molecular pharmaceutics

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Effect of Initial Subcellular Localization of Progesterone Receptor on Import Kinetics and Transcriptional Activity

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Received May 24, 2005

Abstract: Progesterone receptors (PR) are ligand-activated transcription factors that modulate transcription by activating genes. There are two isoforms of PR, PRA and PRB. In most cell contexts, the PRA isoform is a repressor of the PRB isoform. Without hormone induction, PRA is mostly located in the nucleus whereas PRB distributes both in the nucleus and in the cytoplasm. In this paper, a new model system has been used to study the impact of initial subcellular localization, and import rate of progesterone receptor on transcriptional activity. This new model system involves using a mutant version of PRB which is found only in the cytoplasmic compartment of cells in the unliganded state, making the distribution of the receptor more homogeneous to start with compared with the previous model, wild type (wt) PRB, which has a more heterogeneous distribution (nuclear and cytoplasmic even without ligand). Import kinetics has been shown to be one of the major means by which to regulate PR transcriptional activity. Fluorescence microscopy was used to measure green fluorescent protein tagged PRB import rate into the nucleus. Luciferase reporter gene assay was used to measure transcriptional activity of PRB. In addition, a two-hybrid assay was performed to measure the interaction between PRB and importin α. Mutant versions of PRA and PRB with the constitutively active nuclear localization signal removed were created (PRA-NLS_c mutant and PRB-NLS_c mutant). These PR mutants were found to localize mainly in the cytoplasm in the absence of hormone. With addition of hormone, PR mutants translocated to the nucleus, although at a slower rate compared to wt PRB. Our results show that the activation of reporter gene transcription is proportional to the nuclear import rate of PRB-NLS_c mutant, and the difference in import kinetics between wt PRB and the PRB-NLS_c mutant is due to a stronger interaction of wt PRB with importin α. We also show that the hormone inducible NLS in PR, NLS_h, is a weak nuclear localization signal even without hormone and can act as a weak hormone dependent nuclear localization signal when combined with the ligand binding domain of PR. In addition, by changing the initial subcellular localization of PRA from the nucleus to the cytoplasm, this diminished PRA's ability to act as an inhibitor of PRB.

Keywords: Progesterone; luciferase assay; two-hybrid assay; progesterone receptor; import kinetics; transcriptional activity

Introduction

The progesterone receptor is a member of the steroid receptor family. PR is a shuttling protein that actively transports between the nucleus and the cytoplasm.[1] In most cell lines, there are two isoforms of PR, PRA and PRB, which are encoded by the same gene, utilizing two distinct transcriptional start sites.[2] These two proteins are identical except that the human PRA isoform (PRA) is a truncated version of the B isoform (PRB), lacking the first 164 amino acids at the N-terminal region. In most promoter and cell contexts, PRA represses transcription mediated by PRB and

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10.1021/mp0500418 CCC: $30.25
Published on Web 09/08/2005

other nuclear receptors such as glucocorticoid, mineralocorticoid, androgen, and estrogen receptors.[3,4] Another difference between PRA and PRB is that PRA localizes predominantly in the nucleus whereas wt PRB distributes in both the cytoplasm and nucleus in the absence of hormone (although still primarily nuclear).[5] The different subcellular distribution of PRA and PRB reflects different nuclear import and export rates. The mechanism of why PRA mainly distributes in the nucleus is unknown. Studies have been carried out on the subcellular distribution of PR isoforms and possible correlated functions.[6−8] We have also previously reported the link between import kinetics into the nucleus and the transcription activity of PRB.[9]

For import into the nucleus, protein trafficking across the nuclear envelope is tightly regulated. Molecules with size smaller than 40−45 kDa diffuse through nuclear pores easily, whereas molecules greater than 45 kDa require a nuclear localization signal (NLS).[10] Importin α, one of the importin/karyopherin proteins, is found in eukaryotic cells and interacts with SV40 large tumor antigen or SV40 like sequences containing lysine (or arginine) rich sequences (PKKKRKV) that act as nuclear localization signals.[10,11] The classical import pathway uses importin α as an adapter molecule to bind to substrate containing the NLS, along with importin β, the docker molecule. The trimeric complex formed imports the substrate into the nucleus through the nuclear pore complex.[10] There are two possible nuclear localization signals (NLS) in PR: a constitutively active nuclear localization signal, NLS_c, which contains a NLS sequence similar to SV40 NLS and is thought to be imported via the classical importin α/β pathway.[12] The other NLS, a hormone inducible nuclear localization signal, NLS_h, is poorly defined and not well studied.[12]

In this paper we show that NLS_c is necessary for full translocation of PR to nucleus but is not sufficient for full transactivation. After NLS_c is knocked out (PR-NLS_c mutants are created), the import kinetics change dramatically. Enhanced green fluorescent protein was used to study the import kinetics of PRA and PRB-NLS_c mutant compared to wt PRB import rate. To determine why wt PRB and PRB-NLS_c mutant are imported at different rates, mammalian two-hybrid assays between importin α and wt PRB or PRB-NLS_c mutant were conducted. The two-hybrid assay suggests that PRB-NLS_c mutant does not interact well with importin α, resulting in a change in the import kinetics of PRB-NLS_c mutant. In addition, by constructing a cytoplasmically located PRA mutant (called PRA-NLS_c mutant) the transcriptional repression of PRA-NLS_c mutant to wt PRB was studied. Our results suggest that altered cellular distribution may significantly affect PR function.

Materials and Methods

Progesterone was purchased from Sigma Chemical Co. (St. Louis, MO) and was dissolved in absolute ethanol.

Plasmids. The plasmids pEGFPPRB-NLS_c mutant and pEGFPPRA-NLS_c mutant were constructed using Quickchange site directed mutagenesis kit (Stratagene, La Jolla, CA). Mutants of both PRA and PRB were made by knocking out the constitutively active nuclear localization signal (NLS_c) in EGFP-PRA and EGFP-PRB, respectively, using forward primer 5′ CTCTGACTTTATTGAACGCTGCAAATGCTCGACCTCCAAGGACCATGCCAGGC 3′ and back primer 5′ CTGGCATGGTCCTTGGAGGTCGAGCATTTGCAGCGTTCAATAAAGTCAGAG 3′. A double mutation on pCMV-BD (Stratagene, La Jolla, CA) was made to create new restriction enzyme sites, *Bsp*EI and *Kpn*I. The plasmid pBD-PRB was constructed by digesting EGFP-PRB and mutated pCMV-BD with *Bsp*EI and *Kpn*I. The fragment containing PRB was inserted into the mutated pCMV-BD vector, and the new plasmid was named pBD-PRB with size of 7.7 kb. The plasmid pGEX-K1, a kind gift from S. G.

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Nadler (Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA), encodes importin α. The pGEX-K1 plasmid was digested by *Not*I and *Eco*RI. This fragment, containing human importin α, was inserted into the pCMV-AD vector, which was digested by the same restriction enzymes as pGEX-importin α. This new plasmid was named pAD-importin α.

The plasmid pEGFP-NLS$_h$ was constructed by conducting PCR on pEGFP-PRB to obtain the NLS$_h$ fragment (Arg593−Gly636) using forward primer 5′ CCGGAATTCTAGGGCA-ATGGAAGGGCAGCACA 3′ and back primer 5′ACGCGTC-GACACCTCCAAGGACCATGCCAGCC 3′ containing restriction enzyme sites, *Eco*RI and *Sal*I. After digesting with these two enzymes, the PCR product was ligated into pEGFP-C1 vector. The plasmid pEGFP-LBD was constructed by performing PCR on pEGFP-PRB to obtain full length LBD using forward primer 5′ GCGCGGTACCGT-CAGAGTTGTGAGAGCACTGGA 3′ and back primer 5′ GCGCGGATCCCAGTTATCTAGATCCGGTGGATCC 3′. The PCR product was digested with *Kpn*I and *Bam*HI and then inserted into pEGFP-C1 vector digested with the same restriction enzymes. Finally, pEGFP-NLS$_h$-LBD was constructed by inserting the PCR product of NLS$_h$ into the pEGFP-LBD vector.

Cell Culture and Transfections. 1471.1 cells (mouse adenocarcinoma cell line, a kind gift from G. Hager, NIH), which do not express endogenous progesterone receptor, were maintained in Dulbecco's modified Eagle's medium (DMEM, GIBCO-BRL, Grand Island, NY) with 10% fetal bovine serum (FBS, Hyclone Laboratories Inc., Logan, UT), penicillin streptomycin (100 units/mL, GIBCO-BRL), gentamycin (0.5 mg/mL, Hyclone), and L-glutamine (2 mM, Hyclone) at 37 °C with 5% CO_2.

Transient transfection was performed with electroporation as described before.[9] Briefly, 2 μg of plasmid DNA with 8 μg of carrier DNA was transiently transfected into 5×10^6 cells via electroporation. Electroporations were performed at 135 V, 10 ms, and three pulses.[9] Cells were plated into individual wells of a six-well plate with $^1/_{12}$ of the total cells in DMEM with 10% charcoal-treated FBS (Hyclone), which contains no hormone. Twenty-four hours after transfection, fresh medium was added to the cells and hormone was added to cells to make the desired final concentrations. At desired induction times, cells were washed with PBS buffer and fixed with 3% paraformaldehyde for 30 min or imaged directly.

Luciferase Assay. For the luciferase assay 10 μg of firefly luciferase plasmid pMMTV-Luc (a kind gift from G. Hager, NIH) and 20 ng of pRL-SV40 renilla luciferase (Promega Corp., Madison, WI) were cotransfected with functional plasmids (PRB-NLS$_c$ mutant alone, wt PRA with wt PRB, or PRA-NLS$_c$ mutant with wtPRB). Twenty-four hours after transfection, the medium was changed (to DMEM with charcoal-treated FBS as above) and progesterone was added to 2 mL of medium to make a final concentration of 100 nM. Dual Luciferase Assay System (Promega Corp., Madison, WI) was used to measure firefly luciferase activity. Renilla luciferase was used as an internal control according to the manufacturer's instructions.[9]

Two-Hybrid Assay. The mammalian two-hybrid assay kit (Stratagene, La Jolla, CA) was used according to the manufacturer's instructions. Two micrograms each of pBD-PRB and pAD-importin α, 1 μg of pFR-LUC, and 0.02 μg of pRL-SV40 and carrier (total 18 μg) were transiently cotransfected into 1471.1 cells by electroporation.[9] The plasmids pAD-importin α and pCMV-BD were used as a negative control (the corresponding proteins will not interact with each other). Twenty-four hours after transfection, cells were scraped from the plate with passive lysis buffer after 6 h of induction with 100 nM progesterone. Dual Luciferase Assay System was used as described before to determine pFR-Luc reporter gene activity.[9]

Fluorescent Microscopy. An Olympus IX70 inverted microscope (Scientific Instrument Company, Aurora, CO) was used to visualize subcellular localization of proteins expressed from plasmids in the cells. To visualize EGFP, a high-quantity narrow-band GFP filter was used (excitation filter set HQ480/20; emission filter set HQ510/20; with beam-splitter Q495lp). Cells were imaged using neutral-density filters that transmit 25% of the total light and short (500 ms) exposure times in order to minimize photobleaching.

Data Analysis. The details of the analysis method were mentioned in our previous paper.[9] Briefly, all images were analyzed by analySIS software (Soft Imaging System GmbH, Lakewood, CO). The percentage of fluorescence intensity in the nucleus at different time points (t) was calculated, and for each dose the average percentage nuclear intensity vs time was plotted using Microsoft Excel. For analyzing differences between experimental groups, the Tukey−Kramer test was used for multiple comparisons as before.[9] R program (R Development Core Team, Vienna, Austria) was used to fit the import kinetic curves.

Results

All of the plasmids constructed that were visualized kinetically (PRA, PRB, PRA- and PRB-NLS$_c$ mutants, NLS$_h$-LBD) were EGFP tagged. For simplicity, these constructs in the results and following text do not have "EGFP" in the protein or plasmid names.

PRA-NLS$_c$ and PRB-NLS$_c$ Mutants Localize Predominantly in the Cytoplasm with Different Import Kinetics Compared to Wild Type PR. Cytoplasmically localized PR mutant plasmids were constructed by knocking out constitutively active NLS in the hinge region of PR. 1471.1 mouse adenocarcinoma cells were transfected and observed by fluorescence microscopy. Both PRB-NLS$_c$ and PRA-NLS$_c$ mutants distribute homogeneously in the cytoplasm without hormone induction (Figure 1A,B at 0 h). After hormone induction (100 nM progesterone), translocation to the nucleus occurs for PR-NLS$_c$ mutants (Figure 1B and Figure 2B), although in a longer time course compared to wt PRB (Figure 1A), which goes to completion within 30 min. Since transport of PR-NLS$_c$ mutant into nucleus occurs very slowly com-

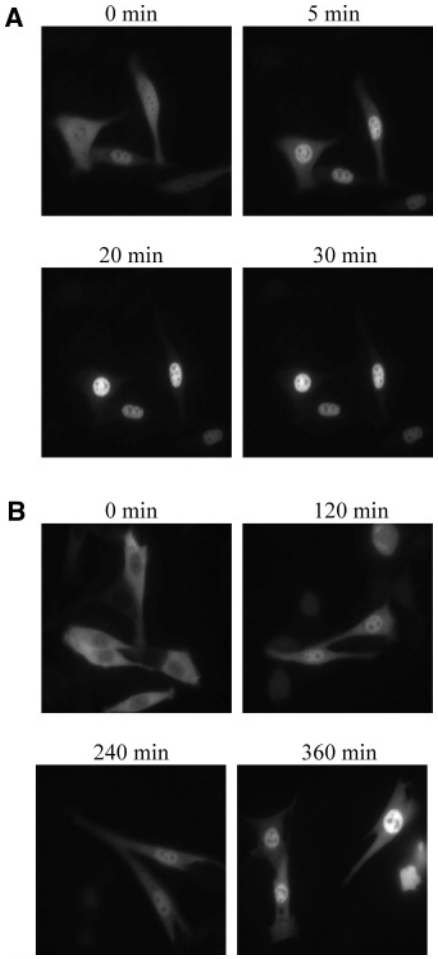

Figure 1. Localization, hormone induction, and subsequent import of wt PRB and PRB-NLS_c mutant. (A) Wt PRB localization at time 0 (no hormone), 20, and 30 min after hormone induction (100 nM progesterone). (B) PRB-NLS_c mutant distributes homogeneously in the cytoplasm at 0 min (no hormone). After hormone induction at 2, 4, and 6 h, PRB-NLS_c mutant translocates slowly into the nucleus. $n \geq 30$, from 3 separate experiments (n = number of cells analyzed).

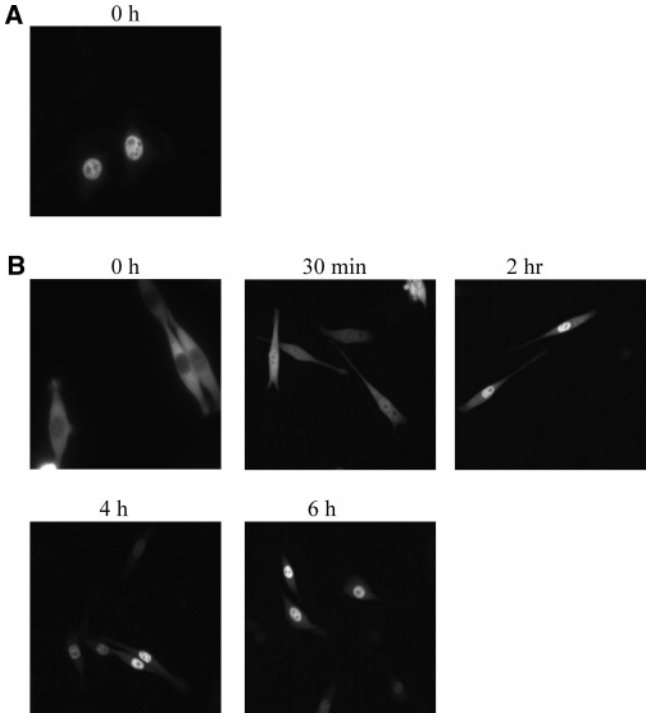

Figure 2. Comparison of import of (A) wt PRA with (B) PRA-NLS_c mutant. Wt PRA localized predominantly in the nucleus in the absence of hormone whereas PRA-NLSc mutant localized mostly in the cytoplasm. Translocation of PRA-NLS_c mutant is noticeable after 30 min of hormone induction and reaches a plateau after about 4 h. $n \geq 30$, from 3 separate experiments (n = number of cells analyzed).

pared to wt PRB, a longer time period was studied (a total of 6 h). An example is shown in Figure 1B and Figure 2B for PRB-NLS_c and PRA-NLS_c mutants at 2, 4, and 6 h. For PRB-NLS_c mutant clear evidence of nuclear accumulation is apparent at 2 h (Figure 1B). The import is very slow and incomplete compared to wt PRB (Figure 1A). For PRB-NLS_c mutant, at the highest dose (1000 nM), around 50% of the receptor−ligand complex is detected in the nucleus at 6 h compared to 25% at 0 h (Figure 3B). Because wt PRA localizes predominantly in the nucleus without hormone, we could only study the import of PRA-NLS_c mutant. The import of PRA-NLS_c mutant is a saturable process. It reaches a plateau around 4 h (Figure 4).

Import kinetics of PR mutants were performed and compared to those of wt PRB. Different doses were chosen for PRA-NLS_c and PRB-NLS_c mutant import kinetics. For PRB-NLS_c mutant import is still observed even after 6 h (Figure 3B) and no saturation occurs during this period. The import rate increases with increasing dose as with wt PRB. Interestingly, there is a significant difference between 1 and 1000 nM ($p < 0.05$) but no significant difference in the import rate between 10 and 1000 nM for PRB-NLS_c mutant. For PRA-NLS_c mutant the plateau arrives at a much later time than for wt PRB comparing the two lower graphs in Figure 3A (diamond and triangle symbols) with the two higher graphs in Figure 4 (circle and square symbols). In addition, at 1000 nM, the nuclear percentage plateau of PRA-NLSc mutant (60%; Figure 4) is lower than wt PRB's plateau, which is 70% (Figure 3A). The kinetic model used for wt PRB[9] was fit for the PRA-NLS_c and PRB-NLS_c mutants as well. The changes of rate constants for PRB-NLS_c mutant (0.0011, 0.0022, 0.068 min^{-1} for 1, 12.5, and 1000 nM) were decreased compared to those for wt PRB (0.076, 0.164, 0.202 min^{-1} at corresponding doses).

Importin α Interacts More Strongly with wt PRB than with PRB-NLS_c Mutant. One question that has not been answered is why the import kinetics (and hence the transcriptional activity) of wt PRB is different from that of PRB-NLS_c mutant. The size of PRB-NLS_c mutant protein is comparable to the size of wt PRB and is well beyond passive diffusion range (45 kDa), so size does not explain differences in localization. However, it has been shown by others that changing lysine residues to alanines in a classical NLS greatly reduces the binding affinity between NLS_c and importin α.[13] So, we hypothesize that wt PRB interacts better

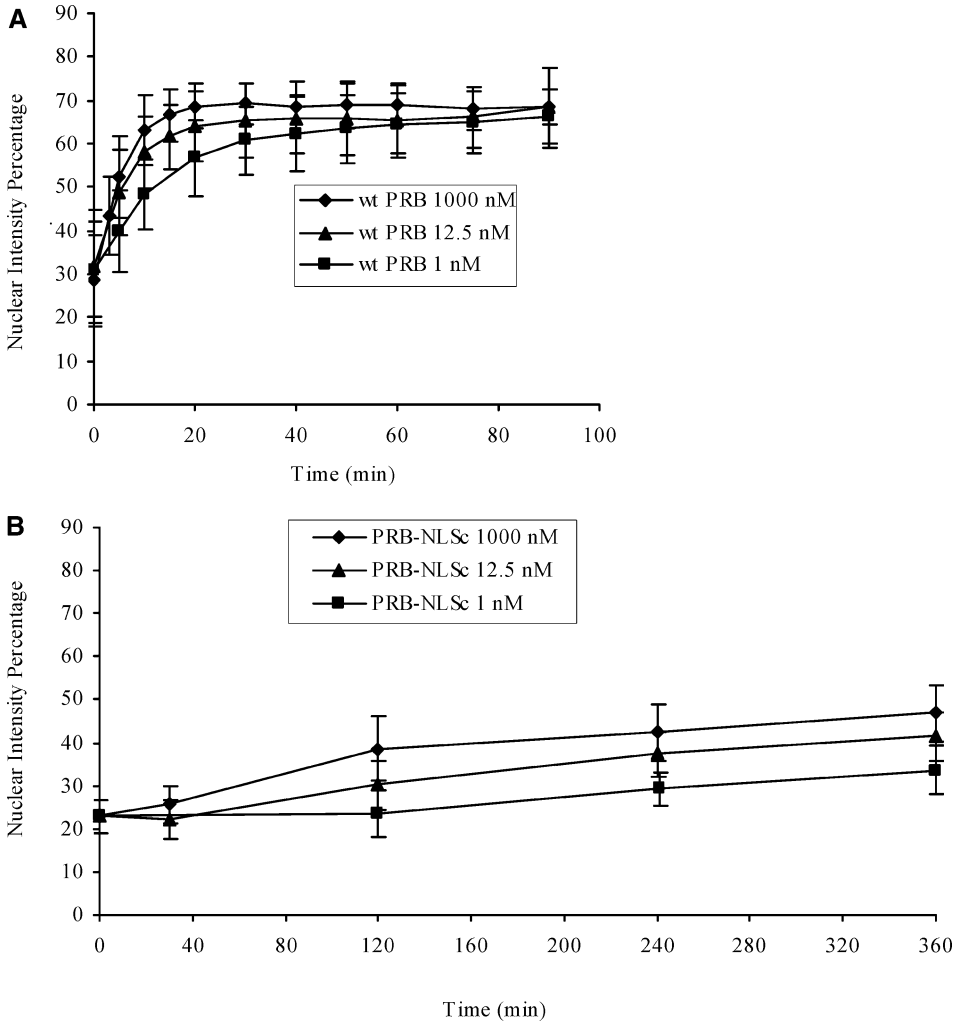

Figure 3. Nuclear import of wt PRB vs PRB-NLS$_c$. (A) wtPRB nuclear intensity increases with time. Representative doses were chosen from our previous paper.[9] (B) PRB-NLS$_c$ mutant translocates into the nucleus on hormone induction. For each dose, $n \geq 30$, from 3 separate experiments (n = number of cells analyzed).

with importin α than PRB-NLS$_c$ mutant. PRB-NLS$_c$ mutant will interact weakly with importin α or not at all. Direct proof of interaction with importin α has been shown for the glucocorticoid receptor (GR). GR's NLS$_1$ (containing an SV40-like NLS) but not NLS$_2$ (hormone dependent NLS) can directly interact with importin α.[14,15] Two-hybrid assays were conducted and confirmed that human importin α can interact directly with wt PRB (Figure 5). The amino acid sequence of PR NLS$_h$ does not contain a well-defined bipartite NLS, so it should not interact well with importin α. Figure 5 shows that interaction of wt PRB with importin α (white bar) is stronger and significantly different ($p < 0.001$) from that of PRB-NLS$_c$ mutant (gray bar). This could explain the markedly reduced rate of nuclear import of PRA-NLS$_c$ and PRB-NLS$_c$ mutants. We also tested the effect of hormone on the interaction of PR with importin α. A high dose of 100 nM progesterone was used in order to test if importin α is involved in the dose response of PR import. Interestingly, hormone induction has no effect on the interaction between wt PRB and importin α (compare no hormone, white bar, to with hormone, dotted bar, in Figure 5). Hormone also has no effect on the interaction between PRB-NLS$_c$ mutant and importin α (compare no hormone, gray bar, to with hormone, striped bar, in Figure 5). This suggests that there is another mechanism responsible for the increase of PRB/PRB-NLS$_c$ mutant import into the nucleus with increasing dose. Compared to the negative control (pAD-PK1 alone, black bar) PRB-NLS$_c$ mutant (gray and striped bars) interacted weakly with importin α (Figure 5). There is a statistical difference from the Tukey−Kramer test between PRB-NLS$_c$ mutant with (striped bar) or without hormone induction (gray bar) and negative control (black

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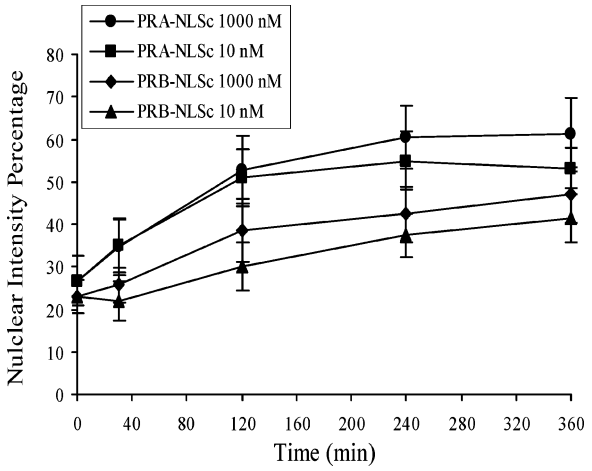

Figure 4. The increase in nuclear intensity for PRA-NLS_c mutant and PRB-NLS_c mutant comparing two different doses over time. The relationship between nuclear intensity and time was fitted to a sigmoidal relationship using nonlinear regression. For each dose, $n \geq 30$, from 3 separate experiments (n = number of cells analyzed).

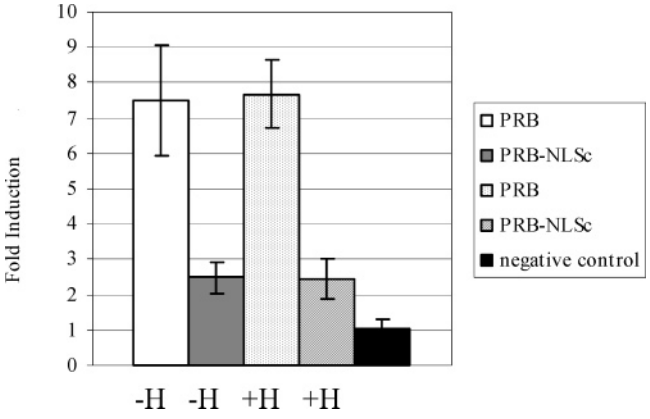

Figure 5. Two-hybrid assay of wt PRB or PRB-NLS_c mutant with importin α. Luciferase assay of pFR-Luc firefly reporter gene was carried out 24 h after transfection followed by no hormone (−H) or 100 nM progesterone treatment for 6 h (+H). Fold induction is relative to the negative control. Error bars represent the standard errors of the means from at least three experiments in duplicate.

bar; $p < 0.01$). This, together with the reduced rate of import of PRB-NLS_c mutant, suggests that importin α still participates in import of PRB-NLS_c mutant; however, other factors may also be involved.[15]

Function of Hormone Inducible Nuclear Localization Signal (NLS_h) in Progesterone Receptor. Milgrom et al.[16] identified a stretch of amino acids in the PR hinge region that could act as a possible nuclear localization signal. We tested this NLS_h out of context by fusing this 43 amino acid stretch (Arg593−Gly636) to EGFP. Since hormone induced

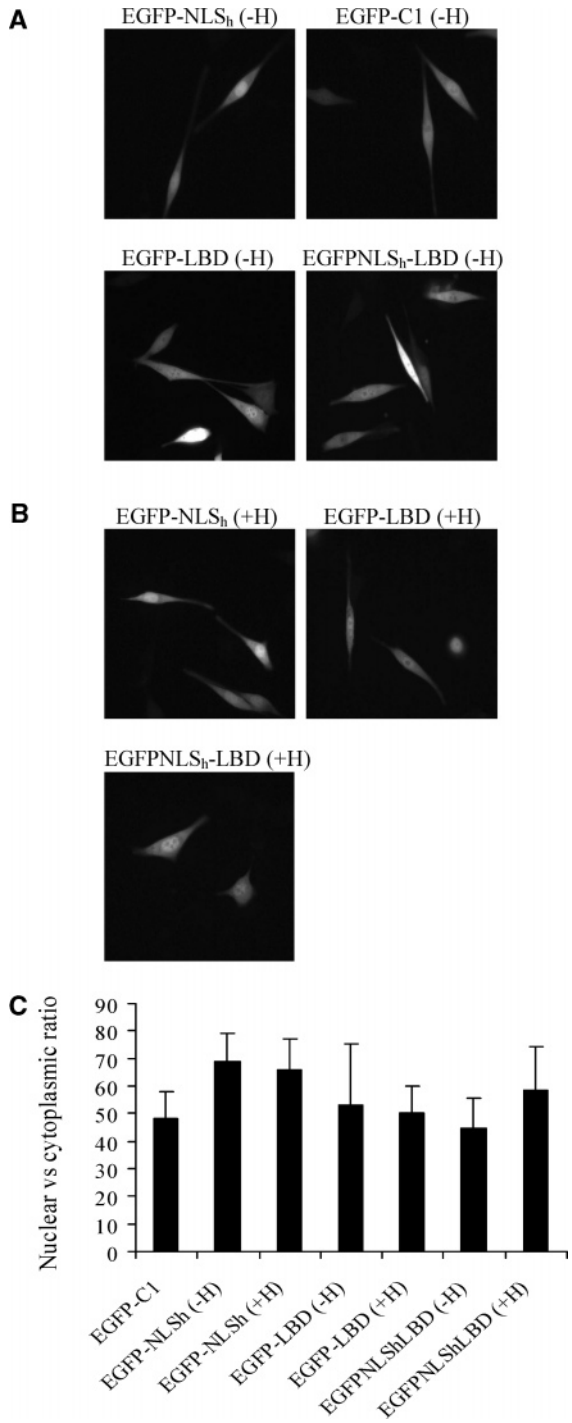

Figure 6. The function of NLS_h in PR. (A) Subcellular distribution of EGFP-NLS_h, EGFP-LBD, and EGFP-NLS_h-LBD without hormone induction. (B) Subcellular distribution of NLS_h, LBD, and NLS_h-LBD after 6 h of 100 nM progesterone treatment. (C) The nuclear vs cytoplasmic ratio of NLS_h was compared with GFP-C1, GFP-LBD, and GFP-NLS_h-LBD before and after progesterone treatment. Tukey−Kramer test was performed for statistical analysis between groups; $n \geq 25$ (n = number of cells analyzed).

nuclear import of PR needs the ligand binding domain to bind to hormone, EGFP-LBD and EGFP-NLS_h-LBD were also constructed. EGFP-C1 (control vector which contains

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EGFP only and no other protein) distributed evenly in the nucleus and cytoplasm (Figure 6A, top right panel). However, EGFP-NLS$_h$ localized more in the nucleus (Figure 6A, top left panel; see Figure 6C for comparisons; $p < 0.001$). There was no significant difference in localization of EGFP-NLS$_h$ with (Figure 6B, top left panel) or without 100 nM progesterone (Figure 6A, top left panel; see Figure 6C for comparisons). When LBD is fused to NLS$_h$, this construct localized more in the nucleus with hormone induction (Figure 6B, bottom panel) compared to no hormone induction (Figure 6A, bottom right panel; see Figure 6C for comparisons, $p < 0.01$). Surprisingly, LBD somehow offsets the weak NLS$_h$ function and EGFP-NLS$_h$-LBD distributes like EGFP-C1 without hormone added to the system (Figure 6C). In order to find out if LBD alone acts as a nuclear import signal, EGFP-LBD was used as a control. EGFP-LBD distributes both in the nucleus and in the cytoplasm in the absence of hormone (Figure 6A, bottom left panel), and there is no statistical difference in localization with (Figure 6B, top right panel) or without progesterone (see Figure 6C for comparisons). We conclude that the second NLS, NLS$_h$, in PR acts as a weak nuclear localization signal without hormone, and it is a hormone dependent NLS with LBD attached.

Subcellular Localization and Kinetics Can Affect Gene Transcriptional Activity. Next, we studied if the transcriptional activity of PRB-NLS$_c$ mutant would follow its import kinetic pattern. Milgrom et al. have reported that a version of PRB without the NLS$_c$ was fully active compared to wild type PRB at high hormone doses.[16] Wt PRB was used as a standard for our assay as it gives high transcriptional activity.[9] As observed previously,[16] the transcriptional activity of PRB-NLS$_c$ mutant in our system is comparable with that of wt PRB at a high progesterone dose (100 nM; Figure 7A,B, circle symbols). At low progesterone concentration (12.5 nM, Figure 7, triangle symbols), the kinetics affects gene activation for PRB-NLS$_c$ mutant more than the 100 nM dose. Furthermore, the time lapse study of PRB-NLS$_c$ mutant provides more detail on how the subcellular occupancy of PR controls the reporter gene transactivation. It was found that time to maximum induction differs between wt PRB and PRB-NLS$_c$ mutant. Wt PRB transcriptional activity saturated at 30 min (Figure 7A); however, for PRB-NLS$_c$ mutant (Figure 7B) the plateau arrived at around 4 h (for 12.5 and 100 nM, triangle and square symbols). For the higher dose of 1000 nM, the plateau of transcriptional activity occurs at around 2 h (Figure 7B, circles). At an even higher dose of 5000 nM, the plateau is the same (data not shown). This means that the initial subcellular distribution (compare Figure 3B and Figure 7B) controls gene transcriptional activity and kinetics.

Since the location of the PRB-NLS$_c$ mutant had an impact on its own transactivation, we next tested if the initial localization of PRA would impact its ability to repress wt PRB. Wild type PRA is known to suppress the transcriptional activity of wt PRB.[17] It may act as an efficient repressor of wt PRB (and other steroid receptors)[17] by primarily being located already in the nucleus, even in the absence of

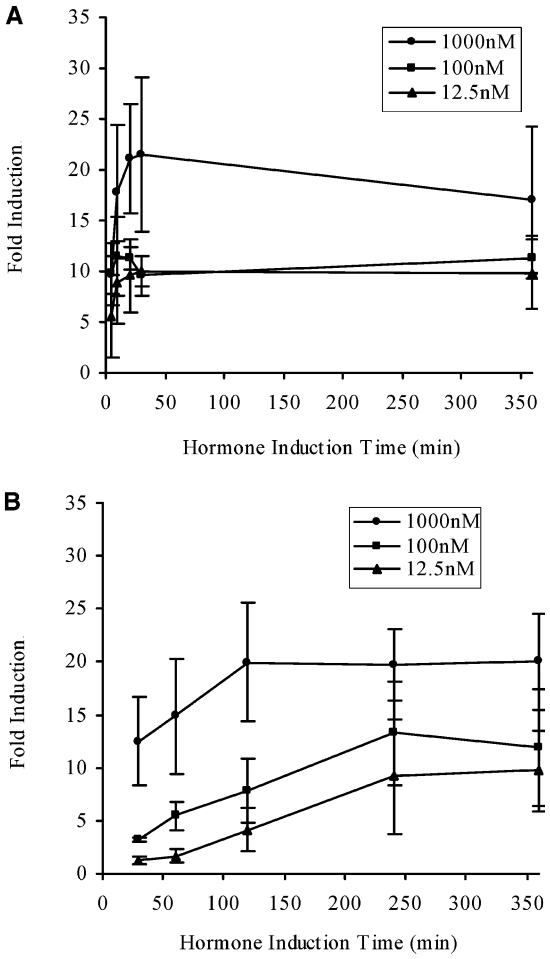

Figure 7. Comparison of transcriptional activity of (A) wt PRB and (B) PRB-NLS$_c$ mutant with time. For wt PRB, representative doses were chosen from Li et al.[9] For each time point, hormone was removed from the system by washing with PBS and luciferase reading was taken at 6 h time point after washing.[9] Error bars are shown for 3 independent experiments performed in duplicate.

hormone. To test the effect of initial localization of PRA on repression of PRB, a cytoplasmically localized mutant version of PRA (PRA-NLS$_c$ mutant) was cotransfected with wt PRB and the transcriptional activity (activation of MMTV-Luc) was compared to that of wt PRA (which is nuclear) cotransfected with wt PRB. A 1:1 ratio of wt PRA or PRA-NLS$_c$ mutant to wtPRB was tested first. As shown in Figure 8A for the 1:1 ratios, wt PRA can repress wt PRB at the 0.5 h time point (white bar vs black bar; $p < 0.05$). At the 6 h time point, this repression is not evident, though ($p < 0.1$). Although the fold of induction of PRA-NLS$_c$ mutant to wt PRB at a 1:1 ratio appears lower compared to wt PRB (compare gray bars and black bars in Figure 8A), there is no significant difference. Compared to wt PRA

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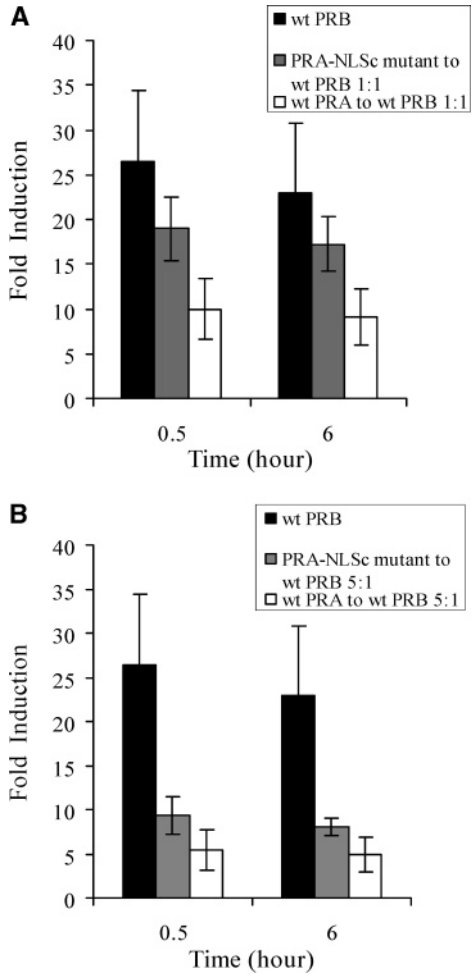

Figure 8. Repression of transcriptional activity of wt PRB by wt PRA or PRA-NLS_c mutant at two time points (0.5 h and 6 h after hormone induction). Two different ratios of PRA-NLS_c mutant to wt PRB were chosen (A, 1:1; B, 5:1). Wt PRB was chosen as a standard. Cells cotransfected with PRA-NLS_c mutant and wt PRB at different ratios were assayed for PRB activaton of MMTV-Luc reporter gene 24 h after 6 h induction with 100 nM progesterone. Error bars are shown for 3 independent experiments performed in duplicate.

(white bars), PRA-NLS_c mutant is not a good repressor of wt PRB (gray bars). Evidence has been shown that at least equimolar levels of wt PRA are required to demonstrate significant inhibition of wt PRB transactivation.[18] Therefore, a 5-fold excess of wt PRA or PRA-NLS_c mutant plasmid to wt PRB plasmid was cotransfected. Figure 8B shows that for the PRA-NLS_c mutant to wt PRB (gray bars) at a 5:1 ratio, there is a 3-fold decrease compared to wt PRB transcriptional activity (black bars) at both time points ($p < 0.001$ at the 0.5 h time point, and $p < 0.05$ at the 6 h time point). There is no significant difference between PRA-NLS_c mutant to wt PRB 5:1 (gray bars) and wt PRA to wt PRB 5:1 (white bars). PRA-NLS_c mutant starts acting like wt PRA at higher PRA-NLS_c mutant to wt PRB ratios but not at equal ratios for short inductions. Without hormone induction, wt PRA localizes predominantly in the nucleus where it can bind to target DNA quickly to suppress other receptors. For PRA-NLS_c mutant, it needs to translocate from the cytoplasm to the nucleus to block the other receptor's activity. It may be a natural way for regulatory factors to modulate gene activity by controlling the subcellular compartmentalization.

Discussion

We have previously studied the import kinetics of wt PRB and the correlation between the import rate and the transcriptional activity.[9] In this study, we have detailed the cytoplasmic version of PRB (PRB-NLS_c mutant) and compared to wt PRB in terms of their import kinetics and transcriptional activity. The effect of changing initial subcellular distribution of PRA on wt PRB was also examined, and a mechanistic reason for the different import kinetics of wt PR and cytoplasmic PRs was studied.

Without hormone induction, mutant PRs distribute mostly in the cytoplasm and translocate into the nucleus after addition of hormone, however, with dramatically different import kinetics compared to those of wt PRB. The distribution of unliganded mutant PRs in the cytoplasm could be a reflection of export rate dominating over import rate. The effect of import rate and subcellular distribution of steroid receptors with NLS_c removed on the transactivation potential has been studied on gluococorticoid receptor (GR) by Lefebvre's group.[19,20] In the case of GR with NLS_c removed, the transcriptional activity was directly proportional to the extent of GR transfer to the nucleus. There was lesser GR mutant translocation into nucleus compared to wt GR, and correspondingly the transcriptional activity was lower after induction with 1×10^{-6} M dexamethasone.[19] For another member of the steroid receptor family, mineralocorticoid receptor (MR), the results were different.[20] For MR, the translocation of MR with NLS_c deleted is not complete compared to wt MR; however, the final transactivation was not affected by the nuclear occupancy of MR even at 1000 nM induction of aldosterone. In this paper, we examined the import kinetics of PR mutants and their effect on the transcriptional activity. Our results show that, at low hormone concentrations, for the PRB-NLS_c mutant the extent of transport into the nucleus is less (Figure 3B) and the transcriptional activity is lower on a PR responsive gene (Figure 7B at initial time points). At high hormone concentration it retains nearly complete transcriptional activity.

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Clearly, NLS$_c$ is required for fast and full agonist dependent import. The import kinetics does impact the activity of progesterone receptor especially at low doses of hormone, which may be more physiologically relevant. In eukaryotic cells, gene transcription and translation take place in separate subcellular compartments; therefore, the regulation of the subcellular distribution of proteins which control gene transcription could play an essential role in control of cell functions. Our results show direct proof that the initial subcellular localization of progesterone receptor can affect the cell function from the transcription stage. This also suggests why PRA naturally localizes predominantly in the nucleus whereas PRB localizes both in the nucleus and in the cytoplasm. Localization of PRA already in its active compartment (the nucleus) could allow fast repression of proteins (like PRB).

Alternatively, if the PRB-NLS$_c$ mutant had a dramatically shorter half-life compared to wt PRB, this could contribute to alterations in kinetics and activity of the mutant receptor. The half-life of PRB-NLS$_c$ mutant could be roughly approximated by taking the total intensity in the cells divided by the total area of the cells, at $t = 0$ and $t = 6$ h, at a given dose of progesterone (1000 nM). At $t = 0$ (no progesterone present), the total intensity divided by total area was 5125; whereas at 6 h, this value was 2661, suggesting a half-life of about 6 h. The half-life of wild type PR in the presence of high dose R5020 (a synthetic progestin) in another breast cancer line (MCF-7) was reported to be 6 h as well.[21] Therefore, the half-life of PRB-NLS$_c$ mutant does not appear to be different than that of wt PRB.

Lefebvre and Yamamoto[15,19] have studied the kinetics of two nuclear localization signals (constitutively active and hormone inducible NLSs) in GR and the import mechanism of these two nuclear localization signals. Importin 7 and 8 were found to interact both with NL1, which has a sequence similar to that of the SV40 NLS, and with NL2, which overlaps with the ligand binding domain and is poorly defined. Importin α was found to selectively interact with NL1. Similarly, the two-hybrid assay in our study showed that wt PRB interacts more strongly with importin α than does RB-NLS$_c$. These results imply that the reason for the kinetic difference between wt PRB and PRB-NLS$_c$ was that wt PRB could bind to importin α better than could PRB-NLS$_c$.

We also showed that NLS$_h$ may be a weak nuclear localization signal out of context even without an SV40-like NLS sequence. Our studies with PR mutants with NLS$_c$ removed, but with NLS$_h$ intact, imply that PR with NLS$_h$ can weakly interact with importin α. EGFP-NLS$_h$ responds to progesterone induction when there is a LBD able to bind to ligand. However, combining LBD with NLS$_h$ causes a slightly less nuclear distribution similar to that of EGFP-C1 control. One of the possible explanations could be that the LBD masks the weak import ability of NLS$_h$.[22] Another possibility is that LBD could contain an export motif.[23] Yet another prospect is that hsp90 (which is known to bind to LBD) could mask NLS$_h$.[19,22] The role of LBD in PR out of context was unexpected. We have found that complete LBD out of context does not respond to progesterone induction even though crystallographic studies have shown that ligand binding domain of PR can interact directly with progesterone.[24] LBD can bind to progesterone, but this in itself does not cause nuclear import.

The import of PRB-NLS$_c$ mutant appears to be a dose dependent process (Figure 3B). One possible ligand dependent pathway is through chaperone heterocomplex machinery. Evidence shows that hsp90 is involved in steroid receptor nuclear import.[7,25] The binding of steroid hormone triggers a dynamic interaction between LBD and the chaperone machinery.[26] The receptor continuously associates with and dissociates from hsp90 and immunophilins. The import rate of PR may be correlated to hsp binding to LBD. In addition, immunophilin cochaperones can control hormone-binding affinity.[27] However, currently the exact mechanism of hormone dependent import of PR-NLS$_c$ mutants is unknown. Yamamoto's group has studied the import of GR with the constitutive nuclear localization signal removed. They suggested that the ligand dependent nuclear import of GR could be a downstream regulated process.[15] This could also be the case for PR. These are just a few possibilities explaining hormone dependent import of PR.

It is not known why import rate of PRA-NLS$_c$ mutant is faster than that of PRB-NLS$_c$ mutant and why the plateau arrived much faster for the PRA-NLS$_c$ mutant. There are several possible explanations based on what is known about

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the wt PRs. First, wt PRA is localized more in the nucleus than wt PRB, suggesting that the import rate of wt PRA is faster than export rate even in the absence of hormone. A second reason could be stronger binding of wt PRB with hsp90 than wt PRA in the cytoplasm.[28] Another possible reason could be the differences in binding affinity of wt PRA and wt PRB with cofactors (coactivators and corepressors).[29] Wt PRA has higher binding affinity with corepressors such as SMRT whereas wt PRB has a higher binding affinity with coactivators such as hSRC-1 and GRIP.[29] Subcellular localization of cofactors has shown that corepressors are localized mostly in the nucleus and coactivators are localized both in the nucleus and in the cytoplasm.[30,31] PR localization therefore may be influenced by differential cofactor binding.

It is clear that the initial subcellular localization of steroid receptors plays a very important role in their cellular biological activity. Our work provides a link between steroid receptor import into the nucleus and transcription regulation and activity.

Abbreviations Used

DMEM, Dulbecco's modified Eagle's medium; EGFP, enhanced green fluorescent protein; FBS, fetal bovine serum; GFP, green fluorescent protein; LBD, ligand binding domain; NLS, nuclear localization signal; NLS_c, constitutively active nuclear localization signal; NLS_h, hormone inducible nuclear localization signal; PBS, phosphate-buffered saline; PR, progesterone receptor; wt PRB, wild type progesterone receptor B isoform; wt PRA, wild type progesterone receptor A isoform; PRA-NLS_c mutant, PRA with constitutive nuclear localization signal knocked out; PRB-NLS_c mutant, PRB with constitutive nuclear localization signal knocked out; PCR, polymerase chain reaction; wt, wild type.

Acknowledgment. This research was funded by a University of Utah College of Pharmacy Research and Graduate Programs Grant, Department of Defense Grant BC996437, and NIH Grant DK070060.

MP0500418

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