptor-associated heat shock and immunophilin proteins (Urda *et al.*, 1989; Cidlowski *et al.*, 1990). After its hormone-dependent translocation to the nucleus, GR targets to distinct subnuclear compartments (Barrack, 1987; Tang and DeFranco, 1996; Yang and DeFranco, unpublished data). The relationship between GR subnuclear trafficking and its transcriptional regulatory capacity remains undefined.

In the process of our fine structure mapping of a composite NLS within the rat GR (rGR) DNA-binding domain (DBD), we have observed a unique defect in subnuclear trafficking of GR that is associated with the mutation of a highly conserved arginine residue at position 496 (i.e., R496). The effect of R496 mutations is not autonomous as subcellular trafficking of hsp70 is likewise altered in transiently transfected cells that express R496 mutant receptors. R496 mistargeting is alleviated upon overexpression of an hsp70 partner (i.e., HDJ-2/HSDJ; Chellaiah *et al.*, 1993; Oh *et al.*, 1993), providing the first demonstration of a nuclear chaperoning function for the hsp70/DnaJ pair. Overexpression of HDJ-2/HSDJ also reverses defective transactivation and transrepression activity of R496 mutant GRs, suggesting that a biologically active conformation can be assumed by these mutant receptors upon activation of the hsp70/DnaJ chaperone system within the nucleus.

MATERIALS AND METHODS

Cell Culture and Transfection

Cos-1 monkey kidney fibroblasts were maintained in DMEM (Life Technologies-Bethesda Research Laboratories, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Irvine Scientific, Santa Ana, CA). Cells grown on 60-mm dishes or on coverslips (22 × 22 mm) in 35-mm Petri dishes were transfected using the calcium phosphate precipitation method (Somers and DeFranco, 1992). Two or 6 μg of DNA were used for transfections with coverslips or 60-mm plates, respectively. In experiments where multiple DNAs were cotransfected, appropriate amounts of carrier DNA were added to keep total DNA constant. In some cases, transfected cells were treated with 1 μM dexamethasone (Sigma, St. Louis, MO) prior to harvesting. After transfection, cells were either fixed with −20°C methanol and processed for indirect immunofluorescence (IIF; Qi *et al.*, 1989) or harvested, lysed, and processed for Western blot analysis (Yang and DeFranco, 1994) or chloramphenicol acetyltransferase (CAT) assays (Somers and DeFranco, 1992).

Plasmids and Mutagenesis

The plasmid p6RGR encodes a full-length rGR cDNA, and VAN525 encodes a carboxyl-terminal deletion of rGR that removes amino acids upstream of residue 525 (Godowski *et al.*, 1987). The plasmid pNL2βgal encodes a chimera possessing the rGR LBD linked to the bacterial β-galactosidase (*βgal*) gene (Picard and Yamamoto, 1987). CAT reporter plasmids, pTAT3CAT and plfG3CAT, possess trimeric simple and composite glucocorticoid response elements (GREs), respectively, linked to a minimal *Drosophila* alcohol dehydrogenase promoter (Diamond *et al.*, 1990). pGRΔ was made by digesting p6RGR, under partial digestion conditions, with *Eco*RI followed by a complete digestion with *Pst*I. Blunt ends were generated at the resulting DNA fragment with T4 DNA polymerase (Boehringer Mannheim, Indianapolis, IN), which removed both 5′ and 3′ overhanging ends. Finally, the blunt-ended plasmid was resealed by ligation to generate pGRΔ, which possesses a deletion of rGR amino acids between 525 and 765.

Plasmids containing R488A, R489K, K490E, and R496S point mutations in the rGR DBD were constructed within the p6RGR backbone as described elsewhere (Thomas, 1992). Using the identical strategy described above, amino acids between 525 and 765 were deleted to obtain pGRΔ derivatives of these point mutants. R496I, R496D, and R496K point mutants were constructed in the pGRΔ backbone by site-directed mutagenesis as described below. Finally, the R498LK499I double mutant was constructed by site-directed mutagenesis using the VAN525 plasmid backbone. The rGR derivative pLS10 possesses an in-frame linker substitution in which rGR amino acids 507–515 have been replaced with the amino acid sequence Pro-Asp-Leu (Godowski *et al.*, 1989). The LBD was deleted from pLS10 to generate pLS10Δ.

All site-directed mutagenesis was done with a transformer mutagenesis kit (Clontech, Palo Alto, CA). Briefly, two oligonucleotide primers were annealed to parental plasmid DNA. One primer, referred to as the mutagenic primer, introduced the desired mutation into the rGR sequence. The second primer (selection primer) introduced a mutation into a restriction site unique to the parental plasmid. After DNA elongation, ligation, and primary selection by restriction digestion, the mixture of mutated and nonmutated DNAs was introduced into a *mutS Escherichia coli* strain defective in mismatch repair. Plasmid DNA prepared from the mixed bacterial population was subjected to a second selective digestion and transformation. The mutated DNA, which was resistant to digestion, shows much higher transformation efficiency than the parental DNA, which is sensitive to digestion. Subsequently, desired mutated plasmids were recovered and mutations were confirmed by dideoxynucleotide sequencing with Sequenase version 2.0 (United States Biochemical, Cleveland, OH).

Plasmids VAN525Δ486–499 and VAN525Δ461–499 were constructed by polymerase chain reaction (PCR)-amplifying DNA segments possessing rGR DNA corresponding to amino acids 1–486, 1–461, or 499–525 from VAN525 DNA. Either a *Bam*HI site or *Kpn*I site was introduced at the ends of PCR primers. After digesting the PCR products with *Bam*HI and *Kpn*I, the DNA segments encoding rGR amino acids 499–525 were ligated to the DNA segments encoding amino acids 1–461 or 1–486. The reconstructed rGR segments, which now possessed two extra amino acids (i.e., glycine and threonine) at the deletion junction, were reinserted into the VAN525 plasmid backbone. The identity of both mutant plasmids was confirmed by DNA sequence analysis.

The expression vector encoding human HDJ-2/HSDJ coding sequences was prepared by first isolating a 1.5-kb *Eco*RI–*Bam*HI fragment of the HDJ-2/HSDJ cDNA (Chellaiah *et al.*, 1993; Oh *et al.*, 1993) and converting its termini to *Sal*I-*Xba*I. This *Sal*I-*Xba*I HDJ-2/HSDJ cDNA fragment was then linked to the plasmid backbone of p6RGR, from which all rGR sequences had been removed by *Sal*I-*Xba*I digestion, to generate a plasmid referred to as pHDJ-2 throughout the remainder of the text. To prepare the HDJ-2/HSDJ deletion mutant pHDJ-2Δ1, amino acids 7–107 of the HDJ-2/HSDJ coding sequence were removed using a PCR-directed approach. This deletion removes the entire J homology domain of HDJ-2/HSDJ (Chellaiah *et al.*, 1993; Oh *et al.*, 1993). The identity of both pHDJ-2 and pHDJ-2Δ1 plasmids was confirmed by DNA sequence analysis.

IIF

IIF was performed essentially as described previously (Qi *et al.*, 1989). BuGR2, a mouse monoclonal antibody (Gametchu and Harrison, 1984), was used in most IIF analyses to detect GR in methanol-fixed cells. In some cases, an anti-GR rabbit polyclonal antibody was used (Affinity BioReagents, Neshanic, NJ). For costaining of GR and hsp70, hsp70 was detected with a rabbit polyclonal anti-Hsp70 antibody (StressGen, Vancouver, Canada), and BuGR2 was used to detect GR. For costaining of GR and βgal, a monoclonal antibody (Sigma) was used to detect βgal, and rGR was detected with a rabbit polyclonal antibody. HDJ-2/HSDJ was visualized in transfected cells with a mouse monoclonal antibody raised against an HDJ-2/HSDJ fusion protein (Neomarkers, Fremont, CA). Either fluorescein- or rhodamine-conjugated goat anti-mouse or anti-rabbit IgG (Boehringer Mannheim) was used as the secondary antibody.

Western Blot Analysis

Cos-1 cells in 60-mm plates were cotransfected with 3 μg of GR expression plasmids (i.e., GRΔ or GRΔR496S) and 0.3 μg of an expression plasmid encoding the bacterial *βgal* gene (Picard and Yamamoto, 1987) to provide an internal control of transfection efficiency. In some cases, 3 μg of pHDJ-2 or pHDJ-2Δ1 DNA were also cotransfected along with GR expression plasmids. For each set of cotransfection experiments, appropriate amounts of carrier DNA was added to keep total DNA amounts constant. Cells were collected 24 h after transfection and whole cell extracts were prepared as described previously (Yang and DeFranco, 1994). Equivalent amounts of total protein were subjected to SDS-PAGE and Western blot analysis as described previously (Yang and DeFranco, 1994).

CAT Assays

Cos-1 cells were cotransfected with 3 μg of GR expression plasmids and 6 μg of either pTAT3CAT or plfG3CAT reporter plasmids. Where indicated, either 0.5 or 3 μg of pHDJ-2 or pHDJ-2Δ1 DNAs were also included in the cotransfections. Equivalent amounts of DNA were used in each cotransfection experiment through the addition of appropriate amounts of carrier DNA. Each set of transfections was performed at least three times and included duplicate samples. CAT assays were performed as described previously (Somers and DeFranco, 1992) with the average fold induction and SD calculated with Microsoft Excel software.

RESULTS

Mapping of a Multicomponent NLS within the rGR DBD

In seminal studies of Picard and Yamamoto (1987), a constitutive NLS was identified within the second zinc finger of the rGR DBD. Although not recognized at the time, the minimal NLS identified in that study (amino acids 498–517) possessed a bipartite basic amino acid motif (Dingwall and Laskey, 1991). Subsequently, NLSs have been mapped within the DBDs of other steroid receptors including the human estrogen receptor (Picard *et al.*, 1990; Ylikomi *et al.*, 1992), chick progesterone receptor (Guiochon-Mantel *et al.*, 1989; Ylikomi *et al.*, 1992), human androgen receptor (Jenster *et al.*, 1993; Zhou *et al.*, 1994), and human GR

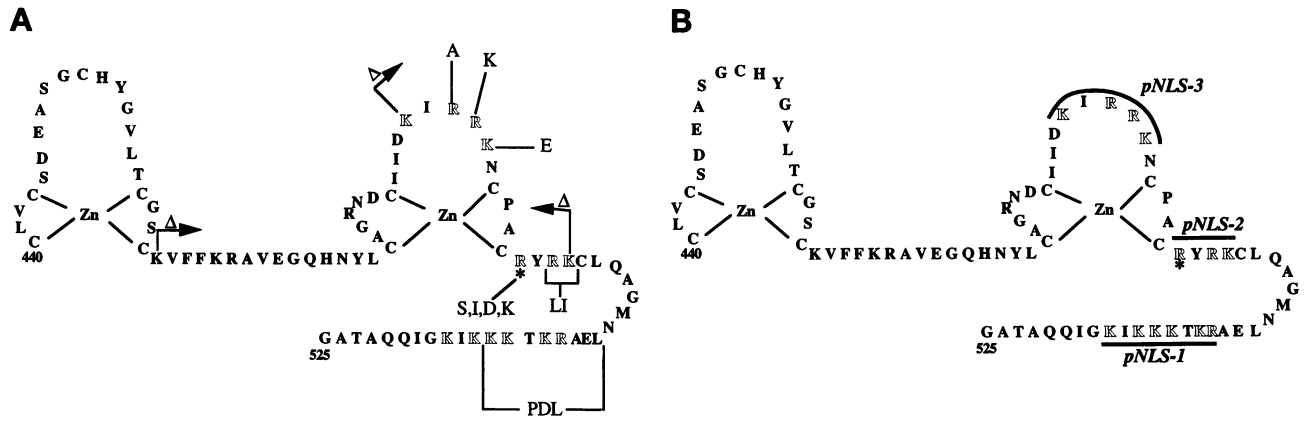

Figure 1. rGR DNA-binding domain. Single-letter code for amino acids 440–525 of the rGR DNA-binding domain. (A) Location of single (R488A, R489K, K490E, and R496S, I, D, or K) and double (R498L K499I) point mutations are indicated as well as the PDL substitution for amino acids 507–515, which defines mutant LS10. End points for amino acids 461–499 and 486–499 internal deletions are indicated. (B) Designation of three proto-NLS segments, pNLS-1, pNLS-2, and pNLS-3.

(Cadepond *et al.*, 1992). To more precisely map the NLS of rGR, a number of point mutations and deletions were constructed within the rGR DBD (Figure 1A). Nuclear import activity of mutant receptors was assessed in transiently transfected Cos-1 cells. To eliminate any potential contribution of the ill-defined hormone-dependent NLS within the rGR LBD (Picard and Yamamoto, 1987), all mutants were tested in the context of a carboxyl-terminal truncation that removed the entire LBD of the receptor (i.e., VAN525, see Godowski *et al.*, 1987, or GRΔ, see MATERIALS AND METHODS). As indicated in Table 1, the basic amino acid stretch just downstream of the second zinc finger (i.e., between amino acids 510 and 517) is absolutely required for NLS activity as deletion of four of six basic amino acids from this region in LS10Δ abolishes its in vivo nuclear import. As long as this segment is intact, single or double point mutations within either one of two basic stretches within the second zinc finger (i.e., between amino acids 486 and 490 and between amino acids 496 and 499) did not affect nuclear import with the exception of a K490E mutation that led to reduced import efficiency (Table 1). Deletion of both basic amino acid segments within the second zinc finger severely compromised the NLS activity of the 510–517 segment (Table 1). Thus, the rGR NLS, in accord with other steroid receptors, possesses a multicomponent redundant NLS as some, but not all, basic amino acid segments are dispensable for complete activity. To be consistent with the paradigm established for chick progesterone receptor and human estrogen receptor by Ylikomi *et al.* (1992), we have designated the three proto-NLSs in rGR as pNLS-1, pNLS-2, and pNLS-3 (Figure 1B).

Table 1. Nuclear import of rGR DBD mutants

Mutant	*Localization*	*Nuclear pattern*
VAN525 (w/t)	N	H
GRΔ (w/t)	N	H
GRΔ/R488A	N	H
GRΔ/R489K	N	H
GRΔ/K490E	N ≥ C	H
GRΔ/R496S(I,D,K)	N	Foci (hsp70+)
VAN525/R498LK499I	N	H
VAN525Δ(461-499)	N = C	H
VAN525Δ(486-499)	C > N	H
LS10Δ	C > N	H
VAN525/R496S + GR LBD-βgal	N	Foci (−hormone) H (+hormone)

Localization and staining pattern of rGR mutants within nuclear (N) or cytoplasmic (C) compartments are indicated. H, homogenous.

Mutation of Arg-496 Leads to Nuclear Mistargeting of GRΔ Receptors

During the course of our rGR NLS mapping studies, we noticed an atypical staining pattern of one NLS mutant. As shown in Figure 2E, carboxyl-terminal truncated rGRs with a serine substituted for an arginine at position 496 (i.e., GRΔ/R496S) localized within large nuclear foci. Confocal microscopic examination of Cos-1 cells transiently transfected with GRΔ/R496S revealed a range of 5–10 foci/cell that were dispersed throughout all planes of the nucleus. It must be emphasized that none of the other GR DBD mutants generated this nuclear staining pattern (Table 1). Since GR expression is comparable in transfections with GRΔ and GRΔ/R496S (Figure 3A), this pattern of nuclear staining is not simply the result of overexpression of this mutant receptor.

To address whether the R496S mutation fortuitously created a high-affinity binding site for some limiting nuclear compartment or structure, this residue was mutated to either a nonpolar amino acid (i.e., isoleucine, R496I), an acidic amino acid (i.e., aspartate, R496D), or another basic amino acid (i.e., lysine, R496K). Remarkably, all carboxyl-terminal rGR derivatives with substitutions at R496 localized within large nuclear foci (Figure 2, E–H), even with the conservative substitution of lysine for R496 (Figure 2G). Thus, there appears to be a strict requirement for arginine at position 496 of the rGR DBD for GRΔ receptors to remain dispersed throughout the nucleus and not cluster within a limited number of large foci. It is important to note that large foci were not observed within the cytoplasm, indicating that R496 mutant receptors do not cluster as they emerge from the ribosome. In fact, R496 mutant receptors remain competent to translocate through the nuclear pore and, by inference, must maintain their capacity to interact via their NLS, which encompasses amino acid 496 (Figure 1B), with appropriate cytoplasmic nuclear transport factors, such as importin/karyopherin α (Görlich *et al.*, 1994; Moroianu *et al.*, 1995). The formation of large nuclear foci will be referred to as receptor mistargeting throughout the remainder of the text to simply further discussions.

GRΔ/R496 mutants do not accumulate within nucleoli, as assessed by costaining with antibodies against nucleolar proteins (our unpublished observations). However, GRΔ/R496 mutant foci coincide with large inclusion bodies that are visible under differential interference-contrast microscopy (Figure 4). Such inclusion bodies were not observed within Cos-1 cell nuclei in the absence of transfected GRΔ/R496 mutants (Figure 4). Finally, GRΔ/R496 mutant proteins resist extraction from nuclei, even under conditions that remove >95% of nuclear protein and DNA and leave behind an RNA-containing nuclear matrix (our unpublished observations).

Hsp70 Colocalizes with GRΔ/R496S within Large Nuclear Foci

Some overexpressed nuclear proteins, such as c-*myc* (Henriksson *et al.*, 1992), v-*myc* (Koskinen *et al.*, 1991), and E1A (White *et al.*, 1988), have been found to accumulate within large granules that can entrap the 70-kDa heat shock protein hsp70. The formation of large nuclear bodies in Cos-1 cells expressing GRΔ/R496 mutants does not correlate with their overexpression (Figure 3) but seems to be a unique property of GRΔ receptors with substitutions at a specific arginine residue (i.e., R496). Nevertheless, the subcellular localization of hsp70 was compared with that of GRΔ/R496S in transiently transfected Cos-1 cells by using anti-hsp70 and anti-GR antibodies to costain individual cells.

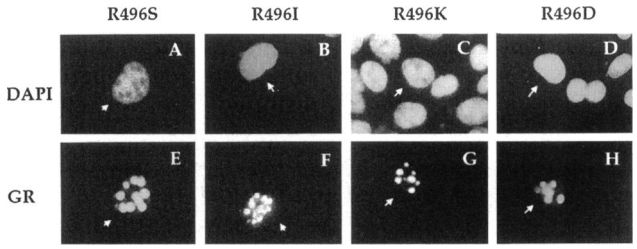

Figure 2. Nuclear import of R496 mutants of rGRΔ. Cos-1 cells were transiently transfected with carboxyl-terminal-deleted rGRs that possessed different point mutations at R496. Nuclei were visualized by DAPI staining (A–D) and GR by IIF with the BuGR2 anti-GR monoclonal antibody (E–H).

As shown in Figure 5B, hsp70 localizes both in the cytoplasm and nucleus of nontransfected Cos-1 cells, in agreement with previous reports (Pelham, 1984; Welch and Feramisco, 1984). However, in cells expressing GRΔ/R496S, hsp70 colocalized with mutant receptors within large nuclear foci (Figure 5). Since we have limited our analysis to transient transfections, it is difficult to assess whether the presence of GRΔ/R496S induced hsp70 protein expression within individual transfected cells. However, the localization of hsp70 to nonnucleolar foci within the nucleus of cells expressing GRΔ/R496S is distinct from the accumulation of hsp70 within nucleoli of cells subjected to various forms of stress (Pelham, 1984; Welch and Feramisco, 1984; Pelham, 1986). GRΔ/R496S remains associated with an insoluble fraction of the nucleus that resists harsh extractions, making it difficult to assess whether the coincident localization of hsp70 is due to

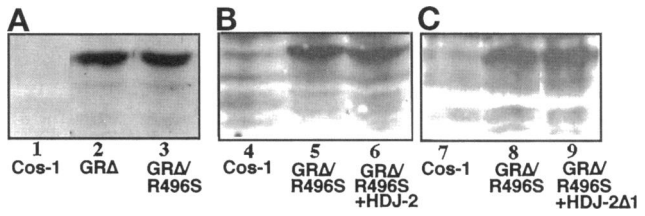

Figure 3. Comparison of GRΔ and GRΔ/R496S expression in transiently transfected Cos-1 cells. (A) Identical amounts of total protein from whole-cell extracts of nontransfected Cos-1 cells (lane 1) or Cos-1 cells transiently transfected with GRΔ (lane 2) and GRΔ/R496S (lane 3) were separated by SDS-PAGE and subjected to Western blot analysis. The BuGR2 anti-GR monoclonal antibody was used to visualize GR derivatives. (B) GRΔ/R496 expression was compared in the absence (lane 5) or presence (lane 6) of cotransfected HDJ-2 DNA. Nontransfected Cos-1 cells are shown in lane 4. Western blot analysis was performed as described in A. (C) GRΔ/R496 expression was compared in the absence (lane 8) or presence (lane 9) of cotransfected HDJ-2Δ1 DNA. Nontransfected Cos-1 cells are shown in lane 7. Western blot analysis was performed as described in A.

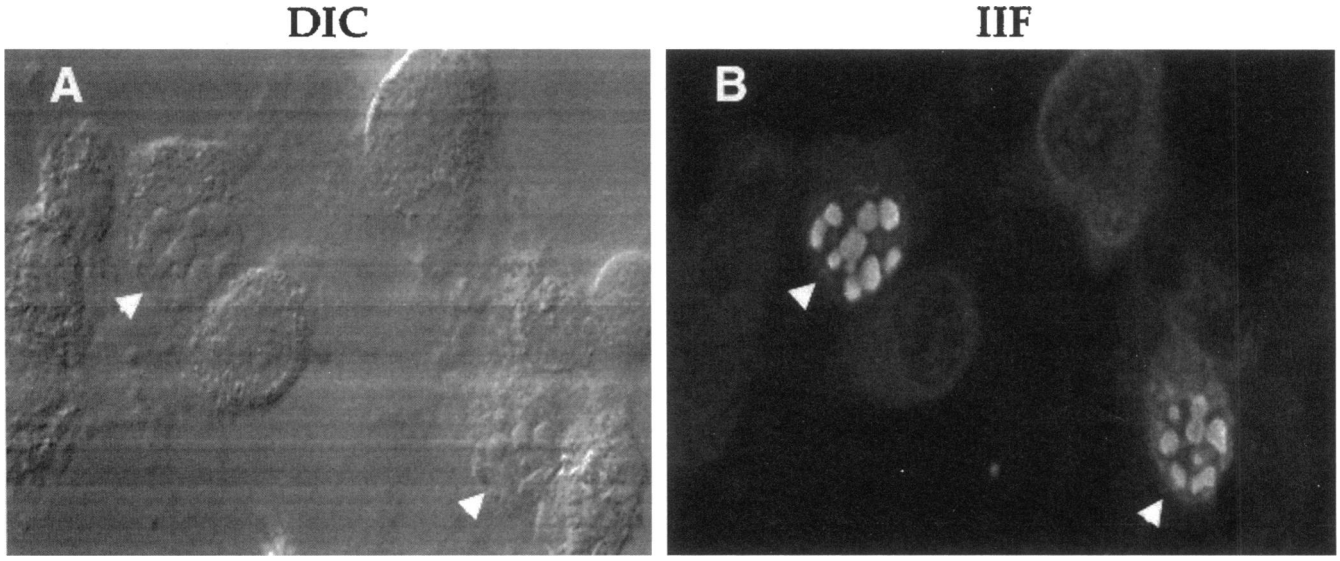

Figure 4. Coincident localization of GRΔ/R496S foci within novel nuclear structures. Differential interference-contrast micrograph (DIC, A) shows nuclear structures that coincide with the location of GRΔ/R496S receptors (B) in transiently transfected Cos-1 cells (arrowheads). GRΔ/R496S was detected by IIF with the BuGR2 anti-GR monoclonal antibody.

direct interactions between hsp70 and the mutant receptor.

Alleviation of GRΔ/R496S Mistargeting by the rGR LBD

rGR NLS mutants, including amino acid substitutions at R496, were assessed in the context of a carboxyl-terminal deleted receptor to eliminate any potential contribution of the hormone-dependent NLS within the LBD (Picard and Yamamoto, 1987). Since the constitutive NLS of GR is unmasked once the LBD is occupied by ligand (Picard and Yamamoto, 1987), it seemed likely that the subnuclear targeting defect associated with R496 mutations would be apparent for ligand-bound full-length rGR. However, when examined in transiently transfected Cos-1 cells, full-length rGRs possessing a R496S substitution were not mistargeted upon translocation to the nucleus but exhibited the homogenous diffuse nuclear staining pattern characteristic of full-length wild-type receptors (Figure 6D). Even more surprising was the observation that this protective effect of the rGR LBD on GRΔ/R496S mistargeting was also exerted in trans. As shown in Figure 7B, GRΔ/R496S exhibited its characteristic mistargeting when cotransfected into Cos-1 cells, in the absence of hormone, with a rGR LBD-βgal chimera (i.e., pNL2-βgal; Picard and Yamamoto, 1987). The staining of cotransfected cells with an anti-βgal antibody confirmed the cytoplasmic localization of the NL2-βgal chimera in cells not treated with hormone (Figure 7C). In contrast, GRΔ/R496S was dispersed throughout the nucleus upon dexamethasone treatment of Cos-1 cells cotransfected with NL2-βgal (Figure 7E). As expected (Picard and Yamamoto, 1987), the NL2-βgal chimera was localized to the nucleus of dexamethasone-treated cells (Figure 7F). GRΔ/R496S expression was not affected by cotransfection with NL2-βgal as revealed by Western blot analysis (our unpublished observations). Thus, mistargeting of GRΔ/R496S receptors is prevented when the rGR LBD is coincidentally localized within nuclei and does not require a direct linkage of the LBD to the mutant receptors. GRΔ/R496S mistargeting was not prevented if the βgal protein alone was directed to the nucleus by virtue of a linked constitutive NLS (Picard and Yamamoto, 1987; our unpublished observations). This result establishes that the βgal portion of the NL2-βgal chimera does not contribute to the hormone-dependent rescue of GRΔ/R496S mistargeting.

Given the hormone dependence of NL2-βgal nuclear localization, we were able to assess the time course of rGR LBD rescue of GRΔ/R496S mistargeting. GRΔ/R496S- and NL2-βgal-cotransfected Cos-1 cells were maintained in hormone-free medium for 24 h to allow sufficient time for the formation of GRΔ/R496S nuclear foci. Cells were then treated for various lengths of time with dexamethasone prior to fixation and IIF analysis. As shown in Figure 8B, within 10 min after dexamethasone treatment, dispersal of GRΔ/R496S nuclear foci was becoming apparent. Within 30 min of dexamethasone treatment, GRΔ/R496S nuclear foci were barely visible because receptors were predominantly distributed throughout the nucleus (Figure 8E). In the field of cells shown in Figure 8H,

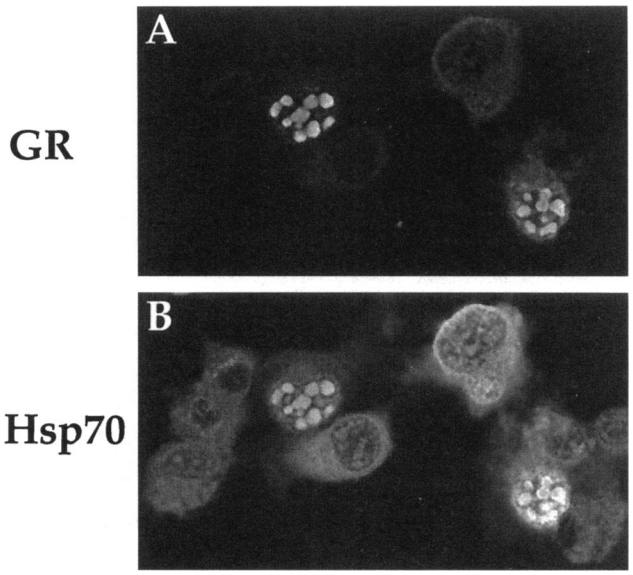

Figure 5. Colocalization of GRΔ/R496S and hsp70. Cos-1 cells transiently transfected with GRΔ/R496S were costained to visualize transfected GRΔ/R496S and endogenous hsp70 by IIF. Confocal microscopic images of one 0.7-μm optical section show GRΔ/R496S (A) and hsp70 (B). An overlay of the two images shown in A and B confirmed the coincidence of GRΔ/R496S and hsp70 foci (our unpublished observations).

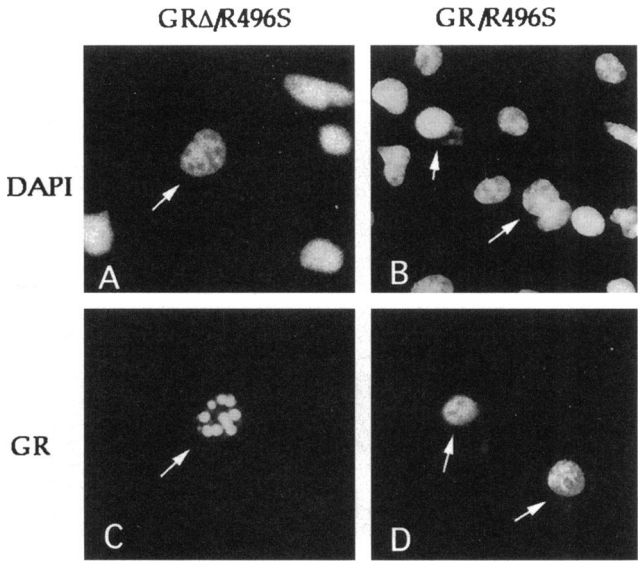

Figure 6. Rescue of GRΔ/R496S nuclear mistargeting, in cis, by a linked rGR LBD. Carboxyl-terminal deleted (A and C) or intact rGR (B and D) possessing R496S mutation was transiently transfected into Cos-1 cells. Nuclei were visualized by DAPI staining (A and B) and GR by IIF with the BuGR2 anti-GR monoclonal antibody (C and D).

GRΔ/R496S nuclear foci were visible in a hormone-treated cell (Figure 8, H and I, arrow) that did not express the LBD-βgal chimera (Figure 8I). Thus, the rGR LBD appears to play an active role in releasing GRΔ/R496S from an inappropriate nuclear compartment rather than to prevent its mistargeting.

Cotransfection with HDJ-2/HSDJ Alleviates GRΔ/R496S Mistargeting

The rGR LBD is generally considered to possess a "protein inactivation" function that is relieved upon hormone binding (Bohen and Yamamoto, 1994). It has been postulated that this activity could reflect the participation of the LBD in conformational transitions of linked protein domains (Bohen and Yamamoto, 1994). In at least one case, this protein inactivation function was supplied in trans toward a dimerization partner of the rGR LBD (Spanjaard and Chin, 1993). If an analogous activity of the LBD accounts for the relief of GRΔ/R496S mistargeting, it must be expressed in the presence of bound ligand and brought about in trans, by interactions between the LBD and carboxyl-terminal-deleted rGR. Although the demonstration of direct interactions between amino- and carboxyl-terminal domains of steroid receptors (Kraus *et al.*, 1995; Langley *et al.*, 1995) provides a mechanism for recruitment of the rGR LBD to sites of GRΔ/R496S accumulation, there are no precedents for the influence of a hormone-bound LBD on folding of covalently or noncovalently linked protein domains.

We therefore considered the possibility that the LBD played an indirect role in alleviating GRΔ/R496S mistargeting and sought to assess whether the recruitment of a specific LBD-associated protein(s) into the nucleus was responsible for this effect. Given the coincident localization of hsp70 and GRΔ/R496S, it seemed logical to examine the potential effects on receptor mistargeting of LBD-associated proteins that directly interact with hsp70. Although a number of proteins of the GR heteromeric complex could contact hsp70 directly (Pratt, 1993), we initially focused on DnaJ homologues, which are known to influence hsp70 ATPase activity (Cyr *et al.*, 1994). One of the DnaJ homologues of *Saccharomyces cerevisiae*, Ydj1p, was recently found to be a component of a rGR heteromeric complex (Kimura *et al.*, 1995).

Subnuclear targeting of GRΔ/R496S was examined in Cos-1 cells cotransfected with an expression plasmid encoding HDJ-2/HSDJ, a DnaJ homologue derived from human cells (Chellaiah *et al.*, 1993; Oh *et al.*, 1993). IIF of nontransfected Cos-1 cells revealed that HDJ-2/HSDJ is localized predominantly within the cytoplasm, with some protein also detected surrounding the nucleus (our unpublished observations). Overexpressed HDJ-2/HSDJ protein also exhibited this same cytoplasmic staining pattern in transfected Cos-1 cells that did not express GRΔ/R496S (Figure 9C). However, cotransfection of GRΔ/R496S and HDJ-2/

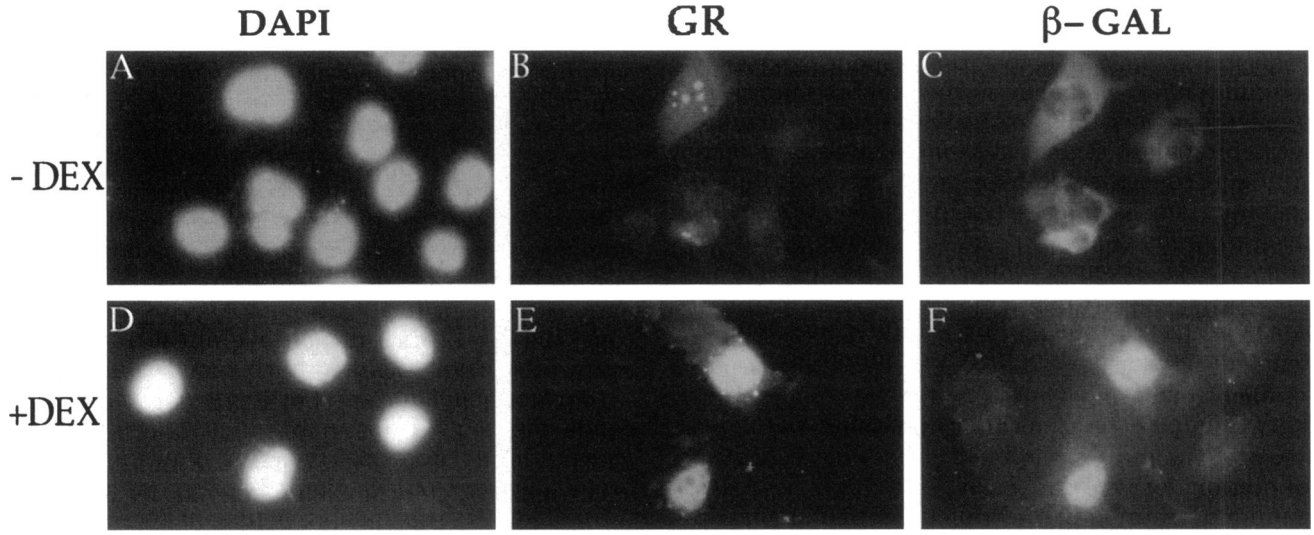

Figure 7. Rescue of GRΔ/R496S nuclear mistargeting, in trans, upon coexpression of a rGR LBD-βgal chimera. Cotransfected Cos-1 cells were costained to visualize GRΔ/R496S (B and E) and a rGR LBD-βgal chimera (C and F) by IIF. Cells were untreated (A–C) or treated with 1 μM dexamethasone (+DEX) for 24 h (D–F). Nuclei were visualized by DAPI staining (A and D).

HSDJ DNAs led to alterations in the nuclear staining pattern of both the mutant receptor and overexpressed HDJ-2/HSDJ. As shown in Figure 9B, GRΔ/R496S did not accumulate within large nuclear foci but was distributed throughout the nucleus in cells that overexpress HDJ-2/HSDJ. GRΔ/R496S expression was not altered in Cos-1 cells upon cotransfection with expression plasmids encoding either HDJ-2/HSDJ (Figure 3B) or a J-domain deletion mutant of HDJ-2/HSDJ (Figure 3C). Thus, alleviation of GRΔ/R496S mistargeting is not the result of HDJ-2/HSDJ effects on expression of the mutant receptor. When overexpressed in cells that contain nuclear GRΔ/R496S, HDJ-2/HSDJ was localized exclusively within nuclei (Figure 9C). Thus, HDJ-2/HSDJ is recruited to the nucleus in response to the accumulation of mistargeted R496 mutant receptors or cotransported into nuclei with these mutant receptors. Overexpressed HDJ-2/HSDJ protein did not accumulate to an appreciable extent within nuclei of Cos-1 cells that do not express GRΔ/R496S (Figure 9C). Overexpression of a deletion mutant of HDJ-2/HSDJ that lacks its J homology domain (i.e., HSDJΔ1) did not alleviate GRΔ/R496S mistargeting (Figure 9E). Interestingly, HSDJΔ1 also localized within nuclei foci coincident with GRΔ/R496S (Figure 9F), indicating that this mutant HDJ-2/HSDJ protein is able to recognize mistargeted nuclear receptors. Overexpressed HSDJΔ1 protein did not form large nuclear foci in cells that did not express GRΔ/R496 but accumulated predominately within the cytoplasm (Figure 9F). Thus, its localization within nuclei required the presence of mistargeted R496 mutant receptors.

Defective Transactivation and Transrepression Activity of Full-Length GR/R496S Is Overcome upon Overexpression of HDJ-2/HSDJ

Although full-length GR/R496S does not exhibit apparent subnuclear targeting defects, its transactivation activity from a simple GRE is severely compromised (Figure 10A, lane 2). This most likely reflects its diminished DNA-binding activity. Interestingly, in transfected Cos-1 cells, GR/R496S activates transcription from a composite GRE (Diamond *et al.*, 1990) that directs wild-type GR to repress transcription from a linked promoter (Figure 10B, lanes 1 and 2). Other mutations have been identified within the GR DBD that lead to misinterpretation of a composite GRE (Starr *et al.*, 1996). It has been postulated that such effects may result from mutant receptors inappropriately adopting a conformation that restricts it to function as a transactivator irrespective of the nature of the GRE (Starr *et al.*, 1996).

The elimination of GRΔ/R496S mistargeting by HDJ-2/HSDJ suggests that this chaperone might directly or indirectly alter mutant receptor conformation and thereby decrease its tendency to collect into large nuclear foci. Can HDJ-2/HSDJ recognize potentially "misfolded" full-length R496S receptors and facilitate their recovery to a biologically active conformational state? To address this question, glucocorticoid effects on both simple and composite GRE CAT reporter plasmids were measured in Cos-1 cells cotransfected with full-length GR/R496S and HDJ-2/HSDJ expression plasmids. For these studies we used CAT reporters that possessed trimeric simple or composite GREs, since R496S receptors were found to bind to these

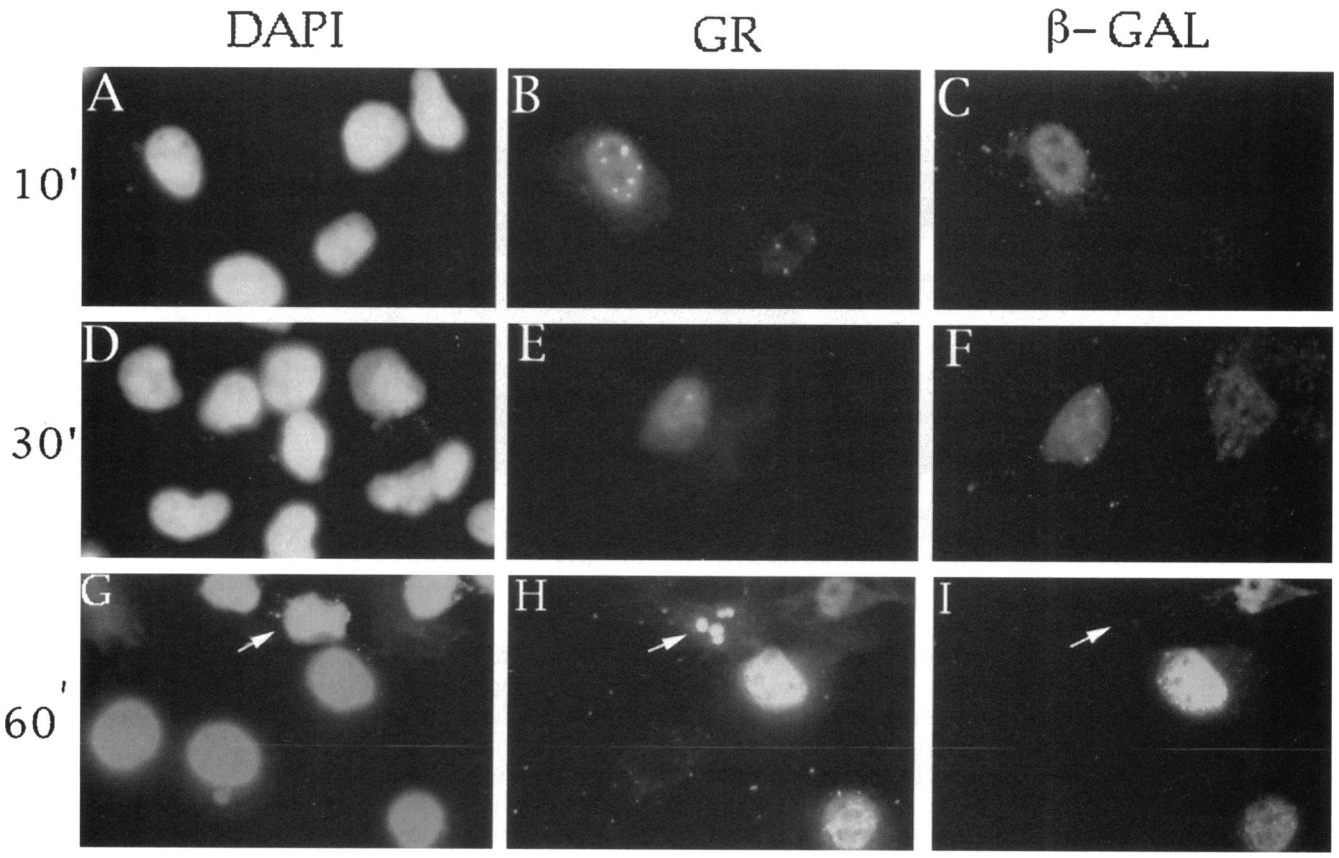

Figure 8. Time course of rGR LBD rescue of GRΔ/R496S mistargeting. Cotransfected Cos-1 cells were subjected to IIF and costained to visualize GRΔ/R496S (B, E, and H) and a rGR LBD-βgal chimera (C, F, and I). DNA was visualized by DAPI staining (A, D, and G). Cells were fixed at 10 min (A–C), 30 min (D–F), and 60 min (G–I) after the addition of 1 μM dexamethasone. Arrows point to the cell that expresses GRΔ/R496S but not the LBD-βgal chimera. Note the persistence of large GR foci in this cell.

elements with a reasonable affinity in vitro (Thomas, 1992). Monomeric GREs displayed little to no binding to R496S receptors in vitro (Thomas, 1992).

As shown in Figure 10A, lanes 4 and 6, dexamethasone-induced transactivation activity of GR/R496S on the TAT3CAT reporter was increased dramatically upon cotransfection of HDJ-2/HSDJ DNA. The 15-fold induction observed upon cotransfection with HDJ-2/HSDJ DNA (Figure 10A, lane 6) was approximately 50% of the level of hormone induction observed with wild-type GR under identical conditions (Figure 10A, lanes 1, 3, 5, 7, and 9). Hormone-dependent transactivation activity of wild-type GR was not significantly affected by cotransfection of HDJ-2/HSDJ DNA (Figure 10A, lanes 3 and 5). Likewise, the basal activity of the TAT3CAT reporter was not affected by overexpression of HDJ-2/HSDJ either in the absence or presence of cotransfected GR expression plasmid (our unpublished observations). Increasing the amount of cotransfected HDJ-2/HSDJ DNA did not increase hormone-dependent transactivation mediated by GR/R496S but in fact diminished the transactivation activity of wild-type receptors (our unpublished observations). As mentioned above, GR/R496S expression was not significantly affected in Cos-1 cells transiently cotransfected with HDJ-2/HSDJ DNA (Figure 3B). Cotransfection with the HSDJΔ1 deletion mutant did not affect the transactivation activity of wild-type or R496S receptors (Figure 10A, lanes 8 and 10). Results from IIF analysis shown above (Figure 9F) demonstrated that HSDJΔ1 protein was expressed in transiently transfected Cos-1 cells.

As shown in Figure 10B, lane 2, full-length GR/R496S misinterprets a composite GRE signal because dexamethasone treatment led to a nearly fourfold induction of transcription from a transiently transfected plfG3CAT reporter. As expected (Diamond *et al.*, 1990), cotransfection of wild-type GR with plfG3CAT into Cos-1 cells led to a 60% repression of transcription (Figure 10B, lane 1). Cotransfection with HDJ-2/HSDJ (Figure 10B, lanes 3 and 5) or the HSDJΔ1 (Figure 10B, lanes 7 and 9) mutant DNAs did not affect the transrepression activity of wild-type GR on plfG3CAT or the basal activity of this reporter (Figure 10B, lanes 11

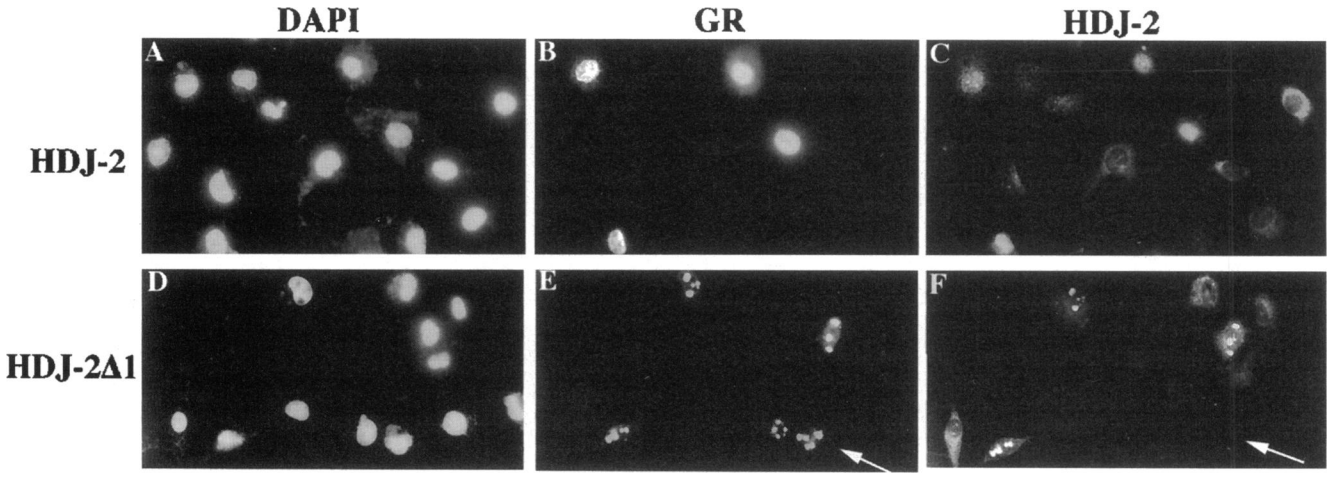

Figure 9. Rescue of GRΔ/R496S mistargeting by HDJ-2/HSDJ. Cos-1 cells were transiently cotransfected with GRΔ/R496S and HDJ-2 (A–C) or HDJ-2Δ1 (D–F) and costained with an anti-GR polyclonal antibody (B and E) and an anti-HDJ-2 monoclonal antibody (C and F). Nuclei were visualized by DAPI staining (A and D). Arrows show a cell that expresses GRΔ/R496S but not HDJ-2Δ1.

and 12). However, the GR/R496S mutant receptor was converted from a transactivator to a transrepressor at the composite GRE upon overexpression of HDJ-2/HSDJ but not HSDJΔ1 (Figure 10B, lanes 3, 5, 7, and 9). Thus, HDJ-2/HSDJ alters the activity of R496 mutant GRs bound to a composite GRE, perhaps restoring them to a conformation required to bring about an appropriate transcriptional response.

DISCUSSION

The NLSs used by steroid receptors to direct their import into nuclei (Picard and Yamamoto, 1987; Guiochon-Mantel *et al.*, 1989; Cadepond *et al.*, 1992; Ylikomi *et al.*, 1992; Zhou *et al.*, 1994) resemble the prototypical bipartite NLS of nucleoplasmin (Dingwall and Laskey, 1991). rGR, like other steroid receptors, possesses redundant proto-NLSs that do not function alone but exhibit full activity when present in unique pairs. At least two of the proto-NLSs of rGR are contained within α helices of the DBD second zinc finger (Luisi *et al.*, 1991; Figure 1B). In the crystal structure of the rGR DBD, several basic residues within these proto-NLSs make specific contacts to the phosphate backbone of DNA at either a specific or nonspecific target site (Luisi *et al.*, 1991). Thus, specific amino acids within the DBD of rGR have the capacity to be involved not only in DNA recognition but also in interactions with cytosolic receptors (i.e., importin/karyopherin α) that are required for nuclear import.

We show herein that the NLS of rGR, in addition to its functioning as a NPC targeting signal, may have an impact on receptor targeting within the nucleus. This presumed dual role of the rGR NLS in nuclear import and compartmentalization is distinguished by point mutations at R496, which although transparent for nuclear import activity of the NLS, exerts dramatic effects on subnuclear targeting of the receptor. Carboxyl-terminal-truncated rGRs with mutations at R496 accumulate within a few large nuclear foci, representing a departure from the characteristic nonrandom mottled nuclear staining pattern of GRs (Martins *et al.*, 1991; van Steensel *et al.*, 1995). The fact that substitution of R496 with another basic amino acid (lysine), an acidic (aspartate), a polar (serine), or a nonpolar (isoleucine) amino acid generated the identical mistargeting of the receptor argues against the fortuitous formation of a novel subnuclear targeting signal. In addition, many other mutations within and surrounding the various components of the NLS, including point mutations at R488, R489, K490, R498, and K499, and deletions of 14 and 39 amino acids of the second zinc finger, did not lead to the accumulation of mutant receptors within large nuclear foci. Thus, the presence of an arginine residue immediately after the final zinc-coordinating cysteine of the rGR DBD appears to be essential for appropriate subnuclear targeting of receptors.

The effect of rGR R496 mutations on subnuclear targeting is not autonomous, because we observed a coincident localization of hsp70 within R496 mutant foci. In this case, there appears to be a redistribution of hsp70, which normally localizes throughout the cell (Pelham, 1984; Welch and Feramisco, 1984), to these nuclear foci. hsp70 has been found to colocalize within nuclear granules which form as a result of overexpression of the E1A or myc proteins (White *et al.*, 1988; Koskinen *et al.*, 1991; Henriksson *et al.*, 1992). Although a stable hsp70/E1A complex could be immunopurified after mild extraction of nuclei (White *et al.*,

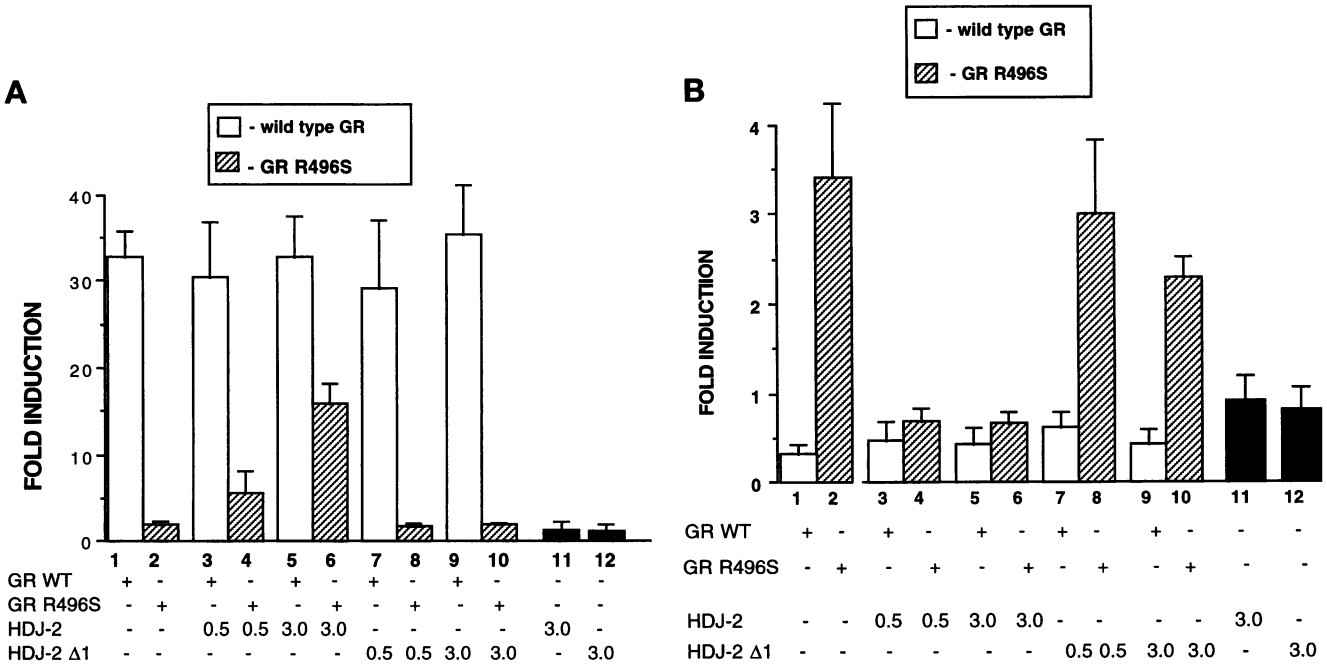

Figure 10. Correction of GR R496S transactivation and transrepression by HDJ-2/HSDJ. Wild-type GR (□) or the rGR/R496S (▨) expression vectors were transfected in Cos-1 cells in the absence of an HDJ-2/HSDJ expression vector (lanes 1 and 2) or presence of various amounts (0.5 or 3 μg) of HDJ-2 (lanes 3–6) or HDJ-2Δ1 (lanes 7–10) expression vectors. Cotransfected reporter plasmids were TAT3CAT (A) or plfG3CAT (B). Cells were treated with and without 1 μM dexamethasone for 16 h, and CAT activity was measured in cell-free lysates. The average fold induction obtained from at least three individual experiments is plotted with the error bars representing the SD. Lanes 11 and 12 present experiments with cotransfected CAT reporters and HDJ-2/HSDJ expression vectors in the absence of rGR expression vectors.

1988), nuclear foci that possess GRΔ/R496S and hsp70 resist even the harshest extraction methods and biochemically partition to an insoluble nuclear compartment. We hypothesize that hsp70 may play a general role in sequestering or shielding "sticky" protein surfaces within the nucleus whose exposure may increase upon overexpression. The ability of hsp70 to shuttle between the nuclear and cytoplasmic compartments provides the means for this chaperone to continuously survey the cytoplasm and nucleus for misfolded proteins. It is important to reiterate that for GRΔ/R496S, the exposure of these potential sticky surfaces does not result from overexpression.

How do GRΔ/R496S foci form? Since R496 makes direct contact with the phosphate backbone at both specific and nonspecific DNA sites (Luisi *et al.*, 1991), the lack of such a stabilizing interaction might unleash a subdomain of the GR DBD that could promote the formation of large nuclear foci. It is noteworthy that R496 is the only amino acid within an α-helical subdomain of the second zinc finger that makes DNA contact (Luisi *et al.*, 1991). The loss of a single phosphate contact does not appear to be solely responsible for mistargeting of carboxyl-terminal-truncated rGRs, since mutations at other amino acids that make phosphate contacts (i.e., R489 and K490) do not lead to receptor mistargeting. R496 is a highly conserved residue in the steroid/thyroid hormone superfamily of nuclear receptors because every member of the superfamily identified to date possesses an arginine at that corresponding position (Freedman, 1992; Mangelsdorf *et al.*, 1995). Since this amino acid serves an identical function in making phosphate backbone contacts in the crystal structures of rGR (Luisi *et al.*, 1991) and other members of the nuclear receptor family (Schwabe *et al.*, 1993; Rastinejad *et al.*, 1995), it will be interesting to examine whether the mutation of this residue within other nuclear receptors also leads to an alteration in subnuclear targeting.

Initially, LBD-truncated receptors were used to simplify our analysis of the rGR NLS. Since ligand binding relieves the dominant inactivation function of the rGR LBD (Bohen and Yamamoto, 1994), we expected full-length R496 mutant receptors to likewise mistarget after hormone-dependent nuclear import. It was therefore surprising that an R496S mutation did not lead to nuclear mistargeting when tested in the context of a ligand-bound intact receptor. The protective function of the ligand-bound rGR LBD applies both in cis, when the LBD is directly linked to the R496S mutant DBD, and in trans, when the mutant DBD and LBD are expressed from separately transfected plasmids. Importantly, since effects of the LBD can be invoked rapidly after its accumulation within the nu-

cleus and act upon preexisting mistargeted receptors, the rGR LBD must supply a function that actively releases R496 mutant receptors from mistargeted sites.

An important clue relating to the mechanism of rGR LBD suppression of GRΔ/R496S mistargeting was provided by the results of cotransfection experiments with an hsp70 partner derived from human cells, HDJ-2/HSDJ. We found that GRΔ/R496S mistargeting in transiently transfected cells was alleviated upon overexpression of HDJ-2/HSDJ. Cotransfection with an HDJ-2/HSDJ mutant that lacks its J-domain (i.e., HDJ-2Δ1) did not relieve GRΔ/R496S mistargeting. Deletion of the J-domain from the *E. coli* DnaJ protein eliminated its ability to mediate protein refolding in vitro, in combination with DnaK and GrpE (Szabo *et al.*, 1996). Thus, HDJ-2/HSDJ may act in an analogous manner, perhaps in combination with hsp70, to refold misfolded or aggregated GRΔ/R496S within the nucleus and restore its appropriate subnuclear targeting. HDJ-2Δ1 possesses an intact cysteine-rich domain (Chellaiah *et al.*, 1993; Oh *et al.*, 1993) that, analogous to its demonstrated role in binding unfolded proteins in vitro (Szabo *et al.*, 1996), may direct HDJ-2/HSDJ to GRΔ/R496S nuclear foci. This could explain the colocalization of HDJ-2Δ1 with GRΔ/R496S foci in transiently transfected cells. The apparent stability of GRΔ/R496S nuclear foci in the absence of cotransfected HDJ-2/HSDJ suggests that the capacity of the normal cellular complement of DnaJ homologues to alleviate GRΔ/R496S mistargeting must be exceeded under these conditions. We postulate that only by supplementing nuclear DnaJ levels upon the introduction of exogenous HDJ-2/HSDJ is a hsp70/DnaJ chaperone system sufficiently activated to either prevent or correct GRΔ/R496S mistargeting.

Isolated eukaryotic DnaJ proteins exhibit chaperone activity in the absence of other partners (Cyr, 1995). The in vitro peptide binding activity of DnaJ, which is clearly distinct from hsp70 (Cyr *et al.*, 1992; Langer *et al.*, 1992; Frydman *et al.*, 1994), is probably used in the context of a multicomponent chaperone system to facilitate protein folding in vivo (Frydman *et al.*, 1994; Höhfeld *et al.*, 1995). An important aspect of the role of DnaJ in this process is its ability to act on preformed hsp70-protein complexes and stimulate ATP-dependent protein release (Cyr *et al.*, 1992). Although we did not specifically test whether HDJ-2/HSDJ brought about the dispersal of preformed GR/hsp70 nuclear foci, the rGR LBD effectively performed this function. If this effect of the rGR LBD is due to the action of an associated DnaJ protein (Kimura *et al.*, 1995), our experiments would provide an in vivo confirmation of an eukaryotic hsp70/DnaJ chaperone cycle that has been elaborated from elegant in vitro studies (Frydman *et al.*, 1994; Höhfeld *et al.*, 1995).

The putative involvement of HDJ-2/HSDJ in refolding mutant GRs in the nucleus extends beyond the presumed disassembly of mistargeted receptors and impacts receptor transactivation and transrepression activities. Thus, the transactivation activity of R496S mutant GRs, which is severely compromised from a trimeric simple GRE, is dramatically increased upon overexpression of HDJ-2/HSDJ. For full-length R496S mutant GRs, defects in subnuclear trafficking are not apparent given their homogenous distribution within nuclei. In addition, full-length R496S mutant receptors are indistinguishable from wild-type GRs in their extractability from nuclei (our unpublished observations), adding further support for the notion that the R496S mutation does not dramatically alter subnuclear targeting in the context of full-length GR. Since the J-domain of HDJ-2/HSDJ was required for R496S mutant receptors to regain transactivation activity, we hypothesize that mutant receptors may be refolded into a conformational state conducive to transactivation in the presence of overexpressed HDJ-2/HSDJ. Currently, it is not known whether this suppression of a transactivation defect applies to other GR DNA-binding or transactivation mutants.

R496S receptors are functional on a composite GRE but misinterpret the signal directed by this element and stimulate transcription from a linked promoter. Transfected wild-type GR in the same cell type represses transcription from the identical promoter linked to this composite GRE. Interestingly, in the presence of overexpressed HDJ-2/HSDJ, the response directed from the composite GRE by R496S receptors is reversed and transcriptional repression nearly indistinguishable from wild-type receptors is obtained. Transcriptional repression mediated by wild-type GR interacting with this composite GRE is not affected by overexpression of HDJ-2/HSDJ. The DNA-binding domain of GR has been found to play an important role in interpreting a composite GRE signal, as specific mutations within this domain change the receptor's response from repressing to activating (Starr *et al.*, 1996). It has been postulated that GR may become "reversibly locked" into an activating or repressing conformation upon binding composite GREs, depending upon the cell context (Starr *et al.*, 1996). If this is indeed the case, it seems reasonable to hypothesize that such conformational transitions may be facilitated by a molecular chaperone system that operates within the nucleus. Our results suggest that DnaJ homologues may be part of a chaperone system that performs this function in the nucleus.

DnaJ homologues in mammalian cells may impact steroid hormone signaling not only at the level of receptor folding in the cytoplasm (Bohen *et al.*, 1995; Caplan *et al.*, 1995; Kimura *et al.*, 1995) but also within the nucleus. This nuclear chaperone function of DnaJ may be required to assure appropriate subnuclear trafficking of receptors or facilitate conformational transitions essential for correct interpretation of DNA-

directed signals. Because DNA binding has an impact on the folding of various transcription factors (Tan and Richmond, 1990; Fujita *et al.*, 1992; Petersen *et al.*, 1995; Misra *et al.*, 1996), nuclear chaperones may play a more critical role in gene regulation than previously appreciated.

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