Molecular chaperones function as steroid receptor nuclear mobility factors

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Live cell imaging has revealed the rapid mobility of steroid hormone receptors within nuclei and their dynamic exchange at transcriptionally active target sites. Although a number of other proteins have been shown to be highly mobile within nuclei, the identity of soluble factors responsible for orchestrating nuclear trafficking remains unknown. We have developed a previously undescribed in situ subnuclear trafficking assay that generates transcriptionally active nuclei, which are depleted of soluble factors required for the nuclear mobility of glucocorticoid (GR) and progesterone receptors (PR). Using this system and a fluorescence recovery after photobleaching technique, we demonstrate that nuclear mobility of GR recovered on incubation with reticulocyte lysate was inhibited by geldanamycin, a drug that blocks the chaperone activity of heat-shock protein 90. Direct proof of molecular chaperone involvement in steroid receptor subnuclear trafficking was provided by the ATP-dependent recovery of nuclear mobility of GR and PR on incubation with various combinations of purified chaperone and/or cochaperone proteins. Additionally, for both receptors, the inclusion of hormone during the recovery period leads to a retardation of nuclear mobility. Thus, our results provide a description of soluble nuclear mobility factors and furthermore demonstrate a previously unrecognized role for molecular chaperones in the regulation of steroid receptor function within the nucleus.

Our understanding of the mechanisms of protein trafficking has been greatly aided by the advent of technologies that allow the movement of individual proteins to be visualized in live cells (1, 2). When applied to the assessment of nuclear protein trafficking, such techniques have uniquely revealed details of protein movement within a single subcellular compartment (3–6). Although protein mobility within the nucleus can be diffusion-limited, low- or high-affinity interactions with specific soluble or solid-state targets can reduce the kinetics of nuclear protein trafficking (7). Nonetheless, nuclear proteins that are components of large macromolecular complexes are highly mobile and possess fast diffusion coefficients (7).

The nuclear receptor superfamily of proteins act as transcription factors with activities regulated by ligand binding (8, 9). Once associated with their target genes through direct interactions with specific DNA sequences or tethering to other DNA-bound factors, nuclear receptors recruit large coregulator complexes to linked promoters (10, 11). These coregulators include coactivator and corepressor complexes that modulate RNA polymerase II activity in part through direct modification of histone proteins within core nucleosomes (12–14). The interaction of specific components (e.g., SRC-1) of coactivator complexes with a ligand-bound nuclear receptor (e.g., estrogen receptor) reduces their mobility within live cell nuclei, perhaps reflecting the assembly of functional coactivator/nuclear receptor complexes on active target genes (15).

Despite the increasing number of nuclear proteins and transcription factors that have been found to be highly mobile, our knowledge of the factors that regulate trafficking and mobility within the nucleus is limited. Live cell imaging (16) techniques reveal that the assembly and disassembly of large nuclear receptor/coactivator complexes on active genes is a very rapid process, yet we know little about the mechanisms used to ensure the efficient turnover of such multisubunit assemblies. Here we report a previously undescribed description of an in situ assay system that allows protein mobility factors to be characterized within transcriptionally active nuclei. Furthermore, we have used this system with purified components of steroid receptor complexes and photobleaching techniques to reveal a role for molecular chaperones in the nuclear mobility of steroid receptors.

Materials and Methods

Cell Growth. GFP-rat glucocorticoid receptor (GR) (3617.4) and enhanced GFP (EGFP)–human progesterone receptor (PR)-B (5953) cells are stably transfected derivatives of a murine mammary adenocarcinoma cell line (3134) that contains multiple copies of a BPV-MMTV-LTR-ras fusion. GFP-GR and EGFP-PR-B expressions are under control of the “Tet-Off” inducible system (17) and were grown in DMEM (BioWhittaker) supplemented with 10% FBS (Gemini Biological Products, Woodland, CA) and 10-μg/ml tetracycline (FisherBiotech, Fair Lawn, NJ). Cells were routinely maintained in an incubator at 37°C, 5% CO₂. For permeabilization, cells were transferred to Lab-Tek II chambers (Nalge) at 40,000 cells per well. At time of transfer, the medium was replaced with DMEM without tetracycline to induce GFP-GR and EGFP-PR-B expression. After 24 h, cells were washed with PBS, and medium was replaced with DMEM supplemented with 10% charcoal-treated serum (to remove hormone) and then cultured for an additional 24 h. Dexamethasone (100 nM) or R5020 (30 nM) was added to the cells for 1 h.

Visualization of Transcription Sites. Sites of active transcription were labeled in situ during a 5-min incubation of 3617.4 cells with 5-bromo-UTP (BrUTP). Cells were then fixed and processed for indirect immunofluorescence microscopy by using an antibromouracil antibody, as described (18). Similar results were obtained by using a protocol that labels the nascent RNA in vivo by microinjection of BrUTP (ref. 18 and data not shown).

Biochemical Extraction. Digitonin-permeabilization and hypotonic buffer extraction of 3617.4 cells were performed essentially as described (19). Briefly, cells were rinsed with PBS and then transport buffer (20 mM Hapes-KOH, pH 7.8/110 mM potassium acetate/5 mM sodium acetate/2 mM magnesium acetate/1 mM EGTA). Transport buffer containing 20 μg/ml digitonin and 1 mM DTT was then added to the cells and incubated on ice for 5 min. The cells were rinsed with transport buffer and then transport buffer containing 10 μg/ml BSA. For hypotonic extraction, digitonin-permeabilized cells were incubated with hypotonic buffer (10

Abbreviations: GR, glucocorticoid receptor; PR, progesterone receptor; FRAP, fluorescence recovery after photobleaching; GA, geldanamycin; BrUTP, 5-bromo-UTP; t₁/₂, half-maximal recovery time.

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FRAP analysis was carried out on a Zeiss 510 laser-scanning confocal microscope by using a ×100/1.3-numerical aperture oil immersion objective and 40-mW argon laser, as described (23). The stage temperature was maintained at 37°C with an ASI 400 Air Stream incubator (Ne Bytek, Burnsville, VA). Five single-imaging scans were acquired followed by a bleach pulse of 160 ms using 458/488/514-nm laser lines at 100% laser power (laser output 75%) without attenuation. Single optical sections from the middle of individual cells (i.e., z sections) were acquired at 1.4-s intervals by using 488-nm laser line with laser power attenuated to 0.1%. Fluorescence intensities in the regions of interest were analyzed, and FRAP recovery curves were generated using LSM software and Microsoft Excel, as described (23). Student’s t test was used to determine the statistical significance of results. All quantitative data values in FRAP recovery kinetics represent averages ± SD from at least 10 cells imaged in three independent experiments.

Results

In Situ Assay to Assess GR Mobility in Transcriptionally Competent Nuclei. We set out to develop an in situ assay for nuclear protein mobility that would maintain the transcriptional competence of isolated nuclei yet provide a system where the effects of specific subnuclear trafficking factors could be assessed. Previously, we used a modified hypotonic buffer extraction of digitonin-permeabilized cells (24) to reveal differential nuclear affinities of ligand-bound vs. unliganded nuclear GR (19). This technique was applied herein to mouse 3617.4 and 5953 cell lines, which contain an integrated copy of a GFP-GR and -PR chimera, respectively, under the control of a tet-regulated promoter (17, 25).

As shown in Fig. 1B, 361.7.4 cells subjected to digitonin permeabilization and hypotonic extraction retained transcriptional activity as assessed by labeling of active transcription sites using an BrUPT incorporation assay (18). This result shows the corresponding FRAP analysis immediately after incubation. In some experiments, 40 μl of reticulocyte lysate (Promega) was added to 160 μl of recovery buffer. Gdalanamycin (GA), when used, was preincubated with complete transport buffer containing reticulocyte lysate for 5 min at 37°C before incubating the mixture with permeabilized and extracted cells. Reactions with purified chaperones used 5 mM ATP in the presence of the ATP regenerating system. Heat shock protein (hsp)90 and hsp70 was expressed in baculovirus-infected Sf9 cells and purified as described (20). The cochaperones Hop, p23, and hsp40 were expressed in Escherichia coli and purified as described (23). His-tagged CHIP (21) and FKBP51 (22) were also expressed in E. coli and purified as described. All proteins were of human origin except hsp40, which was the Ydj1 protein from Saccharomyces cerevisiae. Chaperone proteins were used in the following amounts per 250-μl reaction: hsp70, 20 μg; Ydj-1, 2 μg; Hop, 5 μg; hsp90, 20 μg; p23, 5 μg; FKBP51, 10 μg; and CHIP, 10 μg. These proportions were chosen because they are optimal for the in vitro chaperoning of the progesterone receptor (20), and they resemble chaperone concentrations in cell lysates. No known posttranslational modifications are needed for the activities of these proteins. Extracted cells were subjected to FRAP analysis immediately after the recovery period (i.e., 10-min incubation at 37°C) and never exceeded 30 min total for any data collection.
transcription. Furthermore, permeabilization and extraction did not lead to complete depletion of GFP-GR from hormone-treated 3617.4 cells (Fig. 1D). This is consistent with previously published results where the same treatment was found to extract unliganded but not liganded GR from the nuclei of rat hepatoma cells (19).

We then used photobleaching analysis to assess the mobility of ligand-bound GFP-GR in permeabilized and extracted 3617.4 cells. After laser bleaching of an area within the nucleus of a cell expressing GFP-GR, fluorescence recovery into the bleached area was measured by FRAP. The kinetics of fluorescence signal recovery provide a measure of GFP-GR mobility. Consistent with previously published results (16), nuclear mobility of GFP-GR in live 3617.4 cells was quite rapid. The recovery of GFP-GR was complete (98 ± 1.8% of prebleach level) within 50 s with a half-maximal recovery time ($t_{1/2}$) of 1.5 s (Fig. 1C and E). However, permeabilization and extraction of 3617.4 cells led to severe retardation in GFP-GR mobility, because virtually no fluorescence recovery was observed up to 155 s (Fig. 1D and F). GFP-GR mobility remained blocked when cells were examined 1 h after permeabilization (data not shown). Thus, permeabilization and extraction of 3617.4 cells result in depletion of factors required for GR mobility within the nucleus.

Reticulocyte Lysate Contains Soluble Factors That Restore GR and PR Nuclear Mobility in Permeabilized and Extracted Cells. In an attempt to reconstitute the nuclear mobility of GFP-GR in situ, permeabilized and extracted 3617.4 cells were incubated with rabbit reticulocyte lysate in the presence of ATP. Reticulocyte lysates have routinely been used as a source of soluble factors required for nuclear protein import in vitro (26). As shown in Fig. 2A and D, incubation of permeabilized and extracted 3617.4 cells with reticulocyte lysate in the presence of ATP led to restoration of nuclear GFP-GR mobility. However, incubation in the presence of ATP alone did not lead to significant recovery of GFP-GR mobility (Fig. 2B and D). The recovery of GFP-GR fluorescence in the bleached area by reticulocyte lysates reached 88 ± 3% of prebleach levels within 86 s with a $t_{1/2}$ of 2.5 s (Fig. 2D) and was not as efficient and complete as that observed in
live cells (Fig. 1E; \( P < 0.001 \)). Thus, the reticulocyte lysate may not contain the full complement of mobility factors required to restore \textit{in vivo} rates of GR trafficking within nuclei. Alternatively, modifications in some underlying nuclear substructure brought about by the permeabilization and extraction conditions may also limit the maximum extent of protein mobility.

The recovery of nuclear protein mobility by reticulocyte lysate in permeabilized and extracted 3617.4 cells was not restricted to GR. GFP-PR (B form), rendered immobile by permeabilization and extraction of 5953 cells, regained its mobility on treatment with reticulocyte lysate and ATP. The recovery level and 1/2 were 91 ± 2.9% and 3.4 s, respectively (Fig. 2E). Addition of ATP alone did not lead to a significant recovery of GFP-PR nuclear mobility (Fig. 2E).

Recovery of GR Nuclear Mobility by Reticulocyte Lysate Is Blocked by the hsp90 Inhibitor GA. Reticulocyte lysates have been used extensively in steroid hormone research to reveal the requirements for protein chaperones to generate hormone-binding competent receptors \textit{in vitro} (27). For the hsp90 chaperone, pharmacological agents exist (e.g., GA; ref. 28) that have allowed its role in steroid receptor function to be assessed (29, 30). As shown in Fig. 2C and D, the addition of GA blocked the recovery of GFP-GR fluorescence within the nuclei of permeabilized and extracted 3617.4 cells incubated with reticulocyte lysate and ATP. Therefore, hsp90 is implicated as one of the factors present in reticulocyte lysates that is required for the nuclear mobility of GFP-GR \textit{in situ}.

Recovery of GR and PR Nuclear Mobility by Purified Chaperones and Cochaperones in Permeabilized and Extracted Cells. In addition to hsp90, numerous chaperone proteins and their cochaperone partners function in an ordered assembly reaction to reconstitute steroid receptor hormone-binding activity and transcriptional competence (31, 32). Experiments in yeast also verify the role of chaperone proteins in steroid receptor hormone binding \textit{in vivo}.
partners in the nuclear mobility of GR and PR. Established a role for multiple chaperones and their cochaperone complexes during the rescue of GR and PR mobility. Nonetheless, these results suggest that the conditions may not be optimum for the recovery of mobility factors for both GR and PR.

Chaperone mixture described above, the conditions may not be optimum for the complete extraction of the missing factors during the permeabilization procedure. Because we have not attempted to vary the ratio and amount of chaperone and cochaperone proteins in the various mixtures described above, the conditions may not be optimum for the rescue of GR and PR mobility. Nonetheless, these results establish a role for multiple chaperones and their cochaperone partners in the nuclear mobility of GR and PR.

Hormone Treatment Slows the Recovery Kinetics of GR and PR in Situ.

For all FRAP experiments described above, dexamethasone or R5020, ligands of GR or PR, respectively, was included during the incubation with reticulocyte lysate and purified chaperone/cochaperone complexes. However, hormone was not required for reticulocyte lysate- or chaperone/cochaperone-dependent mobility of GR and PR in situ but actually led to a reduction in GFP-GR and -PR recovery kinetics. Specifically, in permeabilized and extracted 3617.4 cells incubated with the seven-chaperone/cochaperone protein mixture also restored the ATP-dependent mobility of GFP-PR in permeabilized and extracted 5953 cells (Fig. 3F). Thus, molecular chaperones act as nuclear mobility factors for both GR and PR.

The five- and seven-chaperone/cochaperone mixtures were the most efficient at restoring GFP-GR and -PR mobility. When permeabilized and extracted 3617.4 cells were incubated with different combinations of three chaperones or cochaperones, GFP-GR mobility was only partially restored (Fig. 3E). Specifically, a combined mixture of three chaperones, hsp90, hsp70, and Hop, led to a GFP-GR recovery level of 50 ± 2.6% after 85 s (Fig. 3E).

In all cases, the recovery level of GFP-GR by the five-chaperone/cochaperone complex was much lower than the recovery level observed with the five-chaperone/cochaperone complex and required ATP. Finally, experiments with the p23 alone or a combination of Hop and hsp40 cochaperones, the recovery of GFP-GR was minimal, reaching only 15 ± 2% or 21 ± 2.2%, respectively, of the prebleach intensity (Fig. 3E). It is likely that the partial recovery of mobility with the three-chaperone or -cochaperone mixtures resulted from incomplete extraction of the missing factors during the permeabilization procedure. Because we have not attempted to vary the ratio and amount of chaperone and cochaperone proteins in the various mixtures described above, the conditions may not be optimum for the rescue of GR and PR mobility. Nonetheless, these results establish a role for multiple chaperones and their cochaperone partners in the nuclear mobility of GR and PR.

Fig. 4. Hormone treatment retards the mobility of GR and PR within nuclei of permeabilized and extracted cells. Quantitative FRAP analysis of GFP-GR (A–C) and GFP-PR (D) in permeabilized and extracted 3617.4 and 5953 cells, respectively. Cells were treated with 100 nM dexamethasone or 30 nM R5020 for 1 h. Permeabilized and extracted cells were then incubated with combinations of purified molecular chaperone proteins. In all cases, the recovery period was performed either in the absence or presence of 100 nM dexamethasone. For GFP-PR expressing cells, 30 nM R5020 was added during recovery with reticulocyte lysate. All quantitative data values in FRAP recovery kinetics represent averages ± SD from at least 10 cells imaged in three independent experiments.

For all FRAP experiments described above, dexamethasone or R5020, ligands of GR or PR, respectively, was included during the incubation with reticulocyte lysate and purified chaperone/cochaperone complexes. However, hormone was not required for reticulocyte lysate- or chaperone/cochaperone-dependent mobility of GR and PR in situ but actually led to a reduction in GFP-GR and -PR recovery kinetics. Specifically, in permeabilized and extracted 3617.4 cells incubated with the seven-chaperone/cochaperone mixture, GFP-GR recovery level was 95 ± 2.6% with t_{1/2} of 1.3 s in the absence of hormone and 79 ± 2.8% with t_{1/2} of 2.5 s in the presence of hormone (Fig. 4A; P < 0.001). The effect of hormone to retard GFP-GR mobility in situ also occurred when a five- (Fig. 4B) or three- (Fig. 4C) chaperone/cochaperone mixture was used. The kinetics of GFP-PR nuclear mobility also slowed when R5020 was included during incubation of permeabilized and extracted 5953 cells with reticulocyte lysate (Fig. 4D). These results are consistent with the effects of hormone on the reduction of nuclear GR (6) and estrogen receptor (39, 40) mobility in live cells.
Discussion

Molecular chaperones have received considerable attention in the steroid receptor field given their participation in the assembly of hormone-binding competent steroid receptor heteromeric complexes (27). The results presented in this report define a previously unrecognized role for molecular chaperones as nuclear mobility factors for GR and PR. The digitonin-permeabilization and hypotonic extraction conditions we used retain the structural and functional properties required for transcription as assessed by labeling of active transcription sites using a BrUTP incorporation assay. Furthermore, these treatments must either reduce chaperone levels within the nucleus below the threshold required to maintain GR and PR mobility or interfere with the activity of residual chaperones and cochaperones. It remains to be determined whether molecular chaperones function as general nuclear mobility factors or are specific for a subset of mobile nuclear proteins. In fact, in recent experiments, we have been unable to recover mobility of a highly mobile heterochromatin protein in permeabilized and extracted cells with purified chaperones or reticulocyte lysate. The in situ assay we have developed should allow for the identification and characterization of other types of nuclear mobility factors.

Although our results show that multiple chaperone proteins are required to restore GR and PR nuclear mobility, we cannot ascribe the mobility factor activity to any specific chaperone/cochaperone complex. Indeed, multiple distinct chaperone/cochaperone complexes may possess the ability to function as nuclear mobility factors. Conditions for the recovery of nuclear mobility have not been optimized for every combination of chaperone/cochaperone protein tested. Thus it is possible that more complete recovery of GR and/or PR mobility would be observed if chaperone/cochaperone proteins were added in different amounts or ratios. Interestingly, our results indicate the observed if chaperone treatment we have developed should allow for the identification and characterization of other types of nuclear mobility factors.

Although the mechanism responsible for molecular chaperone action in nuclear mobility remains to be determined, this activity appears distinct from the general effects of chaperones on protein folding. Our results show that permeabilized and extracted cells maintain transcriptional competence, thus global protein misfolding cannot account for the immobilization of GR and PR. The macromolecular machinery responsible for efficient transcriptional elongation must still be operating in the permeabilized and extracted cells.

GA disrupts the ATP-dependent chaperone activity of hsp90 (41, 42) and blocks the recovery of nuclear GR mobility elicited by reticulocyte lysate. Thus, hsp90 appears to be an essential component of a multisubunit chaperone machine that functions in the nucleus to maintain the mobility of GR. We had previously used a hormone-withdrawn cells to show the role of hsp90 in chromatin recycling of GR (43). Thus, hsp90 may assist in the assembly and disassembly (43, 44) of the large macromolecular complexes that are recruited to GR-responsive promoters.

The ability of hormone added in situ to retard GFP-GR mobility (ref. 6 and this report), which is restored by purified chaperone/cochaperone mixtures in our experiments, suggests that hormone exchange in the nucleus might also require molecular chaperones. In fact, the status of nuclear receptor-bound hormone during the exchange of receptors at active chromatin templates still remains unresolved. The measurement of hormone effects on GR nuclear mobility in our in situ system provides the most compelling data to date for hormone exchange on a nuclear receptor within transcriptionally competent nuclei.

Chaperone involvement in nuclear functions of steroid receptors has become an emerging concept in recent years. With an in situ system now in place that allows for subnuclear trafficking to be assessed with purified components in transcriptionally active nuclei, the dynamics of assembly and disassembly of the complex transcriptional regulatory machinery will be amenable to biochemical dissection in the context of native chromatin within functional nuclei.

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