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Evidence for NL1-Independent Nuclear Translocation of the Mineralocorticoid Receptor[†]

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ABSTRACT: In the absence of hormone, corticosteroid receptors are primarily located in the cytoplasm, and they rapidly accumulate in the nucleus ($t_{0.5} = 5$ min) upon ligand binding. It is generally believed that the dissociation of hsp90 from the receptor is an absolute requirement for allowing its nuclear translocation. However, recent evidence suggests that hsp90 may remain associated with the glucocorticoid receptor during this process, and thus, the receptor nuclear localization signal (NLS) is not obscured by its presence. To determine the requirements for mineralocorticoid receptor (MR) nuclear transport, it was first shown that in rat kidney collecting duct cells, nuclear localization of MR in the presence of aldosterone was complete in 10 min. Although the hsp90 inhibitor radicicol delayed nuclear translocation, it did not prevent complete nuclear accumulation of MR at longer incubation times ($t_{0.5} = 30$ – 40 min). MR carbamylation generates a non-steroid-transformed receptor that, in contrast to native MR, is very stable in cell-free systems. In contrast to the full nuclear translocation of aldosterone-transformed MR, only a fraction of the carbamylated MR became nuclear in digitonin-permeabilized cells even though its NLS is exposed. Furthermore, while preincubation of permeabilized cells with NL1 peptide or anti-NL1 antibody fully inhibited the nuclear translocation of NL1-tagged albumin, neither treatment fully inhibited MR nuclear translocation. We postulate that there are at least two possible mechanisms for MR nuclear translocation. One of them is hsp90- and NL1-dependent, and the other functions in a manner that is independent of the classical pathway.

The mineralocorticoid receptor (MR)¹ is a ligand-dependent member of the nuclear receptor superfamily. MR mediates the effects of aldosterone on a variety of target tissues such as the distal part of the nephron, the distal colon, the cardiovascular and central nervous systems, and brown adipose tissue (1–3).

In the absence of ligand, native MR is primarily located in the cytoplasm in several cell types (4–7). It has been accepted heuristically that, after steroid binding, the hsp90-based heterocomplex bound to the receptor must be immediately dissociated (a process also called “transformation”), thus triggering the nuclear translocation of the cytoplasmic receptor pool. However, there is no experimental

evidence to support this dogma. On the contrary, in the case of GR, it has been suggested that transformation cannot take place at early steps because binding to the hsp90–immunophilin heterocomplex is in fact required for the movement of the receptor to the nucleus (8–10), which is powered by dynein (11–13). In agreement with this model, both the dissociation of the immunophilin–dynein interaction and the disruption of the GR–hsp90–immunophilin machinery with the hsp90 inhibitor geldanamycin impair movement of the receptor through the cytoplasm toward the nucleus (8, 14). Although GR can still reach the nuclear compartment, it takes longer periods of incubation and the GR is subject to proteasome degradation (15). While nuclear import is very rapid, nuclear export takes several hours after the steroid is withdrawn (16, 17).

Regardless of their primary localization, steroid receptors are constantly shuttling between the nucleus and cytoplasm (18–20). Nuclear import is a mechanism that depends on the nuclear localization signal (NLS). As a common feature for most members of the steroid receptor subfamily, the strongest NLS is a core of basic amino acids (designated NL1) that extends beyond the C-terminus end of the DBD and ensures access to the nuclear compartment (21, 22). Most steroid receptors also possess a second less well characterized motif, NL2, and like other transcription factors, some may contain additional NLSs in other regions of the protein (22, 23).

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¹ Abbreviations: MR, mineralocorticoid receptor; GR, glucocorticoid receptor; NLS, nuclear localization signal; NL1, nuclear localization signal 1; DEPC, diethyl pyrocarbonate; hsp90, 90 kDa heat shock protein.

Both NLS1 and -2 are present in the MR structure, and they are likely to be obscured by the hsp90 complex except in the presence of hormone, the binding of which favors nuclear translocation. In addition to these two known NLSs, it has recently been postulated that there is a potential third NLS located within a serine/threonine rich motif at the N-terminal end of the MR (24).

In this work, we have further examined the requirements for MR nuclear translocation, first by using the hsp90-disrupting agent radicicol and then by transforming the MR by carbamylation of critical histidine residues. The evidence suggests that the exposure of a NLS alone is not sufficient to guarantee efficient MR nuclear translocation and that alternative nuclear import mechanisms that are clearly hsp90- and NL1-independent exist.

EXPERIMENTAL PROCEDURES

Materials. Aldosterone, digitonin, protein A-Sepharose, hydroxylamine, diethyl pyrocarbonate (DEPC), and α -chymotrypsin were obtained from Sigma Chemical Co. (St. Louis, MO). RU38486 was from Amersham (Arlington, IL), and [1,2- 3 H]aldosterone (specific activity of 56.5 Ci/mmol) and 125 I-conjugated counterantibodies were from NEN Life Science Products (Boston, MA). The rabbit polyclonal antiserum against the MR used for immunoprecipitation was kindly provided by G. Litwack (Thomas Jefferson University, Philadelphia, PA). The goat anti-MR antibody used for indirect immunofluorescence was from Santa Cruz Biotechnology (Santa Cruz, CA). The rhodamine-conjugated donkey anti-goat IgG antibody was from Molecular Probes (Eugene, OR). The mouse monoclonal IgG antibody against the 90 kDa heat shock protein was from StressGen (Victoria, BC). Reticulocyte lysate was made in the laboratory according to Merrick's method (25). The rat MR NL1 peptide (ARK-SKKLGKLGK) and the SV40 small T-antigen NL1 peptide (PKKKRKVEDPYGGC) were from Sigma-Genosys. Inasmuch as the results obtained with both oligopeptides were indistinguishable, we show only those obtained with the former (called the "NL1 peptide"). NL1-tagged albumin was made by cross-linking of FITC-BSA (Sigma) and the peptide in the presence of sulfo-SMCC (Apollo Sciences Ltd., Cheshire, U.K.) according to the manufacturer's instructions.

Receptor Preparation. Rat collecting duct cells expressing MR were isolated and cultured as previously described (26). Cells were homogenized in 1 volume of PEGM buffer [25 mM phosphate (pH 7.35), 2 mM EDTA, 10% glycerol, 20 mM Na_2MoO_4 , and a protease inhibitor cocktail]. Homogenates were centrifuged at 100000g for 1 h at 0 °C. The MR was partially purified by adsorption on a hydroxylapatite gel (27), and the resulting cytosol preparation was immediately used for the assays.

Immunoprecipitations. We followed a method previously described (28). Briefly, cell cytosol was first precleared with protein A-Sepharose for 1 h at 0 °C, and the supernatant was then incubated for 2 h with anti-MR antibody precoupled to protein A-Sepharose (or non-immune rabbit serum). The pellet was washed five times with ice-cold PEGM buffer containing 0.02% Nonidet P-40. Subsequently, pellets were washed twice more with further buffers as required for the individual experiments. Immunoprecipitations from soluble fractions of nuclei were performed in a similar manner by

using the nucleoplasmic fraction obtained from isolated nuclei (6), whose insoluble fraction of chromatin and nuclear matrix was pulled down by centrifugation at 30000g for 20 min. Proteins were resolved by SDS-PAGE and visualized by Western blotting.

Steroid Binding Assays. The standard final volume used for binding studies was 200 μL . Binding assays were always performed with 20 nM [3 H]aldosterone at 0 °C for 4 h in PEGM buffer supplemented with 1 mM DTT and 0.1 M RU38486 to prevent possible cross reaction with endogenous GR. Bound steroid was separated from free steroid by addition of 0.25 volume of a suspension of 4% charcoal and 0.4% dextran when cytosol was used or by washing the immune pellets of MR with PEGM buffer. The amount of nonspecifically bound ligand was determined in the presence of a 500-fold excess of radioinert aldosterone, and the values were subtracted from total binding.

Treatment with DEPC. We followed a standard protocol previously described by other laboratories (29–32). Briefly, the immunopurified receptor was treated at 0 °C with DEPC, and the reaction was stopped by adding an imidazole solution at pH 7.3 to a final concentration of 300 μM . The excess of reagents was removed by washing the pellets with ice-cold PEGM buffer. When cytosol preparations were used, reagents were removed by a quick centrifugation in Sephadex G-50 minicolumns (60 s at 6000 rpm) followed by a high-pressure ultrafiltration to rapidly concentrate the samples to the minimal possible volume. Because MR is extremely unstable in cell-free systems, all procedures were always carried out at 4 °C, in the presence of protease inhibitors, and the preparations were not stored but used immediately. The stock solution of DEPC was prepared before each experiment was conducted, and its effective concentration was monitored spectrophotometrically by reaction with a standard solution of histidine. Modification of His residues was confirmed and quantified by the increase in absorbance at 237 nm (33) on solubilized receptor aliquots (as described below).

Circular Dichroism. Immunopurified MR was treated with DEPC, washed, and released from the solid phase with 100 mM glycine at pH 3.0. The supernatants were pooled and centrifuged on Sephadex G-50 minicolumns equilibrated with phosphate/saline buffer at pH 7.4. The samples were concentrated by ultrafiltration and checked by Western blotting and gel staining. The circular dichroism (CD) spectra of unmodified and carbamylated MR were measured with an Aviv-2002 CD spectrometer in the far-UV region of the spectrum (200–260 nm).

Receptor Reconstitution. Immunoprecipitated MR was stripped free of associated proteins with a high ionic strength (0.5 M KCl). After the pellet had been washed, the hsp90-free MR preparation was incubated for 30 min at 30 °C with rabbit reticulocyte lysate in the presence of an ATP-regenerating system (26). The pellets were washed three times with PEGM buffer and used for steroid binding assays or Western blotting.

Limited Chymotrypsinization of MR. We followed a modification of a previously described protocol (26, 34). Rat MR was translated in vitro in a reticulocyte lysate system using the TNT-coupled transcription/translation kit from Promega (Madison, WI) according to the manufacturer's instructions. The ^{35}S -radiolabeled MR was immunoprecipitated and modified with DEPC; control preparations lacked

DEPC. After being washed, pellets were treated with 20 units/mL bovine chymotrypsin in phosphate/saline buffer for 5 min at 0 °C. The reaction was stopped by boiling the pellets with SDS sample buffer; proteins were then resolved via SDS–14% PAGE and autoradiographed.

Cell Permeabilization and Indirect Immunofluorescence Assays. E82.A3 mouse fibroblasts (35) were grown on glass coverslips in DMEM with 10% bovine calf serum. Cells were permeabilized following a modification of a previously used method (34). Coverslips were immersed for 5 min in ice-cold HAMED buffer [20 mM Hepes (pH 7.4), 110 mM KOAc, 5 mM NaOAc, 2 mM Mg(AcO)₂, 1 mM EGTA, and 2 mM DTT] containing 15 μ g/mL digitonin. Then, coverslips were washed with buffer without detergent and incubated at 30 °C under a 95% O₂ atmosphere with 50 μ L of a solution made with up to 40% (v/v) concentrated flag-MR in HAMED buffer, 20% (v/v) duct cell cytosol, and HAMED buffer supplemented with 50 μ g/mL BSA, 15 mM ATP, 15 mM creatine phosphate, 50 units/mL creatine phosphokinase, 1.0 mM GTP, 50 μ M pyruvate, 20 μ M acetyl-CoA, 500 μ M glutathione, and 8 mM glucose. When the native MR·hsp90 complex was used, the nuclear translocation of MR was triggered with 1 μ M aldosterone, whereas the steroid was omitted for the carbamylated MR (DEPC-modified cytosol) on which it has no effect. At the end of each treatment, the cells were rapidly washed with HAMED buffer and fixed with cold (–20 °C) methanol for 15 min. The MR was visualized by indirect immunofluorescence with an Olympus BX60 epi-illumination fluorescence microscope. The cytoplasmic and nuclear intensity of at least 100 cells per condition was quantified for each compartment by image analysis with Zeiss LSM5 Image Examine, and the values were referred to the total fluorescence.

RESULTS

Hsp90-Dependent and -Independent MR Nuclear Translocation in Kidney Duct Cells. Kidney duct cells were incubated on ice with aldosterone to permit binding of aldosterone to the MR, but not nuclear translocation. The hsp90 inhibitor radicicol was added to the medium, and the temperature was increased to 37 °C (zero time) to permit MR nuclear translocation. Figure 1A shows the nuclear translocation rate of the MR, which shows a full nuclear accumulation after 10 min in the presence of hormone. However, when the hsp90-disrupting agent radicicol was added to the medium, MR nuclear localization was significantly impaired; nonetheless, MR reached the nucleus after 50–60 min. These observations imply the existence of two different mechanisms for MR nuclear translocation: a rapid and efficient hsp90-dependent mechanism, which is able to translocate the cytoplasmic MR pool to the nucleus with a $t_{0.5}$ of 5 min, and a second, slower hsp90-independent mechanism ($t_{0.5}$ = 30–40 min). Concentrations of radicicol higher than 2.5 μ M did not strengthen the inhibitory effect. These observations are in agreement with those previously described for GR (8, 36).

Consistent with the hypothesis that MR moves toward the nucleus in an hsp90-dependent mechanism, the chaperone was recovered co-immunoadsorbed with the MR from soluble nucleoplasmic extracts after incubation for 10 min with aldosterone (Figure 1B). At this time, the MR is totally

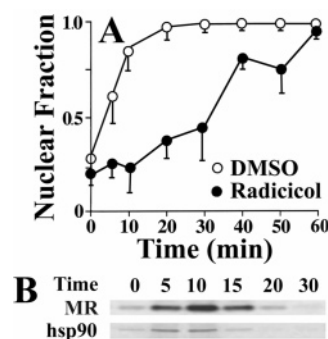


FIGURE 1: Hsp90-dependent and hsp90-independent mechanism of MR nuclear translocation. (A) Kidney duct cells were incubated on ice with 50 nM aldosterone for 1.5 h to allow steroid binding but not nuclear translocation. The hsp90 inhibitor radicicol was added to the medium (2.5 μ M in DMSO) and the incubation on ice continued for 20 min. The medium was then replaced with the same medium prewarmed at 37 °C (time zero). Cells were fixed in cold methanol at the indicated times, and the translocation rate of the MR was semiquantified by scoring 100 cells according to the signal observed by indirect immunofluorescence. Results are the means \pm the standard error of the mean of three independent experiments. (B) MR was immunoprecipitated from the soluble fraction of isolated nuclei after addition of aldosterone. Time is expressed in minutes. Co-immunoprecipitation of hsp90 bound to the nuclear MR was analyzed by Western blotting.

nuclear (Figure 1A). After 20 min in the presence of steroid, the nuclear MR is not longer recovered in the soluble fraction of the nucleus because it was pulled down during the centrifugation process together with the insoluble nuclear fraction of chromatin and nuclear matrix (Western blots not shown).

These results suggest that MR transformation takes place in the nucleus rather than in the cytoplasm. In turn, this implies that the NLS of the MR must be exposed even though hsp90 is still bound to the receptor. Therefore, we decided to explore this hypothesis in digitonin-permeabilized cells, which allowed us to deliver proteins and other membrane impermeable reagents into the cells. This presents an additional problem in the high instability of the transformed MR, which is immediately subject to proteolytic degradation upon hsp90 dissociation. Similar problems were experienced with high-ionic-strength-transformed MR. For this reason, DEPC treatment was used for the N-carboxylation of MR imidazole groups, which led to non-steroid-dependent MR transformation. This modified receptor was quite stable in a cell-free system.

DEPC Abrogates Binding of Aldosterone to the MR. The pH profile for binding of aldosterone to the rat MR yields a maximum at pH 7.3, and the first-derivative function for this curve shows two clear inflection points at pH 6.2 and 8.4 (Figure 2A). The first value is close to pK values of histidine residues in proteins (\sim 6.0). This may indicate that the proper ionization of critical imidazole residues is required to induce an optimal MR conformation for ligand binding. The other inflection point is similar to the pK of cysteine residues (\sim 8.3), which is in agreement with previous reports showing the essential role of MR thiol groups (37–39) and the fact that hsp90 is rapidly dissociated at alkaline pH (26). The inset in Figure 2A demonstrates that the steroid binding capacity of MR fades due to a substantial dissociation of hsp90 from the receptor at extreme pHs, where ionization of thiol groups and imidazole groups is affected.

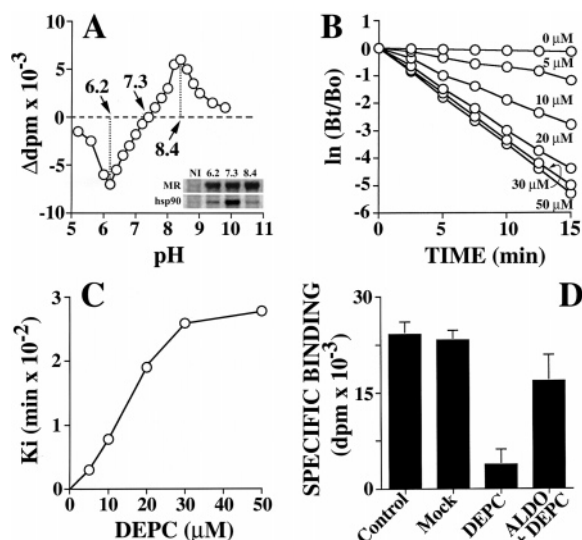


FIGURE 2: Histidine modification impairs binding of aldosterone to the MR. (A) Effect of pH. Binding of aldosterone to the MR was assessed in cytosol made in buffers adjusted to different pHs. Variations of specific binding (Δdpm) were plotted vs pH. Arrows show the inflection points at pH 6.2 and 8.4 and the optimal pH for aldosterone binding at pH 7.3. The inset shows a Western blot for hsp90 after immunoprecipitation of the MR from cytosols made at the indicated pH (NI, non-immune pellet). (B) DEPC- and time-dependent MR inactivation. The MR was immunopurified and washed, and the resultant pellet was incubated at 0 °C with DEPC. The pellet was washed, and the aldosterone binding capacity was measured. The Bt/Bo ratio represents the steroid binding at time t to time zero. (C) Inhibition constant. The pseudo-first-order rate constants (K_i) were obtained from the linear functions depicted in panel B and plotted against the concentration of DEPC. Results are the average of two experiments performed in triplicate. (D) Ligand protection. Immunopurified MR was treated for 15 min at 0 °C with 30 μM DEPC, either prior to (DEPC) or after (ALDO+DEPC) saturation of the MR with [^3H]aldosterone. A mock reaction without DEPC was also carried out prior to the steroid binding assay (Mock), whereas the control was first incubated with the tracer and then a mock reaction without DEPC performed (Control). Bar graphs represent the average mean \pm the standard error of the mean of three independent assays, each performed in triplicate.

Inasmuch as it was likely that His groups were involved in the transformation of MR, immunopurified MR was treated with DEPC, a reagent widely used to specifically modify histidine residues in cell-free systems and intact cells (29–32). Figure 2B depicts the inhibitory effect of increasing concentrations of DEPC and the incubation time with the reagent on binding of aldosterone to the rat MR. Figure 2C depicts the pseudo-first-order rate inhibition constants (K_i) calculated from the linear plots in panel A as a function of the reagent concentration. The second-order rate constant k calculated from the slope of this plot is as high as $1130 \text{ min}^{-1} \text{ M}^{-1}$, which indicates the existence of highly reactive imidazole groups in the rat MR. This suggests that imidazole groups are highly exposed to the aqueous medium.

In spite of their high reactivity, the number of His residues involved in the reaction is limited, which was evidenced by the saturation of the function at concentrations higher than 30 μM (Figure 2C). This indicates that the DEPC-dependent inhibition occurs through modification of specific His sites. Interestingly, steroid binding capacity was preserved when the reaction with DEPC was performed with a preparation of MR presaturated with [^3H]aldosterone (Figure 2D), which suggests that the DEPC-reactive groups may not be available

to the reagent after a conformational change of the MR induced by ligand binding.

Specificity of the N-Carboethoxylation Reaction. N-Carboethoxylation of imidazole groups is a reversible reaction. To test the specificity of the treatment with DEPC, the MR was first inactivated with 30 μM DEPC for 15 min at 20 °C. After the excess reagent had been eliminated, the modified MR was treated with 300 μM hydroxylamine (29–32), the excess of reagents washed out, and the putative restoration of the MR [^3H]aldosterone binding capacity measured. Figure 3A shows that binding of aldosterone to the MR was restored after hydroxylamine treatment, though only partially [Figure 3A (●)] since hydroxylamine itself has an inhibitory effect on steroid binding [Figure 3A (▲)]. This recovery of steroid binding suggests that the reaction with DEPC is specific. Moreover, the presence of a 20-fold molar excess of histidine fully prevented the deleterious effect of DEPC on binding of aldosterone to the MR [Figure 3A (Δ)].

An N-carboethoxyimidazole derivative is expected to exhibit a single peak of absorbance at 235–240 nm (40). Figure 3B depicts the differential absorbance calculated from the spectrum of DEPC-treated MR compared with the profile obtained with the unmodified receptor. In DEPC-treated MR, a single peak was detected at 236 nm, the magnitude of which increased with the incubation time until the reaction was almost complete after incubation for 15 min. This peak also increased in a DEPC concentration-dependent manner (data not shown). These spectra reinforced the idea that DEPC reacted specifically with imidazole groups.

In spite of the fact that the receptor was modified by N-carboethoxylation, both native and DEPC-treated MR exhibited similar CD spectra (Figure 3C). This suggests that the two proteins (native and DEPC-modified) have similar secondary structure. This observation makes unlikely the fact that the altered binding capacity of the DEPC-treated receptor is due to global effects on the structural integrity of the receptor and agrees with the notion that there are local effects at the sites of amino acid modification only.

N-Carboethoxylation of the MR Dissociates the Chaperone Complex. Next we studied the effect of DEPC treatment on the association of hsp90 with modified MR. Duct cell cytosol was treated with DEPC, and MR was immunoprecipitated. Figure 4A shows that the loss of steroid binding in DEPC-modified MR parallels the dissociation of hsp90 from the complex. Consistent with the results shown in Figure 2D, binding of aldosterone prior to DEPC treatment protected MR binding capacity (condition 5 vs condition 4) under conditions where transformation is not favored (e.g., incubations performed at 0 °C in the presence of 20 mM molybdate).

Since the reaction with DEPC was performed using the MR heterocomplex, it is possible that N-carboethoxylation could affect other components of the heterocomplex, such as hsp90, as well as the receptor. To determine whether the receptor was indeed the primarily affected component, MR was immunoprecipitated and stripped of associated proteins by 0.5 M KCl. Figure 4B shows that, as expected, hsp90-stripped MR lacks steroid binding capacity (condition 3). Steroid binding was fully restored by reconstitution of the MR-hsp90 complex with reticulocyte lysate (condition 4). However, when the MR was modified with DEPC prior to the reconstitution step, the reassociation of hsp90 and

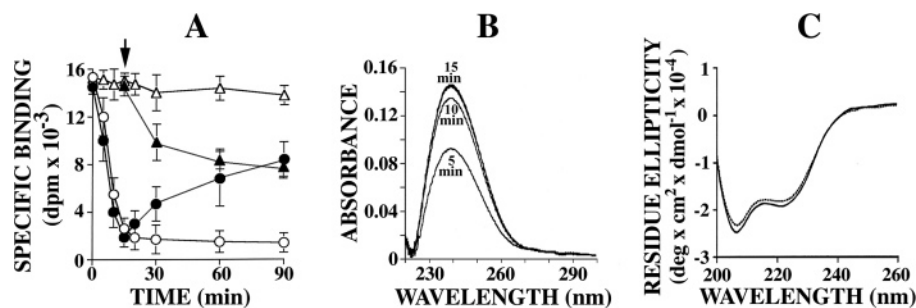


FIGURE 3: Specificity of the reaction with DEPC. (A) Reversion with hydroxylamine. Immunopurified MR was treated with 30 μ M DEPC for 15 min at 20 °C (○), and the pellet was washed with PEGM buffer and reincubated on ice with 300 μ M NH_2OH (●). After washing had been carried out, the steroid binding capacity of the immune pellet was measured. A control treatment with 300 μ M NH_2OH only was also performed (▲). Protection against the inhibitory effect of 30 μ M DEPC was achieved by performing the reaction in the presence of 0.6 mM histidine (△). Results are the average mean \pm the standard error of the mean of three experiments, each performed in duplicate. (B) Difference spectra of the DEPC-modified receptor. Immunopurified MR was treated with DEPC; the pellets were quenched with histidine and washed, and the MR was released from the pellet as described in Experimental Procedures. The final MR solution was scanned between 220 and 300 nm to evaluate the formation of the corresponding N-carbetoxyimidazole derivative. Absorbance values are the difference between DEPC-modified MR and the reference MR sample not treated with DEPC. (C) CD spectroscopy. MR was immunopurified and stripped of associated proteins, and histidine residues were modified by treatment with 30 μ M DEPC at 0 °C for 15 min. The receptor was solubilized, and its structural integrity was analyzed by CD spectroscopy. The CD spectra of the wild-type stripped receptor (—) and the DEPC-treated receptor (···) were superimposed.

subsequent recovery of steroid binding were dramatically impaired (condition 6). These results and those shown in Figure 3 suggest that N-carbetoxylation of MR histidines is responsible for the dissociation of hsp90. Purified hsp90 also reacts with DEPC, but it requires DEPC concentrations higher than 400 μ M when incubated for 30 min at 0 °C to lose its ability to rebinding to the MR (data not shown). Under our experimental conditions for MR, the reaction of purified hsp90 with DEPC is negligible (~5%).

Next, we determined whether there is a direct correlation between the number of modified histidines in stripped MR and the loss of aldosterone binding measured in the MR·hsp90 complex reconstituted with reticulocyte lysate. Figure 4C shows that, under the controlled and mild conditions used for MR carbamylation, the modification of two histidine residues seems to be sufficient to transform the MR and totally abolish steroid binding capacity. Importantly, a Western blot for this modified MR using an anti-NL1 antibody shows that the signal is preserved after MR carbamylation when it was compared to that of unmodified, salt-stripped MR. The inset in Figure 4C shows a Western blot for NL1 after nondenaturing gel electrophoresis. The NL1 bands shown in this figure matched the alignment of the MR bands that were Western blotted in parallel lanes with an anti-MR antibody. Unfortunately, it was not possible to analyze by Western blotting NL1 of the nontransformed MR since this large complex is retained on the top of even a 4.5% gel.

If DEPC-modified MR undergoes a structural modification, it would be possible to demonstrate such conformational change by comparing the fragments of MR generated by limited proteolysis (26, 34). The receptor was translated *in vitro*, immunopurified, stripped of associated proteins, and digested with chymotrypsin under controlled conditions. Figure 4D shows that carbamylation of the MR did not affect the efficiency of its immunoprecipitation by the anti-MR antibody. Limited proteolysis yielded a dramatically different pattern for native MR and modified MR. While the unmodified MR was totally degraded by chymotrypsin, N-carbetoxylation of MR shows three resistant fragments (69, 31, and 10 kDa). Therefore, even though N-carbetoxylation trans-

forms MR by an efficient dissociation of hsp90, it makes the receptor more resistant to degradation. In fact, DEPC-modified MR was much more stable than native MR transformed by either steroid binding or high-ionic strength treatment. A similar resistance to degradation was monitored by incubation of carbamylated MR in cell cytosols incubated at room temperature as compared to native MR, which is totally degraded after 10 min (data not shown). Therefore, we used the carbamylated MR as a tool to further study the role of the NL1 in the mechanism for MR nuclear import.

Nuclear Import of the MR. Because DEPC fully transforms the MR (Figure 4A) and consequently exposes its NLS (Figure 4C), we asked whether this non-steroid-dependent transformation of MR affects its subcellular localization. We used permeabilized E82.A3 cells to deliver the MR in its untransformed or transformed state. In this cell line, which is derived from L929 fibroblasts, GR expression is knocked out (35) and the cells show no detectable amounts of other steroid receptors. Flag-MR was purified by immobilizing the receptor on flag-Sepharose beads followed by receptor elution with the flag peptide. These flag-MR fractions were concentrated to the minimal possible volume by ultrafiltration and incubated with digitonin-permeabilized cells. Control binding assays demonstrated that the presence of the flag peptide in the medium has no effect on the steroid binding capacity of the MR (data not shown).

Figure 5A shows the indirect immunofluorescence for the MR delivered into permeabilized cells. The receptor was cytoplasmic in the absence of steroid and was efficiently translocated to the nucleus by aldosterone. When the permeabilized cells were preincubated with an excess of 1 mM NL1 oligopeptide and then with aldosterone, the hormone-dependent nuclear translocation of the MR was significantly impaired. Nevertheless, Figure 5A also shows that the inhibition was only partial and the receptor was almost equally distributed between cytoplasm and nucleus. This distribution was not further changed by higher concentrations of the peptide. Cotreatment with the hsp90-disrupting agent radicicol also did not abolish in full the nuclear translocation of the MR, nor was any potentiation observed. This suggests an alternative mechanism for MR nuclear

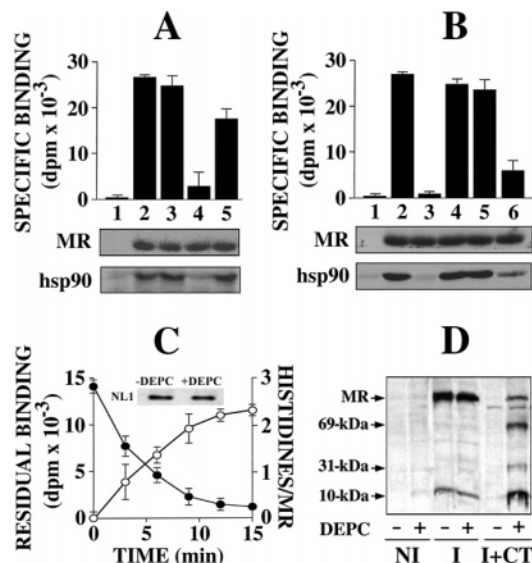


FIGURE 4: Histidine modification promotes MR transformation and induces a differential conformational change in the receptor. (A) Treatment of soluble MR. Duct cell cytosol obtained in buffer supplemented with 20 mM molybdate was treated with 30 μ M DEPC for 15 min at 0 °C, the reaction quenched with histidine, and the excess of reagents cleared by centrifugation in Sephadex G-50 minicolumns at 4 °C equilibrated with buffer supplemented with 20 mM molybdate. The MR was immunoprecipitated in duplicate with either an anti-MR antibody (conditions 2–5) or a rabbit non-immune antibody (condition 1). One sample was used for measuring [³H]aldosterone binding capacity (bar graphs are the means \pm the standard error of the mean; $n = 3$), and the other sample was used for performing Western blots for the MR and hsp90: (1) non-immune pellet and (2–5) immune pellets from cytosol (2) not treated or treated with (3) mock reaction without DEPC, (4) 30 μ M DEPC, or (5) aldosterone first bound to the MR, followed by the cytosol being treated with 30 μ M DEPC. (B) Treatment of immunopurified stripped MR. MR was immunoprecipitated from the duct cell cytosol, and the associated proteins were stripped with 0.5 M KCl. Stripped pellets were first treated with DEPC, and the hsp90 heterocomplex was reconstituted with reticulocyte lysate. Both steroid binding capacity (mean \pm the standard error of the mean; $n = 3$) and MR transformation were analyzed as in the previous panel: (1) non-immune pellet treated with reticulocyte lysate, (2) nonstripped immune pellet, (3) 0.5 M KCl-stripped MR, (4) stripped MR reconstituted with reticulocyte lysate, (5) a mock reaction first carried out with stripped MR followed by the heterocomplex being reconstituted with reticulocyte lysate, and (6) stripped MR first treated with DEPC followed by the heterocomplex being reconstituted with reticulocyte lysate. (C) MR carbamylation and loss of steroid binding capacity relationship. The immunopurified MR was stripped with 0.5 M KCl, treated with 30 μ M DEPC, and reconstituted with reticulocyte lysate. The plot depicts the reactivity of histidine groups (○) and aldosterone binding capacity (●) as a function of the reaction time with DEPC. Modified histidines were quantified by spectrometry at 237 nm as described in Experimental Procedures, and the number of MR binding sites was measured by [³H]aldosterone binding assuming a 1:1 molar ratio. The inset shows a Western blot for immunopurified MR transformed by steroid binding (–DEPC) and DEPC-transformed MR (+DEPC) resolved by nondenaturing gel electrophoresis and Western blotted with the anti-NL1 antibody. Results are the means \pm the standard deviation of four experiments performed in duplicate. (D) Limited proteolysis. The ³⁵S-labeled MR translated in vitro using a reticulocyte lysate translation–transduction system was immunoprecipitated with anti-MR antibody (I) or a non-immune antibody (NI). Pellets were stripped with high ionic strength and treated with DEPC (+) or with a mock reaction (–). After being washed, the immune pellets were incubated with chymotrypsin (CT). Proteins were resolved by gel electrophoresis and autoradiographed with a film sensitive to ³⁵S. These results were reproduced three times.

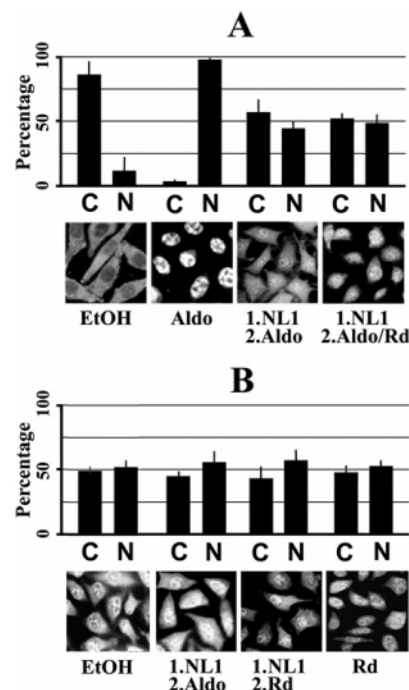


FIGURE 5: Nuclear import of native MR and carbamylated MR. Digitonin-permeabilized E82.A3 fibroblasts were preincubated for 10 min at 30 °C with HAMED buffer or 1 mM NL1 oligopeptide (where indicated). After being preincubated, cells were treated for 20 min at 30 °C with the purified flag-MR·hsp90 heterocomplex supplemented with vehicle (EtOH) or 50 nM aldosterone (Aldo) or a preincubation with 1 mM NL1 oligopeptide followed by an incubation with 50 nM aldosterone and 0.1% (v/v) DMSO (1.NL1, 2.Aldo) or 50 nM aldosterone and 2.5% radicicol (1.NL1, 2.Aldo/Rd). The native MR (A) and carbamylated MR (B) were visualized by indirect immunofluorescence performed with a mouse IgG followed by a goat anti-mouse IgG labeled with rhodamine. Bar graphs represent the percentage of cytosolic (C) and nuclear (N) receptor (means \pm the standard error of the mean of three experiments) after counting \sim 250 cells per experiment.

translocation that may not be related to hsp90 and NL1. This hsp90-independent mechanism of nuclear translocation resembles the effect of radicicol in intact cells (Figure 1).

When MR was transformed in a non-steroid-dependent manner by DEPC treatment, the carbamylated MR was primarily localized in the nucleus in the absence of steroid (Figure 5B), and aldosterone had no effect (data not shown), as expected in view of the inability of carbamylated MR to bind steroid (Figures 2 and 4). This condition was used as an internal control to test the efficiency of the treatment with DEPC. Although the receptor was efficiently transformed by N-carbethoxylation, it did not fully translocate into the nucleus (compare the complete translocation observed with aldosterone in Figure 5A vs the partial MR nuclear translocation shown in Figure 5B). Interestingly, saturation of the cells with the NL1 oligopeptide did not affect that fraction of the receptor that is able to move into the nucleus, suggesting that this pool of MR also translocates in an NL1-independent manner. Consistent with the results shown in Figure 5A, radicicol had no effect.

Taken together, these observations suggest that the exposure of the NLS alone does not ensure MR nuclear translocation and that there are at least two different mechanisms for MR nuclear import, one NL1- and hsp90-dependent and the other NL1- and hsp90-independent.

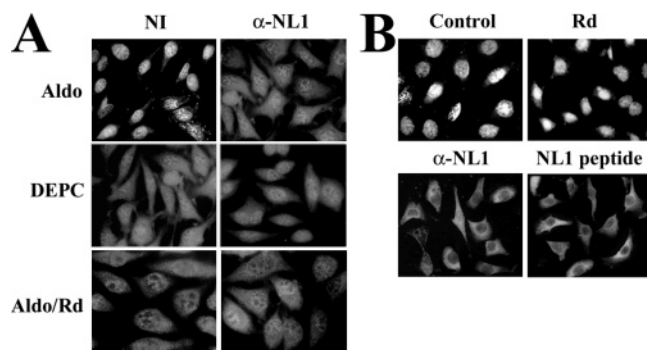


FIGURE 6: Effect of an anti-NL1 antibody in the nuclear translocation of MR and NL1-tagged BSA. (A) Nuclear translocation of flag-MR. The DEPC-treated MR or the native MR bound to aldosterone was preincubated for 10 min at 30 °C with 1% (v/v) non-immune rabbit antibody (NI) or anti-NL1 rabbit antibody (α-NL1). Then, the receptor was delivered into E82.A3 fibroblasts as described in the legend of Figure 5. Aldosterone (50 nM) was added to the medium when steroid–MR complexes were used (Aldo). DEPC represents carbamylated MR, and Aldo/Rd indicates that the carbamylated MR was preincubated with 50 nM aldosterone and delivered into the cells in the presence of 2.5 μM radicicol. After 20 min at 30 °C, the cells were fixed in cold methanol, incubated with anti-flag mouse IgG followed by a goat anti-mouse rhodamine-conjugated IgG, and visualized by indirect immunofluorescence. (B) Nuclear translocation of NL1-tagged FITC BSA. Preparations containing 250 μM NL1-BSA-FITC in HAMED buffer were preincubated for 10 min at 30 °C with either buffer alone (Control), 2.5 μM radicicol (Rd), 1% (v/v) anti-NL1 antibody (α-NL1), or 1 mM NL1 oligopeptide (NL1 peptide). Then, the mixture was incubated with permeabilized cells for 20 min at 30 °C, which were fixed in cold methanol, and the fluorescence of BSA was visualized with a fluorescence microscope. The results shown in both panels of this figure were reproduced four times.

MR Nuclear Translocation Is Impaired by an Anti-NL1 Antibody. Native MR bound to aldosterone or DEPC-treated MR was preincubated with 1% anti-NL1 antibody or 1% non-immune antibody. Then, the receptor was delivered into digitonin-permeabilized cells (Figure 6A). Blocking the NL1 signal in this way partially prevented nuclear translocation of aldosterone–MR complexes, suggesting again the existence of an NL1-independent mechanism for nuclear translocation. On the other hand, the antibody had no effect on the fraction of carbamylated MR that was imported in a non-steroid-dependent manner. In other words, carbamylation seems to inhibit the fraction of MR that translocates via NL1 only, whereas the alternative mechanism appears to be unaffected by the modification of MR imidazole groups.

Under the same experimental conditions used for the MR in Figure 6A, the anti-NL1 antibody and the NL1 oligopeptide were both able to abolish the nuclear localization of fluorescent NL1-tagged BSA, demonstrating that the lack of full inhibition seen for MR could not be attributed to experimental conditions. Radicicol did not affect nuclear translocation of NL1-tagged BSA, demonstrating that the nuclear import mechanism of the cell is not generally affected by the hsp90 inhibitor. This observation agrees with a previous publication which demonstrated that impairment of GR nuclear translocation by geldanamycin was unable to influence the nuclear translocation of a GFP–STAT5b chimera upon cell stimulation with growth hormone (14). In short, Figure 6B validates the results observed in Figure 6A with the MR.

DISCUSSION

In spite of the fact that a given member of the nuclear receptor family may be primarily located in the cytoplasm or the nucleus, it is almost certainly not likely to be confined to either cell compartment in a static manner. Thus, it is accepted that receptors move in and out of the nucleus in a highly dynamic shuttling process (10, 41). Therefore, the final subcellular localization of steroid receptors reflects the steady-state equilibrium between nuclear import and nuclear export, itself a regulatory mechanism critical for biological function in all members of the superfamily (42).

It is possible that the subcellular localization of MR is regulated in a manner more complex than previously thought. Although the digitonin-permeabilized cell system is a superb tool for delivering membrane-impermeable compounds into cells, transformed MR preparations were highly unstable in this experimental system and the MR was immediately subject to degradation. Consequently, it was necessary to develop a reliable system in which the hsp90-free MR is stable. This problem was solved by modifying the MR with DEPC; the resultant N-carbethoxylated MR was resistant to degradation and lost the ability to bind hsp90, and its NLS was exposed. In characterizing the properties of the carbamylated MR, we found that two imidazole residues may be key residues involved in receptor transformation.

Interestingly, the conformation acquired by the carbethoxylated receptor allows MR nuclear translocation by an NL1-independent mechanism. This alternative mechanism was also observed in intact cells treated with hsp90 inhibitors (Figure 1).

It is also evident that exposure of the NLSs alone does not automatically result in MR nuclear translocation (Figure 5). The conventional view hitherto has been that, in receptors primarily located in the cytoplasm such as MR or GR, NLSs are hidden when hsp90 forms part of the complex. Therefore, it has always been believed that receptor transformation must be the first mandatory step prior to nuclear translocation. However, there is evidence that conflicts with this dogma. First, the presence of hsp90 bound to the GR is critical for its link with the cytoplasmic machinery that moves the receptor toward the nucleus (8, 43). Second, hybrid molecules between the PR (primarily nuclear) and hsp90 constructs reveal that the receptor may be relocated in the cytoplasm in a manner that is not altered by the exposure of its NLS (44). Third, there is evidence that hsp90 can be cotransported with the steroid receptor into the nucleus, keeping the nonliganded receptor inactive but poised for transcriptional regulation (45). Fourth, in the studies presented here, hsp90 was recovered bound to the MR immediately after its nuclear translocation, suggesting that the complex remained intact during the process (Figure 1B). There is, therefore, no clear relationship between NLS availability and nuclear translocation. Instead, it is likely that nuclear translocation is the result of a concerted mechanism between the strong NL1 sequence and perhaps the weak and diffuse NL2 signal, and/or unknown sequences remaining to be identified or characterized. This mechanism could be regulated by the ability of the receptor to change its conformation according to the stimulus that promotes its transformation and, possibly, recruitment of other factors that may participate in the nuclear import mechanism as well (46). There are reports describing

how some transcription factors gain access to the nucleus through cotransport with other factors (47, 48). Importantly, there is also evidence that hsp90-binding high-molecular weight immunophilins such as FKBP52 and FKBP51 switch in the GR complex upon ligand binding (11). Recently, we have also found a similar response according to the nature of the steroid–MR complex for both immunophilins and the immunophilin-like phosphatase PP5 (L. Gallo, A. Ghini, G. Piwien Pilipuk, and M. Galigniana, manuscript submitted for publication). Such selective recruitment of different regulatory factors may explain the specific biological response triggered by a given ligand when it is bound to the same receptor.

Our results agree with the notion that the nuclear translocation of the MR is hsp90-dependent. This is supported by the experiments shown in Figure 1. When radicicol is present, MR nuclear translocation is significantly delayed. Whether it depends on the putative NL2-like NLS motif present in the ligand binding domain is uncertain. Unfortunately, due to the diffuse characterization of such NLSs, we were unable to study this hypothesis. Nuclear import of liganded GR is also mediated by the NL1 motif and correlates with binding to α -importin. However, the α -importin-independent translocation observed for a GR mutant in which NL1 was deleted agrees with the speculation that nuclear import may be NL2-dependent (16) and mediated by separate pathways. Interestingly, the nuclear translocation rate of GR in that NL1-deleted mutant is identical to the rate shown in Figure 1A for the MR in cells treated with radicicol.

A similar observation has also been reported for a PR mutant where the active NLS is absent. As expected, this PR mutant is cytoplasmic in a medium without steroid. With addition of hormone, the PR mutant translocates to the nucleus, although at a slower rate compared to that of native PR (49). The authors show that the slower nuclear import kinetics observed with the PR mutant is due to its lack of interaction with α -importin.

In the two examples discussed above, both GR and PR reach the nucleus in a less efficient, NLS-independent, manner. This parallels the property described for the MR in this work, which suggests a similar alternative mechanism for nuclear translocation for at least some members of the steroid receptor family.

It has been known for a long time that α -importin is responsible for recognizing the cargo NLS sequence and β -importin accounts for the targeting to the nuclear pore. However, recent studies have also identified novel pathways in which β -importin binds directly to cargoes without interacting with α -importin (50, 51). Nevertheless, it is unlikely that this may explain the alternative nuclear import pathway evidenced in our work since a recent study showed that MR and α -importin simultaneously move into the nucleus upon activation with ligand, but not β -importin, which undergoes no change and remains in the perinuclear region with a dotlike pattern (52).

It has been reported that unconventional NLS sequences can be generated at random with an unexpectedly high frequency, and they have been described on a case by case basis (53). Therefore, it is entirely possible that the conformational change generated in the MR-hsp90 complex by treatment of cells with radicicol, or that induced by N-carbethoxylation of MR in a cell-free system, is responsible

for exposing unknown or atypical motifs that may function as NLSs. In this sense, the chemically modified variant of transformed MR described here may mimic the properties of MR in intact cells treated with hsp90 inhibitors, so the N-carbethoxylated receptor may be a useful tool for elucidating the molecular mechanisms underlying MR subcellular localization, a critical factor in the mineralocorticoid signal transduction pathway and the cellular response to corticosteroids.

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