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Nucleocytoplasmic shuttling of the progesterone receptor

Anne Guiochon-Mantel, Pierre Lescop, Sophie Christin-Maitre, Hugues Loosfelt, Martine Perrot-Applanat and Edwin Milgrom

Hormones et Reproduction (INSERM U 135), Faculté de Médecine Paris-Sud, 94275 Le Kremlin Bicêtre Cedex, France

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The nuclear localization of the progesterone receptor is mediated by two signal sequences: one is constitutive and lies in the hinge region (between the DNA and steroid binding domains), the other is hormone dependent and is localized in the second zinc finger of the DNA binding domain. The use of various inhibitors of energy synthesis in cells expressing permanently or transiently the wild-type receptor or a receptor mutated within the nuclear localization signals, demonstrated that the nuclear residency of the receptor reflects a dynamic situation: the receptor diffusing into the cytoplasm and being constantly and actively transported back into the nucleus. The existence of this nucleo-cytoplasmic shuttle mechanism was confirmed by receptor transfer from one nucleus to the other in heterokaryons. Preliminary evidence was obtained, using oestrogen receptor, that this phenomenon may be of general significance for steroid receptors.

Key words: nuclear localization signals/nucleocytoplasmic shuttle/progesterone receptor

Introduction

Interest for steroid hormone receptors has stemmed not only from the desire to understand at the molecular level the mechanism of action of these hormones but also from the fact that they constitute one of the best available models for the study of the regulation of gene transcription (Evans, 1988; Green and Chambon, 1988). After binding their ligand, receptors interact with enhancer-like elements leading to increased (or decreased in some cases) gene transcription (Yamamoto, 1985; Beato *et al.*, 1989). Extra genomic effects of steroids have also been described but remain controversial (Liao *et al.*, 1980).

The subcellular localization of the receptors was initially thought to be cytoplasmic in the absence of hormone and nuclear in its presence (Gorski *et al.*, 1968; Jensen *et al.*, 1968). This conclusion was based on cell homogenization and fractionation studies. Availability of specific monoclonal antibodies and immunocytochemical analysis showed, however, the oestrogen and progesterone receptors to be intranuclear even in the absence of their ligands (King and Greene, 1984; Perrot-Applanat *et al.*, 1985). This was generalized to most steroid receptors (Husmann *et al.*, 1990) except for the glucocorticoid receptor (Fuxe *et al.*, 1985; Wikström *et al.*, 1987) and recently mineralocorticoid receptor (Lombès *et al.*, 1990; Farman *et al.*, 1991) which seem to be cytoplasmic or both nuclear and cytoplasmic in the absence of hormone and to concentrate in the nucleus in its presence (Fuxe *et al.*, 1985). Cloning of the receptors and *in vitro* mutagenesis studies led to the description of karyophilic signals in glucocorticoid (Picard and Yamamoto, 1987), oestrogen (Picard *et al.*, 1990) and progesterone receptors (Guiochon-Mantel *et al.*, 1989). In the latter case we identified two regions involved in nuclear localization of the receptor: one, constitutively active, was similar to the well known signal of SV40 large T antigen (Kalderon *et al.*, 1984; Lanford *et al.*, 1986). A second mechanism of nuclear localization could be observed after deletion of this karyophylic signal. Deletion mutants showed it to reside in the DNA binding region of the receptor and it could be made active either by binding of hormone or by deletion of the steroid binding domain (this procedure has previously been shown to yield a constitutive receptor) (Guiochon-Mantel *et al.*, 1988). Its activity thus paralleled very closely that of the DNA binding function: one interpretation of these findings was that the receptor could passively cross the nuclear membrane, bind to the DNA, and accumulate in the nucleus by this mechanism. Alternatively it was possible that a second karyophylic signal was intermingled with the DNA binding domain and could be unmasked by exactly the same mechanisms as those involved in the accessibility of the DNA binding site. Moreover another puzzling observation was made in the same study: oligomers were found to be formed between a cytoplasmic mutated receptor and a nuclear wild-type receptor. Thus both receptor monomers had to contact each other. This could be due to the passive diffusion through the nuclear membrane of a fraction of the 'cytoplasmic' receptor. Alternatively the 'nuclear' receptor could be shuttling out of and into the nucleus. In several systems, it has previously been shown that transport of proteins through the nuclear membrane is active, i.e. energy dependent (Newmeyer and Forbes, 1988; Richardson *et al.*, 1988). Thus we used inhibitors of energy synthesis to try to answer the questions raised by the intranuclear localization of the receptor. These experiments yielded observations suggesting that the nuclear residency of the progesterone receptor reflects a dynamic situation: the receptor diffusing into the cytoplasm and being constantly and actively transported back into the nucleus.

Results

Energy depletion and progesterone receptor entry into the nucleus

Previous studies have shown that deletion of five amino acids in the major constitutive karyophylic region of the progesterone receptor yields a cytoplasmic mutant (mutant Δ638–642) which can be shifted into the nucleus by administration of the hormone (Guiochon-Mantel *et al.*, 1989). Two mechanisms were possibly involved in this phenomenon: either the mutated receptor passively diffused

through the nuclear membrane and became trapped in the nucleus due to its binding to DNA, or the mutated receptor contained a second karyophylic signal which was unmasked only after binding of hormone. Experimentally, energy depletion could allow a distinction between these two mechanisms since DNA binding does not involve any energy dependent step whereas proteins imported through karyophylic signals are known to first interact with nuclear pores and thereafter be transported into the nucleus through an energy dependent step (Newmeyer and Forbes, 1988; Richardson *et al.*, 1988).

We used a permanent cell line established in L cells and containing the Δ638−642 mutant. These cells were incubated with deoxyglucose and sodium azide in conditions previously shown to inhibit the transport of other nuclear proteins (Richardson *et al.*, 1988). The cells were then challenged with hormone. As shown in Figure 1 the nuclear translocation of the receptor was completely inhibited. Since sodium azide may interact with metals such as zinc (Hewitt and Nicholas, 1963) and the latter is involved in receptor binding to DNA we tested other inhibitors known to act through completely different mechanisms. Inhibition of nuclear localization of receptor was also obtained with oligomycin, antimycin A and atractyloside. Moreover nuclear shift of receptor was also inhibited when cells were kept at 4°C (Figure 1). These experiments thus suggested the presence of another (hormone dependent) karyophylic signal in the DNA binding domain of the receptor.

Energy depletion and progesterone receptor efflux from the nucleus

Since energy depletion was able to prevent Δ638−642 receptor entrance into the nucleus we wondered if it might impair receptor maintenance in the nucleus once it has entered this organelle. Thus cells were incubated for 4 h with hormone to shift the receptor into the nucleus and thereafter deoxyglucose and sodium azide were added. This treatment provoked an efflux of the receptor from the nucleus into the cytoplasm visible already after 30 min and which was complete in 4 h (Figure 2). Similar results were obtained in transient expression experiments using mutant Δ638−642 cDNA and COS-7 cells (not shown).

These observations suggested that despite its size the Δ638−642 receptor could diffuse from the nucleus into the cytoplasm. Since its size was only negligibly smaller than that of the wild-type receptor (925 versus 930 amino acids) it appeared likely that similar mechanisms could be also effective for the latter.

To test this hypothesis we used another permanent L cell line transformed with the wild-type receptor and submitted it to the action of various inhibitors (sodium azide, antimycin A and oligomycin). All three inhibitors provoked an extranuclear localization of the receptor: oligomycin showing the strongest and sodium azide the weakest effect (not shown). Transient expression experiments using the wild-type receptor and COS-7 cells gave similar results (Figure 2).

Energy synthesis inhibitors might have acted through a

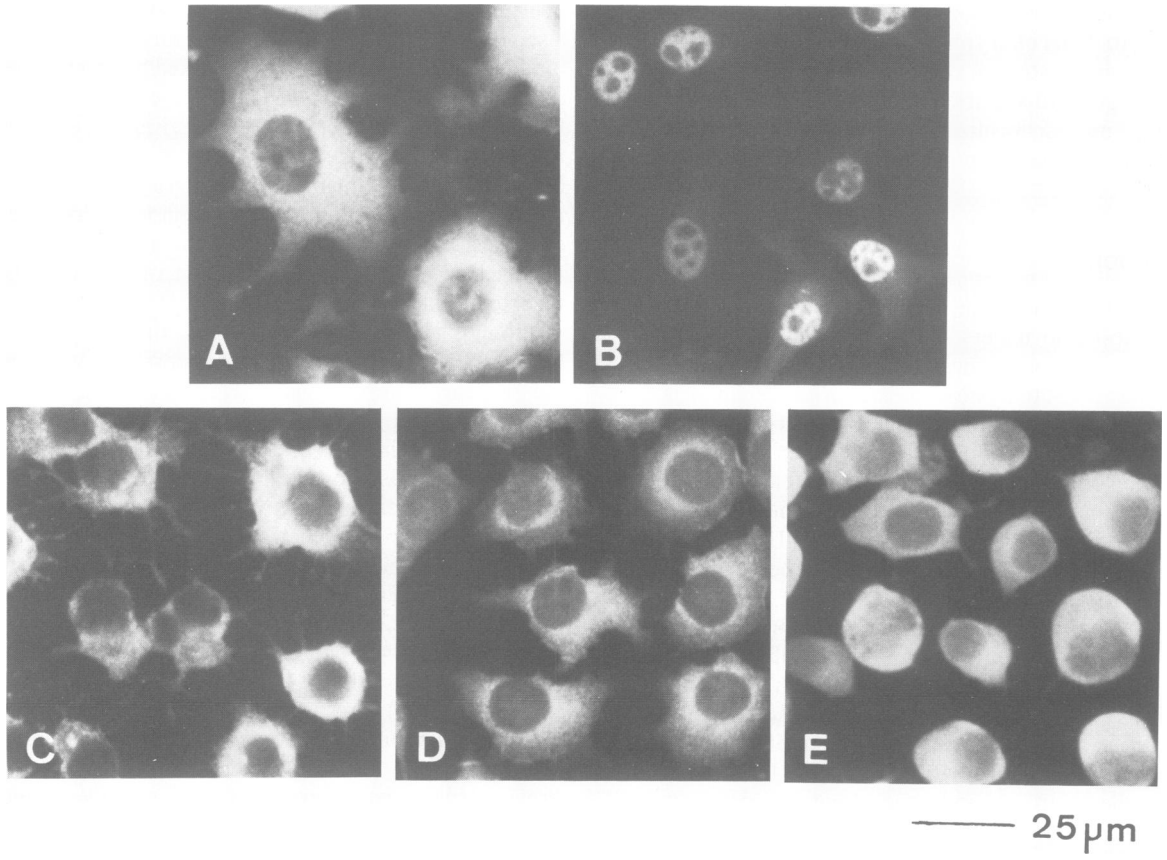

Fig. 1. Inhibition by energy depletion of hormone dependent nuclear transfer of Δ638−642 mutant progesterone receptor. (**A**) L Δ638−642 cells incubated at 37°C, (**B**) L Δ638−642 cells incubated at 37°C with hormone for 4 h; (**C**) L Δ638−642 cells incubated at 37°C with hormone and sodium azide and 2-deoxyglucose; (**D**) L Δ638−642 cells incubated at 37°C with hormone and atractyloside and 2-deoxyglucose; and (**E**) L Δ638−642 cells incubated at 4°C with hormone.

non-specific mechanism i.e. a modification of the permeability properties of the nuclear pore. To test this possibility L cells were microinjected into the nucleus with fluorescent rabbit immunoglobulins and treated or not by oligomycin (in conditions previously shown to provoke receptor exit from the nucleus). In both cases the microinjected protein remained in the nucleus without any observable passage into the cytoplasm (Figure 3).

Differences in the effect of energy depletion on various cell types and on various receptor mutants

During these experiments, differences were observed in the effect of various inhibitors depending on the one hand on the cell type which was used (Table I) and on the other hand on the receptor species which was under study (Table II). For instance, atractyloside inhibited completely nuclear localization of the Δ638−642 mutant in L cells but very weakly in COS-7 cells (Table I). These effects were probably due to the known variability of penetration of different inhibitors into different cells and also to the fact that various metabolic pathways do not contribute to the same extent to energy formation in different cells (Hewitt and Nicholas, 1963; Klingenberg, 1989).

Moreover differences were also observed in the effect of energy depletion on various receptor species (wild-type or mutants). For instance when sodium azide was incubated with COS-7 cells during transient transfection experiments, nuclear localization of Δ638−642 mutant in the presence of hormone was completely inhibited whereas wild-type receptor incubated with hormone was entirely nuclear. In the same conditions the wild-type receptor in the absence of hormone was partially cytoplasmic (Table II).

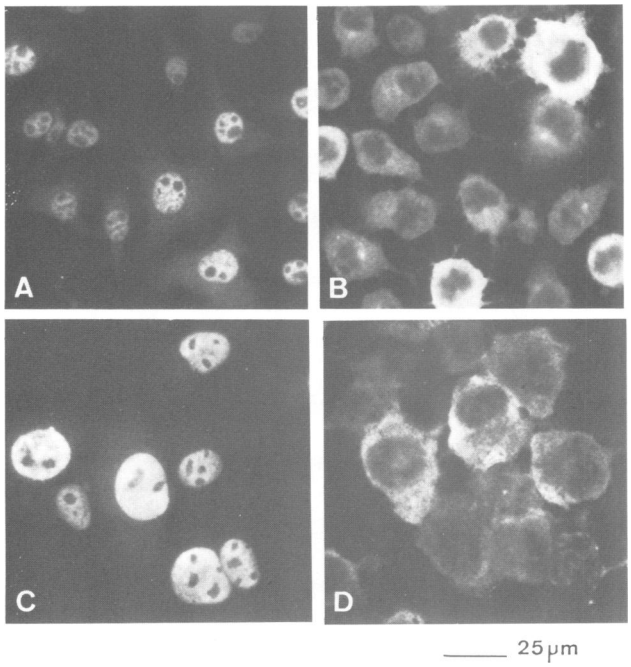

Fig. 2. Energy depletion provokes efflux of nuclear progesterone receptor into the cytoplasm. (**A**) L cells permanently expressing Δ638−642 mutant receptor were incubated for 4 h with hormone to shift the receptor into the nucleus. The incubation was continued for 4h . (**B**) Identical to (A) except that during the last 4 h period cells were cultured in the presence of 2-deoxyglucose and sodium azide. (**C**) COS-7 cells transfected with the plasmid encoding for the wild-type progesterone receptor. (**D**) Identical to C, except that cells were cultured for 4 h in the presence of deoxyglucose and sodium azide.

These results are probably related to the fact that proteins carrying two karyophylic signals (as in the case of wild-type receptor in the presence of hormone) are more efficiently transported into the nucleus than proteins carrying a single karyophylic signal (mutant Δ638−642 in the presence of hormone, or wild-type receptor in the absence of hormone) (Lanford *et al.*, 1986; Dworetzky *et al.*, 1988). Thus partial inhibition of energy formation has a more pronounced apparent effect in the latter than in the former case. Moreover, the fact that wild-type receptor in the absence of hormone (only the hinge region karyophylic signal is then active) is more readily transported into the nucleus than mutant Δ638−642 in the presence of hormone (only the second finger karyophylic signal is active) suggests that the hinge region karyophylic signal is more potent than the second zinc finger signal.

Experiments with constitutive receptors (deleted of the steroid binding region) led to similar conclusions. Mutant Δ663−930 which contains the two karyophylic signals remained essentially nuclear in the presence of sodium azide. The double mutant (Δ638−642, 663−930) which contains only the signal present in the second zinc finger was shifted into the cytoplasm in the same conditions (Table II).

Nucleocytoplasmic exchanges and progesterone receptor oligomerization

We took advantage of the differences in the extent of inhibition of nuclear localization of various mutated forms

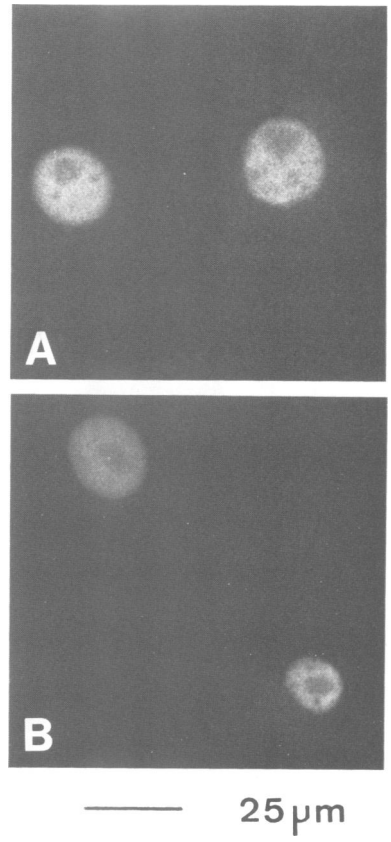

Fig. 3. Effect of energy depletion on the permeability of the nuclear pore. (**A**) L cells were microinjected into the nuclei (see Materials and methods) with fluorescein-conjugated immunoglobulins and further cultured for 4 h prior to fixation. (**B**) L cells were treated as described in (A) except that after microinjection they were cultured for 4 h in the presence of 2-deoxyglucose and oligomycin (50 μM).

Table I. Effect of different energy inhibitors on entry into and efflux from the nucleus of progesterone receptor in different cell lines

Cell type	L cells			Cos-7 cells[a]		
Receptor mutant	Δ638–642	Δ638–642	Wild-type	Δ638–642	Δ638–642	Wild-type
Inhibitor	Entry**	Efflux**		Entry**	Efflux**	
None	100N*	100N*	100N	100N*	100N*	100N
Oligomycin (50 μM)	100C	100C	100C	100C	100C	100C
Antimycin A (50 μM)	100C	100C	20N > C 80C	100C	100C	20N > C 80C
Sodium azide (10 mM)	100C	100C	100N > C	100C	100C	80N > C 20C
Atractyloside (50 μM)	100C	100N*	ND	100N*	100N*	ND

100N: receptor detected only in nucleus, in all cells.
xN > C, yC: predominantly nuclear staining accompanied by a clear cytoplasmic staining in x% of cells, receptor detected only in the cytoplasm in y% of cells.
100C: receptor detected only in the cytoplasm, in all cells.
100N*: Strong nuclear staining in all cells, accompanied by a slight cytoplasmic labeling in some cells.
Entry**: Cells containing Δ638–642 receptor were first incubated with inhibitor, then nuclear entry of receptor was elicited by hormone administration.
Efflux**: Δ638–642 receptor was first shifted into the nucleus by administration of hormone, then nuclear efflux was induced by inhibitor administration.
[a]L cells stably transfected either with the Δ638–642 mutant or the wild-type progesterone receptor and COS-7 cells transiently transfected with expression vectors encoding the Δ638–642 mutant or the wild-type receptor were used (see Materials and methods). Δ638–642 was cytoplasmic in the absence of hormone and was shifted into the nucleus after 4 h incubation with progesterone (see Materials and methods). Wild-type receptor was nuclear. Cells were incubated with energy inhibitors as described in Materials and methods.

Table II. Differences in the inhibition by sodium azide of nuclear localization of various forms of progesterone receptor (wild-type or deletion mutants)

	Nuclear localization signals which are active	Receptor localization in the absence of sodium azide (% cells)	Receptor localization in the presence of sodium azide (% cells)[a]
Wild-type receptor – H	NL_1	100N	80N > C 20C
Wild-type receptor + H	NL_1 + NL_2	100N	100N
Δ638–642 receptor – H	none	100C	100C
Δ638–642 receptor + H	NL_2	100N*	100C
Constitutive mutant (Δ663–930)	NL_1 + NL_2	100N	100N
Constitutive mutant with Δ638–642 deletion (Δ638–642,663–930)	NL_2	100N*	100C

H, hormone.
For other symbols see footnote to Table I.
[a]COS-7 cells were transfected with expression vectors encoding wild-type or Δ638–642 receptor. The cells were cultured either in the absence or in the presence of hormone and/or sodium azide. In the wild-type receptor only the constitutive hinge region nuclear localization signal (NL_1) is active in the absence of hormone. In the presence of hormone both NL_1 and NL_2 (hormone dependent nuclear localization signal present in the second zinc finger) are active. In the Δ638–642 mutant no karyophylic signal is active in the absence of hormone. In the presence of hormone NL_2 is active. In the constitutive mutant (Δ663–930) both NL_1 and NL_2 are active. In the double constitutive mutant (Δ638–642,663–930) only NL_2 is active.

of receptor to try to understand the mechanism of receptor oligomerization inside the cell.

In previous studies (Guiochon-Mantel *et al.*, 1989) we have shown that if cotransfection experiments were used to introduce in the same cells a 'nuclear' form of receptor (for instance wild-type receptor) and a 'cytoplasmic' form of

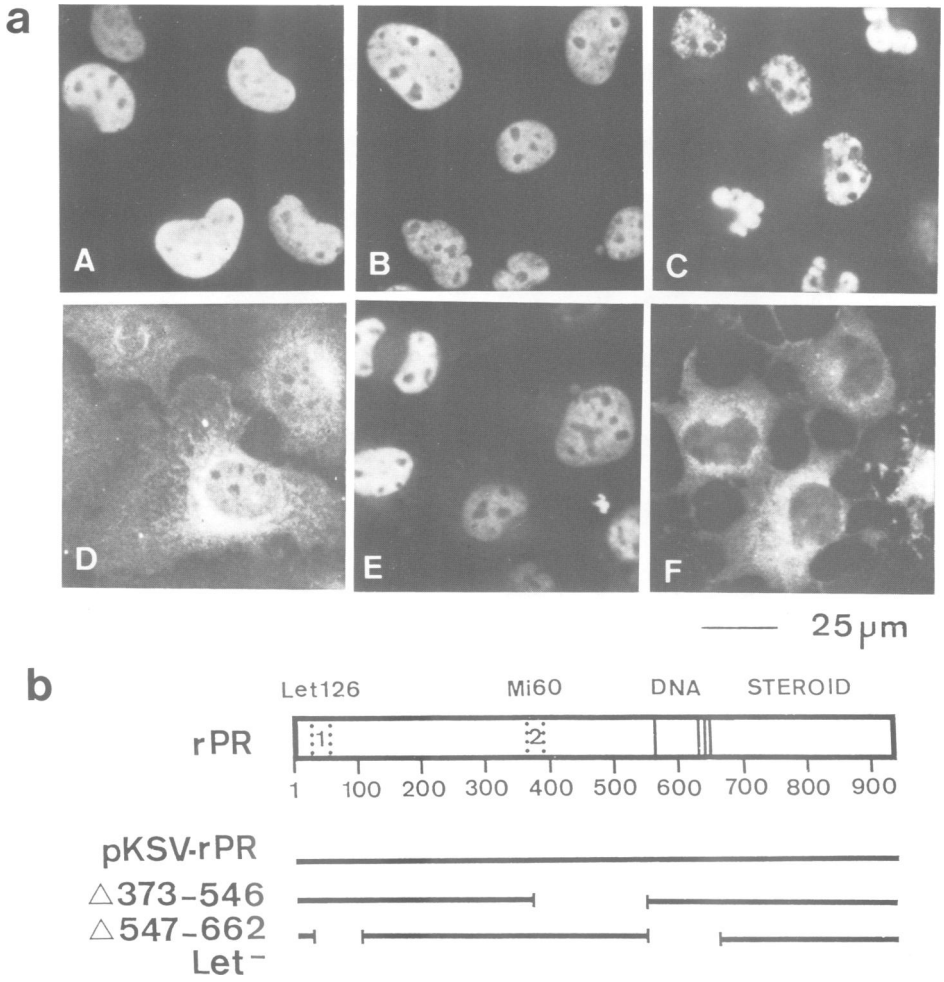

Fig. 4. Energy depletion and hormone induced oligomerization between 'nuclear' and 'cytocomplasmic' forms of the progesterone receptor. (**a**) COS-7 cells were cotransfected with expression vectors encoding wild-type Mi60$^-$ (Δ373−546) ('nuclear') and Δ547−662, Let$^-$ ('cytoplasmic') progesterone receptors. Let126 antibody was used to detect the former and Mi60 the latter. (A) and (D) Incubation (4 h) in the absence of hormone and sodium azide. Detection of the nuclear receptor (**A**) and cytoplasmic mutant (**D**). (B) and (E) Incubation (4 h) in the presence of hormone. Detection of the 'nuclear' receptor (**B**) and 'cytoplasmic' mutant (**E**). The 'cytoplasmic' mutant is transported into the nucleus. (C) and (F) Incubation in the presence of sodium azide (30 min) and hormone and sodium azide (4 h). Detection of the 'nuclear' receptor (**C**) and 'cytoplasmic' mutant (**F**). The transport of the 'cytoplasmic' mutant is inhibited. (**b**) Structure of progesterone receptor deletion mutants used to study oligomerization. The 930 amino acid long rabbit progesterone receptor (rPR) is schematically represented at the top of the figure. Boxes corresponding to the DNA binding region (DNA) and the steroid binding region (STEROID) are indicated above. The constitutive nuclear localization signal is shown by two vertical bars. The regions recognized by the antibodies Let126 (1) and Mi60 (2) are boxed with dotted lines. The wild-type pKSV-rPR and the mutants are represented below with a thick line, interrupted by a gap corresponding to the deleted amino acids.

receptor (for instance a mutant deleted of the two karyophylic signals but retaining the steroid binding region), after hormone was administered oligomers were formed and the 'cytoplasmic' receptor was transported piggy-back into the nucleus. The apparent paradox of these experiments was thus that 'nuclear' and 'cytoplasmic' forms of receptor could get into contact. This could be explained by two mechanisms: either the 'cytoplasmic' form of receptor could passively diffuse to some extent into the nucleus but could not remain there except if a 'nuclear' form was present and formation of oligomers led to trapping of the mutant receptor in the 'nuclear' compartment. Alternatively contact between 'nuclear' and 'cytoplasmic' monomers of receptor might have been due to the fact that the nuclear receptor was continuously shuttling between the cytoplasm and the nucleus and was actively accumulated there. During this shuttle it could contact the cytoplasmic monomer.

Partial inhibition of energy formation in conditions where a major fraction of the wild-type receptor remained in the nucleus could allow us to distinguish between both possibilities. If the 'cytoplasmic' receptor passively diffused through the nuclear membrane and was trapped in the nucleus by interaction with the 'nuclear' monomer this process had no reason to be impaired by energy depletion. On the contrary, if the 'nuclear' monomer diffused into the cytoplasm where it formed a hetero-oligomer which then had to be actively transported back into the nucleus, this mechanism would probably be impaired by energy depletion. Wild-type Mi60$^-$ receptor ('nuclear') was cotransfected into COS-7 cells with the mutant Δ547−662, Let$^-$ ('cytoplasmic') (Deletion of epitopes for either Let126 or Mi60 allowed independent observation of each receptor monomer in the same cell). When hormone was administered the latter was shifted into the nucleus as described previously (Figure 4E). However, if the cells were preincubated with sodium azide before hormone administration no such transfer occurred (Figure 4F). An alternative experimental approach consisted in cotransfecting the two receptors, administering

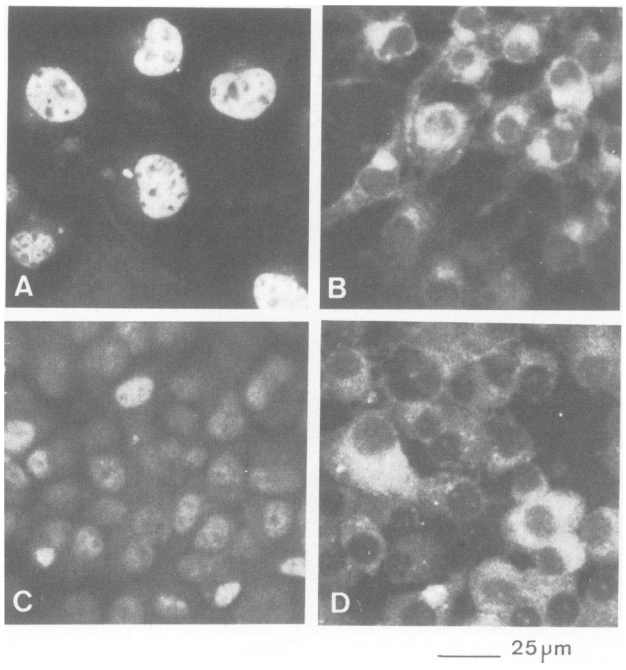

Fig. 5. Energy depletion and oestrogen receptor efflux from the nucleus. COS-7 cells were transfected with an expression vector encoding the human oestrogen receptor. The cells were incubated either in the absence (**A**) or in the presence (**B**) of sodium azide. Breast cancer MCF-7 cells were incubated in the absence (**C**) or in the presence (**D**) of oligomycin. Oestrogen receptor was detected using an Abbott ERICA kit.

hormone for a sufficient time to localize Δ547−662, Let⁻ mutant in the nucleus and thereafter partially blocking energy formation with sodium azide. If the 'cytoplasmic' receptor had diffused into the nucleus and was trapped there by interaction with 'nuclear' monomers it should be insensitive to energy depletion. On the other hand, if the wild-type 'nuclear' receptor was continuously recycled through the cytoplasm and if partial energy depletion prevented hetero-oligomers (where only one monomer carried karyophylic signals) being retransported into the nucleus whereas homopolymers of wild-type receptor (where both subunits carried karyophylic signals) could still be retransported one would expect an appearance of Δ547−662, Let⁻ in the cytoplasm. The latter experimental result was actually observed (data not shown). All these experiments thus suggested that the mutated receptor devoid of karyophylic signals cannot cross the nuclear membrane whereas nuclear receptor species are continually recycled through the cytoplasm.

Energy depletion and oestrogen receptor efflux from the nucleus

To evaluate how general this phenomenon was, we transfected COS-7 cells with an expression vector encoding wild-type oestrogen receptor, incubated these cells with sodium azide, oligomycin or antimycin A and examined the distribution of oestrogen receptor using monoclonal antibodies. As shown in Figure 5 energy depletion resulted in the efflux of oestrogen receptor from the nucleus into the cytoplasm.

Moreover, when MCF-7 cells were incubated with sodium azide, oligomycin or antimycin A the endogenous nuclear receptor was shifted into the cytoplasm (oligomycin being the most potent and azide the least potent inhibitor in these conditions) (Figure 5).

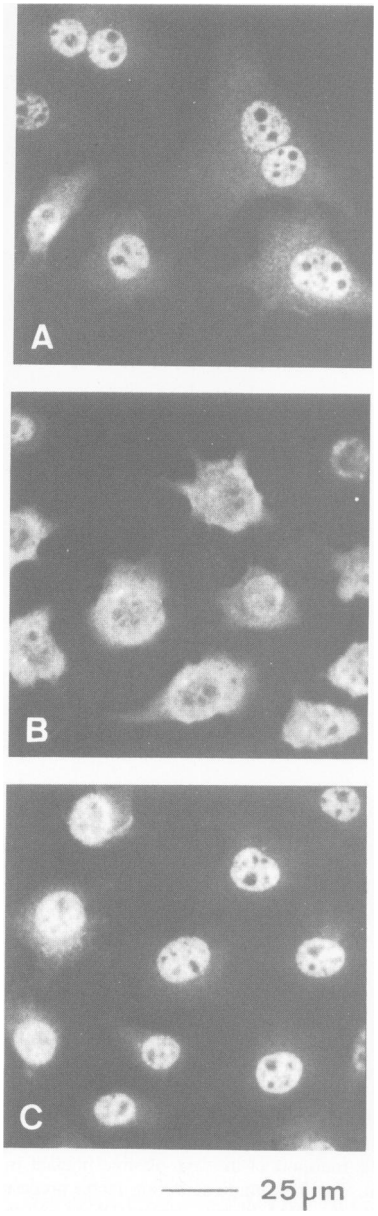

Fig. 6. Progesterone receptor can repeatedly shuttle through the nuclear membrane. L cells containing the Δ638−642 mutant were cultured for 4 h and 30 min in the presence of progesterone. Cycloheximide was added for the last 30 min (**A**) and maintained during the following steps. Cells were then cultured in the presence of deoxyglucose and sodium azide for 90 min (**B**). The cells were transferred into a medium containing glucose and devoid of sodium azide for 4 h (**C**).

Receptor synthesis and intracellular traffic

It has previously been suggested that the steroid receptors undergo a specific cycle in the cells and that nuclear transfer is linked to receptor biosynthesis (Horwitz *et al.*, 1983). A role for the interaction with heat shock proteins and elements of the cytoskeleton has been postulated (Pratt, 1990). It has also been proposed that the receptor is degraded after having bound hormone and having exerted its biological activity (Wei *et al.*, 1987). On the other hand, if the nuclear signals which lead the protein to the nuclear pore are the major

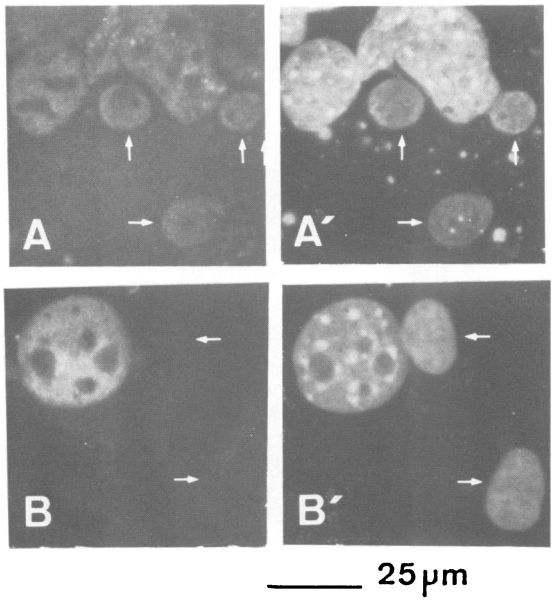

Fig. 7. Transfer of receptor from mouse to human nuclei in heterokaryons. (A) and (A') Mouse L cells containing wild-type progesterone receptor were fused with 293 human cells devoid of receptor (see Materials and methods). Heterokaryons were observed. Cycloheximide was administered to prevent neosynthesis of progesterone receptor. Progesterone receptor was labelled by immunofluorescence 12 h after the fusion (**A**). Human (arrows) and mouse nuclei could be distinguished by fixation of Hoechst 33258 (**A'**) and also to some extent by their size. After the fusion, progesterone receptor appears in 293 human cell nuclei. (B) and (B') Control experiment, in which cells have not been fused. Progesterone receptor was labelled by immunofluorescence (**B**). Colouration of Hoechst 33258 allows distinction between the two different cell types (**B'**). 293 human cells are devoid of receptor (arrow).

factors in nuclear localization of receptor it should be possible to provoke transfers of receptor back and forth from the nucleus into the cytoplasm and again into the nucleus. Presence of protein synthesis inhibitors should ensure that these transfers actually involve the same receptor molecules and not neosynthesized receptors.

The cells containing the Δ638−642 mutant were incubated with hormone and submitted to the action of cycloheximide. The receptor was localized in the nucleus (Figure 6A). Cycloheximide was maintained and cells were then cultured in the presence of deoxyglucose and sodium azide: the receptor was shifted into the cytoplasm (Figure 6B). When the cells were returned to a medium containing glucose and devoid of sodium azide the receptor re-entered the nucleus (Figure 6C).

Migration of progesterone receptor between nuclei in interspecies heterokaryons

To confirm the existence of a nucleocytoplasmic shuttle of receptor we used a method previously employed in the case of nucleolar proteins (Borer *et al.*, 1989). Heterokaryons were formed by polyethylene glycol fusion of mouse L cells containing wild-type progesterone receptor and human 293 cells devoid of receptor. Treatment by cycloheximide prevented synthesis of new receptor molecules and receptor distribution was analysed by immunocytochemistry. Mouse nuclei were identified by fluorescent staining with Hoechst 33258 (Figure 7).

Twelve (Figure 7A and A') and 18 h (not shown) after fusion, receptor could be identified in human nuclei giving a signal of equivalent magnitude to that observed in the mouse nuclei. When cells have not been fused the receptor is only present in mouse nuclei (Figure 7B and B'). Thus the receptor has migrated from one nucleus to the other implying a shuttle through the cytoplasm.

Discussion

Previous studies (Guiochon-Mantel *et al.*, 1989) have shown the presence in the progesterone receptor of a constitutively active karyophylic signal located around amino acids 638−642. When this signal was deleted the receptor became cytoplasmic, however, administration of hormone led to its accumulation in the nucleus. This phenomenon could have been related to a passive diffusion of the receptor through the nuclear membrane followed by its entrapment in the nucleus due to binding to DNA. Alternatively there might have existed a second karyophylic signal located (as shown by deletion mutants) in the DNA binding domain of the receptor. Use of inhibitors of energy synthesis favoured the second hypothesis. Examination of the sequence of this domain shows two stretches of basic amino acids which are candidates for being nuclear localization signals: amino acids 614−618 and 624−627. We are presently using these amino acid sequences to try to direct heterologous proteins into the nucleus. The existence of multiple karyophylic signals is a frequent phenomenon for nuclear proteins (Roberts, 1989; Silver and Goodson, 1989). The glucocorticoid receptor also contains two signals, however, both are hormone dependent and one of them resides in the steroid binding domain (Picard and Yamamoto, 1987).

The residency in the nucleus of the progesterone receptor seems to be a dynamic phenomenon resulting from the continuous active transport into the nucleus counterbalance by some diffusion into the cytoplasm. It is not known if this diffusion is a totally passive phenomenon or if it necessitates the presence of a nuclear localization signal in the protein (Mandell and Feldherr, 1990). Interaction with specific protein(s) in the pore could then take place during nuclear exit of the protein. The preferential localization in the cytoplasm of receptor in the presence of energy formation inhibitors suggests a possible interaction with some cytoplasmic component.

Shuttle mechanisms have recently been proven for two nucleolar proteins (Borer *et al.*, 1989) and discussed in other cases (Rechsteiner and Kuehl, 1979; Goldstein and Ko, 1981; Madsen *et al.*, 1986; Bachmann *et al.*, 1989) including the steroid receptors (Nigg, 1990). Other proteins like nucleoplasmin having once entered the nucleus seem to remain there (Dingwall *et al.*, 1982). This may be due to the fact that they form tight complexes with intranuclear components preventing any nuclear efflux (Dilworth *et al.*, 1987). This mechanism of nuclear localization of the receptor explains some previous observations. After homogenization ligand-free progesterone or oestrogen receptors, which reside in the nucleus (Perrot-Applanat *et al.*, 1985; King and Greene, 1984) are found in the cytosol (Gorski *et al.*, 1968), even when nuclear structures have been preserved. This is probably due to diffusion through nuclear membranes in conditions where active transport is blocked by dilution and low temperature. Incidentally association of receptors with nuclei after homogenization of cells at 25°C has been described (Molinari *et al.*, 1985). Moreover, localization of

ligand-free glucocorticoid receptor in cytoplasm (Govindan, 1980; Papamichail *et al.*, 1980) or both cytoplasm and nuclei (Wikström *et al.*, 1987) has been considered as a complete difference from oestrogen or progesterone receptors 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 glucocorticoid receptor may be only quantitatively and not qualitatively different from that of sex steroid receptors. Less effective constitutive karyophylic signals would lead to an increased time of residency of the receptor in the cytoplasm and to an apparent distribution between cytoplasmic and nuclear compartments. The understanding of receptor function may also be modified by the fact that receptors shuttle between nucleus and cytoplasm. For instance, considerable speculations have been published on the problem of receptor interaction with the heat shock protein of 90 kDa which is mainly a cytoplasmic component (Lindquist and Craig, 1988). This fact has led in some cases to the conclusion that receptor binding to heat shock protein of 90 kDa (hsp90) was an artefact due to receptor extraction from the nucleus during cell homogenization (Bailly *et al.*, 1986). On the contrary other reports have tried to prove that a small fraction of the hsp90 is intranuclear (Gasc *et al.*, 1990). Obviously such discussions are meaningless if receptors cycle between cytoplasm and nucleus. Moreover, this mechanism is compatible with receptor exerting biological activities in the cellular cytoplasm. Such effects have indeed been described (Liao *et al.*, 1980; Verdi and Campagnoni, 1990). 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 expanding number of proteins implicated in the regulation of gene transcription.

Materials and methods

Plasmids

Nomenclature: derivatives denoted with a Δ lack the receptor segment delineated by the numbered amino acids. When the epitope recognized by the monoclonal antibody Let126 is deleted (amino acids 25–103), the mutant is called Let$^-$. When the epitope recognized by the monoclonal antibody Mi60 is deleted (amino acids 373–546) the mutant is called Mi60$^-$. Plasmids encoding the rabbit progesterone receptor cDNA (pKSV-rPR) and mutants Δ638–642, Δ663–930, Δ638–642, 663–930, Δ547–662, Δ373–546, Δ25–103 have been previously described (Guiochon-Mantel *et al.*, 1988, 1989). Mutant Δ547–662, Let$^-$ was constructed by cleavage of the mutant Δ547–662 at the proper restriction sites, purification of the restriction fragment encompassing the deletion, cleavage of the wild-type Let$^-$ receptor (Δ25–103) at the same restriction sites, and ligation of the purified restriction fragment with the wild-type Let$^-$ receptor. The plasmid encoding the human oestrogen receptor cDNA (pKSV-hER) has been previously described (Savouret *et al.*, 1991).

Cell culture, DNA transfection and immunofluorescence studies

COS-7 cells (Gluzman, 1981), 293 cells (Graham *et al.*, 1977), L mouse cells and MCF7 cells (Horwitz *et al.*, 1975) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. DNA transfections were performed as described (Guichon-Mantel *et al.*, 1989), using the calcium phosphate precipitate method (Graham and Van der Eb, 1973). Progesterone 10^{-6} M was added during 4 h where indicated. Immunofluorescence studies were performed as described (Guiochon-Mantel *et al.*, 1989). Monoclonal anti-progesterone receptor antibodies Mi60 (Logeat *et al.*, 1983) and Let126 (Lorenzo *et al.*, 1988) have previously been described (see Perrot-Applanat *et al.*, 1985, 1987). Monoclonal anti-oestrogen receptor antibody was from the Abbott ERICA Kit. A fluorescein-conjugated rabbit anti-mouse antibody (Dakopatts) was added at a dilution of 1:40 (for 1 h at room temperature). Photographs were taken on a Leitz microscope, with Kodak type Tmax 400 ASA film.

Energy inhibitors

The different inhibitors were always used with glucose minus DMEM supplemented with 2-deoxyglucose 6 mM (Richardson *et al.*, 1988). The fetal calf serum used was dialysed and charcoal stripped.

Sodium azide 10 mM (Merck), atractyloside 50 μM (Sigma), oligomycin 50 μM (Sigma) and antimycin A 25 μM or 50 μM (Sigma) were added where indicated.

Permanent cell lines

L mouse cells were cotransfected with the plasmid encoding either for the wild-type receptor (pKSV-rPR) or the Δ638–642 mutant and with the plasmid pSV*neo* conferring resistance to the antibiotic G418 (Southern and Berg, 1982). Clones resistant to G418 (Geneticin, Sigma) were selected and screened for expression of the receptor by immunocytochemistry. L pKSV-rPR is the cell line expressing permanently the wild-type receptor. L Δ638–642 is the cell line expressing permanently the mutant Δ638–642. These clones have now been studied for more than 20 passages and stably express the corresponding form of receptor.

Heterokaryon formation

Human 293 cells were plated on 'chamber/slides' (Nunc Inc). 24 h later, L pKSV-rPR were plated onto the same chamber/slide. Cells were fused 24 h later. Fusions were carried out essentially as described (Davidson and Gerald, 1976). Prewarmed 50% polyethylene glycol 6000 (PEG) (Serva), in calcium–magnesium-free Hanks balanced solution (HBSS) was added. After 4 min the PEG was removed and the cells were washed extensively with prewarmed HBSS. The cells were then incubated in the prewarmed culture medium containing 10 μM cytosine arabinoside (Borer *et al.*, 1989). Cycloheximide (10 μg/ml) was added where indicated.

Immunofluorescence studies were performed as described for transfection experiments. For staining of DNA, Hoechst 33258 (Sigma) was added at 1 μg/ml at the time of incubation with secondary antibody.

Microinjection experiments

L cells were plated on glass coverslips. 24 h later, cell nuclei were injected with a solution of fluorescein-conjugated rabbit immunoglobulins (Dakopatts) using Eppendorf Femtotips. Injection pressure was generated by an Eppendorf microinjector 5242. Microinjection was monitored under a Zeiss inverted microscope Axiovert 35. Cells were then incubated at 37°C in DMEM minus glucose, supplemented with 2-deoxyglucose 6 mM containing 50 μM oligomycin for 4 h, or incubated at 37°C in standard DMEM medium for 4 h before fixation. They were directly observed on a Leitz microscope.

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