Pergamon

Biochemical Pharmacology, Vol. 47, No. 1, pp. 21–24, 1994.

0006-2952/94 $6.00 + 0.00

CYTOPLASMIC-NUCLEAR TRAFFICKING OF PROGESTERONE RECEPTOR

IN VIVO STUDY OF THE MECHANISM OF ACTION OF ANTIPROGESTINS

ANNE GUIOCHON-MANTEL, KARINE DELABRE, PIERRE LESCOP, MARTINE PERROT-APPLANAT and EDWIN MILGROM*

Hormones et Reproduction, Unité de Recherche Inserm 135, Faculté de Médecine Paris-Sud, 94275 Le Kremlin Bicetre, France

Abstract—The signal responsible for the nuclear localization of the progesterone receptor has been characterized. The study of the mechanism of this nuclear localization has revealed that the receptor continuously shuttles between the nucleus and the cytoplasm. The receptor diffuses into the cytoplasm and is constantly and actively transported back into the nucleus. Preliminary evidence suggests that the same mechanism exists for estradiol and glucocorticoid receptors. Experiments designed to study the traffic of steroid hormone receptors have been applied to the determination of the molecular mechanism of action of antisteroids. Using these techniques, we have shown that two major antiprogestins, RU486 and ZK98299, act at the same point in the cell as the hormone.

The subcellular localization of steroid hormone receptors has been the subject of many studies for over 20 years. Initially, it was observed that when cell homogenates were prepared from animals lacking in hormone, the corresponding receptor was recovered in the soluble fraction. After hormone administration, the activated steroid–receptor complexes were found attached to chromatin. The hypothesis was then put forward that the receptor has a cytoplasmic location and migrates into the nucleus under the effect of hormone [1, 2]. The development of monoclonal antibodies against ER†, PR and GR allowed immunocytochemical studies to be undertaken.

These studies reported exclusive nuclear localization of the ER [3], the PR [4] and the AR [5] even in the absence of endogenous hormone and were in agreement with biochemical studies on enucleated cells [6] showing that unoccupied ER was almost exclusively in the nucleoplast fraction. Thus, this group of steroid hormone receptors (ER, PR, AR) is transported, after its synthesis, from the cytoplasm to the nucleus where the receptors remain in an inactive state. When subsequently the hormone arrives it triggers an interaction of receptors with high affinity DNA-binding sites where the primary events involved in transcription activation occur.

NLSs of PR

The nuclear localization of proteins has been shown to occur through two mechanisms [reviews in Refs. 7–9]. In the first, the protein diffuses through the nuclear membrane and is then trapped by binding to an intranuclear component. The second is an active process; it is temperature dependent, requires ATP and displays saturation kinetics. Active transport into the nucleus requires that proteins contain suitable NLS. They are mostly short basic sequence motives, rich in arginines and lysines. The first to be described was the SV40 large T antigen signal [10, 11]. Since then, numerous signals have been identified and it has been shown, in the case of nucleoplasmin, that two interdependent basic domains may constitute a bipartite signal [12]. These NLSs are recognized by NLS-binding proteins (NBP) which are thought to function as adaptor molecules between the karyophylic protein and the transport machinery of the nuclear pore complex. Several candidate NBPs have been identified, by affinity methods. Active nuclear import of proteins takes place in at least two steps. The first step is the interaction between the protein and the nuclear pore complex through the NLS. The second step is the translocation to the nucleus. Only the latter requires ATP. Cloning of the receptors and *in vitro* mutagenesis studies led to the description of karyophylic signals in GR [13], PR [14, 15] and ER [16].

We have studied the signals responsible for the nuclear localization of rabbit PR using two approaches. The first approach was to study the subcellular localization of a series of deletion mutants [14]. We have shown that its main NLS is a stretch of amino acids located in the hinge region around position 638–642 which bears similarities to the NLS present in the SV40 large T antigen. This putative signal is constitutive (acts in the absence of hormone) and if it is deleted, the ligand-free receptor becomes

* Corresponding author. Tel. (33) 1 45 21 33 29; FAX (33) 1 45 21 27 51.

† Abbreviations: GR, glucocorticoid receptor; ER, estrogen receptor; PR, progesterone receptor; AR, androgen receptor; NLS, nuclear localization signal; HREs, hormone responsive elements.

cytoplasmic. Homologous sequences are found at exactly the same position (10 amino acids after the last conserved cysteine) in all other steroid hormone receptors except in the case of the ER where a homologous sequence is found 11 amino acids after the last conserved cysteine. For a given steroid receptor, this region is highly conserved in the diferent species studied. A mutant deleted of the first NLS (mutant Δ 638–642) is cytoplasmic in the absence of hormone and is translocated to the nucleus in the presence of hormone. This mutant retains a complete biological activity when saturated with hormone. Thus it can be used as a conditional receptor.

Using *in vitro* mutagenesis we have shown that there is a second NLS, located in the second zinc finger [14]. This NLS is masked and unmasked by identical mechanisms to those regulating the activity of the DNA binding function. It bears the same properties as previously described NLSs; its activity is blocked by energy depletion. It has been shown in different models that proteins carrying two NLSs are more efficiently transported into the nucleus than proteins carrying a single NLS [11, 17]. The existence of multiple NLSs is a frequent feature of nuclear proteins [18, 19].

The second approach was to characterize the minimum NLS able to mediate the nuclear localization of a cytoplasmic protein like β-galactosidase. Using this approach we have shown that this NLS is a complex signal (unpublished data). It is not a simple NLS, like SV40 large T antigen NLS because the constitutive NLS (638–642) is unable to localize β-galactosidase to the nucleus. It is not a bipartite NLS, like nucleoplasmin NLS, because a sequence encompassing two clusters of basic amino acids including the constitutive NLS is also unable to localize β-galactosidase to the nucleus. The two NLSs (constitutive and hormone-dependent) defined by deletion experiments are necessary to locate β-galactosidase to the nucleus. This complex NLS encompasses several stretches of basic amino acids. Using a deletion mutant approach, it has been shown that this signal is composed of multiple proto-signals that cooperate together [15].

Interactions between receptor monomers during nuclear transport

The interaction of dimeric receptors with palindromic HREs is an important step in hormone action. Such a process has been observed *in vitro* using band-shift electrophoretic analysis of receptor–DNA interactions [20, 21]. *In vitro* studies using purified receptors are, however, controversial because of artifacts.

We have devised an experimental design allowing us to study oligomerization *in vivo*. We have constructed a receptor mutant in which epitope for antibody Mi60 was deleted (this mutant will be called Mi−). We have constructed a cytoplasmic mutant, devoid of both constitutive and hormone-dependent NLSs, in which epitope for antibody Let 126 was deleted (this mutant will be called Let−). Preliminary experiments showed that these deletions, both in the N-terminal part of the protein, did not modify the main physiological properties of the receptor. The cDNA encoding for Mi− wild-type receptor was then co-transfected with cDNA for Let− cytoplasmic receptor. The antibody Mi60 was used to detect Mi+ cytoplasmic receptor without interference by the Mi− nuclear receptors. In these conditions, we observed that, in the absence of hormone the Mi+ cytoplasmic receptor remained in the cytoplasm. However, if hormone was added, the cytoplasmic mutant was shifted to the nucleus. Thus, in the absence of ligand, the receptor is transferred into the nucleus as a monomer. Hormone binding provokes an interaction between receptor monomers (oligomerization) allowing the form capable of nuclear transfer to carry along the form initially localized in the cytoplasm. Using different receptor mutants, we have shown that this interaction occurs through the steroid binding domain of both receptors and is independent of any DNA binding event.

This oligomerization between a cytoplasmic and a nuclear monomer, in the absence of protein synthesis, implies that both monomers are not rigidly confined to their respective compartments. A dynamic situation must exist in which they may contact each other.

Nucleocytoplasmic traffic of steroid hormone receptors

(1) *Energy depletion and nuclear receptor efflux.* Energy depletion prevents the mutant Δ 638–642, devoid of the constitutive NLS, to enter into the nucleus by blocking the hormone-dependent NLS. We wondered if it might impair receptor maintenance in the nucleus, once it has entered this organelle. We have administered the same treatment to hormone activated mutant Δ 638–642 and to the wild-type nuclear receptor and we have observed an efflux of the receptor [22]. This phenomenon can be generalized to steroid hormone receptors, because we have observed the same result in the case of estradiol receptor. It suggests that steroid hormone receptors despite their size, exit continuously from the nucleus to the cytoplasm and are constantly and actively transported back into the nucleus.

(2) *Nucleocytoplasmic shuttle of steroid hormone receptor.* To confirm directly the existence of a nucleo-cytoplasmic shuttle of receptor we have studied the migration of progesterone receptor between nuclei in interspecies heterokaryons. We fused a mouse L cell line permanently expressing the progesterone receptor with human 293 cells devoid of receptor, in the presence of protein synthesis inhibitors. We observed the presence of the receptor in human nuclei 12 hr after the fusion. Thus the receptor had migrated from one nucleus to the other, indicating a shuttle through the cytoplasm.

The residency of the progesterone receptor in the nucleus is a dynamic phenomenon resulting from the continuous active transport into the nucleus counterbalanced by some diffusion into the cytoplasm. It is not known whether this diffusion is a totally passive phenomenon or if it necessitates the presence of a NLS in the protein. Interaction with specific protein(s) in the pore could then take place during nuclear exit of the protein. Shuttle mechanisms have recently been demonstrated for

nucleolar proteins, the inner nuclear membrane protein p55 and proteins of the hsp70 family. Similar experiments have been performed on ligand-bound GR showing the same results [23].

This mechanism of nuclear localization of the receptor explains some previous observations; ligand-free PRs or ERs, which reside in the nucleus, are found in the cytosol after homogenization even when nuclear structures have been preserved. In these conditions, active transport is blocked by dilution and low temperature. So, the exit of receptors from the nucleus to the cytoplasm is not counterbalanced by active entry into the nucleus. Incidently, association of receptors with nuclei after homogenization of cells at 25° has been described [24]. Moreover, localization of ligand-free GR in cytoplasm or both cytoplasm and nuclei has been considered as completely different to that of ERs or PRs which are located in the nucleus. Many authors have been puzzled by the fact that proteins having such similar properties may exhibit such differences in their subcellular localization. However, if receptor continually shuttles between nucleus and cytoplasm the case of the GR may be only quantitatively and not qualitatively different from that of sex steroid receptors. The ligand-free hormone binding domain could mask the NLS region [15] leading to an increased time of residency of the receptor in the cytoplasm and to an apparent distribution between cytoplasmic and nuclear compartment.

The understanding of receptor function may also be modified by the fact that receptors shuttle between nucleus and cytoplasm. Receptors could thus interact with cytoplasmic components and exert biological activities in the cellular cytoplasm. Such effects have indeed been described [25, 26].

The mechanisms of receptor translocation across the nuclear envelope and those of its traffic within the cytoplasm remain to be elucidated. Co-localization of cytoskeletal networks on the one hand and of GR or heat shock protein 90 on the other hand have been described [27, 28]. It has been proposed that steroid hormone receptors "creep" along these networks to enter the nucleus. We have used the Δ 638–642 PR deleted of the constitutive NLS to examine its localization in the cytoplasm. Normal or detergent permeabilized cells were used and examined by optical and confocal laser microscopy. In all cases there was no co-localization of receptor and actin, tubulin or vimentin [29]. The functional involvement of microtubule, microfilament or intermediate filament networks was studied by administration of specific inhibitors (nocodazole, demelcocine or cytochalasin) either separately or in combination. After disruption of the cytoskeletal network(s), hormone was administered to cells in order to activate the hormone-dependent signal and to provoke the transfer to Δ 638–642 PR into the nuclei. None of the inhibitors impaired this transfer or even slowed its rate. Similar results were obtained with wild-type receptor which had been shifted into the cytoplasm under the effect of energy deprivation [29]. These experiments thus suggest that the cytoskeletal networks are not involved in the cellular localization mechanisms of the PR.

The study of the cellular traffic of steroid receptors is of interest not only for the understanding of the mechanism of action of these hormones but also as a model to understand the mechanisms underlying the nuclear localization of the increasing number of proteins implicated in the regulation of gene transcription.

Mechanism of action of antiprogestins

Recently, there has been a great interest in the study of antiprogestins for two main reasons. Firstly, they have been shown to have a great therapeutic potential; interruption of early pregnancy, post-coital contraception, triggering of labour and treatment of hormone-dependent tumors. Secondly, antiprogestins have also been used as potent tools to decipher the molecular mechanisms of action of the hormone.

Antiprogestins can act at all the steps of hormone action: activation, dimerization or binding of the dimers to HREs. The experimental designs described above can be used to study the first two steps of hormone action. The hormone-dependent NLS, located in the DNA binding region, is masked and unmasked by mechanisms identical to those regulating the activity of the DNA binding function and can thus be used to study the agonist or antagonist provoked activation. We have also devised *in vivo* experiments allowing us to study agonist or antagonist induced oligomerization.

The last step of hormone action is the binding to HREs and we are able to study this step *in vivo*. A receptor, deleted from the entire ligand binding region becomes constitutively active, at about 60% of the maximal activity of wild-type receptor. It is of course totally unaffected by the presence of antiprogestins [30]. If activated antagonist-wild-type receptors bind to HREs, they will compete with the constitutive receptor.

We have studied the effect of different antiprogestins on these steps of hormone action. We have shown that RU 486, the only antiprogestin used in therapeutics, activates PR, induces its *in vivo* oligomerization [14] and that the RU 486–wild-type PR complexes do bind *in vivo* to HREs [30]. Thus RU 486 acts at a distal step of hormone action (failure of trans-activation by RU 486–receptor complexes bound to HREs).

Recently, a different antiprogestin molecule, ZK 98299 has been characterized. Studies of ZK 98299–PR complexes failed to detect *in vitro* binding to HREs [31]. This led to the suggestion that ZK 98299 may be an example of a second class of antiprogestins which act by preventing the formation of DNA–PR complexes.

Using experimental designs described above we have demonstrated that this compound in fact does activate *in vivo* the PR, that it induces *in vivo* oligomerization and that ZK 98299–PR complexes do bind *in vivo* to HREs. In each situation, we have observed that RU 486 and ZK 98299 differ only quantitatively and not qualitatively, RU 486 being 10-fold more potent that ZK 98299. This is correlated with their differences in affinity for the receptor, measured *in vivo* [32].

Thus, the experiments described in this study show

a method of general interest which allows the analysis of effects of hormone antagonists *in vivo*, in intact cells. It should also facilitate the design of new, more efficient antiprogestins.

REFERENCES

1. Jensen T, Suzuki T, Kawashima T, Stumpf WE, Jungblut PW and DeSombre ER, A two-step mechanism for the interaction of oestradiol with the rat uterus. *Proc Natl Acad Sci USA* **59**: 632–638, 1968.
2. Gorski J, Toft D, Shyamala G, Smith D and Notides A, Hormone receptors: studies on the interaction of estrogen with the uterus. *Rec Prog Horm Res* **24**: 45–81, 1968.
3. King WJ and Greene GL, Monoclonal antibodies localize oestrogen receptor in the nuclei of target cells. *Nature* **307**: 745–747, 1984.
4. Perrot-Applanat M, Logeat F, Groyer-Picard MT and Milgrom E, Immunocytochemical study of mammalian progesterone receptor using monoclonal antibodies. *Endocrinology* **116**: 1473–1484, 1985.
5. Tan J, Joseph DR, Quarmby VE, Lubahn DB, Sar M, French FS and Wilson EM, The rat androgen receptor: primary structure, autoregulation of its messenger ribonucleic acid, and immunocytochemical localization of the receptor protein. *Mol Endocrinol* **12**: 1276–1285, 1988.
6. Welshons WV, Lieberman ME and Gorski J, Nuclear localization of unoccupied oestrogen receptors. *Nature* **307**: 747–749, 1984.
7. Davis LI, Control of nucleocytoplasmic transport. *Curr Opinion Cell Biol* **4**: 424–429, 1992.
8. Gerace L, Molecular trafficking across the nuclear pore complex. *Curr Opinion Cell Biol* **4**: 637–645, 1992.
9. Dingwall C and Laskey R, The nuclear membrane. *Science* **258**: 942–947, 1992.
10. Kalderon D, Roberts BL, Richardson WD and Smith AE, A short amino acid sequence able to specify nuclear location. *Cell* **39**: 499–509, 1984.
11. Lanford RE, Kanda P and Kennedy RC, Induction of nuclear transport with a synthetic peptide homologous to the SV40 antigen transport signal. *Cell* **46**: 575–582, 1986.
12. Robbins J, Dilworth SM, Laskey RA and Dingwall C, Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. *Cell* **64**: 615–623, 1991.
13. Picard D and Yamamoto KR, Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. *EMBO J* **6**: 3333–3340, 1987.
14. Guiochon-Mantel A, Loosfelt H, Lescop P, Sar S, Atger M, Perrot-Applanat M and Milgrom E, Mechanisms of nuclear localization of the progesterone receptor: evidence for interaction between monomers. *Cell* **57**: 1147–1154, 1989.
15. Ylikomi T, Bocquel MT, Berry M, Gronemeyer H and Chambon P, Cooperation of proto-signals for nuclear accumulation of estrogen and progesterone receptors. *EMBO J* **11**: 3681–3694, 1992.
16. Picard D, Kumar V, Chambon P and Yamamoto KR, Signal transduction by steroid hormones: nuclear localization is differentially regulated in estrogen and glucocorticoid receptors. *Cell Reg* **1**: 291–299, 1990.
17. Dworetzky SI, Lanford RE and Feldherr CM, The effects of variations in the number and sequence of targeting signals on nuclear uptake. *J Cell Biol* **107**: 1279–1287, 1988.
18. Roberts B, Nuclear location signal-mediated protein transport. *Biochim Biophys Acta* **1008**: 263–280, 1989.
19. Silver P and Goodson H, Nuclear protein transport. *Crit Rev Biochem Mol Biol* **24**: 419–435, 1989.
20. Tsai SY, Carlstedt-Duke J, Weigel NL, Dahlman K, Gustafsson JA, Tsai MJ and O'Malley BW, Molecular interactions of steroid hormone receptor with its enhancer element: evidence for receptor dimer formation. *Cell* **55**: 361–369, 1988.
21. Kumar V and Chambon P, The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. *Cell* **55**: 145–156, 1988.
22. Guiochon-Mantel A, Lescop P, Christin-Maitre S, Loosfelt H, Perrot-Applanat M and Milgrom E, Nucleocytoplasmic shuttling of the progesterone receptor. *EMBO J* **10**: 3851–3859, 1991.
23. Madan AP and DeFranco DB, Bidirectional transport of glucocorticoid receptors across the nuclear envelope. *Proc Natl Acad Sci USA* **90**: 3588–3592, 1993.
24. Molinari AM, Medici N, Armetta I, Nigro V, Montcharmont B and Puca GA, Particulate nature of the unoccupied uterine estrogen receptor. *Biochem Biophys Res Commun* **128**: 634–642, 1985.
25. Liao S, Smythe S, Tymoczko JL, Rossini GP, Chen C and Hiipakka RA, RNA-dependent release of androgen and other steroid receptor complexes from DNA. *J Biol Chem* **255**: 5545–5551, 1980.
26. Verdi JM and Campagnoni AT, Translational regulation by steroids. Identification of steroid modulatory element in the 5′-untranslated region of the myelin basic protein messenger RNA. *J Biol Chem* **265**: 20314–20320, 1990.
27. Akner G, Sundqvist KG, Denis M, Wikström AC and Gustafsson JA, Immunocytochemical localization of glucocorticoid receptor in human gingival fibroblasts and evidence for a colocalization of glucocorticoid receptor with cytoplasmic microtubules. *Eur J Cell Biol* **53**: 390–401, 1990.
28. Miyata Y and Yahara I, Cytoplasmic 8 *S* glucocorticoid receptor binds to actin filaments through the 90-kDa heat shock protein moiety. *J Biol Chem* **266**: 8779–8783, 1991.
29. Perrot-Applanat M, Lescop P and Milgrom E, The cytoskeleton and the cellular traffic of the progesterone receptor. *J Cell Biol* **119**: 337–348, 1992.
30. Guiochon-Mantel A, Loosfelt H, Ragot T, Bailly A, Atger M, Misrahi M, Perricaudet M and Milgrom E, Receptors bound to antiprogestin form abortive complexes with hormone responsive elements. *Nature* **336**: 695–698, 1988.
31. Klein-Hitpass L, Cato ACB, Henderson D and Ryffel GU, Two types of antiprogestins identified by their differential action in transcriptionally active extracts from T47D cells. *Nucleic Acids Res* **19**: 1227–1234, 1991.
32. Delabre K, Guiochon-Mantel A and Milgrom E, *In vivo* evidence against the existence of antiprogestins disrupting receptor binding to DNA. *Proc Natl Acad Sci USA* **90**: 4421–4425, 1993.