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A New First Step in Activation of Steroid Receptors

HORMONE-INDUCED SWITCHING OF FKBP51 AND FKBP52 IMMUNOPHILINS*

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We have identified a new first step in the hormonal activation of the glucocorticoid receptor (GR). Rather than causing immediate dissociation of the cytoplasmic GR heterocomplex, binding of hormone-induced substitution of one immunophilin (FKBP51) for another (FKBP52), and concomitant recruitment of the transport protein dynein while leaving Hsp90 unchanged. Immunofluorescence and fractionation revealed hormone-induced translocation of the hormone-generated GR·Hsp90·FKBP52·dynein complex from cytoplasm to nucleus, a step that precedes dissociation of the complex within the nucleus and conversion of GR to the DNA-binding form. Taken as a whole, these studies identify immunophilin interchange as the earliest known event in steroid receptor signaling and provide the first evidence of differential roles for FKBP51 and FKBP52 immunophilins in the control of steroid receptor subcellular localization and transport.

The glucocorticoid receptor (GR)[1] is a hormone-activated transcription factor that requires hormonally driven movement to its site of action within the nucleus. In the absence of hormone, the GR is recovered in the cytosolic fraction of cells as an oligomeric complex containing one molecule of receptor and two molecules of heat shock protein 90 (Hsp90), to which the receptor binds directly (for review see Ref. 1). An intriguing recent development, however, is that hormone-free receptor is not found as a single, well defined complex but exists as a mixture of complexes. Although all of these complexes contain receptor and Hsp90, each contains only one molecule of either FKBP52, FKBP51, Cyp40, or PP5. The latter proteins have been classified as TPR domain proteins based on the presence of several tetratricopeptide repeat domains that are their sites of interaction with Hsp90 (2, 3). FKBP52, FKBP51, and Cyp40 are also members of the immunophilin family of proteins (4–6). Thus, it is now clear that up to four distinct receptor heterocomplexes are possible, even within the same cell or tissue; yet almost nothing is known about the differential roles served by each immunophilin in steroid receptor responses.

It is generally accepted that the first event in hormonal activation of GR is dissociation of hormone-bound GR from Hsp90 and the TPR proteins, followed by nuclear translocation of the GR and all other downstream events. Hormone-induced dissociation of the complex is a rapid event, occurring both in the intact cell (7) and in cytosolic preparations (8). In cytosols, dissociation of GR complexes has been shown to require warming (typically 20–25 °C) in addition to hormone, and this process can be blocked by molybdate and other transition metal oxyanions (8, 9). Indeed, because of the ability of molybdate to effectively block dissociation, it has been assumed that molybdate-stabilized receptors are more or less "frozen" in their native, untransformed state even when GR is bound with hormone. In this work, we have examined this assumption by measuring the effect of hormone on the immunophilin content of GR heterocomplexes. In so doing, we have identified a new first step in hormonal activation of steroid receptors, *i.e.* hormone-induced switching of FKBP51 and FKBP52 within the complex, showing that this event leads to movement of the newly generated complex to the nucleus prior to its final dissociation.

EXPERIMENTAL PROCEDURES

Immunoadsorption of GR Complexes—Mouse L929 cells were grown in Dulbecco's modified Eagle's medium containing 10% charcoal-stripped calf serum (Hyclone Laboratories, Inc.). Cells were ruptured by Dounce homogenization in HEMG buffer (10 mM HEPES, 3 mM EDTA, 20 mM sodium molybdate, 5% glycerol, pH 7.4). Lysates were centrifuged at 16,000 × *g* for 30 min. All cytosols were used without freezing or storage. In Fig. 1, aliquots (typically 300 μl) of cytosol were treated with dexamethasone (Sigma), RU486 (gift from Daniel Philibert, Hoechst Marion Roussel, Inc.), or appropriate vehicle controls followed by the addition of FiGR, the monoclonal antibody to GR (gift from Jack Bodwell (10)), or nonimmune mouse IgG2A (Sigma) on ice for 6 h. Samples were rotated with 20 μl of protein A-Sepharose at 4–8 °C overnight. The pellets were washed three times with TEG (10 mM Tris, 3 mM EDTA, 10% glycerol, 50 mM NaCl, 20 mM sodium molybdate, pH 7.4) followed by elution of GR complexes with 2× SDS. In Figs. 2 and 3, all steps were the same, except hormone-treatment occurred at the intact cell.

Gel Electrophoresis and Western Blotting—Samples were resolved on denaturing SDS gels using a 7–14% acrylamide gradient to achieve maximal separation between the immunophilins and antibody heavy chains. Transfer of the samples to Immobilon-P® membranes (Millipore Corp.) and quantitative immunoblotting were performed as described previously (8, 11). The BuGR2 monoclonal antibody against GR (Affinity Bioreagents) was used to probe for receptor, and various antibodies were used to probe for Hsp90 (H38220, Transduction Laboratories, Inc.), FKBP52 (UPJ56 (12)), FKBP51 (PA0-021, Affinity Bioreagents), and dynein intermediate chain (monoclonal antibody 1618, Chemicon International, Inc.). The blots were then incubated with appropriate peroxidase- and ^{125}I-conjugated counter-antibodies followed by color development and autoradiography.

Immunofluorescence and Fractionation—L929 cells grown on coverslips were incubated at 4 °C for 3 h or 37 °C for 1 h with dexamethasone or vehicle control. Immunofluorescence was preformed by fixation with buffered 3% formaldehyde solution at 4 °C for 12 h followed by permeabilization with 0.3% Triton X-100. Permeabilized cells were incubated with FiGR monoclonal antibody against GR or H38220 antibody against Hsp90 at a 1:100 dilution followed by fluorescein-conjugated secondary antibody (Calbiochem) at a 1:20 dilution. Cells were visualized using a

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[1] The abbreviations used are: GR, glucocorticoid receptor; Hsp90, heat shock protein 90; TPR, tetratricopeptide repeat; Dex, dexamethasone; FKBP, FK506-binding protein.

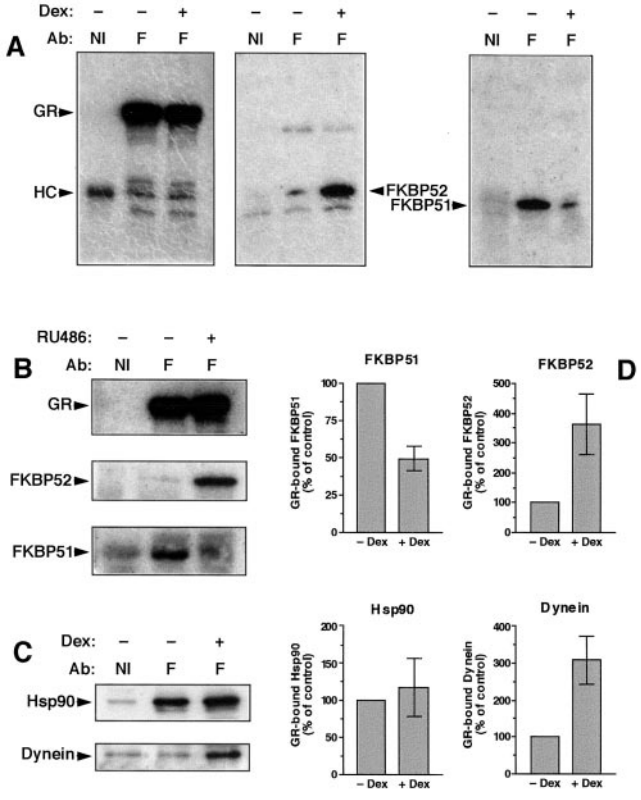

FIG. 1. **Hormone induces reciprocal exchange of FKBP51 and FKBP52 and co-recruitment of dynein within the GR heterocomplex *in vitro*.** Aliquots (300 μl) of L929 cell cytosol were treated on ice for 3 h with 100 nM dexamethasone (*panels A* and *C*), 100 nM RU486 (*panel B*), or vehicle controls followed by immunoadsorption with FiGR antibody (*Ab*) against GR (*F*) or nonimmune mouse IgG (*NI*). Samples were split and analyzed by Western blotting with BuGR2 antibody against GR, UPJ56 antiserum against FKBP52, PA0-021 antiserum against FKBP51, H38220 mouse monoclonal antibody against Hsp90, and mouse monoclonal antibody 1618 against dynein intermediate chain. *HC*, IgG heavy chain. *D*, quantitation of relative changes in protein levels within the GR heterocomplexes was accomplished by densitometric scanning of the autoradiograms followed by subtraction of nonimmune (*NI*) values and normalization to amount of GR protein in each condition.

100× objective on a Nikon Eclipse E800 microscope. Photographs were taken with a Sensys digital camera. Fractionation and analysis of GR complexes was performed on L929 cells subjected to the same conditions. Cytosolic and nuclear fractions were prepared by Dounce homogenization as described above, except that nuclear pellets were extracted with 500 mM NaCl for 1 h on ice.

RESULTS AND DISCUSSION

To test the effect of hormone on cytosolic GR complex composition, we first measured alterations to the FKBP52 content (Fig. 1*A*). The results show almost no GR-associated FKBP52 in the absence of Dex but a large increase in FKBP52 for the hormone-bound receptor. Because the interaction of FKBP52 with the GR occurs through the TPR-binding domain of Hsp90 and because this domain can accommodate only one TPR protein (13), we reasoned that increased FKBP52 could result from one of three mechanisms: 1) stabilization of the GR-Hsp90 interaction (leading to increased yields of Hsp90 and all other Hsp90-bound components), 2) binding of FKBP52 to unoccupied Hsp90 within the GR complex (leaving both Hsp90 and other TPR protein levels unchanged), or 3) displacement of other TPR proteins from Hsp90 by FKBP52 (leaving Hsp90 levels unchanged but the levels of other TPR proteins decreased). To test these alternatives, we measured the amounts of FKBP51 bound to the GR in response to hormone. The results show much higher levels of GR-bound FKBP51 in the absence of hormone than in its presence (Fig. 1*A*). Thus, it appears that the hormone binding event can cause a swapping of FKBP immunophilins within the GR complex, presumably at the Hsp90 TPR-binding domain.

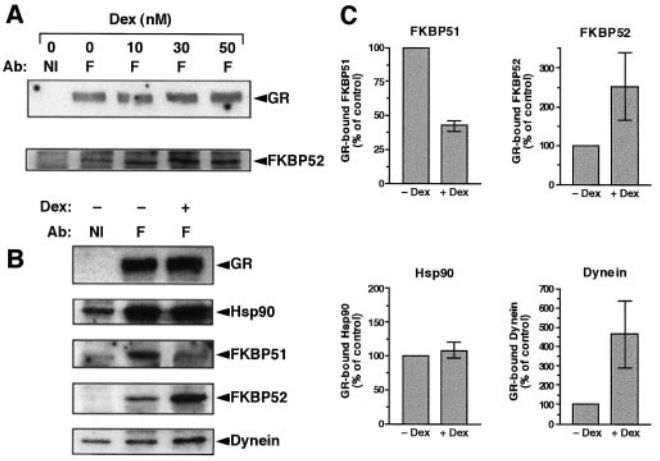

FIG. 2. **Hormone-induced switching of FKBP immunophilins and recruitment of dynein to GR heterocomplexes occurs in the intact cell.** L929 cells were incubated at 4 °C for 3 h with increasing concentrations of dexamethasone (*A*) or with 30 nM dexamethasone or vehicle control (*B*). After the cells were washed, cytosols were prepared and analyzed for GR-associated components as described in the legend for Fig. 1. Immunoadsorptions were performed with FiGR antibody (*Ab*) against GR (*F*) or nonimmune mouse IgG (*NI*). Western blotting was performed with antibodies against GR, FKBP52, FKBP51, Hsp90, and dynein intermediate chain. *C*, quantitation of GR-associated proteins was performed as described in the legend for Fig. 1.

To determine whether this response occurs with antagonist, we incubated cytosols with 100 nM RU486 (Fig. 1*B*). Here, too, the results were the same: an apparent swapping of FKBP52 for FKBP51 in response to hormone. Thus, RU486 is not an antagonist at this early stage of GR action. Although the above results suggest that the primary effect of hormone is not to increase yields of GR-associated Hsp90, we tested this directly (Fig. 1*C*). The results show that Hsp90 levels within the GR complex are only marginally affected by hormone. As a further test of the FKBP interchange hypothesis, the amounts of dynein associated with the GR complex were measured in the same samples (Fig. 1*C*). Like FKBP52, dynein levels in the GR complexes went from almost undetectable in the absence of hormone to clearly detectable in its presence. Thus, the binding of hormone to GR not only replaces FKBP51 with FKBP52 but also causes co-recruitment of a protein, dynein, that is known to directly bind FKBP52 within its peptidylprolyl isomerase domain (14).

To test this response in the intact cell, we needed hormone treatment conditions that would not cause dissociation of the GR complex and concomitant conversion of the GR to the tight nuclei-bound state. Moreover, we reasoned that if swapping of immunophilins did occur in the intact cell, the event had to be a short-lived intermediate, as prior studies had shown that translocation of GR and conversion to tight nuclear binding occurred within 20 min of hormone addition in cells maintained at 37 °C (15). To detect such an intermediate, we simply exposed intact cells to hormone at 4 °C. In an initial experiment (Fig. 2*A*), a concentration-dependent increase in the amount of GR-associated FKBP52 was observed, with maximal effect occurring at ~30 nM Dex. This concentration of hormone was then used in the *in vivo* experiment of Fig. 2*B*, in which changes to the major components of the GR complex were measured. As expected, equal amounts of GR were recovered in the absence or presence of hormone, as well as equal amounts of receptor-associated Hsp90. In contrast, the pattern of recep-

tor-bound immunophilins was similar to that seen under *in vitro* conditions, namely, hormone-induced loss of FKBP51 and gain of FKBP52.

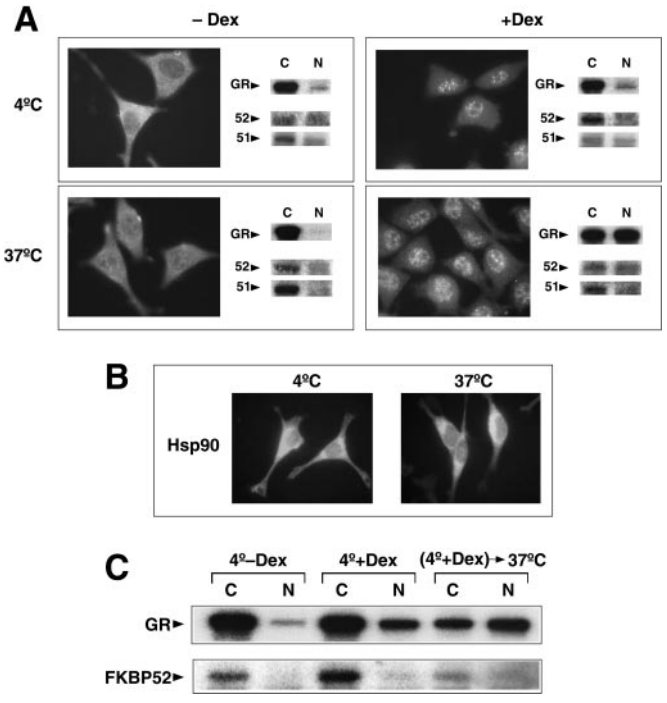

FIG. 3. **Hormone converts the cytoplasmic FKBP51-containing GR heterocomplex to a nuclear FKBP52-containing intermediate that is a precursor of the fully transformed GR.** *A*, analysis of GR-immunophilin interactions by immunofluorescence and fractionation. Immunofluorescence analysis of L929 cells at 4 °C (3 h) or 37 °C was performed as described under "Experimental Procedures." Analysis of GR heterocomplexes from cytosolic (*C*) and nuclear extracts (*N*) was performed on L929 cells subjected to the same conditions. Immunoadsorptions were done with FiGR antibody against GR and Western blotting with antibodies against GR, FKBP52, and FKBP51. *B*, cold shock does not cause nonspecific accumulation of proteins in the nucleus. L929 cells exposed to 4 °C for 3 h or maintained at 37 °C were analyzed for subcellular localization of Hsp90 by immunofluorescence. *C*, the hormone-induced FKBP52-containing intermediate converts to tight nuclear binding upon warming. Individual flasks of L929 cells were incubated at 4 °C for 3 h with 1 μM dexamethasone or vehicle control. A third flask was incubated at 4 °C for 3 h with 1 μM dexamethasone and was then warmed to 37 °C for an additional 1 h. Fractionation, immunoadsorption, and Western blots were performed as described above.

Given the well documented role of dynein in retrograde transport of proteins and vesicles (16), we reasoned that hormone-directed recruitment of FKBP52 and dynein to the GR may cause transport of the GR as a complex to the nuclear compartment. To test this possibility, we performed indirect immunofluorescence using FiGR antibody (Fig. 3*A*). As expected, in cells maintained at 37 °C, treatment with 30 nM Dex caused a shift of GR from cytoplasm to nucleus. Surprisingly, a similar Dex-induced shift of GR to the nucleus was observed in cells maintained at 4 °C, suggesting that hormone binding was indeed causing translocation of the GR to the nucleus even at this low temperature. Interestingly, movement of GR to the nucleus at 4 °C was not seen until 3 h of hormone treatment, whereas translocation at 37 °C was much faster, occurring as soon as 20 min (data not shown).

If hormone could cause the GR to move to the nucleus at 4 °C, was it actually moving as a complex containing FKBP52 and dynein? To test this possibility directly, cells subjected to the same treatments used in the fluorescence studies were lysed by Dounce homogenization to yield cytosolic and nuclear extract fractions, followed by analysis of GR complex composition in each fraction (Fig. 3*A*). As expected, cells maintained at 4 and at 37 °C, but not exposed to hormone, yielded GR that was recovered in the cytosolic fraction as a complex containing FKBP51 but little or no FKBP52. Also as expected, GR from cells maintained at 37 °C cells and incubated with hormone was recovered predominantly in the nuclear pellet fraction without bound immunophilin, demonstrating that this treatment caused dissociation of the GR complex and conversion of the receptor to its high affinity nuclei-bound state. In contrast, GR from cells maintained at 4 °C and incubated with hormone was found in the cytosolic fraction as a complex containing FKBP52 rather than FKBP51, demonstrating that this GR, although localized to the nucleus, is not tightly bound to this cellular compartment and is released into the cytosolic fraction as a complex upon cell rupture. To test whether Dex-induced accumulation of GR in the nucleus was the result of damage to or compromised function of the nuclear pore complex at 4 °C, we examined localization of Hsp90, which is known to reside predominantly in the cytoplasm of cells (Fig. 3*B*). The results show cytoplasmic localization of Hsp90 at both 4 and 37 °C.

The above results indicate that hormonally induced acquisition of FKBP52 and dynein may indeed be the event that causes movement of the GR complex to this compartment.

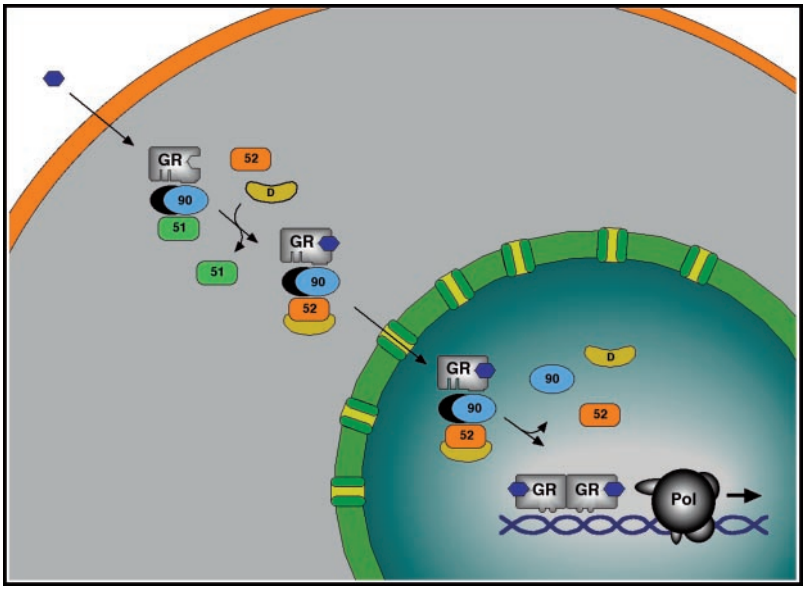

FIG. 4. **Model for hormonal activation of the glucocorticoid receptor.** This model presents immunophilin interchange as the first consequence of hormone binding to the cytoplasmically localized receptor (*GR*). This event is followed by concomitant recruitment of dynein and GR translocation to the nucleus as a complex prior to the final dissociation of the complex within the nucleus to generate the DNA-binding competent form of the receptor.

If so, this would suggest that the hormone-bound GR·Hsp90·FKBP52·dynein complex found in the nucleus at 4 °C represents an intermediate stage in the hormonal activation of GR. Further evidence to support this conclusion is the fact that when cells are first bound with hormone at 4 °C (generating the FKBP52-containing GR complex within the nucleus), subsequent warming to 37 °C causes the release of GR from the FKBP52-containing complex and conversion to the high affinity nuclei-bound state (Fig. 3*C*). Thus, the GR·Hsp90·FKBP52·dynein complex behaves like an intermediate in that it can proceed to the next step in the pathway.

Based on our observations, we now propose a model for the early stages of GR signaling in which immunophilin interchange is the first hormone-directed event, followed by movement of the hormone-altered complex to the nucleus prior to its final dissociation in that compartment (Fig. 4). In this model, we depict the hormone-induced FKBP interchange (Fig. 4) as a process in which FKBP52 directly interacts with the Hsp90 that is already bound to GR. It is possible, however, that the hormone-bound receptor recruits a distinct Hsp90 complex containing FKBP52 as opposed to FKBP51. The model also does not depict whether Cyp40 and PP5 are involved in this response. Because the stoichiometry of the FKBP exchange is unknown, it is possible that Cyp40 and/or PP5 are either displaced by FKBP52 or are similarly recruited by hormone to the sites vacated by FKBP51. Yet, we have not been able to detect Cyp40-containing GR complexes in L929 cells, either in the absence or presence of hormone (data not shown). Thus, in L929 cells, hormone-induced recruitment of FKBP52 had little effect on the Cyp40 content of receptors.

An important question that arises from these results is how the hormone-generated GR·Hsp90·FKBP52·dynein complex is targeted to the nucleus. An obvious candidate process is microtubule-based transport exploiting the recruitment of dynein to the complex. Consistent with this speculation is the fact that the form of dynein observed here is the dynein intermediate chain, a component of dynein motor complexes responsible for cargo binding activity (16). Yet how can this process occur at 4 °C? Obviously, we have not yet answered this question. Therefore, it remains a possibility that movement of the complex at this temperature is through passive diffusion, even though movement at physiological temperatures may occur by active transport. Transport to the nucleus aside, is it possible for GR to move into the nucleus as a complex or to reassemble on the other side? It appears that this process can occur, as a variety of hormone-free receptors are found associated with Hsp90 whether or not they reside in the cytoplasm or the nucleus (17). Moreover, it is now clear that hormone-free (18) and hormone-bound receptors (19) can freely shuttle between the nuclear and cytoplasmic compartments and that the equilibrium of these movements determines whether any given receptor is predominantly in the cytoplasm or the nucleus. Yet almost nothing is known about the factors that control this equilibrium. Our results may now provide the basis for understanding this process, as the initial function of hormone may simply be to alter the shuttling equilibrium in favor of nuclear retention of the complex, a process in which FKBP52 is responsible for nuclear localization of receptor, whereas FKBP51 directs GR to the cytoplasm.

In conclusion, we present evidence that the hormone binding event causes an interchange of FKBP51 and FKBP52 immunophilins within the GR heterocomplex that in turn appears to control the intracellular trafficking of GR. This observation may have implications for many target substrates of the Hsp90-based chaperone complex such as other steroid receptors, unrelated transcription factors (*e.g.* HSF1), protein kinases (*e.g.* Src and Raf), and a variety of proteins and structures (*e.g.* proteosome and $G_{\beta\gamma}$ complexes) (20–23). One such implication is that the Hsp90 chaperone complex is needed not only for proper folding of substrate proteins but also for subcellular trafficking of these substrates, a function that may be regulated by differential immunophilin content.

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