Subnuclear Trafficking of Glucocorticoid Receptors In Vitro: Chromatin Recycling and Nuclear Export

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Abstract. We have used digitonin-permeabilized cells to examine in vitro nuclear export of glucocorticoid receptors (GRs). In situ biochemical extractions in this system revealed a distinct subnuclear compartment, which collects GRs that have been released from chromatin and serves as a nuclear export staging area. Unliganded nuclear GRs within this compartment are not restricted in their subnuclear trafficking as they have the capacity to recycle to chromatin upon rebinding hormone. Thus, GRs that release from chromatin do not require transit through the cytoplasm to regain functionality. In addition, chromatin-released receptors export from nuclei of permeabilized cells in an ATP- and cytosol-independent process that is stimulated by sodium molybdate, other group VI-A transition metal oxyanions, and some tyrosine phosphatase inhibitors. The stimulation of in vitro nuclear export by these compounds is not unique to GR, but is restricted to other proteins such as the 70- and 90-kD heat shock proteins, hsp70 and hsp90, respectively, and heterogeneous nuclear RNP (hnRNP) A1. Under analogous conditions, the 56-kD heat shock protein, hsp56, and hnRNP C do not export from nuclei of permeabilized cells. If tyrosine kinase inhibitors genistein and tyrphostin AG126 are included to prevent increased tyrosine phosphorylation, in vitro nuclear export of GR is inhibited. Thus, our results are consistent with the involvement of a phosphotyrosine system in the general regulation of nuclear protein export, even for proteins such as GR and hnRNP A1 that use distinct nuclear export pathways.

THE glucocorticoid receptor (GR)[1] is a member of a nuclear receptor superfamily that includes steroid hormone receptors, the retinoid, thyroid and vitamin D receptors, and a growing number of "orphan" receptors whose natural ligands remain largely unknown (Yamamoto, 1985; Evans, 1988; Mangelsdorf et al., 1995). Members of this receptor superfamily participate in a wide variety of physiological processes, primarily through their functioning as regulated transcription factors for distinct sets of target genes (Yamamoto, 1985; Tsai and O'Malley, 1994). While the transcriptional regulatory activities of nuclear receptors are most often regulated by hormonal ligand, ligand-independent activation of steroid receptors has been observed (Denner et al., 1990; Power et al., 1991; Somers and DeFranco, 1992; Zhang et al., 1994) and may be relevant in particular physiological settings (Mani et al., 1994).

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1. *Abbreviations used in this paper*: DAPI, 4′,6-diamidino-2-phenylindole; GR, glucocorticoid receptor; hnRNP, heterogeneous nuclear RNP; IIF, indirect immunofluorescence; NES, nuclear export signal sequence; NLS, nuclear localization signal sequence; NPC, nuclear pore complex.

Ligand binding to steroid hormone receptors initiates their transformation from a weak to tight DNA-binding form (Pratt, 1987). For GRs, this transformation is often accompanied by hormone-induced nuclear import of cytoplasmic receptors (Picard and Yamamoto, 1987; Wikström et al., 1987; Qi et al., 1989; Cidlowski et al., 1990). In contrast, for receptors that localize predominantly within the nucleus when unliganded (i.e., estrogen and progesterone receptors), ligand binding increases nuclear affinity of the receptors in the apparent absence of cytoplasmic to nuclear translocation (Welshons et al., 1984; Guiochon-Mantel et al., 1989; Picard et al., 1990*b*). The dissociation of hormone from steroid receptors decreases their affinity for nuclei, and, for GR in particular, this reduced nuclear affinity is associated with its efflux from the nucleus and reappearance within the cytoplasm (Orti et al., 1989; DeFranco et al., 1991; Sackey et al., 1996).

Nuclear import of steroid receptors, like other karyophilic proteins, occurs through nuclear pore complexes (NPCs) that are embedded within the nuclear envelope (Akey and Radermacher, 1993; Doye and Hurt, 1995). Although NPCs accommodate both inward and outward trafficking of various macromolecules, it was initially thought that these transport processes were unidirectional for a given transporting substrate. However, in recent years, a number of proteins have been found to have the

capacity to shuttle between the cytoplasmic and nuclear compartments (Nigg et al., 1991; Gerace, 1992; Newmeyer, 1993). The list of proteins that exhibit this nucleocytoplasmic shuttling property includes not only steroid receptors (Guiochon-Mantel et al., 1991; Chandran and DeFranco, 1992; Dauvois et al., 1993; Madan and DeFranco, 1993), but also other nuclear proteins such as the heterogeneous nuclear RNP (hnRNP) A1 (Pinol-Roma and Dreyfuss, 1992), nucleolar proteins nucleolin and B23/No38 (Borer et al., 1989), the heat-stable inhibitor of cAMP-dependent protein kinase (Fantozzi et al., 1994), the HIV Rev protein (Meyer and Malim, 1994), and the 70-kD heat shock protein, hsp70 (Mandell and Feldherr, 1990). As these proteins exhibit a wide range of activities, different functional consequences may be imparted by bidirectional nuclear transport.

Extensive studies on nuclear protein import have led to the identification of soluble and NPC-associated proteins (i.e., nucleoporins) that function in distinct steps in the nuclear import process. Karyopherin/importin α comprises one subunit of the receptor for nuclear localization signal sequences (NLSs) and, along with karyopherin/importin β, mediates the binding of import-competent proteins to NPC docking sites (Görlich et al., 1995; Moroianu et al., 1995; Rexach and Blobel, 1995; Hurt, 1996). A GTP-binding protein, Ran/TC4 (Melchior et al., 1993; Moore and Blobel, 1993), and an associated protein, pp10/NF2 (Moore and Blobel, 1994; Nehrbass and Blobel, 1996), are required for the translocation of proteins through the NPC. As the hydrolysis of Ran-bound GTP is required for nuclear import and export (Schlenstedt et al., 1995; Rush et al., 1996), both Ran-specific GTPase activating proteins (e.g., rna-1) (Corbett et al., 1995) and guanosine nucleotide exchange proteins (e.g., RCC1) (Kadowaki et al., 1993; Tachibana et al., 1994) have an impact on nuclear transport.

While a unifying mechanism of nuclear protein import seems to be emerging, current views of nuclear protein export remain disparate. For example, it has been suggested that protein nuclear export may be restricted primarily by retention within specific nuclear compartments. In these cases, nuclear protein export may not require a specific signal sequence, but merely proceed via a default pathway into which released nuclear proteins are channeled (Schmidt-Zachmann et al., 1993). In direct contrast, specific nuclear export signal sequences (NESs) have been identified within hnRNP A1 (Michael et al., 1995), protein kinase inhibitor (Wen et al., 1995), and the HIV Rev protein (Fischer et al., 1995), demonstrating that nuclear protein export can also be a signal-mediated, active process. Finally, it has also been suggested that an NLS may serve a dual role in nuclear protein trafficking functioning as both an NLS and NES (Guiochon-Mantel et al., 1994; Moroianu and Blobel, 1995). These hypotheses may not be mutually exclusive, as multiple pathways of nuclear protein export may exist and use distinct soluble factors or nucleoporins.

In contrast to the rapid hormone-dependent nuclear import of GR ($t_{1/2}$ ~5–10 min) (Picard and Yamamoto, 1987; Yang and DeFranco, 1994), nuclear export of GR that follows hormone withdrawal appears to be a relatively slow process (Madan and DeFranco, 1993; Sackey et al., 1996). This protracted export is not related to the rate of hormone dissociation from the receptor, as previous studies have shown that the off-rate of natural glucocorticoids from GR upon hormone withdrawal is quite rapid, having a $t_{1/2}$ of ~10 min (Munck and Foley, 1976). Moreover, it has been reported that glucocorticoid-induced chromatin reorganization and transcriptional activation of the tyrosine aminotransferase gene (*TAT*) are rapidly reversed after hormone withdrawal (Reik et al., 1991). Thus, the rapid dissociation of hormone from the receptor, and the subsequent rapid inactivation of the receptor's transcriptional regulatory activity, are not directly coupled to a corresponding rapid release of receptors from nuclei.

We report here the results of in vitro studies of GR subnuclear trafficking using digitonin-permeabilized cells. In situ biochemical extractions were used to define a distinct subnuclear compartment that collects chromatin-released receptors and serves as a putative nuclear export staging area. The subsequent export of these poised receptors is stimulated in vitro upon treatment of permeabilized cells with compounds that increase overall protein tyrosine phosphorylation. The corresponding stimulated export of select nuclear proteins under these conditions suggests that some aspects of NPC function may be influenced, either directly or indirectly, by a protein phosphotyrosine system(s).

Materials and Methods

Cells and Cell Culture

The GrH2 rat hepatoma cell line, which expresses elevated levels of GR (Howard et al., 1990), was maintained at 37°C in DME (Life Technologies, Bethesda, MD) supplemented with 5% FBS (Irvine Scientific, Santa Ana, CA). HeLa cells were maintained in DME containing 10% FBS. VAN556/CHO, a CHO cell line expressing a stably transfected carboxyl-terminal deletion mutant of GR (Tang and DeFranco, 1996), was maintained in DME plus 10% FBS, in the presence of 400 μg/ml G418 (GIBCO BRL, Gaithersburg, MD).

Antibodies

The BuGR2 mAb (Gametchu and Harrison, 1984) and BL.11.124 rabbit antiserum (Yang and DeFranco, 1994) were used to detect GR. TSTA (Yang and DeFranco, 1994) and UPS56 (Czar et al., 1995) rabbit antiserum were used to detect hsp90 and hsp56 proteins, respectively. Ab-1 is a mouse mAb against the NuMA nuclear matrix protein (Oncogene Science, Cambridge, MA). The anti-hnRNP C mAb, 4F4, and anti-hnRNP A1 mAb, 9H10, were kindly provided by Dr. G. Dreyfuss (University of Pennsylvania School of Medicine, Philadelphia). The PY20 anti-phosphotyrosine mAb was purchased from Transduction Laboratories, Inc. (Lexington, KY).

Hormone Withdrawal

GrH2 cells were grown in DME plus 5% charcoal-stripped FBS for at least 16 h before treatment with 10^{-6} M corticosterone (Sigma Chemical Co., St. Louis, MO) for 1 h. Cells were briefly rinsed three times in DME plus 5% stripped FBS, and then incubated with hormone-free medium for lengths of time indicated.

Cell Permeabilization

Cells were grown on glass coverslips (22 × 22 mm) in 35-mm-diam plastic petri dishes in 2 ml of indicated medium. After hormone withdrawal, cells were washed twice with ice-cold transport buffer (20 mM Hepes, pH 7.3, 110 mM KOAc, 5 mM NaOAc, 2 mM $Mg(OAc)_2$, 1 mM EGTA, 2 mM DTT, and 1 μg [each] of protease inhibitors aprotinin, leupeptin, and pepstatin A per ml) (Yang and DeFranco, 1994), and then permeabilized by immersing the coverslips in ice-cold transport buffer containing 40 μg of digitonin (Sigma Chemical Co.) per ml for 5 min. All traces of buffer were

then removed by aspiration, and the coverslips were washed twice with ice-cold transport buffer.

In Situ Nuclear Extraction

Hypotonic Buffer Extraction. Permeabilized cells were immersed briefly in water before treatment with 2 ml of hypotonic (Hypo) buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.1% Triton X-100, 1 μg/ml of each of protease inhibitors) (Tang and DeFranco, 1996) for 3 min at 0°C. Cells were then washed twice with transport buffer and fixed with cold (−20°C) methanol.

Cytoskeletal Buffer Extraction. Permeabilized cells were treated for 5 min on ice with 2 ml of cytoskeletal (CK) buffer (10 mM Pipes, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, and protease inhibitors). Cells were then washed twice with transport buffer and fixed with methanol.

Nuclear Extraction from Cell Suspensions

Cells were grown in 60-mm tissue-culture dishes to ~60–70% confluency. After hormone addition and subsequent hormone withdrawal, cells were harvested and washed twice with ice-cold transport buffer. Cells were then permeabilized for 7 min on ice in 1 ml transport buffer containing 80 μg/ml digitonin, after which the suspension was diluted 10-fold with ice-cold transport buffer. Intact nuclei were recovered by centrifugation at 600 *g* and resuspended in the same buffer. Each nuclear suspension was aliquoted as indicated. One aliquot (~$5–8 \times 10^5$ nuclei) was incubated with 300 μl of ice-cold Hypo buffer for 3 min. An identical aliquot of nuclei was incubated with 300 μl of ice-cold CK buffer for 5 min. The Hypo or CK buffer extracted nuclei, as well as an aliquot of untreated nuclei, were washed twice with transport buffer and dissolved in high salt lysis buffer (10 mM Hepes, pH 7.0, 450 mM NaCl, 5 mM EDTA, 0.05% SDS, 1% Triton X-100, 2 mM DTT, and protease inhibitors). The lysates were mixed with 4× SDS sample buffer (132 mM Tris-HCl, pH 6.8, 20% glycerol, 10% SDS, 10.4% β-mercaptoethanol, 0.02% pyronin Y), boiled for 10 min, and then subjected to SDS-PAGE.

Chromatin Mini-Cycle

For in vivo mini-cycle experiments, GrH2 cells were treated with 10^{-6} M corticosterone for 1 h, withdrawn from hormone for 30 min, and then refed with hormone-containing medium for 10 min. Cells were permeabilized using digitonin either on coverslips or in suspension as described above, and then subjected to Hypo buffer extraction.

For in vitro mini-cycle experiments, permeabilized cells were incubated with 50 μl of transport mixture (Yang and DeFranco, 1994) that contained 30% HeLa cytosol diluted in transport buffer, 10 mg/ml BSA, 2 mM ATP, 5 mM creatine phosphate (Sigma Chemical Co.), 20 U/ml of creatine phosphokinase (Sigma Chemical Co.), and 1 μM corticosterone where indicated. The reaction mixture was incubated at 30°C for 20 min. Intact nuclei were subjected to either Hypo or CK buffer extraction, as described above.

In Vitro Nuclear Export

For in vitro nuclear export assays, permeabilized cells were incubated with 50 μl of transport mixture that contained 10 μg/ml BSA, 2–4 mM ATP, 5 mM creatine phosphate, 20 U/ml creatine phosphokinase, and 20 mM sodium molybdate (Na_2MoO_4). In some experiments, sodium molybdate was replaced by either 20 mM sodium tungstate (Na_2WO_4), 7.5 mM sodium vanadate (Na_2VO_4), or 50 μg/ml heparin (Sigma Chemical Co.). Likewise, ATP was sometimes replaced by 4 mM GTP. In addition, 0.25 mM genistein (Sigma Chemical Co.), 0.2 μg/ml WGA (Sigma Chemical Co.), 1 mM tyrphostin AG126 (Sigma Chemical Co.), or a 1:500 dilution of the Ab-1 anti-NuMA antibody were also included where indicated. AMP-PNP (Sigma Chemical Co.) or GTPγS, when included, were used at a final concentration of 4 mM. After a 20-min incubation at 30°C, intact nuclei on coverslips were washed twice in transport buffer and fixed with methanol, and GR was visualized by indirect immunofluorescence (IIF). For suspension assays, intact nuclei were washed twice with transport buffer supplemented with 150 mM NaCl and 20 μM of cytochalasin B (Sigma Chemical Co.), and then dissolved in high salt lysis buffer for Western blot analysis.

Indirect Immunofluorescence

GR in methanol-fixed cells was visualized by IIF, typically using BuGR2 as previously described (Yang and DeFranco, 1994). For double staining of GR and NuMA, methanol-fixed cells were incubated with the BL11.124 anti–GR rabbit serum and the Ab-1 anti-NuMA mAb. A TRITC-coupled anti–rabbit IgG antibody (Sigma Chemical Co.) and an FITC-coupled anti–mouse IgG antibody (Boehringer Mannheim Biochemicals, Indianapolis, IN) were used as secondary antibodies to detect GR and NuMA, respectively. For double staining of hnRNP A1 and hsp56, or double staining of hnRNP C and hsp90, cells were fixed with 4% paraformaldehyde at room temperature for 15 min, washed three times in PBS, permeabilized with −20°C acetone for 5 min, and finally washed three times with PBS. Primary staining was carried out using the 9H10 anti–hnRNP A1 mAb and UPS56 anti–hsp56 rabbit serum, or the 4F4 anti–hnRNP C mAb and TSTA anti–hsp90 rabbit serum. 4′,6-diamidino-2-phenylidole (DAPI; Sigma Chemical Co.) was included in all secondary incubations to stain DNA. Stained cells were observed by fluorescence microscopy through an Optiphot-2 microscope (Nikon Inc., Garden City, NY) and photographed with T-Max 400 film (Eastman-Kodak Co., Rochester, NY).

Western Blot Analysis

GRs from intact or extracted nuclei were detected by Western blot analysis using BuGR2 as previously described (Yang and DeFranco, 1994). As internal controls, NuMA and hnRNP A1 were also detected on the same blots using Ab-1 and 9H10 antibodies, respectively. Phosphotyrosine-containing proteins were detected using the PY20 mAb. In this case, the immobilon membrane was blocked with 5% BSA after Western transfer. Autoradiographs obtained from enhanced chemiluminescence (ECL; Amersham Intl., Amersham, UK) detection were quantified using an XRS 3cx scanner (Millipore Corp. Imaging Systems, Ann Arbor, MI).

Results

GRs Are Rapidly Released from Chromatin upon Hormone Withdrawal and Accumulate within a Biochemically Distinct Subnuclear Compartment

Unliganded cytoplasmic GRs undergo rapid nuclear import upon ligand binding (Picard and Yamamoto, 1987; Yang and DeFranco, 1994). While this regulated translocation through the NPC is reversed upon hormone withdrawal, the rate of GR nuclear export is considerably slower than that of receptor import (Madan and DeFranco, 1993; Sackey et al., 1996). As the dissociation of natural hormone ligands from GR, such as corticosterone, is quite rapid upon hormone withdrawal (Munck and Foley, 1976), hormone release is not kinetically coupled to receptor nuclear export. We have therefore used a coupled biochemical/cell biological approach to investigate the mechanisms that might operate to limit GR nuclear export. Two types of in situ extractions were used to distinguish GR subpopulations with alternative nuclear affinities. Hypo buffer was used to extract nuclear receptors bound with low affinity, while CK buffer was used to extract tightly bound nuclear receptors. CK buffer is commonly used as the first extraction step in nuclear matrix preparations (Tang and DeFranco, 1996). Importantly, by assessing nuclear localization of receptors in intact and permeabilized cells, nuclear receptors could be visualized, irrespective of the relative strength of their interactions with nuclei.

GrH2 rat hepatoma cells were treated with hormone for 1 h, and then withdrawn from hormone for various lengths of time. We used the natural glucocorticoid hormone, corticosterone, rather than synthetic analogs such as dexamethasone, since natural hormones rapidly dissociate from receptors after hormone withdrawal (Munck and Brinck-Johnsen, 1968). Hormone-withdrawn cells were

permeabilized with digitonin, and the resultant semiintact cells were either fixed directly or subjected to different in situ extractions. GRs were detected by either IIF or Western blot analysis using the BuGR2 antibody. As internal controls, NuMA and hnRNP A1 proteins were also detected on the same Western blots. NuMA is a component of nuclear matrix (Lyderson and Pettijohn, 1980), while hnRNP A1 is an RNA-binding protein that is also associated to some extent with the nuclear matrix (Dreyfuss et al., 1993).

Fig. 1 shows the differential extraction of GR from nuclei of hormone-treated vs hormone-withdrawn cells. GRs accumulated in nuclei after a 1 h of corticosterone treatment (Fig. 1 *a*, *A*, and Fig. 1 *b*, lane *1*) and remained nuclear after 30 min of hormone withdrawal (Fig. 1 *a*, *D*, and Fig. 1 *b*, lane *4*). While Hypo buffer extraction of permeabilized cells removed ~20% of the nuclear GR from hormone-treated cells (Fig. 1 *a*, *B*; Figs. 1 *b* and *c*, lane *2*), 80% of nuclear GRs were extracted by Hypo buffer from hormone-withdrawn cells (Fig. 1 *a*, *E*; Fig. 1, *b* and *c*, lane *5*). Thus, although a brief hormone withdrawal does not apparently alter the nuclear localization of GRs, unliganded and liganded nuclear receptors differ dramatically in their nuclear affinity. Importantly, these results also establish that GR nuclear export is not merely restricted by high affinity binding of GR to nuclei. GRs in hormone-

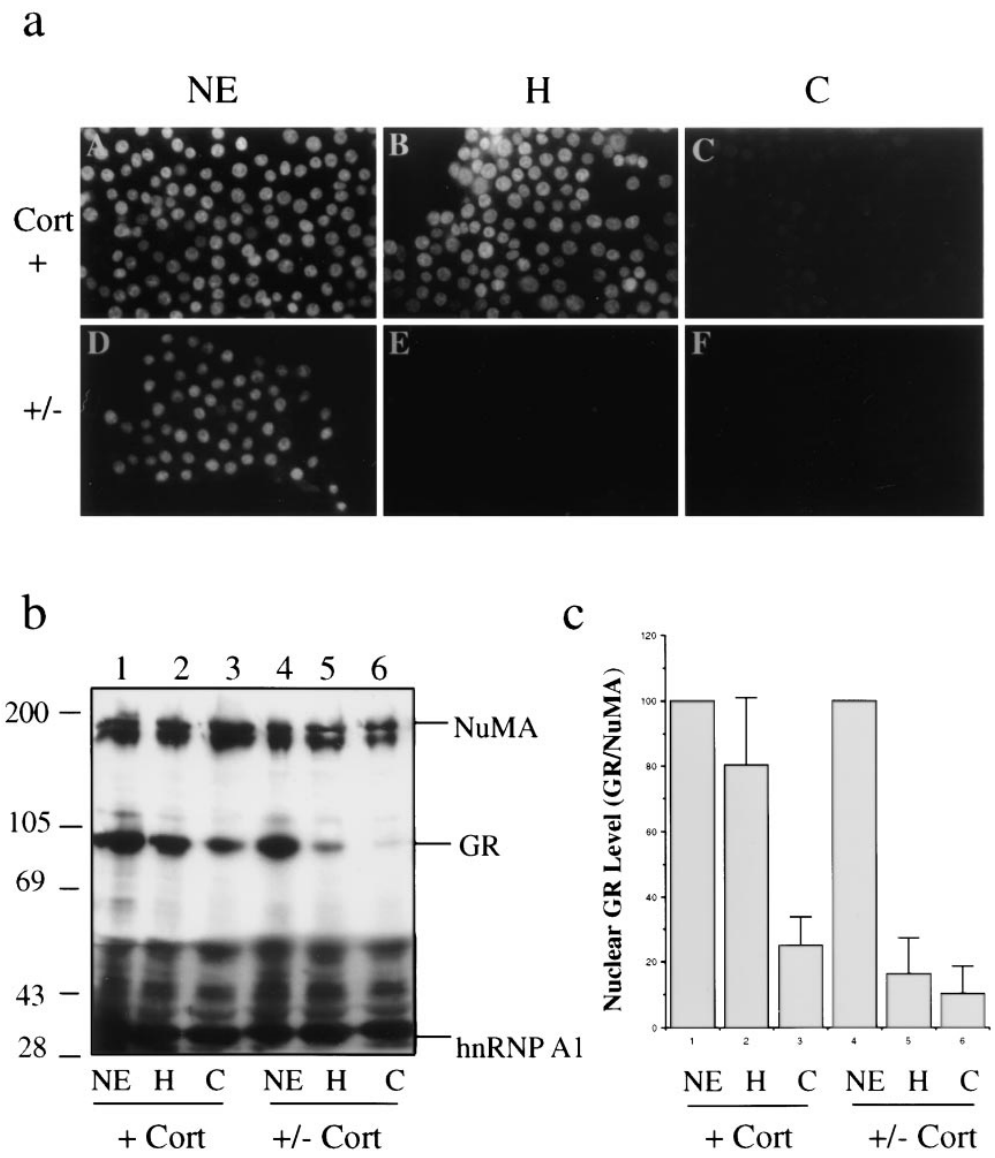

Figure 1. Differential extraction of GR from nuclei of hormone-treated vs hormone-withdrawn cells. GrH2 cells grown in hormone-free medium were treated with 10^{-6} M corticosterone (*Cort*) for 1 h, and then either incubated with hormone for an additional 30 min (*a*, *A–C*; *b*, lanes *1–3*) or withdrawn from hormone for 30 min (*a*, *D–F*; *b*, lanes *4–6*). Cells were either permeabilized using digitonin (*a*, *A* and *D*; *b*, lanes *1* and *4*), or permeabilized and then subjected to extractions with either Hypo buffer (*a*, *B* and *E*; *b*, lanes *2* and *5*) or CK buffer (*a*, *C* and *F*; *b*, lanes *3* and *6*). (*a*) In situ extraction of cells grown on coverslips. GR was visualized by IIF using BuGR2. (*b*) Differential extraction of GR from cells in suspension. After permeabilization and extraction in suspension, nuclear proteins were resolved by SDS-PAGE. GR, NuMA, and hnRNP A1 were detected by Western blot analysis and ECL using BuGR2, the Ab-1 anti-NuMA, and the 9H10 anti–hnRNP A1 mAbs, respectively. (*c*) Quantification of results in *b* by densitometry (mean ± SD of four experiments). The relative ratio of GR to NuMA in intact nuclei (*NE*) was set at 100. *NE*, intact GrH2 nuclei after permeabilization, not extracted; *Hypo* and *H*, nuclei after permeabilization and Hypo buffer extraction; *CK* and *C*, nuclei after permeabilization and CK buffer extraction.

treated cells are not artificially trapped within nuclei by our permeabilization conditions, as a high salt, detergent wash (i.e., CK buffer) efficiently extracts 80% of nuclear GR (Fig. 1 *a*, *C* and *F*; Fig. 1, *b* and *c*, lanes *3* and *6*). DAPI staining confirmed that nuclei remained intact after this extraction (not shown). The residual amount of GR that resists CK extraction (Fig. 1, *b* and *c*, lane *3*) may represent nuclear matrix–associated receptors (Tang and DeFranco, 1996).

Hormone-bound GRs are known to associate with chromatin upon their import into nuclei (Yamamoto and Alberts, 1976). To confirm that hormone-bound nuclear GRs in Hypo buffer–extracted cells were chromatin bound, hormone-treated, permeabilized cells were subjected to a DNase I digestion after the Hypo buffer extraction. In agreement with our previous results, GRs in hormone-treated, permeabilized cells were resistant to Hypo buffer extraction (Fig. 2 *A*). However, treatment of Hypo buffer–extracted cells with DNase I led to the loss of nearly all GRs from most nuclei (Fig. 2 *E*). If the extent of DNase I digestion was varied, the relative amount of hormone-bound GR that remained nuclear in Hypo buffer–extracted cells was correlated with the relative amount of DNA that remained within nuclei (Fig. 2, *C* and *D*). The nuclear matrix protein NuMA was not significantly extracted by either Hypo buffer or DNase I digestion (not shown), indicating that the nuclear matrix remained intact under these conditions. Thus, since chromatin-bound receptors in hormone-treated cells are predominantly resistant to hypotonic extraction, it appears likely that the sensitivity of GRs to Hypo buffer extraction in hormone-withdrawn cells is due to their rapid release from chromatin.

To examine the kinetics of GR release from chromatin upon hormone withdrawal, we performed a time course study of GR sensitivity to hypotonic extraction. As shown previously, GRs in hormone-treated cells are relatively resistant to Hypo buffer extraction (Fig. 3 *A*). However, within 10 min of hormone withdrawal, most GRs were extracted by Hypo buffer (Fig. 3 *C*). Nuclear GRs appeared to be maximally extracted within 30 min of hormone withdrawal (Fig. 3 *E*). As noted above, in cells permeabilized after a 30-min hormone withdrawal, GRs localized within nuclei (Fig. 3 *G*). These results suggest that, while GRs are rapidly released from chromatin after dissociation of hormone (i.e., within 30 min), they remain associated with nuclei. Importantly, in hormone-withdrawn cells, the kinetics of bulk GR dissociation from chromatin corresponds precisely with the reversal of glucocorticoid-induced nuclease hypersensitivity within chromatin of the glucocorticoid-responsive *TAT* gene (Reik et al., 1991). Thus, hormone withdrawal leads to release of both bulk GRs from chromatin, and GRs bound to chromatin at specific target genes. While the possibility that unliganded nuclear GRs remain loosely bound to chromatin cannot be excluded, we will refer to these receptors as localizing within a distinct subnuclear compartment, with the caveat that this has only been defined biochemically and may not represent a unique structural component of the nucleus. As will be re-

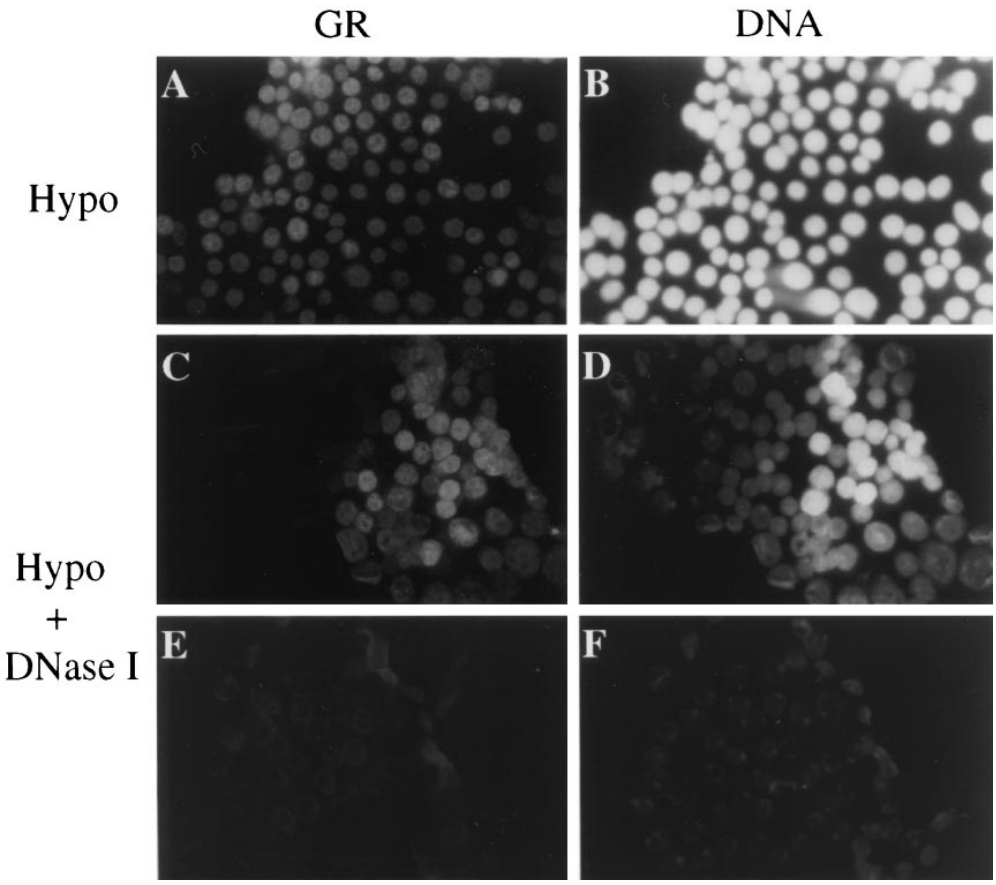

Figure 2. Hormone-bound nuclear GR in Hypo buffer–extracted cells is associated with chromatin. GrH2 cells grown on coverslips were treated with 1 μM of corticosterone for 1 h. After permeabilization with digitonin, nuclei were either extracted with Hypo buffer (*A* and *B*), or extracted and then incubated with 100 μg/ml of RNase-free DNase I for 30 min at room temperature (*C–F*). GR (*A*, *C*, and *E*) and DNA (*B*, *D*, and *F*) in fixed cells were visualized by IIF using BuGR2 and DAPI staining, respectively.

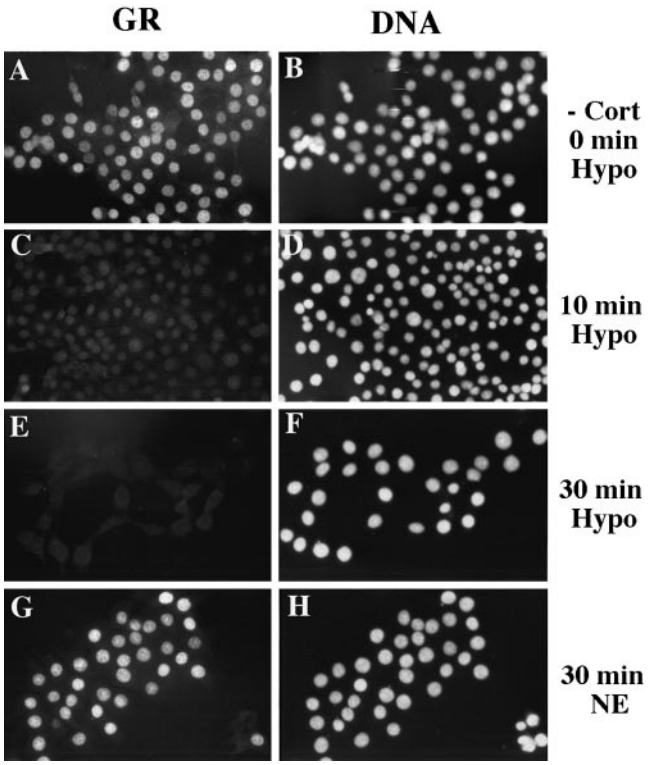

Figure 3. Time course of GR sensitivity to Hypo buffer extraction. GrH2 cells grown on coverslips were treated with 1 μM corticosterone (*Cort*) for 1 h. Cells were then withdrawn from hormone for either 0 min (*A* and *B*), 10 min (*C* and *D*), or 30 min (*E–H*). After permeabilization, cells were either fixed directly (*G* and *H*) or subjected to Hypo buffer extraction before fixation (*A–F*). (*A*, *C*, *E*, and *G*) GR staining detected by IIF. (*B*, *D*, *F*, and *H*) DNA detected by DAPI staining.

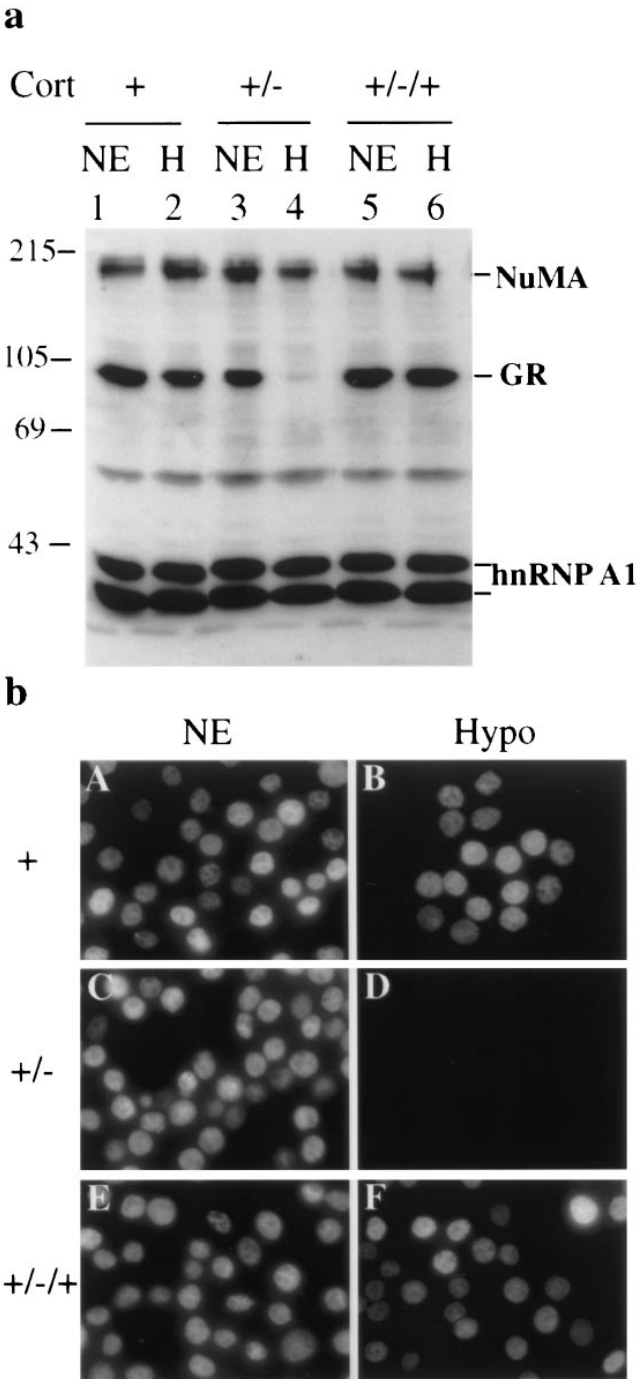

Figure 4. In vivo recycling of nuclear GRs. GrH2 cells were either treated with corticosterone (*Cort*) for 1 h (*a*, lanes *1* and *2*; *b*, *A* and *B*), withdrawn from hormone for 20 min after the 1-h hormone treatment (*a*, lanes *3* and *4*; *b*, *C* and *D*), or treated with hormone for an additional 10 min after the 20-min withdrawal (*a*, lanes *5* and *6*; *b*, *E* and *F*). (*a*) Suspension assay: cells were harvested and either permeabilized and not extracted (*NE*) or permeabilized and then extracted with Hypo buffer (*H*) in suspension. Nuclear GRs were detected by Western blot analysis. NuMA and hnRNP A1 proteins were also visualized on the same blot. (*b*) Coverslip assay: cells grown on coverslips were directly permeabilized and either not extracted (*NE*; *A*, *C*, and *E*) or extracted with Hypo buffer after permeabilization (*Hypo*; *B*, *D*, and *F*). Cells were fixed and GRs were detected by IIF.

vealed below, the subnuclear trafficking of these receptors is distinguished from that of chromatin-bound receptors.

Unliganded Nuclear GRs Rapidly Regain Their Capacity to Reassociate with Chromatin upon Rebinding Hormone

Since the rapid release of GR from chromatin upon hormone withdrawal is not coupled to its rapid nuclear export, receptors appear to be held within a distinct, low affinity subnuclear compartment. Upon prolonged hormone withdrawal, nuclear GRs redistribute to the cytoplasm where they eventually regain competence to respond to a secondary hormone challenge (Qi et al., 1989). Is cytoplasmic reentry obligatory for recycled receptors to regain functionality? Given the effectiveness of the hypotonic extraction in distinguishing between chromatin-bound and -released receptors, we asked whether GRs could regain high affinity chromatin binding if charged with hormone while still resident within nuclei. We therefore applied a secondary hormone treatment to GrH2 cells, which had previously been treated with corticosterone for 1 h but briefly withdrawn from hormone (i.e., 20 min). Nuclear GRs were detected by either Western blot analysis (Fig. 4 *a*) or IIF (Fig. 4 *b*) after either permeabilization and no extraction (*NE*), or permeabilization and Hypo buffer ex-

traction (*Hypo*; *H*). As shown previously, a 1-h hormone treatment led to the accumulation of nuclear GRs that were not extracted by Hypo buffer (Fig. 4 *a*, lanes *1* and *2*; Fig. 4 *b*, *A* and *B*). In addition, 20 min after hormone withdrawal, GRs remained nuclear (Fig. 4 *a*, lane *3*; Fig. 4 *b*, *C*) but were extracted by Hypo buffer (Fig. 4 *a*, lane *4*; Fig. 4 *b*, *D*), indicating that most GRs were released from chromatin. Interestingly, when hormone-withdrawn cells were briefly exposed to a 10-min secondary hormone treatment, GRs regained their resistance to hypotonic extraction (Fig. 4 *a*, lanes *5* and *6*; Fig. 4 *b*, *E* and *F*). Thus, nuclear GRs that have been released from chromatin during hormone withdrawal can rebind chromatin upon a secondary hormone exposure.

Since GR nuclear export is extremely slow, we did not expect considerable redistribution of receptors from the nucleus to the cytoplasm during the 20-min hormone withdrawal and 10-min secondary hormone treatment. However, to exclude the possibility that the redistribution of nuclear GRs under our limited hormone withdrawal (i.e., 20 min) was proceeding undetected, we performed the secondary hormonal stimulation in permeabilized cells. GrH2 cells were treated with hormone for 1 h, withdrawn from hormone for 20 min, and then permeabilized and washed extensively. Hormone was then added along with cytosol from receptor-negative HeLa cells, ATP, and an energy-regenerating system, and the permeabilized cells were incubated for 20 min at 30°C. We found that cytosol and ATP were required to prevent the quantitative association of GRs with the nuclear matrix that occurred upon in vitro 30°C incubation of permeabilized cells in buffer alone (not shown). If hormone was not included upon the incubation of permeabilized cells with cytosol and ATP, GRs remained nuclear yet were extractable by Hypo buffer (Fig. 5, *A* and *B*). However, under an identical incubation conducted in the presence of hormone, nuclear GRs regained their resistance to Hypo buffer extraction (Fig. 5, *C* and *D*), indicating that the receptors were now tightly bound to chromatin. Therefore, nucleocytoplasmic shuttling of GR includes not only a nuclear to cytoplasmic cycle (Orti et al., 1989; DeFranco et al., 1995; Sackey et al., 1996), but also a nuclear mini-cycle that permits receptors that release from chromatin to reassociate with chromatin upon hormone binding without an obligatory passage through the cytoplasm.

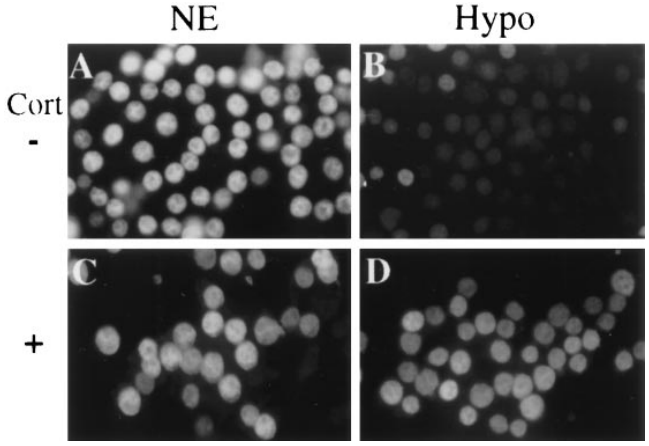

Figure 5. In vitro recycling of nuclear GRs. GrH2 cells grown on coverslips were treated with corticosterone (*Cort*) for 1 h, and then withdrawn from hormone for 30 min. Cells were then permeabilized and intact nuclei were incubated with 50 μl transport mixture containing HeLa cytosol, ATP, and an ATP-regenerating system for 20 min at 30°C, with (*C* and *D*) or without (*A* and *B*) 1 μM corticosterone present. Nuclei were then either directly fixed without extraction (*A* and *C*) or extracted with Hypo buffer before fixation (*B* and *D*). GRs were detected by IIF.

Stimulation of In Vitro GR Nuclear Export by Molybdate

The observation that, upon successive rounds of hormone treatment, withdrawal, and restimulation, GR can cycle between chromatin and a distinct low affinity nuclear compartment raises questions regarding the status of GR association with heat shock proteins. In cytosolic extracts, the binding of hormone to GR requires its association with the 90-kD heat shock protein, hsp90 (Bresnick et al., 1989; Pratt, 1993). In vivo, the association between hsp90 and GR is dynamic and influences both ligand binding and nucleocytoplasmic shuttling (Yang and DeFranco, 1996). An essential aspect of our hypothesized GR nuclear minicycle is the rebinding of hormone to unliganded nuclear receptors that have been released from chromatin. Is hsp90 binding required for nuclear GR to regain the competence to bind hormone?

To test whether hsp90 plays a role in a GR nuclear minicycle, we used sodium molybdate in our permeabilized in vitro system to alter the dynamics of steroid receptor/hsp90 complex formation. This compound and related group VI-A transition metal oxyanions such as tungstate and vanadate have been shown to stabilize GR/hsp90 complexes (Dahmer et al., 1984). Thus, the GR nuclear minicycle might be disrupted if dynamic interactions between GR and hsp90 were altered by molybdate. GrH2 cells subjected to an identical hormone withdrawal regimen as described previously were permeabilized and then incubated with cytosol and an ATP-regenerating system in the presence of sodium molybdate. To our surprise, nuclear GR levels were dramatically reduced when sodium molybdate was included in the incubation of hormone-withdrawn permeabilized cells with cytosol and ATP (not shown). In fact, a 20-min incubation of hormone-withdrawn permeabilized cells at 30°C with 20 mM sodium molybdate and ATP, in the absence of cytosol, led to a reduction of GR nuclear staining (Fig. 6 *B*). DAPI staining confirmed the integrity of treated nuclei (data not shown).

It must be emphasized that these permeabilized cells have not been extracted and, as shown in Fig. 6 *A*, GR is effectively retained within nuclei of permeabilized cells incubated under identical conditions (i.e., 20-min incubation at 30°C, with ATP) in the absence of sodium molybdate. Molybdate exerted no apparent effect on GR nuclear retention at 0°C (Fig. 6 *C*) or in the absence of ATP (Fig. 6 *D*). Nonhydrolyzable analogs ATPγS and AMP-PNP cannot substitute for ATP (data not shown), demonstrating that ATP hydrolysis is required for the molybdate effects on nuclear efflux of GR. We believe that the loss of nuclear GR in the presence of ATP and sodium molybdate repre-

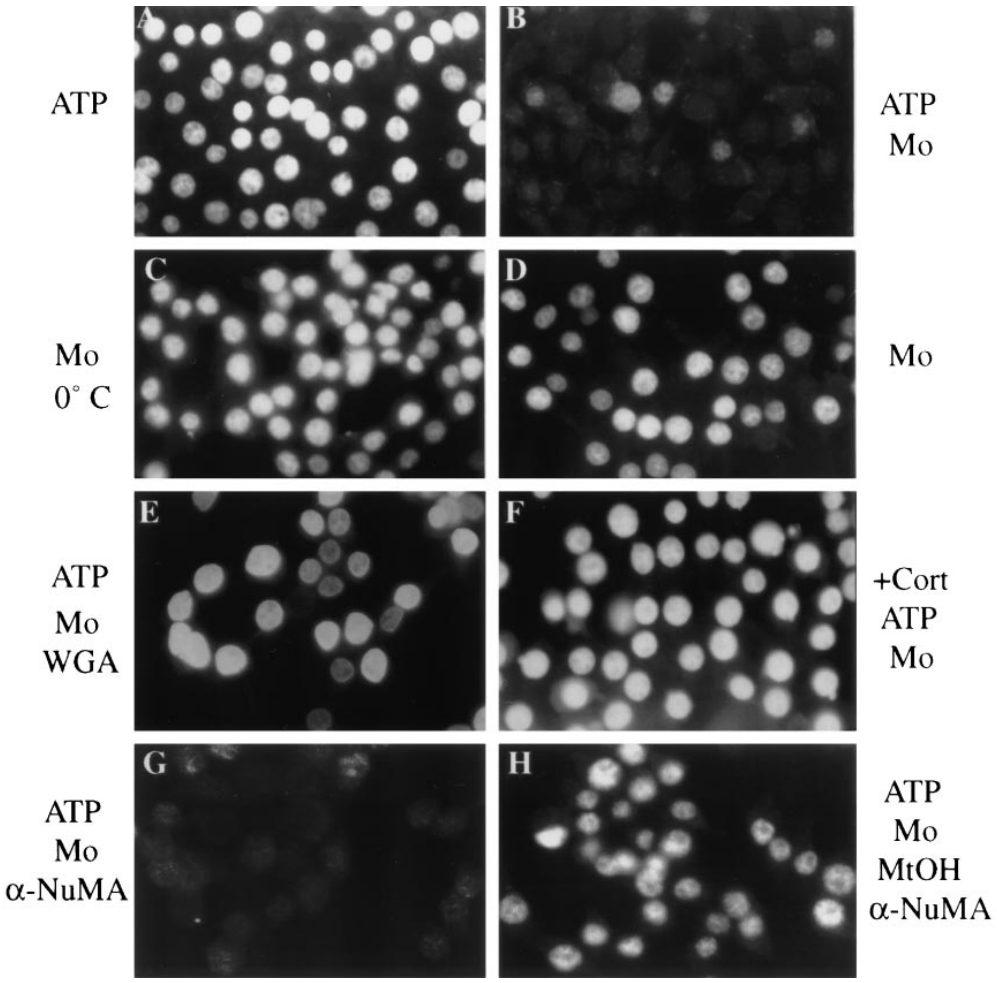

Figure 6. Stimulation of in vitro GR nuclear export by sodium molybdate. Hormone-withdrawn GrH2 cells (*A–E*, *G*, and *H*) were permeabilized and then incubated with 10 mg/ml of BSA in transport buffer at 30°C (*A*, *B*, and *D–H*) or 0°C (*C*) with the following additions: (*A*) ATP; (*B* and *C*) ATP and 20 mM sodium molybdate; (*D*) 20 mM sodium molybdate; (*E*) 0.2 mg/ml WGA, ATP, and 20 mM sodium molybdate; (*F*) corticosterone-treated GrH2 cells were permeabilized and then incubated for 20 min at 30°C with ATP and 20 mM sodium molybdate; (*G*) ATP, 20 mM sodium molybdate, and the Ab-1 anti-NuMA antibody; and (*H*) ATP and 20 mM sodium molybdate. In *A–F*, cells were fixed with methanol and subjected to IIF to detect GR. In *G*, permeabilized cells were fixed after the incubation described above and treated with a FITC-coupled secondary antibody to detect the Ab-1 anti-NuMA primary antibody. In *H*, ATP- and molybdate-treated permeabilized cells were fixed and then subjected to IIF with the Ab-1 antibody to detect nuclear NuMA.

sents the export of receptors through the NPC, given its sensitivity to WGA (Fig. 6 *E*). In some cells, GR accumulated at the nuclear rim in the presence of molybdate, ATP, and WGA (Fig. 6 *E*), which could represent receptors trapped at the NPC in the process of export. If GrH2 cells were maintained in hormone-containing medium before permeabilization, GRs remained predominantly nuclear after molybdate and ATP treatment (Fig. 6 *F*). Thus, molybdate stimulation of GR nuclear export requires that receptors are released from chromatin. In addition, this result establishes that nuclear GR is not merely leaking from nuclei that may have been damaged by molybdate treatment. The inability of an anti-NuMA antibody to enter the nucleus of ATP and molybdate-treated permeabilized cells (Fig. 6 *G*) provides independent confirmation of the integrity of the nuclear envelope. As shown in Fig. 6 *H*, this same antibody gained access to nuclei if permeabilized cells were fixed with methanol after ATP and molybdate treatment.

The results of the IIF assay were confirmed by Western blots, which analyzed GR nuclear export from permeabilized cells maintained in suspension. This assay provided a more quantitative assessment of in vitro GR nuclear export that was particularly useful for dose–response analysis of the various compounds tested (Table I). GrH2 cells were treated with hormone for 1 h, and then either withdrawn from hormone for 20 min (Fig. 7, lanes *1–8*) or maintained in hormone-containing medium for an additional 20 min (Fig. 7, lanes *9–12*). After permeabilization, intact nuclei were incubated with BSA at 30°C for 20 min, with or without sodium molybdate and/or ATP. Nuclear suspensions were split into two identical samples after the in vitro incubation. SDS sample buffer was added to one sample, which was immediately subjected to SDS-PAGE and Western blot analysis to reveal overall GR levels and the integrity of the receptor (Fig. 7, lanes *1–4*). The other sample was washed, and GR remaining within nuclei was visualized by Western blot analysis (Fig. 7, lanes *5–12*). Similar amounts of intact GR were recovered under all conditions (Fig. 7, lanes *1–4*), indicating that the reduction in nuclear GR levels that occurred upon sodium molybdate treatment (Fig. 7, lanes *6* and *7*) resulted from active GR nuclear export and not degradation. 20 mM sodium molybdate was chosen for subsequent assays since it was effective in the stimulation of GR nuclear export (Table I) and did not generate abnormal nuclear morphology some-

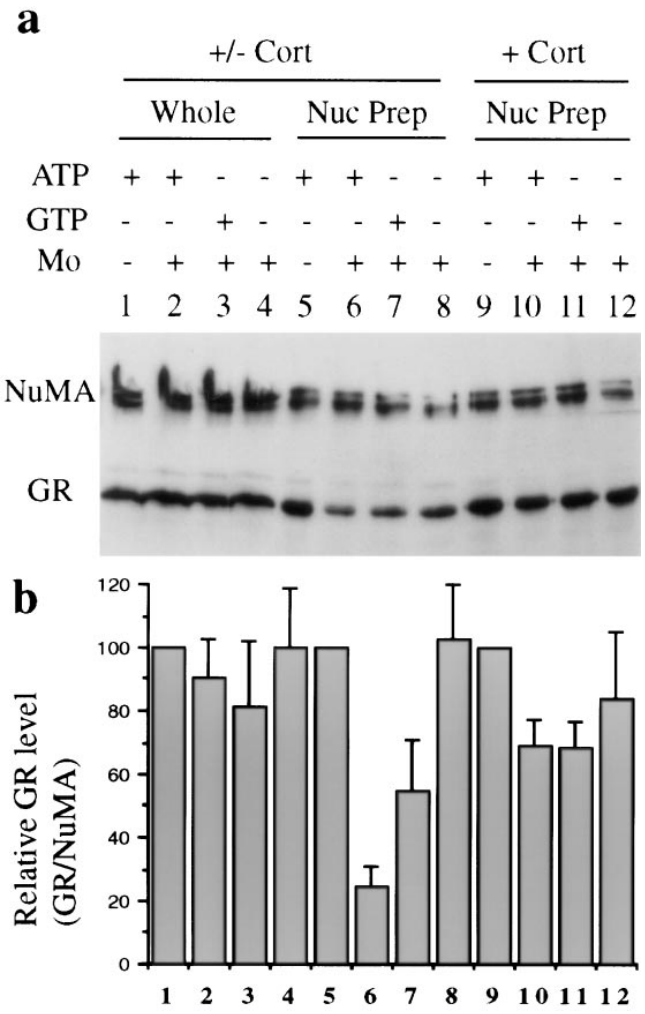

Figure 7. (*a*) Western blot analysis of molybdate-stimulated in vitro GR nuclear export. (Lanes *1–8*) GrH2 cells treated with corticosterone (*Cort*) for 1 h and then withdrawn from hormone for 20 min. (Lanes *9–12*) GrH2 cells treated with corticosterone for 80 min. Harvested cells were permeabilized in suspension, and aliquots of intact nuclei were incubated with 50 μl of reaction mixture containing 10 mg/ml BSA in transport buffer, 4 mM ATP (lanes *1*, *2*, *5*, *6*, *9*, and *10*), or 4 mM GTP (lanes *3*, *7*, and *11*) with an energy-regenerating system, and 20 mM sodium molybdate where indicated (lanes *2*, *3*, *4*, *6*, *7*, *8*, *10*, *11*, and *12*). After a 20-min incubation at 30°C, each nuclear suspension was split into two identical samples. One sample was subjected to SDS-PAGE directly (lanes *1–4*, *Whole*). The other identical sample was washed, and nuclei were recovered and subjected to SDS-PAGE for detection of the remaining nuclear GR (lanes *5–12*, *Nuc Prep*). (*b*) Quantification of GR levels observed in Western blots by densitometry. GR levels were normalized to the internal control NuMA protein. (Bars *1–8*) Average of four experiments; (bars *9–12*) average of two experiments. *Whole*, whole reaction mix; *Nuc Prep*, nuclear pellets.

Table I. Dose–Response Analysis of Protein Tyrosine Phosphatase and Kinase Inhibitor Effects on In Vitro GR Nuclear Export

Treatment	Concentration	Nuclear GR levels relative to NuMA
None		1
Na_2MoO_4	5 mM	0.64 ± 0.16
	20 mM	0.47 ± 0.05
	40 mM	0.39 ± 0.12
Heparin	25 μg/ml	0.80 ± 0.10 (n = 2)
	50 μg/ml	0.38 ± 0.12 (n = 2)
Na_2MoO_4 (20 mM)	0	0.45 ± 0.08 (n = 4)
+ genistein	125 μM	0.60 ± 0.17
	250 μM	0.83 ± 0.35 (n = 4)
	500 μM	0.98 ± 0.30 (n = 4)
Na_2MoO_4 (20 mM)	0	0.41 ± 0.07
+ tyrphostin AG126	50 μM	0.40 ± 0.02 (n = 2)
	200 μM	0.69 ± 0.04 (n = 4)
	1000 μM	0.84 ± 0.29

Hormone-withdrawn GrH2 cells were permeabilized with digitonin, and then incubated for 20 min at 30°C with 10 mg/ml BSA and 4 mM ATP with an ATP-regenerating system (see Materials and Methods). During the incubation period, either no additions were made, or heparin, Na_2MoO_4, genistein, or tyrphostin AG 126 was added at the indicated concentrations. The amount of GR remaining in nuclear fractions was quantified from Western blot analysis. GR levels are expressed relative to the amount of the nuclear matrix protein NuMA recovered in nuclear fractions, with GR levels in cells treated with ATP alone arbitrarily set to 1. Each value represents an average of three independent experiments ± SD unless otherwise indicated.

times associated with higher doses. In the suspension assay shown in Fig. 7, sodium molybdate treatment in the presence of ATP led to the export of ~80% of nuclear GR (lane *6*) while, in the presence of GTP, ~40% of nuclear GR was exported (lane *7*). Molybdate alone did not induce GR export in the absence of ATP or GTP (Fig. 7, lane *8*), or in the presence of nonhydrolyzable ATP or GTP analogs (i.e., ATPγS, AMP-PNP, or GTPγS, respectively, not shown). Finally, in cells that were not withdrawn from hormone so that GRs remained tightly bound to chromatin (Fig. 7, lanes *9–12*), molybdate exerted a limited effect on GR nuclear export. Thus, energy-dependent, in vitro nuclear export of GRs is most effective when receptors are released from chromatin.

To more precisely define the nucleotide requirement for in vitro nuclear export of GRs, different combinations of nucleotides and nonhydrolyzable nucleotide analogs were added. AMP-PNP effectively blocked molybdate-stimulated GR nuclear export observed in both ATP- (not shown) and GTP-treated permeabilized cells (Fig. 8 *D*), suggesting that export is mainly an ATP-driven process. This was corroborated by the fact that GTPγS was ineffective in preventing ATP-dependent GR nuclear export in molybdate-treated permeabilized cells (Fig. 8 *C*).

Molybdate Stimulation of GR Nuclear Export Does Not Reflect Cotransport of GR-hsp90 Complexes But a Generalized Effect on Nuclear Export

Since molybdate has been commonly used to stabilize steroid receptor–hsp90 complexes, we set out to determine whether this effect was related to the in vitro stimulation of GR nuclear export. When hormone-withdrawn, permeabilized GrH2 cells were treated with sodium molybdate, rapid ATP-dependent nuclear export of hsp90 and GR was observed (not shown), suggesting that GR and hsp90 might be coexported from nuclei. However, hsp90 nuclear export was not dependent on coexport of GR since hsp90

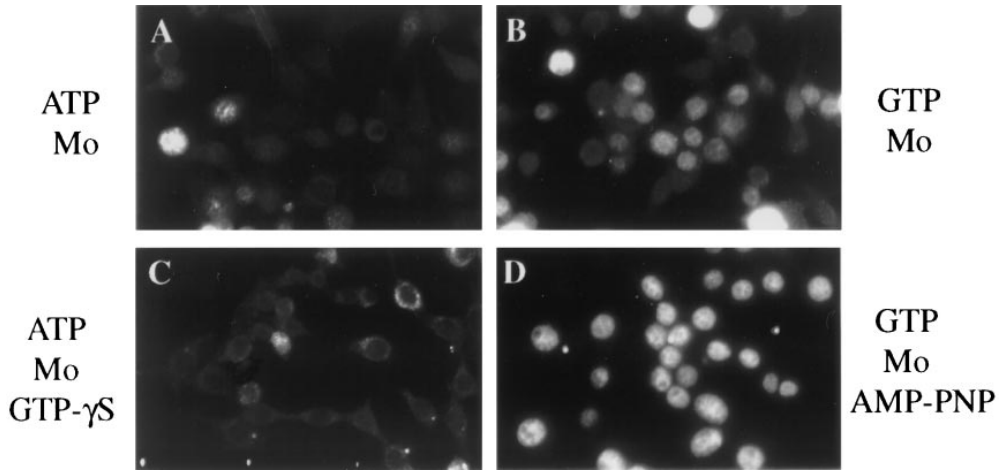

Figure 8. In vitro nuclear export of GR is an ATP-driven process. Hormone-withdrawn GrH2 cells were permeabilized and then incubated with 10 mg/ml of BSA in transport buffer containing 20 mM sodium molybdate at 30°C with the following additions: (*A*) 4 mM ATP; (*B*) 4 mM GTP; (*C*) 4 mM each of ATP and GTPγS; (*D*) 4 mM each of GTP and AMP-PNP. GR in fixed cells was detected by IIF.

still showed significant export in hormone-treated GrH2 cells in which GRs were not effectively exported (not shown). We therefore addressed the issue of GR/hsp90 coexport by examining the export of a GR carboxyl-terminal deletion mutant that does not associate with hsp90 (i.e., VAN556; Tang and DeFranco, 1996). As shown in Fig. 9, VAN556 expressed in stably transfected CHO cells also exhibited ATP-dependent in vitro nuclear export upon sodium molybdate treatment (Fig. 9 *C*). hsp90 nuclear export was also stimulated by sodium molybdate in the same cells (Fig. 9 *D*). These data strongly suggest that the rapid, energy-dependent in vitro nuclear export of unliganded GR and hsp90 brought about by sodium molybdate treatment is not due to coexport of GR–hsp90 complexes.

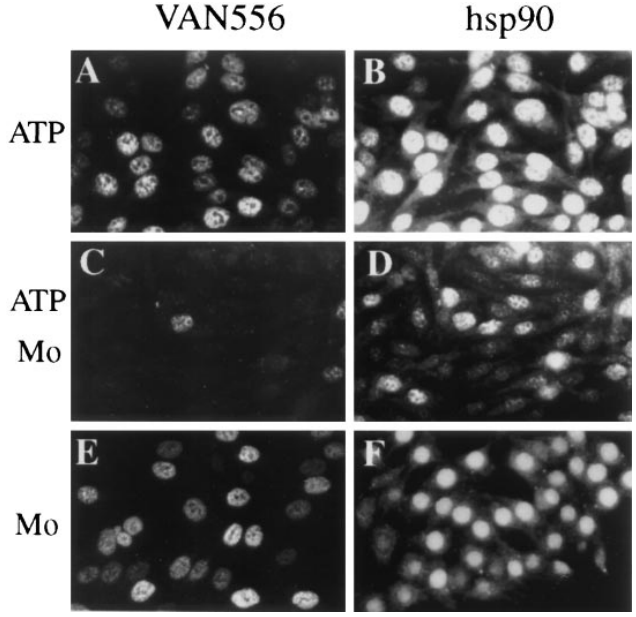

Figure 9. Molybdate-stimulated in vitro nuclear export of VAN556 GR and hsp90. CHO cells stably transfected with VAN556 GR were permeabilized and then incubated with 50 μl of 10 mg/ml BSA in transport buffer for 20 min at 30°C with the following additions: (*A* and *B*) 4 mM ATP; (*C* and *D*) 4 mM ATP and 20 mM sodium molybdate; (*E* and *F*) 20 mM sodium molybdate. GR (*A*, *C*, and *E*) and hsp90 (*B*, *D*, and *F*) were detected in fixed cells by costaining with the BuGR2 mAb and TSTA rabbit serum.

Since nuclear export of unliganded GR and that of hsp90 appear to be independent events which are both triggered by sodium molybdate, we expanded our analysis to include hsp56, another GR associated protein, and distinct members of the heterogeneous ribonuclear protein family that differ in their nucleocytoplasmic shuttling properties (i.e., hnRNP A1 and hnRNP C) (Pinol-Roma and Dreyfuss, 1992). In our assessment of in vitro nuclear export of hnRNP A1, hnRNP C, and hsp56, we used tungstate, a group VI-A transition metal oxyanion that was found to be more effective than molybdate. As shown in Fig. 10, in the absence of ATP, all four proteins were retained within nuclei of permeabilized cells after a 20-min incubation with sodium tungstate (Fig. 10, *A*, *C*, *E*, and *G*). However, in the presence of ATP, hnRNP A1 (Fig. 10 *B*) and hsp 90 (Fig. 10 *F*) efficiently exported from the nuclei after incubation with sodium tungstate while, in the same field of the cells, hsp56 (Fig. 10 *D*) and hnRNP C (Fig. 10 *H*), respectively, were retained within nuclei. Tungstate also induced rapid in vitro nuclear export of hsp70 (not shown). hsp90 is distributed in both the cytoplasm and nucleus (Gasc et al., 1990), indicating that it might be a shuttling protein, although this has not been experimentally verified. hsp56 is found predominantly within the nucleus (Czar et al., 1995) and its nucleocytoplasmic shuttling properties have not been assessed. The fact that molybdate and tungstate effects are exerted on hsp70, a shuttling protein, and a shuttling hnRNP protein (i.e., hnRNP A1) suggests that some component of the nuclear export machinery used by shuttling proteins may be targeted by metal oxyanions.

Molybdate Effects on In Vitro Nuclear Export Correlate with its Stimulation of Protein Tyrosine Phosphorylation

In addition to stabilizing GR–hsp90 complexes, metal oxyanions such as molybdate and tungstate exert multiple effects on cellular functions. Molybdate has been found to stimulate protein degradation (Modarress et al., 1994), disrupt GR association with specific RNAs (Rossini, 1987),

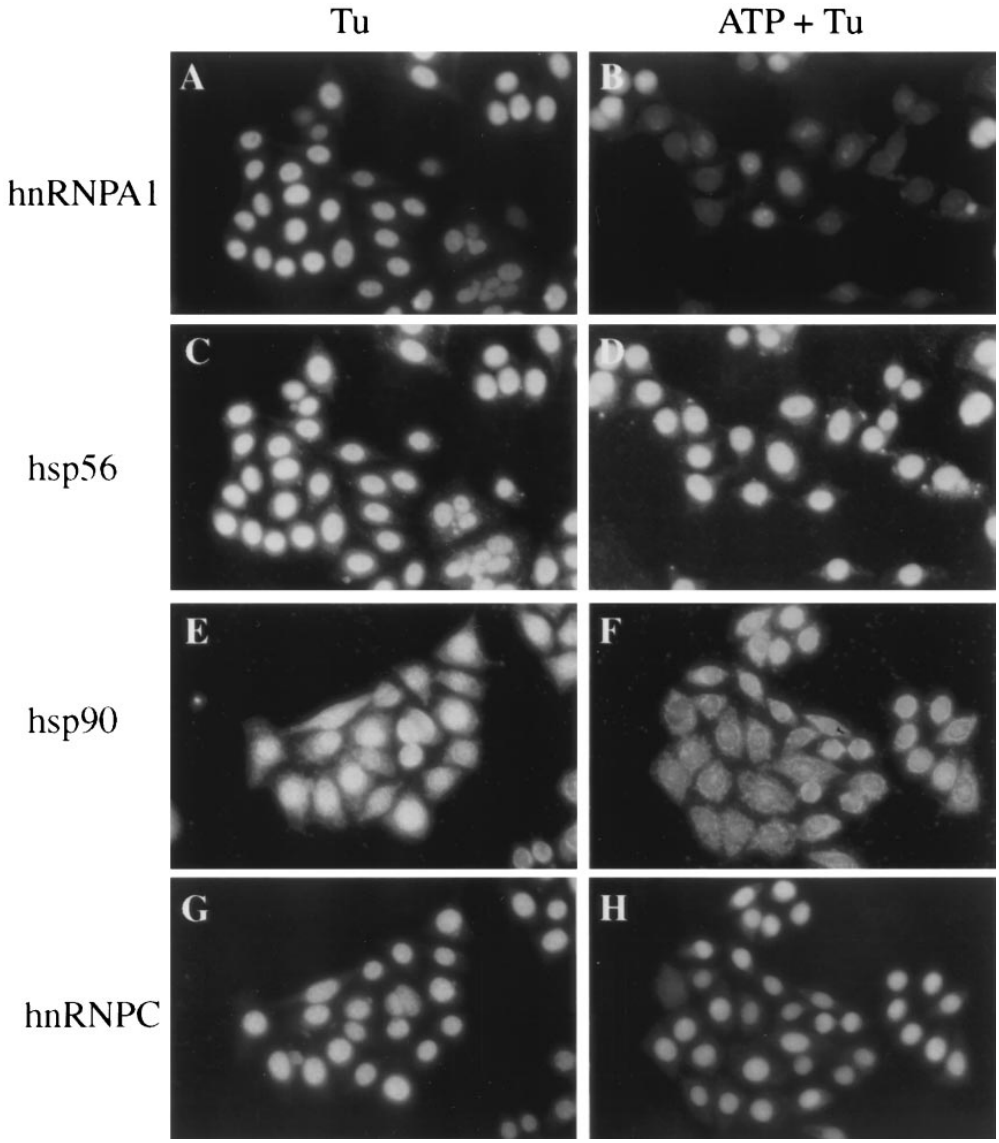

Figure 10. Selective effects of tungstate on in vitro nuclear protein export. Permeabilized HeLa cells were incubated for 20 min at 30°C with BSA in transport buffer containing 20 mM sodium tungstate, in the absence (*A*, *C*, *E*, and *G*) or presence of 4 mM ATP (*B*, *D*, *F*, and *H*). Fixed cells were subjected to costaining to detect either hnRNP A1 (*A* and *B*) and hsp56 (*C* and *D*), or hnRNP C (*G* and *H*) and hsp90 (*E* and *F*) in the same fields of cells.

and increase cellular cGMP levels by stimulating guanylate cyclase activity (Barsony and McKoy, 1992). So far, we have excluded these possibilities as potential reasons for molybdate-induced, energy-dependent in vitro nuclear export of GR, by observing that: (*a*) significant degradation of GR did not occur after molybdate treatment (Fig. 7, lanes *1–4*); (*b*) RNase A treatment of permeabilized cell in the absence of molybdate treatment did not lead to GR nuclear export while efficiently depleting hnRNP A1 from nuclei (not shown); and (*c*) addition of cGMP to permeabilized cells exerted no significant effect on GR nuclear export (not shown).

Molybdate and tungstate are also potent inhibitors of tyrosine phosphatases and, as a result, their treatment leads to increased protein tyrosine phosphorylation. Two other tyrosine phosphatase inhibitors, vanadate and heparin, also induced rapid in vitro nuclear export of GR (Fig. 11, *C* and *E*), while protein serine-phosphatase inhibitors microcystin (Fig. 11 *G*) and okadaic acid (not shown) were ineffective. As shown in Table I, 50 μg/ml heparin was most effective in stimulating in vitro nuclear export of GR, while analogous dose–response analysis showed that 7.5 mM sodium vanadate was an optimal concentration for stimulation of GR export (not shown). Given these results, we were intrigued by the possibility that molybdate effects on energy-dependent nuclear export may correlate with its stimulation of protein tyrosine phosphorylation. To test this hypothesis, we examined whether molybdate effects on nuclear export were blocked if tyrosine phosphorylation was inhibited. As shown in Fig. 11, molybdate-, vanadate-, or heparin-induced, ATP-dependent in vitro nuclear export of GR was inhibited by tyrosine kinase inhibitors genistein (Fig. 11, *B*, *D*, and *F*) or tyrphostin AG126 (Fig. 11 *H*). Dose–response analyses of genistein and tyrphostin AG126 effects on molybdate-stimulated GR nuclear export are shown in Table I. This inhibitory effect was specific for protein tyrosine kinase inhibitors, as a protein serine kinase inhibitor, staurosporin, was ineffective in blocking molybdate effects on GR nuclear export (not shown).

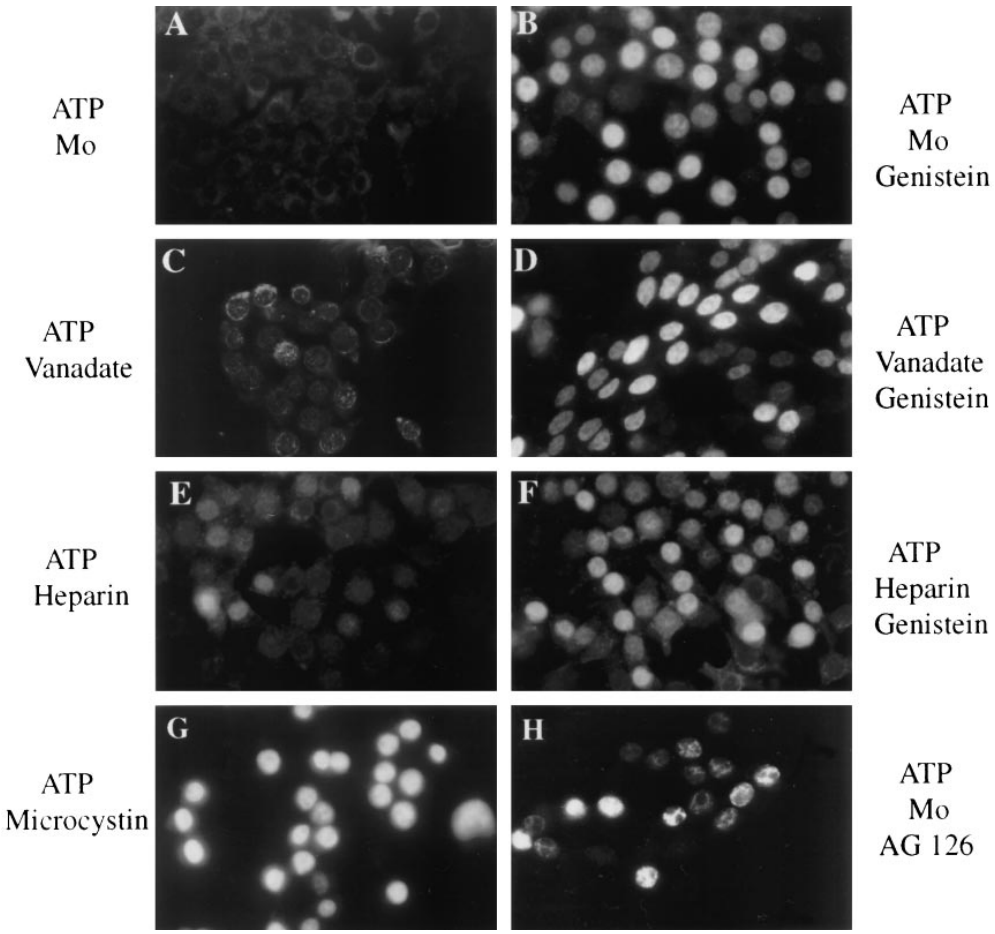

Figure 11. In vitro nuclear export of GR stimulated by tyrosine phosphatase inhibitors is blocked by the tyrosine kinase inhibitors. Permeabilized, hormone-withdrawn GrH2 cells were incubated with BSA in transport buffer containing 4 mM ATP for 20 min at 30°C, in the absence (*A*, *C*, *E*, *G*, and *H*) or presence (*B*, *D*, and *F*) of 0.2 mM genistein. 20 mM sodium molybdate was included in *A*, *B*, and *H*, 7.5 mM sodium vanadate in *C* and *D*, 50 μg/ml of heparin in *E* and *F*, 1 μM microcystin in *G*, and 1 mM tyrphostin AG126 (*AG 126*) in *H*. GR was detected in fixed cells by IIF.

To confirm that genistein blocked molybdate-stimulated protein tyrosine phosphorylation, we performed Western blots with an anti-phosphotyrosine antibody. These blots were also costained with GR and NuMA to confirm GR nuclear export visualized by IIF in Fig. 11. As shown in Fig. 12, relatively low levels of protein tyrosine phosphorylation were observed in permeabilized cells treated with either ATP (Fig. 12, lane *1*) or sodium molybdate alone (Fig. 12, lane *4*). Under these conditions, GR is effectively retained within nuclei. Treatment of permeabilized cells with ATP and molybdate dramatically increased the overall level of protein tyrosine phosphorylation, and correspondingly stimulated nuclear export of GR (Fig. 12, lane *2*). Thus, increased tyrosine phosphorylation induced by molybdate corresponds with the stimulation of GR nuclear export. In agreement with our IIF data (Fig. 11), genistein blocked molybdate-stimulated GR nuclear export (Fig. 12, *middle*). In addition, genistein also decreased the extent of molybdate-induced protein tyrosine phosphorylation in permeabilized cells (Fig. 12, lane *3*). Thus, our results are consistent with the possible involvement of some protein phosphotyrosine system in the nuclear export of GR and perhaps other shuttling proteins.

Discussion

In this report we have used an in vitro approach to analyze GR nuclear export. Steroid receptors have been shown to export from nuclei in vivo (Guiochon-Mantel et al., 1991; Chandran and DeFranco, 1992; Dauvois et al., 1993; Madan and DeFranco, 1993), but before our studies, this transport process had not been recapitulated in vitro. It has been known for many years that the tight nuclear binding that accompanies ligand binding to steroid receptors is rapidly reversed upon the dissociation of hormone (Munck and Foley, 1976). While transient heterokaryon assays established that GRs have the capacity to export from nuclei, this translocation appeared to proceed with much slower kinetics (Madan and DeFranco, 1993; Sackey et al., 1996) than the rapid hormone-induced nuclear import of receptors that occurs both in vivo (Picard and Yamamoto, 1987) and in vitro (Yang and DeFranco, 1994). This protracted nuclear export of GR appeared to be inconsistent with the rapid loss of high affinity nuclear binding of the receptor upon hormone dissociation (Munck and Foley, 1976). However, our results establish that, although GRs are rapidly released from chromatin upon hormone withdrawal, they are held in a distinct low affinity nuclear compartment that appears to serve as a nuclear export staging area. Unliganded GRs that collect within this staging area have alternative trafficking fates, as they have the capacity to either export from nuclei or reassociate with chromatin upon rebinding hormone.

The kinetics of nuclear GR chromatin cycling is rapid

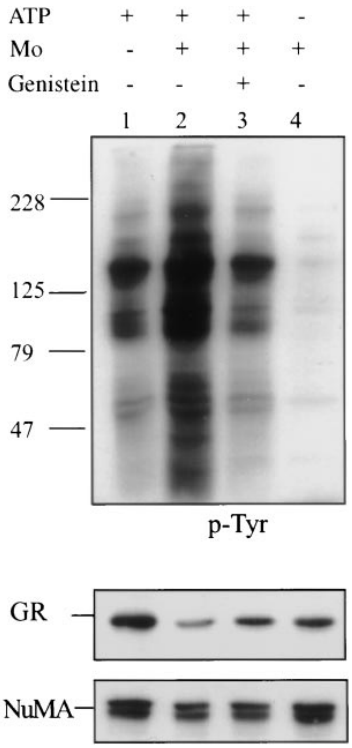

Figure 12. Genistein blocks molybdate stimulation of protein tyrosine phosphorylation and in vitro nuclear export of GR. Hormone-withdrawn GrH2 cells were permeabilized in suspension, and recovered nuclei were incubated with the following components: 4 mM ATP (lane *1*); 4 mM ATP and 20 mM sodium molybdate (lane *2*); ATP, molybdate, and 0.2 mM genistein (lane *3*); and molybdate (lane *4*). After a 20-min incubation at 30°C, nuclei were recovered and nuclear proteins were subjected to SDS-PAGE. Half of the sample was subjected to Western blot analysis to detect phosphotyrosine (*p-Tyr*) using the PY20 anti-phosphotyrosine mAb, while the other half of the sample was subjected to Western blot analysis to detect GR and NuMA.

both in vivo and in vitro. In addition, the reversible chromatin binding of bulk GRs, as monitored by our studies, is consistent with the kinetics of GR association with and dissociation from chromatin of specific target genes (Reik et al., 1991; Mymryk and Archer, 1995). Thus, it does not appear obligatory for unliganded GRs, which have been released from chromatin, to return to the cytoplasm to regain their hormone- and chromatin-binding capacity. The energy dependence of this chromatin cycle is consistent with GR recycling models elaborated by Munck and coworkers (Orti et al., 1989). However, the reuse of nuclear receptors in the apparent absence of cytoplasmic transport raises questions about the role of hsp90 in a nuclear cycle of hormone dissociation and reassociation. It is well established that GRs must be associated with hsp90 to have the capacity to bind hormone in vitro (Bresnick et al., 1989). This requirement may also apply in vivo, given the impact of disruptions in hsp90 function on GR hormone binding in yeast (Picard et al., 1990*a*; Bohen and Yamamoto, 1993). The nucleus possesses a number of chaperone proteins that participate in steroid receptor folding (Csermely et al., 1995), but whether these chaperones are required to reconstitute functional nuclear receptors is unknown. Unliganded nuclear steroid receptors released from chromatin in vivo may either maintain their competence to rebind hormone or use a distinct protein chaperone system to assist in folding transitions that impact their hormone-binding activity.

During our attempts to develop an in vitro nuclear export system for GRs, we made the surprising discovery that treatment of permeabilized cells with molybdate, a group VI-A transition metal oxyanion, induced a rapid, temperature- and ATP-dependent nuclear export of unliganded GRs. The molybdate-induced in vitro nuclear export of GR was greatly facilitated by the release of receptors from chromatin, as minimal effects of molybdate on GR nuclear export were observed if cells were not withdrawn from hormone before permeabilization. Thus, once released from chromatin, unliganded GR may be held in a low affinity, nuclear export staging area until directed to the NPC for export. Tungstate and vanadate, two other group VI-A transition metal oxyanions, were even more effective in stimulating in vitro GR nuclear export. The fact that our assays revealed nuclear export of GR was confirmed by the lack of molybdate effects on GR degradation and the sensitivity of this apparent nuclear export to WGA. A significant fraction of exporting GRs appeared to be trapped at the nuclear envelope in the presence of WGA, given the appearance of prominent nuclear rim staining.

Metal oxyanions are known to stabilize the association between GR and hsp90, but this appears unlikely to be responsible for the in vitro stimulation of GR nuclear export for a number of reasons. First, molybdate and tungstate treatment of permeabilized cells also stimulated the energy-dependent nuclear export of a GR mutant that does not interact with hsp90. Second, metal oxyanion effects on in vitro nuclear export were not restricted to GR, but also noted for hsp90, hsp70, and hnRNP A1. Not all nuclear proteins in permeabilized cells were exported upon metal oxyanion treatment in the presence of ATP, as hsp56 and hnRNP C were found to be retained within the nuclei of cells that supported the export of hnRNP A1 and hsp90, respectively. The differential sensitivity of hnRNP A1 vs hnRNP C nuclear export to metal oxyanions is noteworthy, as these proteins differ in their nucleocytoplasmic shuttling properties; i.e., hnRNP A1 is a shuttling protein, while hnRNP C is not (Pinol-Roma and Dreyfuss, 1992). While the nucleocytoplasmic shuttling properties of other proteins tested in our assay system, notably hsp90 and hsp56, have not been established, it is intriguing to consider the possibility that metal oxyanions may be acting to affect a component of the nuclear export pathway that is exclusively used by shuttling proteins. GR and hnRNP A1 use different nuclear export pathways, yet export of both proteins is analogously affected by metal oxyanions in our in vitro assay system.

While a number of biochemical processes are known to be affected by group VI-A transition metal oxyanions, the analogous effects of heparin on GR nuclear export focused our attention on the protein tyrosine phosphatase inhibitory properties of these compounds. Indeed, stimulation of in vitro nuclear export of unliganded GR was correlated with increased tyrosine phosphorylation of a number

of proteins present within the permeabilized cell preparation. An inhibition of protein tyrosine phosphorylation with tyrosine kinase inhibitors, genistein and tyrphostin AG126, abolished molybdate effects on GR nuclear export, further strengthening the correlation between stimulated nuclear export and tyrosine phosphorylation. Protein phosphorylation has been shown to affect nuclear import, but, in many cases, this effect is direct as phosphorylation of some substrates can impact the efficiency of their nuclear import (Rihs and Peters, 1989; Rihs et al., 1991). Examples of both serine/threonine (Rihs and Peters, 1989; Rihs et al., 1991) and tyrosine (Fu, 1992; Shuai et al., 1993) phosphorylation effects on nuclear import have been observed. It appears unlikely that direct phosphorylation of exporting substrates is responsible for accelerated nuclear export in our assays since GR is not tyrosine phosphorylated in vivo (Bodwell et al., 1991) and under our in vitro assay conditions (data not shown). Furthermore, since sodium molybdate treatment also accelerated the energy-dependent nuclear export of a GR mutant with serine to alanine substitutions at its seven predominant phosphorylation sites (data not shown), it appears unlikely that indirect activation by sodium molybdate of downstream protein serine/threonine kinases acting on GR is responsible for this effect.

The ATP dependence of nuclear export observed in our in vitro system differs from the recently observed GTP- and ATP-independent nuclear export of NLS-conjugates in a similar digitonin-permeabilized cell system (Moroianu and Blobel, 1995). The discrepancy between this report and ours may reflect the usage of different exporting substrates. Since we examined the export of native shuttling proteins, ATP hydrolysis may be required for additional steps in the export pathway, such as release from some retention compartment, which are not obligatory for an NLS-conjugate. GR NLS-conjugates, while importing efficiently into nuclei, do not associate with the nuclear matrix, even under conditions that lead to dramatic increases in GR matrix binding (Tang and DeFranco, 1996). Thus, the subnuclear trafficking of NLS-conjugates clearly differs from that of the native protein from which the NLS was derived.

Since GTPγS did not block ATP-dependent in vitro export of GR, while AMP-PNP inhibited the partial export observed in GTP-treated permeabilized cells, a role for Ran in molybdate-stimulated nuclear export appears unlikely. This is consistent with the lack of a cytosolic requirement for our in vitro nuclear export and also differs from the observed Ran-dependent nuclear export of an NLS-conjugate in permeabilized cells (Moroianu and Blobel, 1995). The addition of nuclear import competent cytosol (which contains high levels of Ran) to our permeabilized cells did not lead to in vitro nuclear export of GR in the absence of molybdate treatment (data not shown), implying that nuclear protein export may not only be limited to a Ran-dependent pathway. Future studies will be directed toward revealing whether a Ran-independent protein nuclear export pathway operates under specific conditions in vivo.

The fact that accelerated in vitro nuclear export applied to proteins that use distinct mechanisms for nuclear export suggests that this may represent a generalized effect on NPC function. In fact, the kinetics of hormone-induced in vitro nuclear import of GR was also found to be stimulated by molybdate treatment (Yang, J., and D.B. DeFranco, unpublished observations), suggesting that inward and outward trafficking through the NPC may be sensitive to molybdate effects. The in vitro system that we have developed should be amenable to further biochemical and molecular experiments that may ultimately identify the factors regulating NPC function in response to molybdate treatment. Finally, this regulation of subnuclear compartmentalization provides a novel level of cross talk between distinct signal transduction pathways that could have an important impact on the activity of diverse nuclear proteins that function in transcription, DNA replication, or RNA splicing.

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