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Intracellular distribution of a cytoplasmic progesterone receptor mutant and of immunophilins cyclophilin 40 and FKBP59: effects of cyclosporin A, of various metabolic inhibitors and of several culture conditions

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Abstract

The effect of cyclosporin A (CsA) on the intracellular distribution of a mutated NLS minus rabbit progesterone receptor (PRm) and the receptor-associated immunophilins, cyclophilin 40 (Cyp40) and FKBP59, was tested in Lcl3 cells by indirect immunofluorescent staining. PRm, which is cytoplasmic in absence of progesterone, is shifted to the nucleus by the hormone as well as by CsA, but not by FK506 or Rapamycin [I. Jung-Testas, M.-C. Lebeau, E.E. Baulieu. C.R. Acad. Sci. Paris 318 (1995) 873-878]. However the time course of nuclear import due to CsA and its sensitivity to *N*-ethyl maleimide (NEM) and to a calmodulin inhibitor (W7) was different from those observed for the hormonal effect.

Cyp40 in Lcl3 cells is localized mainly in the nucleoli. CsA treatment increased nucleolar staining, while NEM and W7 caused it to decrease; after actinomycin D (1 μM) nucleolar staining of Cyp40 disappeared.

FKBP59 is mainly cytoplasmic and concentrated in the perinuclear region, never in the nucleoli. CsA, actino D and W7 treatment did not influence FKBP59 localization. In serum-deprived medium FKBP59 was cytoplasmic, but when the culture medium was enriched (20% serum, insulin and EGF) FKBP59 became perinuclear and hsp 86 was partly shifted to the nucleus, but PRm remained cytoplasmic.

CsA has an effect on PRm distribution, while it does not influence Cyp40 and FKBP59 localization. In presence of actino D the labelling of Cyp40 disappears from the nucleoli, while the distribution of PRm and FKBP59 is unaffected. Growth factors influence FKBP59 but not PRm or Cyp40. These results suggest that these proteins shuttle independently and that their association is transient.

1. Introduction

In target tissues and in the absence of hormone, steroid hormone receptors are associated with a number of proteins, which modulate their activity by stabilizing them in a non-DNA binding conformation and which participate in their intracellular trafficking. In the presence of hormone, these proteins dissociate from the receptors, which can then dimerize and interact with specific elements on the DNA, promoting the transcriptional response to the hormone [1,2].

Among these associated proteins, heat shock proteins 90 and 70, immunophilins FKBP 52/59 and 54 [3] and Cyclophilin 40 [4], as well as a few proteins of yet unknown function, p23, p48 and p60 [5], have been described. The immunophilins which were found associated with steroid hormone receptors have peptidyl-prolyl-isomerase activity which is inhibited by their cognate immunosuppressant drug [6,7]. They interact with hsp90 via their tetratricopeptide (TPR) repeats [8,9] and can bind calmodulin [4,10]. These proteins could therefore be involved in protein folding and chaperoning and could play a role in Ca^{2+} mediated events. Moreover, the immunosuppressant drugs which bind to the immunophilins, FK506 and Cyclosporin A

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(CsA), can also modulate responses to steroid hormones, potentiating or inhibiting receptor-mediated gene expression according to the cell line examined [11–14].

The effect of these drugs on the intracellular distribution of a steroid hormone receptor was tested in mouse fibroblasts, stably transformed with a plasmid containing a progesterone receptor mutant (PRm) [15]. This progesterone receptor mutant, which is deleted of its main constitutive nuclear localization signal (NLS), is cytoplasmic in the absence of progesterone, contrary to the wild type receptor which is nuclear in these conditions. The mutant receptor is rapidly shifted to the nucleus in the presence of progesterone [16]. We observed that CsA also promotes a nearly complete relocalization of this cytoplasmic receptor to the nucleus, although this takes a longer time than after the hormone [17]. Trying to understand the type of mechanism involved in this transfer, we examined the effects of different culture conditions and of metabolic inhibitors such as *N*-ethylmaleimide, calmodulin antagonists and actinomycin D on the distribution of the progesterone receptor mutant in parallel with that of the immunophilins, Cyclophilin 40 and FKBP59.

2. Materials and methods

2.1. Cells and cell culture

The mouse L-cells, which permanently express the PR-NLS mutant D638-642 (PRm), had been co-transfected with the plasmid pSVneo, conferring resistance to the antibiotic geneticin (G418). They were kindly given by A. Guiochon-Mantel [16]. These cells (Lcl3) were grown at 37°C in DMEM (Gibco BRL, Life Technologies LTD) supplemented with 10% heat-inactivated calf serum (Eurobio), penicillin (100 IU/ml), streptomycin (100 μg/ml) and G418 (0.2 mg/ml, Sigma). For hormone withdrawal, cells were cultured in 10% charcoal-treated calf serum (CS-CX) for 2 weeks before the experiments, during which the concentration of CS-CX was reduced to 5%. For starvation experiments, Lcl3 cells were maintained in the absence of serum for 3–4 days and then incubated in serum-free medium (SF) for the indicated lengths of time. Upon release of starvation, intense cell division was obtained in DMEM supplemented with 20% fetal calf serum (Eurobio), EGF (20 ng/ml, Sigma) and insulin (4.8 μg/ml, Organon) (rich medium).

2.2. Immunofluorescence staining

For immunofluorescence staining, Lcl3 cells were plated at the desired dilution on glass coverslips (14 mm diameter), placed in Petri dishes and cultured under the described conditions. After incubation with either drug or hormone, the coverslips were transferred to PBS-containing Petri dishes, washed thoroughly to eliminate cell debris and fixed for 20 min in paraformaldehyde (Sigma) 4% in PBS. After washing in PBS, the cells were permeabilized in a 1% solution of Triton X-100 (Boehringer, Mannheim) for 4 min. After 3 washes in PBS, the cells were incubated with the appropriate first antibody for 2 h at room temperature, or overnight at 4°C, in a humid atmosphere. After 3 washes, the corresponding fluorescein-coupled second antibody was added for 1 h at room temperature. The washed coverslips were mounted in Moviol (Merck) and examined under a Leitz Laborlux D photomicroscope.

2.3. Antibodies

The mouse monoclonal anti-progesterone receptor antibody Let 126 [18] was used to detect PRm. The rabbit polyclonal anti-FKBP59 (C-terminal) antibody was used to detect FKBP59 [19] and the cyclophilins 40 (Cyp40) and 18 (Cyp18, CypA) were detected using the rabbit polyclonal anti-Cyp40 (C-terminal) and anti-CypA, respectively. The rabbit polyclonal antibodies raised against heat shock proteins 84 and 86 (N-terminal) were used to detect mouse hsp 90 b and a, respectively. All the rabbit polyclonal antibodies were from Affinity BioReagents (Neshanic Station, NJ, USA).

2.4. Hormones and drugs

Progesterone (Roussel-UCLAF) was diluted in ethanol at the required concentration, never exceeding 0.1% ethanol, final concentration.

Immunosuppressant drugs cyclosporin A (Sandimmun), FK506 and Rapamycin were gifts from Sandoz (Basel, Switzerland), Fujisawa GmbH (Munich, Germany) and Wyeth-Ayerst (Princeton NJ, USA), respectively. They were diluted in ethanol and the stock solutions kept at −20°C. *N*-ethylmaleimide (NEM), the calmodulin antagonist W7 (ref A3281) and actinomycin D were from Sigma. Phenoxybenzamine (POBA) was a gift from SmithKline Beecham Pharmaceuticals (England). All these drugs were diluted in ethanol and added to the culture medium at the concentrations indicated.

3. Results

3.1. Cyclosporin A, alone or with N-ethylmaleimide or calmodulin antagonists: effect on PRm localization

In mouse Lcl3 cells, the receptor mutant PRm is

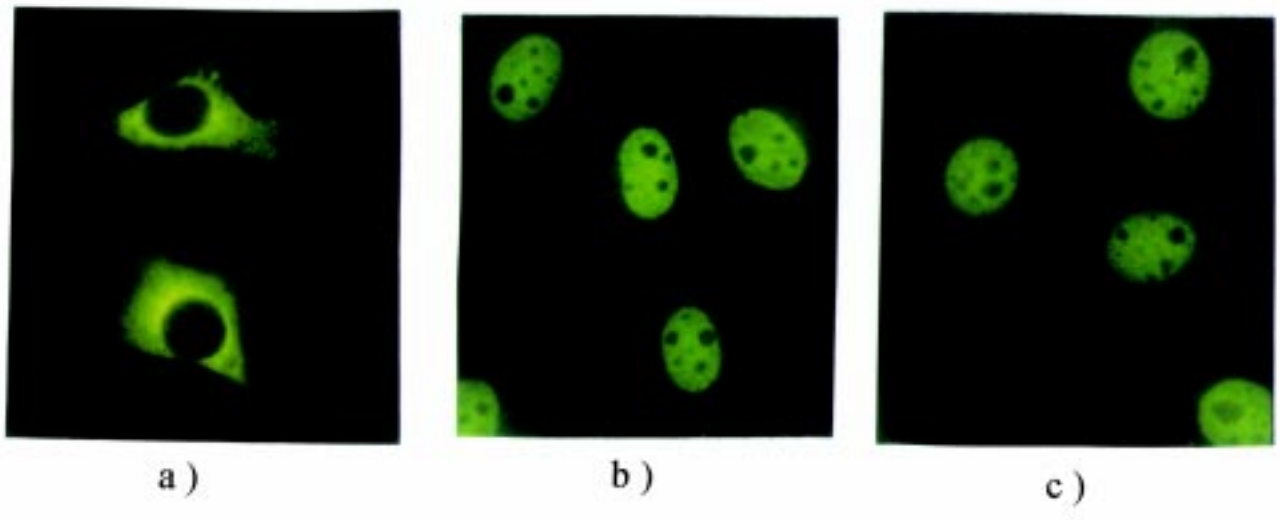

Fig. 1. Subcellular localization of PRm: effect of progesterone (Prog) and cyclosporin A (CsA). Mouse Lcl3 cells were cultured in DMEM, 5% CS-CX on glass coverslips, either in absence of hormone (a), or for 18 h in presence of 10 nM Prog (b) or 10 μM CsA (c). The glass coverslips were then recovered, fixed and immunostained with the anti-PR antibody Let 126, as described in Material and methods (×1600).

localized in the cytoplasm in the absence of progesterone and is shifted to the nucleus in presence of the hormone (10 nM) (Fig. 1a and b). When the cells were cultured in the presence of CsA (10 nM–10 μM), the PRm was also transferred to the nucleus in a drug concentration- and time-dependent fashion [17]. The nuclear transfer of the receptor mutant, which began after 2 h of culture in presence of CsA (10 μM), increased gradually and was complete after 18 h (Fig. 1c).

Once the PRm was shifted to the nucleus in presence of CsA and the cells were then cultured in absence of the drug for as long as 2 weeks, the receptor mutant remained localized in the nucleus.

Other immunosuppressant drugs such as FK506 or Rapamycin (10 μM) had no effect on the localization of PRm [17].

When energy sources were blocked by substituting 2-deoxyglucose for glucose and adding sodium azide to the medium, the nuclear shift caused by CsA was inhibited [17], as it is in the presence of progesterone [16].

The effect of *N*-ethylmaleimide (NEM), an inhibitor of the docking step of nuclear protein import [20] and a blocker of sulfhydryl groups, was also examined on the nuclear transfer of PRm. After 20 h of culture in the presence of NEM alone (0.8 μM), no change of PRm localization (C) was seen (Fig. 2a) and NEM did not affect the progesterone-induced transfer of the receptor mutant (N) (Fig. 2b). However, the CsA-

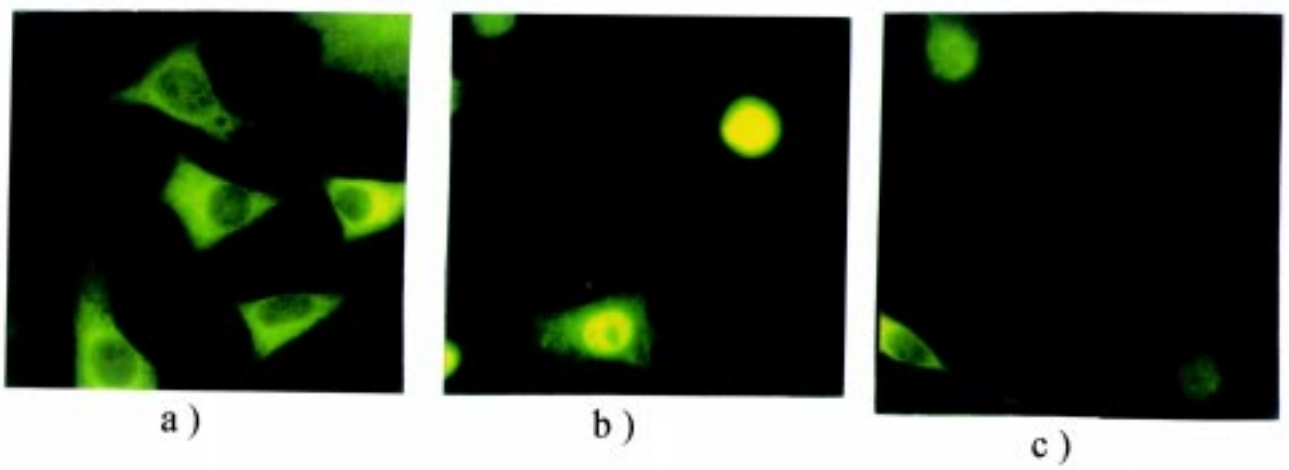

Fig. 2. Subcellular localization of PRm: effect of *N*-ethylmaleimide (NEM). Lcl3 cells were cultured as described in Fig. 1 and treated for 20 h with NEM (0.8 μM), alone (a), or together with 10 nM Prog (b), or together with 10 μM CsA (c). Immunofluorescence staining was with antibody Let 126, as above (×1000).

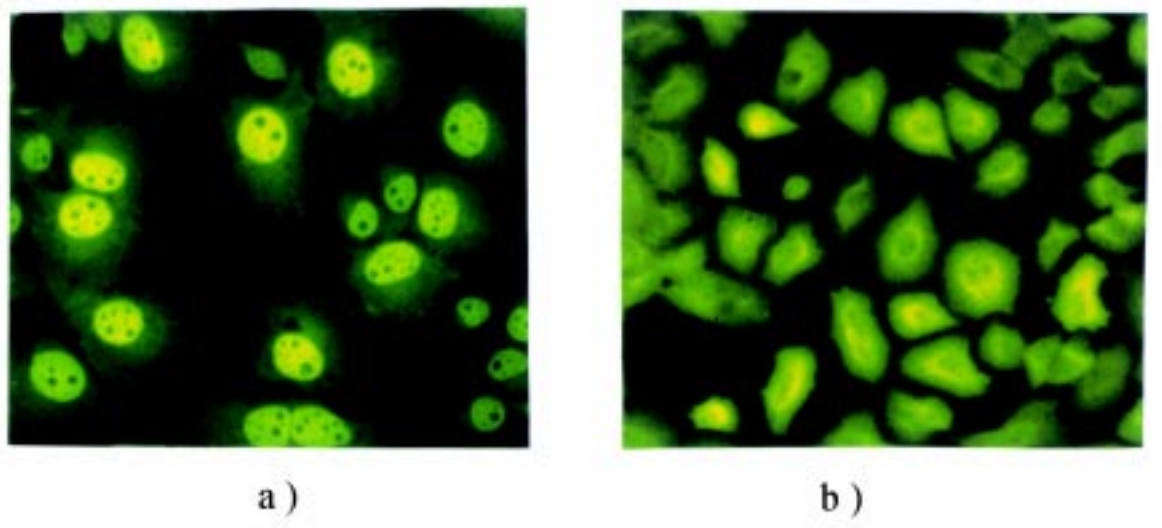

Fig. 3. Subcellular localization of PRm: effect of *N*-(6-aminohexyl)-5-chloro-1-naphtalenesulfonamide (W7). Lcl3 cells were cultured as described in Fig. 1 and treated for 30 min with W7 (25 μM). Then, Prog (10 nM) was added to the cultures in (a) and CsA (10 μM) to the cultures in (b) and they were kept in culture for 18 h. The coverslips were then recovered and immunostained with antibody Let 126 (×1000).

induced transfer of PRm was partly prevented after incubating Lcl3 cells for 20 h in presence of NEM and CsA (C> or =N) (Fig. 2c), showing a greater sensitivity to NEM of the CsA-induced transfer. This inhibition was specific for NEM, since cycloheximide [17] or actinomycin D did not prevent the CsA- induced shift of PRm.

The effect of calmodulin antagonists on the relocalization of PRm was also examined. Calmodulin binds to both immunophilins Cyp40 [4] and FKBP59 [10], as well as to hsp90 [21] and has also been shown to interact with the estrogen (ER) [22] and glucocorticosteroid (GR) [23] receptors. Moreover, when CsA is bound to cyclophilin18 (Cyp18), it inhibits the activity of calcineurin, a Ca^{2+}-calmodulin dependent protein phosphatase [24] and consequently the nuclear translocation of the transcription factor $NFAT_p$ [25]. It was therefore interesting to see if blocking calmodulin would affect the shift of the receptor mutant we observed in presence of CsA.

We first tested the effect of the calmodulin antagonist phenoxybenzamine (POBA). It was reported to reduce hormone binding to GR and hence to inhibit hormone-induced receptor translocation to the nucleus and receptor mediated gene expression [26]. The POBA concentration used in these experiments (100 μM) was toxic for Lcl3 cells, particularly in the presence of 10 μM CsA, so a 20 μM concentration of POBA was chosen for the experiments described here. At this concentration, POBA did not affect progesterone induced nuclear transfer of PRm, however it prevented complete translocation of PRm by CsA (not shown).

A more radical effect was observed when treating Lcl3 cells with *N*-(6-aminohexyl)-5-chloro-1-naphtalenesulfonamide (W7), an antagonist which binds specifically to calmodulin in the presence of Ca^{2+} [27]. The cells were exposed to W7 (25 μM) 30 min before adding the hormone or the immunosuppressant drug to the culture medium. After 18 h incubation with both W7 and progesterone (10 nM), all the PRm was visualized in the nucleus, there was no inhibition of the nuclear transfer by W7 (Fig. 3a). In contrast, when cells were cultured in the presence of CsA (10 μM) plus W7, PRm remained mainly cytoplasmic and was concentrated around the nucleus. It was rarely found in the nuclei (Fig. 3b). These results indicate again that different mechanisms are involved in PRm nuclear transfer due to progesterone or to the immunosuppressant CsA.

3.2. Intracellular localization of cyclophilin40, cyclophilin18 and FKBP59

In the cell, CsA is bound to Cyp18, the major and best known cyclophilin [28], as well as to other less abundant cyclophilins, among which the steroid receptor-associated Cyp40 [3,4]. The intracellular localization of Cyp18 and Cyp40 was checked in Lcl3 cells, in parallel with that of PRm and FKBP59 as a control. The latter protein could not be responsible for the nuclear transfer of PRm, since its ligands FK506 and Rapamycin had no effect [17]. However FKBP59 is an hsp90-binding protein, like Cyp40, which contains tetratricopeptide (TPR) domains [8] and calmodulin binding sites [10]. It was therefore interesting to follow its intracellular localization in comparison with the other proteins.

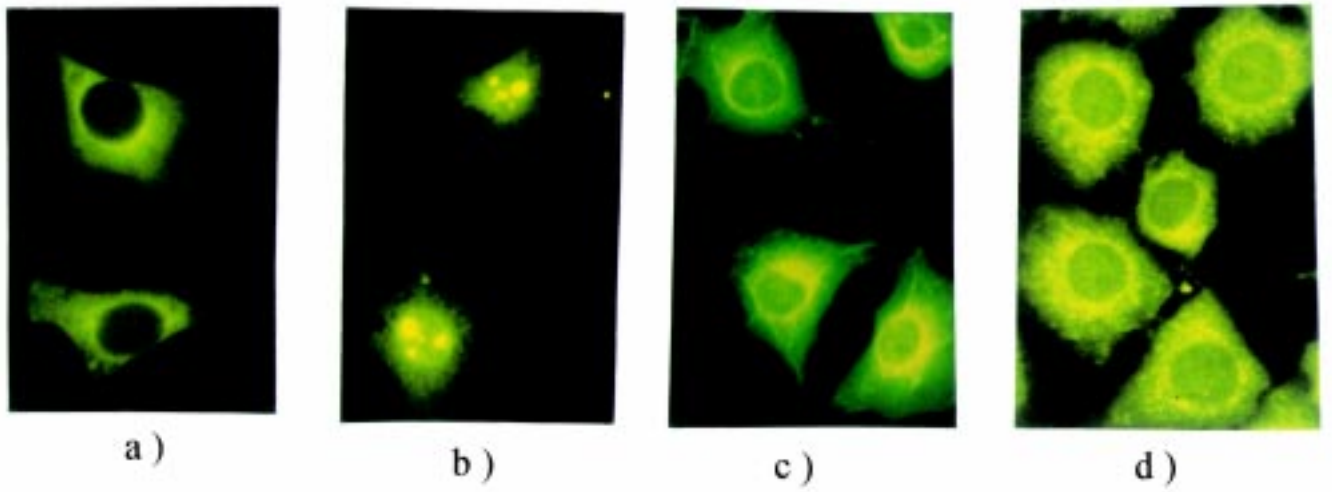

Fig. 4. Intracellular localization of PRm, Cyp40, Cyp18 and FKBP59. Lcl3 cells were cultured in absence of hormone as described in Fig. 1 and immunostained with anti-PR antibody Let 126 (a), or anti-Cyp40- (b), or anti-Cyp18- (c), or anti-FKBP59- (d) antibodies, as described in Material and methods (×1600).

In standard culture conditions, in absence of hormone, PRm was cytoplasmic (Fig. 4a), as were Cyp18 (Fig. 4c) and FKBP59 (Fig. 4d). In contrast, Cyp40 was nucleolar, as already observed by others [29], with some background staining in the nucleoplasm and the cytoplasm (Fig. 4b). In the presence of CsA, the nucleolar staining of Cyp40 increased, while NEM (0.8 μM) or W7 (25–40 μM) caused it to decrease (not shown), but Cyp40 remained in the nucleoli. In contrast, after an overnight culture (18 h) in the presence of actinomycin D (100 nM), the nucleolar localization of Cyp40 diminished considerably and at a higher actinomycin D concentration (1 μM) the staining in the nucleoli completely disappeared and only background staining was visible in the nucleoplasm (Fig. 5b).

When the localization of PRm was examined in parallel, in absence of hormone and in presence of actinomycin D, the receptor mutant remained cytoplasmic. After addition of progesterone, the PRm was transferred to the nucleus, despite the presence of actino D and was mostly localized at the nuclear membrane (not shown). PRm was never found in the nucleoli, whatever the experimental conditions.

After removal of actinomycin D from the culture medium and several medium changes during 3 days, Cyp40 co-localized again with the nucleoli.

As for FKBP59, in standard culture conditions it

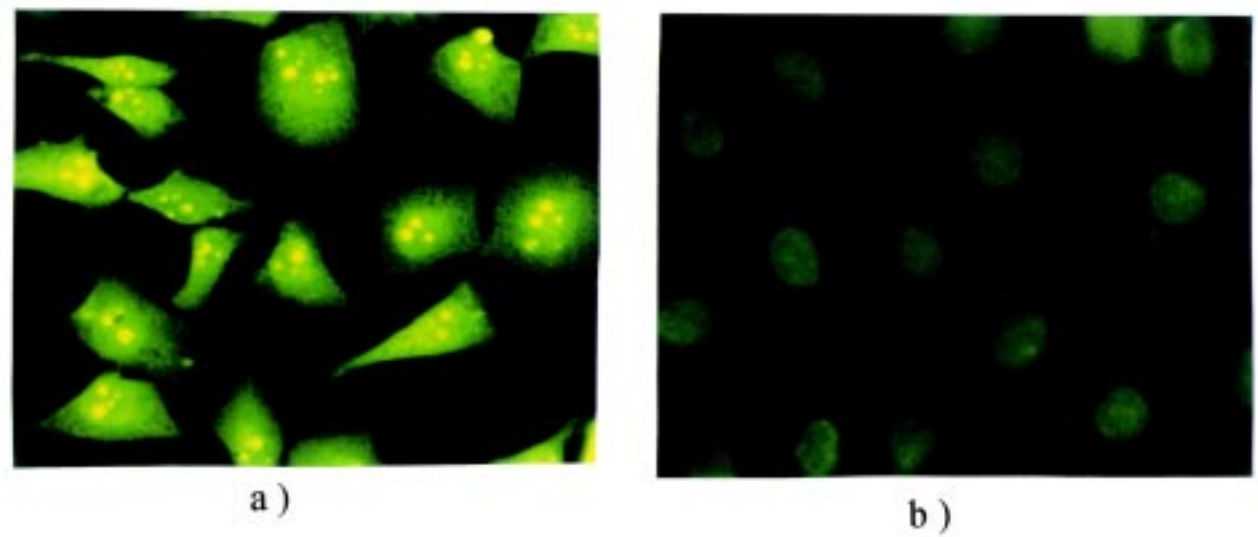

Fig. 5. Lcl3 cells were cultured in absence (a) or in presence (b) of actinomycin D (1 μM) for 18 h. Immunofluorescence staining of Cyp40 was then done with anti-Cyp40 antibody, as described in Material and Methods (×1600).

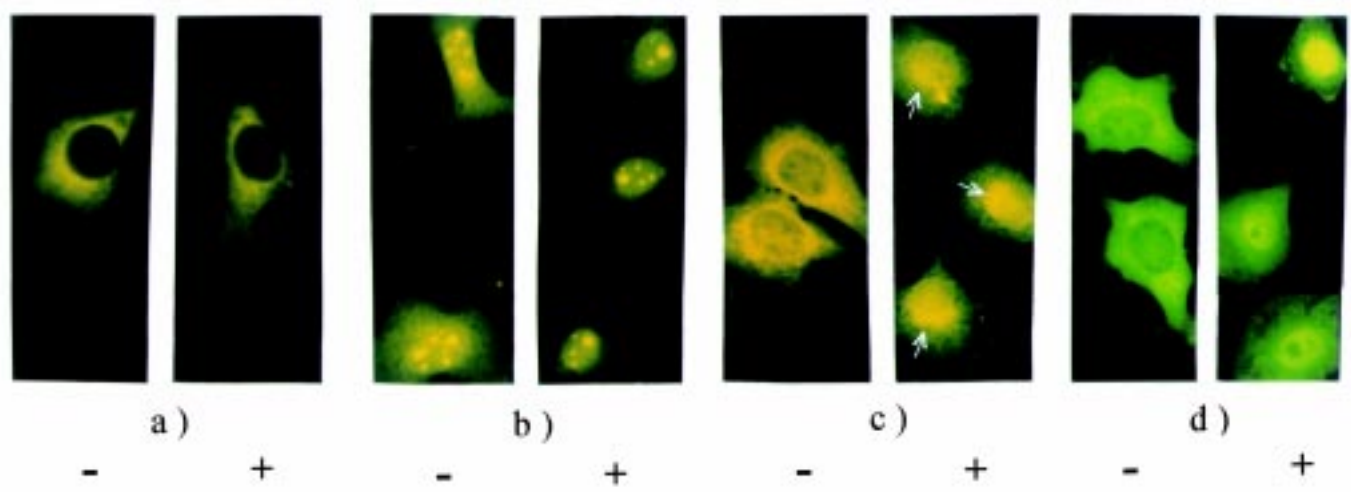

Fig. 6. Subcellular localization of PRm, Cyp40, FKBP59 and hsp86 in absence of serum (poor medium) or rich medium. Lcl3 cells were cultured for 3–4 days in DMEM, in absence of serum (minus sign). They were then shifted to DMEM containing 20% fetal calf, serum, EGF (20 ng/ml) and insulin (4.8 μg/ml) and kept in culture for 18 h (plus sign). Immunofluorescence of PRm (a) was done with the anti- PR antibody Let 126, of Cyp40 (b) was with the anti-Cyp40 antibody, of FKBP59 (c) was with anti-FKBP59 antibody and of hsp86 (d) with the anti-hsp86 antibody, as described in Material and methods. Arrows (c-right) indicate the Golgi area (×1600).

was mainly visualized in the cytoplasm (Fig. 4d), where it had a fibrous and punctuate appearance. It was uniformly distributed in presence of NEM plus CsA and moved to the perinuclear region in presence of actino D. It was never found in the nucleoli.

Our data therefore support previous observations that Cyp40 and FKBP59 form separate complexes with hsp90 [13,30–32] and are located in different compartments of the cell [29].

3.3. Influence of culture conditions on the localization of PRm, Cyp40, FKBP59 and hsp 86 and 84

Cell culture conditions were varied in an attempt to see how these proteins traffick in quiescent and dividing cells. To obtain quiescent cells, they were cultured for 3–4 days in absence of serum or growth factors and PRm, Cyp40, FKBP59 and hsp 86 (mouse hsp90a) were visualized by indirect immunofluorescence staining with the appropriate antibodies. In absence of either hormone or CsA, PRm remained in the cytoplasm at all times (Fig. 6a left). Cyp40 was mainly concentrated in the nucleoli, with some diffuse immunostaining all over the cell (Fig. 6b left). FKBP59 was mostly cytoplasmic, with denser staining around the nucleus (Fig. 6c left). Hsp86 was more cytoplasmic than nuclear (Fig. 6d left). After 3–4 days of starvation, the cells were transferred to a rich medium containing 20%FCS, insulin (4.8 μg/ml) and EGF (20 ng/ml) and the same proteins were observed 18 h later. PRm remained always in the cytoplasm (Fig. 6a right) and Cyp40 in the nucleoli, where the immunostaining became sharper. The diffuse fluorescence staining seen all over the cell disappeared and became localized in the nucleoplasm (Fig. 6b right). FKBP59 was concentrated in the perinuclear region, possibly the Golgi area and the cytoplasm appeared empty (Fig. 6c right). Growth-dependent expression of FKBP59, at the mRNA and protein level, has been demonstrated in a hamster fibroblastic cell line CCL39 [33].

Hsp86 was partly shifted to the nucleus in enriched medium, (Fig. 6d right), while on the contrary, hsp84 (mouse hsp90b) remained in the cytoplasm under all conditions observed.

3.4. Effect of heat shock

When Lcl3 cells were submitted to 1 h of heat shock at 43°C, in the usual medium without progesterone, PRm remained in the cytoplasm (Fig. 7 top). At the same time, Cyp 40 remained in the nucleoli, while all the low cytoplasmic staining disappeared (Fig. 7 middle). The nucleoli were enlarged and the components appeared to be segregating. As for FKBP59, after 1 h of heat shock, there was a spectacular concentration of fluorescence staining around the nucleus due to its perinuclear localization (Fig. 7 bottom).

4. Discussion

CsA and FK506 on one hand and Rapamycin on the other, cause immunosuppression by inhibiting distinct signalling pathways. Both CsA and FK506, in the presence of their cognate intracellular receptors cyclophilin A and FKBP12, block T-cell activation by inhibiting calcineurin phosphatase activity. Rapamycin

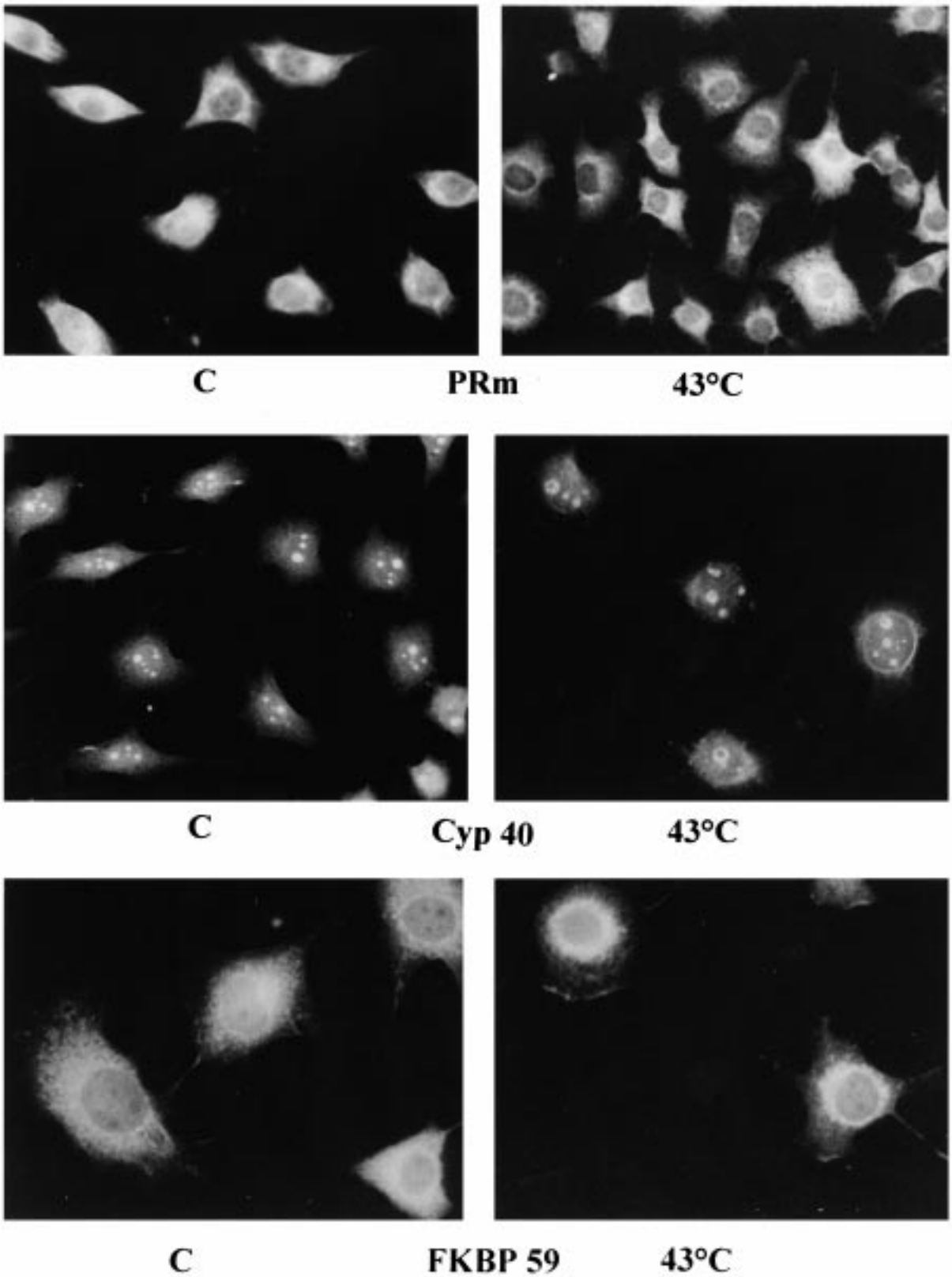

Fig. 7. Effect of heat-shock on the subcellular localization of PRm, Cyp40 and FKBP59. Lcl3 cells were cultured in DMEM containing 5% CS-CX, in absence of hormone at 37°C, as usual (control C), or for 1 h at 43°C. The cells were then fixed and immunostained as described in Material and methods, with the antibodies corresponding to PRm, Cyp40 or FKBP59 (×1600).

inhibits Il-2 dependent T-cell proliferation and interferes with the activation of certain kinases [34]. All 3 immunosuppressant drugs and a number of their analogs (immunosuppressant or not) also potentiate the steroid hormone induced expression of certain genes [11–14]. This could be an indirect effect due to inhibition by the drugs of membrane steroid transporters [35], such as the products of the mdr genes [36], resulting in nuclear accumulation of the hormone and increased gene expression.

The specificity of action of CsA on the re-localization of the mutant PR indicates that yet another mechanism is involved. A number of metabolic inhibitors were tested in our system and their effects were observed on either progesterone or CsA induced nuclear transfer of the receptor.

The 3 inhibitors of cell function that we examined, NEM, POBA and W7, have distinct effects on the nuclear transfer of PRm due to progesterone and that due to CsA. This observation supports the idea, already suggested by the different time-courses observed for the two effectors [17], that CsA and progesterone cause nuclear transfer of the receptor via different pathways. The inhibitory effect of NEM could indicate that CsA acts at the level of an NLS receptor, at the docking step of nuclear import [20]. In fact, RanBP2, a protein which is part of the nuclear pore complex, contains a cyclophilin-like domain which binds CsA [37] and could be responsible for the drug-dependent nuclear import of PRm. Alternatively, the calmodulin antagonist W7, also inhibits CsA induced PRm relocalization, suggesting that a Ca^{2+}-calmodulin dependent step is involved.

We confirm the separate localizations of Cyp40 and FKBP59 in our experiments and show that the immunophilins do not shuttle like PRm. PRm does not transfer to the nucleus and remains cytoplasmic in rich medium, while both FKBP59 and Cyp40 are depleted from the cytoplasm. Different localizations have already been reported for FKBP59 and PR, since the immunophilin has been shown to co-localize with microtubules [38,39], while no co-localization of PRm or WT-PR with cytoskeletal components could be demonstrated [40]. It has been suggested that an acidic region in the first hinge of FKBP59 could interact with the NLS1 of GR (510-RKTKKKIK-517), targeting it to the nucleus [41] and that FKBP59 and GR co-localize in the nucleus [38]. Our experiments with PRm do not confirm or infirm these observations, since the mutant receptor lacks its main constitutive NLS (638-RKFKK-642).

Although immunofluorescence staining does not provide quantitative data, the experiments described here suggest that even if these different proteins were isolated in complexes together, the associations they form in vivo must be transient, since they traffick independently.

Our results also suggest that the two large immunophilins Cyp40 and FKBP59 play different roles in the cell, in spite of the fact that they both have PPIase activity inhibited by their cognate immunosuppressive ligand and can associate with hsp90a and calmodulin. Due to its nucleolar localization, Cyp40 could be involved in ribosome formation or export, or the folding of ribosomal proteins, as suggested for yeast FPR3 [42]. FPR3 is a nucleolar FK506 and Rapamycin binding protein (also called NPI46) which has a C-terminal bearing PPIase activity and an N-terminal similar to nucleolin [42,43]. In *E. coli*, trigger factor, also found to have PPIase activity, is associated with the ribosomes [44,45]. The characteristic localization of these proteins could correspond to a conserved biological function. Indeed, the distribution of Cyp40 and its behaviour in the presence of actino D which resembles that of nucleolin [46], support the idea that Cyp40 plays a role in ribosomal function.

FKBP59, on the other hand, could play a role in the course of mitosis. It co-localizes with structural elements of the cell, such as microtubules in the cytoplasm and the mitotic apparatus in mitotic cells [38,39]. In Lcl3 cells switched from poor to enriched medium and which are beginning to divide, FKBP59 moves partly to the nucleus, partly focuses to a particular locus at the nuclear membrane which resembles the Golgi apparatus [39]. FKBP59, which is multifunctional and is both a PPIase and a calmodulin binding protein, could be implicated in the regulation of microtubule assembly and the establishment of mitotic structures, or it could act as a chaperone at the Golgi site. Indeed, there may be a need for PPIase activity during cell division since a PPIase essential for the regulation of mitosis has been described in human cells [47]. This protein, called Pin 1, is a member of the parvulin family and does not resemble FKBP59. However, immunophilins are being found more and more to be involved in fundamental steps of cell function. Indeed, Cyp40 has recently been shown to negatively regulate the DNA-binding activity of the transcription factor c-Myb, which regulates cell growth, differentiation and transcription in a number of cells [48].

Both Cyp40 and FKBP59 contain TPR repeats, which are found in proteins which can interact with other proteins and transport them across membranes, or which are involved in transcription repression or anaphase promoting complexes [49]. The localizations of the two large immunophilins are compatible with these functions and it is noteworthy that Cyclophilin18, which lacks the TPR- and calmodulin-binding regions of Cyp40, is cytoplasmic while Cyp40 is mostly nucleolar.

In conclusion, the work reported here shows that

the nuclear shift of PRm caused by progesterone and that caused by CsA are differently sensitive to several metabolic inhibitors and presumably obey different mechanisms. We also show that several proteins which have been identified in complexes with steroid hormone receptors indeed shuttle independently and we confirm the distinct localizations of Cyp40 and FKBP59. While Cyp40 is found in nucleoli and could play a role in ribosome function, FKBP59 appears to associate with microtubules and cytoskeletal structures and could be involved in cell division, since it responds to growth factors by re-localizing to the mitotic apparatus.

Acknowledgements

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