The Cytoskeleton and the Cellular Traffic of the Progesterone Receptor

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Abstract. Previous studies on glucocorticoid receptors have suggested the existence of interactions between the receptor and microtubule or actin networks. It was hypothesized that such interactions may contribute to the guidance of steroid hormone receptors towards the nucleus.

We used a permanent L cell line expressing the Δ638–642 progesterone receptor. This mutant has all the characteristics of the wild type receptor except that the deletion of five amino acids inactivates the constitutive karyophilic signal. Consequently, the receptor is cytoplasmic in the absence of hormone but is shifted into the nucleus when administration of hormone activates the second karyophilic signal. Optical microscopy and confocal laser microscopy were used in intact cells or in cells depleted of soluble elements by permeabilization with detergents. By immunofluorescence, the receptor was found to be mainly concentrated in the perinuclear area. A small fraction of progesterone receptor (PR) persisted in this region after Triton X100 treatment. These observations suggested that the receptor could interact with some insoluble constituent(s) of the cytoplasm. However, careful colocalization studies showed that this heterogenous distribution was not due to interactions with microtubule, microfilament, or intermediate filament networks.

Functional involvement of these networks in the translocation of the receptor into the nucleus was studied after cell treatment with cytoskeletal drugs such as nocodazole, demecolcine and cytochalasin. None of these compounds prevented or even delayed the hormone-dependent transfer of Δ638–642 PR into the nucleus. Similar conclusions were reached with the wild type receptor expressed by transfection in Cos-7 cells. PR was shifted from the nucleus into the cytoplasm by administration of energy-depleting drugs. After disruption of the various cytoskeletal networks normal nuclear reaccumulation of the receptor was observed when these drugs were removed.

The results thus suggest that the progesterone receptor is not colocalized with the main cytoskeletal components. Disruption of the cytoskeletal networks does not prevent its nuclear translocation. Thus, karyophilic signals and interactions with the nuclear pore seem to be the primary determinants of the cellular traffic of the progesterone receptor.

THE progesterone receptor (PR)[1] is an intracellular protein which upon binding to the hormone, interacts with enhancer-like elements involved in the regulation of gene transcription (3). From cell homogenization and fractionation studies, the cellular localization of PR and other steroid hormone receptors was initially thought to be cytoplasmic in the absence of hormone and nuclear in its presence (12, 17). However, enucleation experiments and immunohistochemical studies with mAbs against estrogen and progesterone receptors (ER and PR) have revealed that receptor molecules are exclusively located in the nucleus, whether they are hormone-free or -occupied (19, 29, 43). The same phenomenon was observed with most steroid receptors, including androgen receptors (15). By contrast, the glucocorticoid (11, 44) and mineralocorticoid (9, 25) receptors seem to be either cytoplasmic or both cytoplasmic and nuclear in the absence of hormone, and to accumulate in the nucleus in its presence (11).

The mechanisms of the nuclear localization of steroid receptors have begun to be analyzed in recent years (13, 31, 32). Karyophilic signals involved in the nuclear translocation of PR have been characterized using in vitro mutagenesis of receptor cDNA (13). One signal, constitutively active and present in the hinge region of the receptor, was similar to the karyophilic signal of SV-40 large T antigen (18, 22). After deletion of this karyophilic signal, a second nuclear localization signal (NLS) was found to reside in the second zinc finger region and to be activated by binding to the hormone (13). Recently, analysis of the subcellular distribution of

1. *Abbreviations used in this paper*: GR, glucocorticoid receptor; hsp, heat shock protein; NLS, nuclear localization signal; PR, progesterone receptor.

transfected receptor mutants in energy-depleted cells and cell fusion experiments have provided evidence for a nucleocytoplasmic shuttle of the PR (14). As a consequence, the "nuclear" location of wild-type PR reflects a dynamic equilibrium between active nuclear import and outward diffusion. However, the mechanisms of receptor translocation across the nuclear envelope and those of its traffic within the cytoplasm, remain to be elucidated.

Hypotheses have been made that steroid receptor displacement toward and within the nucleus involves a protein transfer system. It has recently been suggested that the transfer of glucocorticoid receptors (GR) into the nucleus may involve translocation along cytoskeletal scaffolds (33). Immunofluorescent studies have revealed an association of both GR and receptor-associated heat shock protein (hsp90) with microtubules (2, 36). Other biochemical (27) and immunofluorescent (20) studies have reported the association of GR with microfilaments, via its interaction with hsp 90. No similar study has been performed for other steroid receptors. Moreover, the question of whether such a colocalization of GR with cytoskeletal networks indicates an active participation of the cytoskeleton in guiding the receptor to the nucleus, has never been investigated.

The present study was initiated in order to determine: (*a*) if PR interacts with elements of the cytoskeleton before its transfer into the nucleus; and (*b*) if an intact cytoskeleton is required for receptor translocation. Taking into account that PR mainly resides in the nucleus, whether or not the hormone is present (29), we have used a PR mutant (Δ638–642) with five amino-acids deleted from part of the constitutive nuclear localization signal. This mutant accumulates in the cytoplasm in the absence of hormone and migrates into the nucleus in its presence. Since all the functional properties of this mutated receptor are preserved, except for nuclear accumulation in the absence of hormone (13, 14), we hoped to observe functional interactions with cytoplasmic components. We have, therefore, compared the distribution by immunofluorescence of this mutated form of PR with those of various elements of the cytoskeleton. Moreover, the hormone-dependent nuclear translocation of the mutant PR in cells treated by cytoskeleton disrupting drugs was investigated. Similar experiments were also performed with wild-type PR after its shift into the cytoplasm under energy-depleted conditions (14). Results show that the PR labeling pattern is not similar to that of any of the three cytoskeletal networks and that the translocation of PR into the nucleus is not affected by disruption of any of these networks.

Materials and Methods

Cell Culture

Mouse L cells (NCTC 929) (fibroblast-like cells, derived from subcutaneous areolar and adipose tissue) were used as the recipient cell for the study of PR expression. A cell line permanently expressing a Δ638–642 mutant of the PR was established as described elsewhere (14). This mutant can be shifted into the nucleus by administration of hormone. Progesterone or agonist R5020 (10^{-6} M) in culture medium was used for 4 h where indicated. These cells were subcultured as monolayers at weekly splits of 1:6 in a DME. DME was supplemented with 10% stripped FCS (treated by dextran-coated charcoal and dialyzed). Cell culture was performed in a moist chamber atmosphere of 5% CO_2 and 95% air at +37°C. The cells were plated on "Chamber slides" (Nunc Inc., Roskilde, Denmark), each well (18 × 18 mm) receiving 5×10^4 cells in a total volume of 1.5 ml.

Cos-7 cells were grown in DME medium supplemented with 10% FCS. Transfections were performed with PR expression vector using the calcium phosphate precipitate method, as previously described (13).

Antibodies

Two monoclonal mouse antirabbit progesterone receptor designated Mi60 (IgG_2a) (24) and Let 126 (IgG_1) (26) were used. They were diluted to a final concentration of 12 μg/ml IgG for immunofluorescence studies. The characteristics of these antibodies have been extensively described (29, 30). They recognize a region on PR separate from the ligand and DNA-binding domains (26).

The following antibodies directed against the cytoskeleton elements were used: (*a*) a monoclonal mouse antichicken brain β tubulin (dilution 1:300; Amersham International, Amersham, UK) and a monoclonal rat antitubulin (dilution 1:20; Biosys, Compiègne, France); two polyclonal rabbit anti-sea urchin tubulin or antibovine brain tubulin (dilution 1:10; Dakopatts, Glostrup, Denmark); (*b*) a polyclonal rabbit antichicken actin (dilution 1:10; Biomedical Technologies, Stoughton, MA); (*c*) A polyclonal goat antimouse fibroblast vimentin (dilution 1:40; Miles Laboratories Inc., Naperville, IL). Rhodamine phalloidin was also used in this study to label microfilaments.

The following secondary polyclonal antibodies were used: (*a*) a FITC-conjugated goat (or rabbit) anti-mouse IgG (dilution 1:60; Dakopatts); (*b*) FITC or rhodamine-conjugated goat anti-rabbit IgG (dilution 1:40 and 1:80, respectively; Nordic); (*c*) FITC or rhodamine-conjugated rabbit anti-goat IgG (dilutions 1:40 and 1:80, respectively; Nordic); (*d*) biotinylated goat anti-rat Ig and rhodamine-streptavidin (dilution 1:100; Amersham International).

Drug Treatments

Demecolcine (=N-deacetyl-N-methyl-colchicine, colcemid), nocodazole, cytochalasin B and D, phalloidin, and acrylamide were obtained from Sigma Chemical Co. (St. Louis, MO). The cell cultures were treated on the second to fourth day of subculture. The following substances were used at 37°C: 2–5 μM demecolcine (2–6 h), 10 μM nocodazole (1–2 h), 20 μM cytochalasin B (15 min–6 h) or 2 μg/ml cytochalasin D (1 h), 10 μm phalloidin (1–3 h), 5 mM acrylamide (4 h) or a mixture of 2 μM demecolcine for 6 h and 20 μM cytochalasin B for 1 h. The cells, still in the presence of the drugs, were further incubated with progesterone (10^{-6} M in DME; Sigma Chemical Co.) for 4 h at 37°C. Cells were then fixed, processed, and analyzed by indirect immunofluorescence. Control experiments consisted of cell cultures incubated in the absence of either progesterone or cytoskeleton-disrupting drugs. Reversibility of the effect was assessed by incubating cells with cytoskeleton-disrupting drugs, then removing the drug (2 h to overnight). The cells were lastly incubated in DME plus progesterone for 4 h. 10 mM sodium azide (Merck) was added for 4 h where indicated. This inhibitor was always used with DME supplemented with 6 mM 2-deoxyglucose instead of glucose, as previously described (14).

Fixation Procedures

The fixation procedures were used before indirect immunofluorescence microscopy.

Fixation of Unextracted Cells. The cells were rinsed twice with PBS, and treated for 15 min at 20°C as follows: (*a*) 4% (wt/vol) paraformaldehyde in PBS, pH 7.4; (*b*) picric-acid paraformaldehyde, pH 7.4; (*c*) periodate-lysine-paraformaldehyde, pH 7.4 (30). After fixation, the cells were washed twice with PBS and permeabilized with 0.2% Triton X100 (Sigma Chemical Co.) for 4 min; (*d*) 0.2% glutaraldehyde in PBS containing 0.2% Triton X100 for 10 min at 4°C followed by sodium borohydride (3 × 10 min); (*e*) methanol fixation. The cells were rinsed twice with PBS, and treated for 10 min with methanol at −20°C.

Paraformaldehyde Fixation of Detergent-extracted Cells. To remove soluble proteins the cells were exposed to several concentrations of Triton X100 (0.05–0.5% in PBS) or of saponine (7.5–75 × 10^{-3} mg/ml in PBS) before fixation. In other experiments, the cells were lysed with detergent according to the procedure described for the study of the cytoskeletal framework: the cells were first washed with buffer A (0.1 M Pipes, pH 6.9 containing 1 mM EGTA, 2.5 mM GTP, and 4% polyethylene glycol). They were then incubated for 4 min at room temperature with the same buffer containing 0.2% Triton (28). For the visualization of intermediate and microfilaments, the cells were first briefly rinsed with buffer B (10 mM Hepes, pH 6.8, containing 100 mM KCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$, 200 mM sucrose). They were then treated for 2 min with buffer B containing

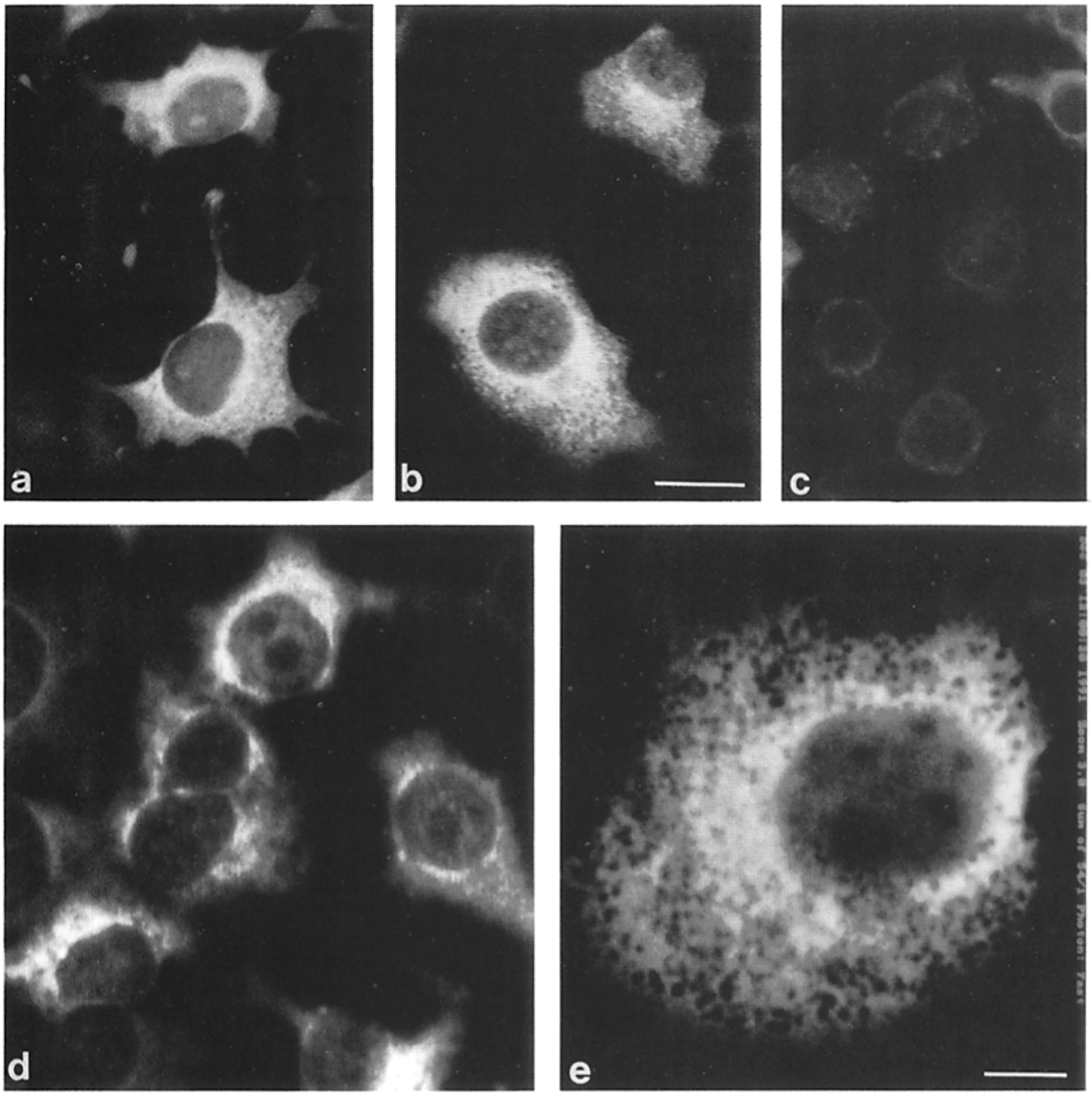

Figure 1. Distribution of Δ638-642 progesterone receptor in the cytoplasm of L cells. Interphase mouse fibroblast cells were stained for PR using immunofluorescence and conventional microscopy (*a–c*) or confocal laser microscopy (*d* and *e*). Note the strong fluorescence in regions near the nucleus and the additional low, diffuse fluorescence extending toward the cell periphery. Confocal microscopy at high magnification shows the appearance of a perinuclear network with outward extensions (*e*). In *d*, the selected plane of focus shows either a large perinuclear accumulation of fluorescence or a thin perinuclear ring, depending on the cell. Some regions of the cytoplasm are nearly devoid of labeling. In *c*, PR staining is observed by conventional microscopy in Triton-extracted cells (0.05% for 2 min). Note the clustering of fluorescence around the nucleus and within the cytoplasm after removal of soluble proteins. Similar patches of fluorescence were observed using confocal microscopy (not shown). Bars: (*a–d*) 20 μm; (*e*) 10 μm.

0.5% Triton and 0.15 mg/ml PMSF (10). The cells were then rinsed in PBS and fixed in 4% paraformaldehyde for 15 min at room temperature. In addition to indirect immunofluorescence, Triton-permeabilized cells were also stained with the immunoperoxidase method (29).

Indirect Immunofluorescence Staining

Fixed cells were rinsed three times in PBS and preincubated in goat serum diluted 1:40 in PBS. The cells were incubated with mouse Mi60 anti-PR antibody (12 μg/ml) for 12 h at 4°C, and rinsed three times over a period of 20 min. The cells were then incubated for 60 min with FITC-conjugated secondary antibody (goat anti-mouse IgG), washed three times with PBS, and mounted in PBS/glycerol. Controls were carried out by an identical procedure, except that the primary antibody was replaced by a nonimmune serum or a PR unrelated mAb.

Double immunofluorescence studies were performed on aldehyde (4% paraformaldehyde) or methanol-fixed cells. For receptor and actin staining, the cells were incubated overnight with a mixture of mouse anti-PR and rabbit antiactin (or rhodamine-conjugated phalloidin) for 12 h at 4°C. Cells were then incubated with a mixture of FITC-conjugated goat anti-mouse IgG and rhodamine-conjugated goat anti-rabbit IgG for 1 h at room temperature. For the simultaneous detection of the receptor and vimentin, the cells were successively incubated with mouse anti-PR, FITC-conjugated rabbit anti-mouse IgG, goat antivimentin and rhodamine-conjugated rabbit anti-goat IgG. For the simultaneous detection of receptor and tubulin, the cells were successively incubated under two different conditions, depending on which antigen (receptor or tubulin) was first revealed: (*a*) mouse anti-PR, FITC-conjugated rabbit anti-mouse IgG, then rat antitubulin, biotinylated goat anti-rat IgG and rhodamine-streptavidin; or (*b*) rat antitubulin, biotinylated goat anti-rat IgG, rhodamine-streptavidin, then mouse anti-PR and FITC-conjugated rabbit anti-mouse IgG. In these conditions, no cross reactivity or spillover was observed after omitting the antireceptor or any

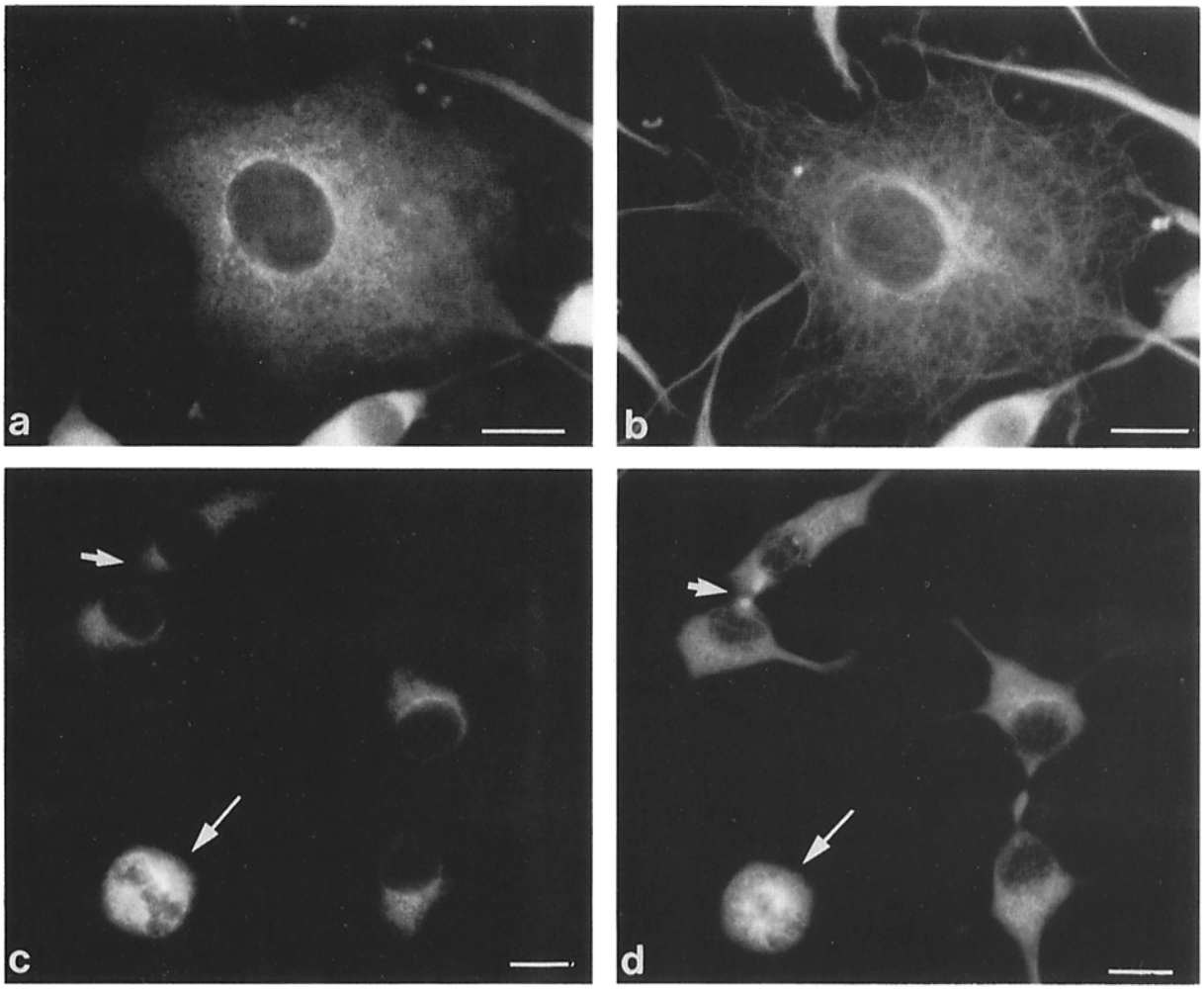

Figure 2. Double-labeling immunofluorescence with anti-PR and antitubulin antibodies. Conventional immunofluorescent microscopy. Interphase cells expressing Δ638–642 PR were double stained with anti-PR antibody (*a*) and antitubulin antibody (*b*). PR does not appear to be colocalized along cytoplasmic microtubules. (*c* and *d*) Mitotic cells were double stained for PR (*c*) and tubulin (*d*). The mitotic apparatus (*thin arrow*) and midbodies (*thick arrow*) well stained with antitubulin antibody are devoid of PR staining. Methanol fixation had to be used for these experiments, in order to observe both PR and microtubules staining. Bars, 25 μm.

of the antitubulin, antiactin, or antivimentin antibodies. Observations were made on a Leitz orthoplan microscope equipped with epifluorescence. Color and black and white photographs were taken with Fuji 800 ASA or Kodak T Max 400 using a 40× objective.

Confocal Microscopy

Confocal laser scanning microscopy was performed using a BioRad MRC-600 (BioRad Laboratories, Palo Alto, CA), mounted on an Optiphot II Nikon microscope equipped with a 60× objective (plan apochromatic NA 1.4). An Argon ion laser adjusted at 488 nm wave length was used for the analysis of fluorescein, and an Helium-Neon ion laser adjusted at 543 nm was used for the analysis of rhodamine. The emitted light was separated by a dichroic mirror (DR565); a 540DF30 band pass filter was placed in front of the photomultiplier collecting the fluorescein emission, and a EF600LP long pass filter was placed in front of the photomultiplier collecting the rhodamine emission. The adjustment of the confocal system allows a field depth of ~0.5 μm. Double fluorescence images were acquired in two passes, fluorescein first, rhodamine second. The emitted signal was digitalized by Kalman filter collection, and each section was scanned eight times. Color and black and white pictures from screen images were taken on Fujichrome 100 and Kodak TMax 100, respectively.

Results

Nonrandom Cytoplasmic Distribution of Δ638–642 Progesterone Receptor in Transfected Cells

A PR mutant (Δ638–642) which fails to accumulate into the nucleus in the absence of hormone has been used to investigate the cytoplasmic distribution of PR. This mutant is characterized by the lack of five amino acids within the constitutive karyophilic NLS. All other functional properties of this mutated form of PR were preserved (14).

Indirect immunofluorescence, with both conventional light microscopy and confocal laser microscopy, and various fixation conditions (see Materials and Methods) were used. In the absence of hormone, the Δ638–642 receptor expressed in L cells was always found in the cytoplasm, as shown in Fig. 1. Using conventional light microscopy (Fig. 1, *a* and *b*), immunofluorescence was very bright and diffuse in a large perinuclear area. In the lamellipodes the intensity of fluorescence was faint, whereas staining was rather intense

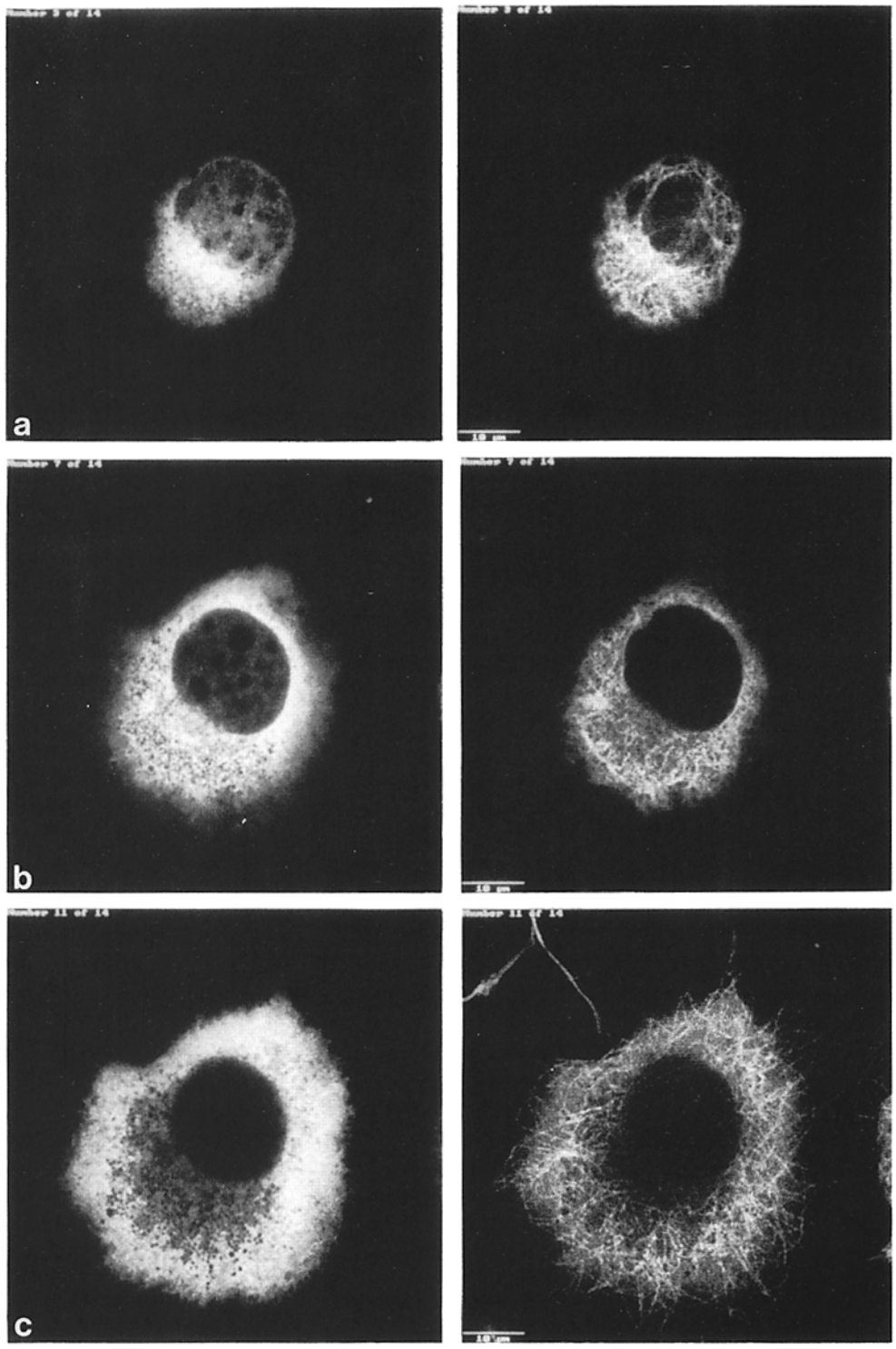

Figure 3. Double-labeling immunofluorescence with anti-PR (*left*) and antitubulin (*right*) antibodies. Confocal laser scanning microscopy was used. Serial optical sections were obtained at 1-μm increments. The figure shows a series of three optical 0.5-μm thick transversal sections at 4-μm intervals through the whole cell. The images were scanned in the sequence *a–c*, i.e., starting from the top of the cell (*a*) and moving toward the bottom (*c*). Different distributions of PR and tubulin are apparent in each section.

in ruffles. Confocal laser microscopy producing 0.5-μm thick optical sections, showed that the perinuclear accumulation pattern was not due to the increased depth of cytoplasm around the nucleus (Fig. 1 *d*). Confocal laser microscopy also showed a fluorescence pattern having the appearance of a lattice and forming a discontinuous perinuclear ring (Fig. 1 *e*).

The location of the Δ638–642 receptor was also studied after removing soluble proteins by permeabilization of the cells before paraformaldehyde fixation. Cells were first incubated in conditions that are usually used to analyze Triton-insoluble structures of the cytoskeleton (0.2–0.5% Triton X100 for 2 min). In these conditions, nearly all PR molecules were extracted indicating that most of the receptor was not tightly bound to insoluble cell structures. However, when a lower concentration of Triton X100 (0.05% for 30 s–2 min) was used, the perinuclear ring remained visible by either immunofluorescence or the indirect peroxidase method (Fig. 1

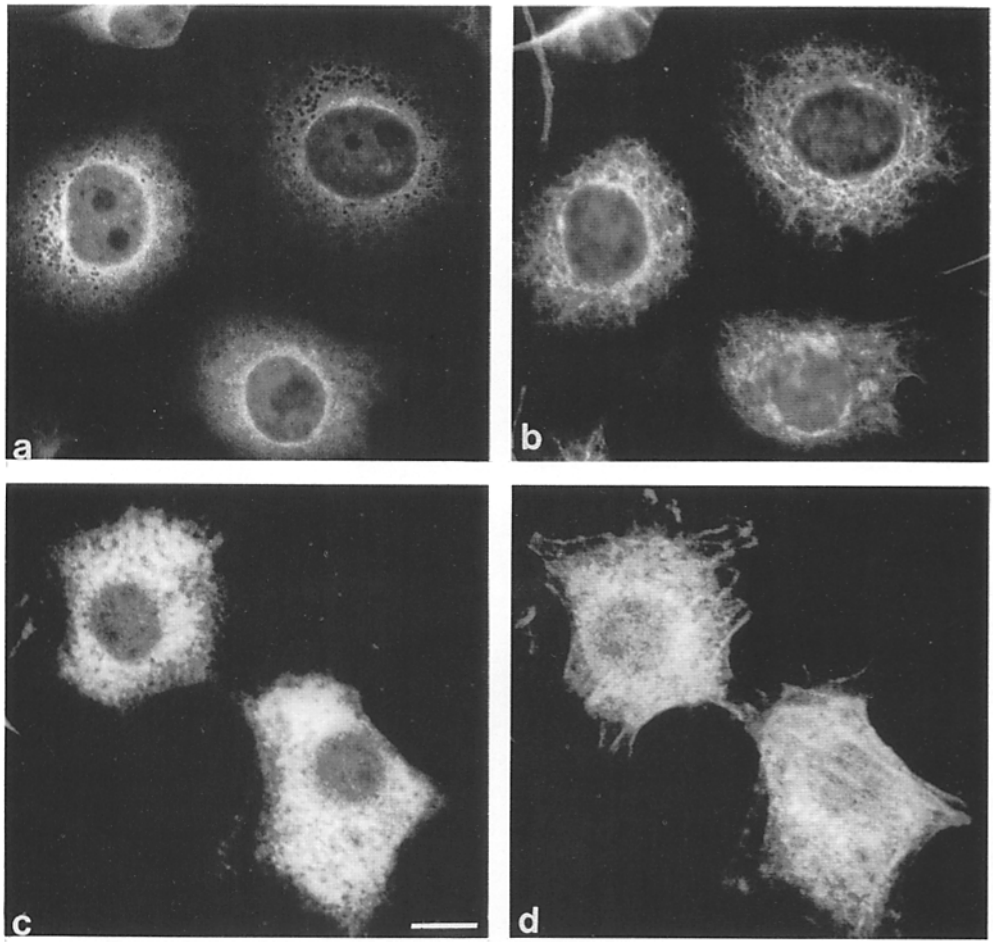

Figure 4. Double-labeling immunofluorescence with anti-PR (*left*) and antivimentin or antiactin (*right*) antibodies. (*a* and *b*) The same cells were double stained with anti-PR antibody (*a*) and antivimentin (*b*) antibodies using conventional light microscopy. (*c* and *d*) The same cells were double stained with anti-PR (*c*) and antiactin (*d*) antibodies using confocal laser microscopy. Bars, 25 μm.

c). Also, after this treatment, confocal laser microscopy detected patches of fluorescence within the cytoplasm (not shown).

Comparison between the Distribution of PR and of Cytoskeletal Elements

The pattern of distribution of the Δ638–642 receptor described above suggested the possibility that PR molecules were interacting with an organized network. The distribution of PR was thus compared to that of the three main constituents of the cytoskeleton (microtubules, microfilaments, and intermediate filaments).

L cells with a flat morphology (i.e., well-spread cells) were preferentially examined because they provide a large area of cytoplasm that is optimal for the visualization of cytoskeleton elements. Antitubulin and antivimentin antibodies stained wavy fibers extending throughout the cytoplasm. Antitubulin labeling (Fig. 2 *b*) radiated from a focal point near the nucleus (centrosome) towards the cell periphery through all regions of the cytoplasm with no special aggregation. Antivimentin staining (see Fig. 4 *b*) showed marked perinuclear accumulation with filaments extending both to the nucleus and the cell periphery. Actin was localized to thin filaments (microfilaments) and to the periphery of the cell at the level of submembranous domains and ruffles (see Fig. 7 *e*). The cells were also characterized by a diffuse perinuclear stain for actin and by the presence of few stress fibers (as shown with antiactin antibodies and with phalloidin). Therefore, the pattern of PR immunostaining did not resemble the filamentous staining pattern observed for microtubules or intermediate filaments. As well, actin distribution in the cell periphery was clearly different from that of PR. Around the nucleus, the very dense immunolabeling made comparison difficult; however, the width of the perinuclear accumulation of PR seemed greater than that observed for actin.

To compare more precisely the distribution of PR with that of the cytoskeletal elements, double-labeling experiments were also performed using both conventional and confocal microscopy. The same cells were treated with antibodies against PR on one hand and tubulin, actin, or vimentin on the other hand. Varying experimental conditions were tested, such as the use of methanol or aldehyde fixation. In some experiments, detergents were also used before fixation to remove soluble proteins. The staining patterns of PR and of the various cytoskeletal elements were different using both conventional and confocal laser microscopy (Figs. 2–4). This was especially visible in the cell periphery (i.e., the thinner part of the cells) where microtubules, intermediate filaments, or microfilaments were not decorated with PR (Figs. 2, *a* and *b* and 4). The centrosomal region did not show accumulation of PR. In contrast, a strong fluorescence was observed in the perinuclear area with both anti-PR antibodies and anticytoskeletal antibodies. This led to a lack of resolution of cytoskeletal elements which did not allow for the determination of whether fluorescein-decorated PR was associated with some cytoskeleton elements. With confocal

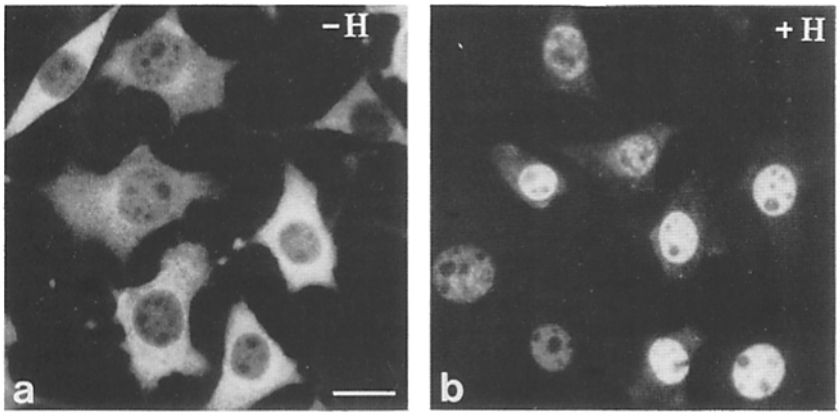

Figure 5. Hormone-induced translocation of Δ638–642 progesterone receptor. Immunofluorescent receptor is cytoplasmic in the absence of hormone (*a*), but is shifted into the nucleus under progesterone administration (10^{-6} M, 4 h) (*b*). Bar, 25 μm.

microscopy (Figs. 3 and 4, *c* and *d*), the different networks were more clearly contrasted in the perinuclear region and appeared non coincident with PR staining. At high magnification, most of the PR fluorescence appeared as irregular large patches located between microtubules, intermediate filaments and actin microfilaments (not shown). In permeabilized cells, the persistent cytoplasmic fluorescent PR patches and the discontinuous PR perinuclear ring did not decorate cytoskeleton scaffolds (not shown).

Mitotic fibroblasts were also examined. The staining for PR was stronger in mitotic cells when compared to the surrounding interphase cells. PR fluorescence was homogeneously distributed throughout the cells. However, PR fluorescence was not present in the chromosome region or on structures such as the mitotic spindle or midbodies which are known to contain tightly packed microtubules (Fig. 2, *c* and *d*). This was confirmed by confocal laser microscopy in different sections of mitotic cells at all stages of mitosis (not shown).

In conclusion, results obtained in the various experiments indicated a nonrandom distribution of Δ638–642 PR in the cytoplasm, suggesting its interaction with some cytoplasmic constituent(s). However, the majority of PR was soluble or easily extractable and its distribution was not similar to that of microtubules, microfilaments, or intermediate filaments. These observations could not exclude the possibility that a small fraction of PR molecules interacted transiently with the cytoskeleton, being in equilibrium with a large pool of soluble PR. Such an interaction could have preceded the interaction of the receptor with components of the nuclear pore complex in the process of translocation into the nucleus. If this was the case, nuclear accumulation of PR should be blocked by agents known to disrupt the cytoskeleton.

Nuclear Accumulation of Δ638–642 Progesterone Receptor in the Presence of Cytoskeleton-disrupting Drugs

L cells expressing the Δ638–642 PR were incubated with progesterone 10^{-6} M for time intervals varying between 30 min and 6 h. A 4-h time period was necessary to obtain maximal nuclear translocation of PR from the cytoplasm to the nucleus (Fig. 5). We then asked whether the receptor would localize in the nucleus of cells containing a disrupted cytoskeleton.

Nocodazole, demecolcine (colcemid), and cytochalasin B were used to disrupt the microtubular, intermediate filament, and microfilament networks. These drugs have well-documented effects on the three major cellular cytoskeleton networks (7, 16, 23, 39, 42). However, it was necessary to determine optimal conditions for the disruption of these networks in mouse L fibroblasts (not shown).

After 1 h of treatment with nocodazole (10 μM), the overall cell shape became more rounded. Staining for tubulin showed that the microtubular network had disappeared (compare Figs. 6 *b* and 2 *b*). A small coiling of vimentin filaments was still observed around the nucleus. Staining for PR showed that the Δ638–642 PR was cytoplasmic as in control cells incubated in the absence of nocodazole (Fig. 6 *a*). After addition of hormone, the time course of nuclear translocation was not changed in the nocodazole-treated cells (Fig. 6 *c*). All morphological changes induced by the treatment with nocodazole were reversible after changing the cells to a normal medium for 2 h (Fig. 6 *d*). The nuclear staining of PR was unchanged in these conditions.

On treatment with demecolcine (2 μM for 6 h), the cells were rounded and the fibrillar aspect of microtubules was no longer observed, giving a diffuse pattern of staining with antitubulin antibodies. The vimentin containing filaments appeared clumped into thicker bundles and took up coiled forms. An intense ring of staining was present around the nucleus (Fig. 6 *f*). Pretreatment with demecolcine (2–10 h) did not alter the penetration of PR into the nucleus in the presence of progesterone (Fig. 6 *e*).

Treatment of the fibroblasts with cytochalasin B at 20 μM induced in 30 min a typical arborization pattern, exhibiting total disruption of the actin-containing microfilaments and bulging of the nucleus. The same aspect was observed after 6 h of drug treatment (Fig. 7 *c*). The filamentous network of microtubules and intermediate filaments was less marked but immunostaining with antitubulin and antivimentin antibodies was observed in the center of the cells and extending into the various projections. After addition of progesterone, the cytochalasin B–treated cells (30 min to 1 h) exhibited also a nuclear localization of Δ638–642 PR, indicating that cytochalasin B did not prevent the nuclear accumulation of the receptor (Fig. 7, *a* and *b*). Similar results were obtained with cytochalasin D (not shown). This translocation of PR from the cytoplasm into the nucleus was also observed in cells exposed for long periods of time to cytochalasin (up to 18 h). The removal of the drug and the subsequent incubation of the cells in a normal medium for 3 h resulted in the maintenance of the nuclear PR staining and a gradual restoration of the typical pattern of staining observed for microfilaments (Fig. 7, *d* and *e*). Phalloidin, which is known to stabilize ac-

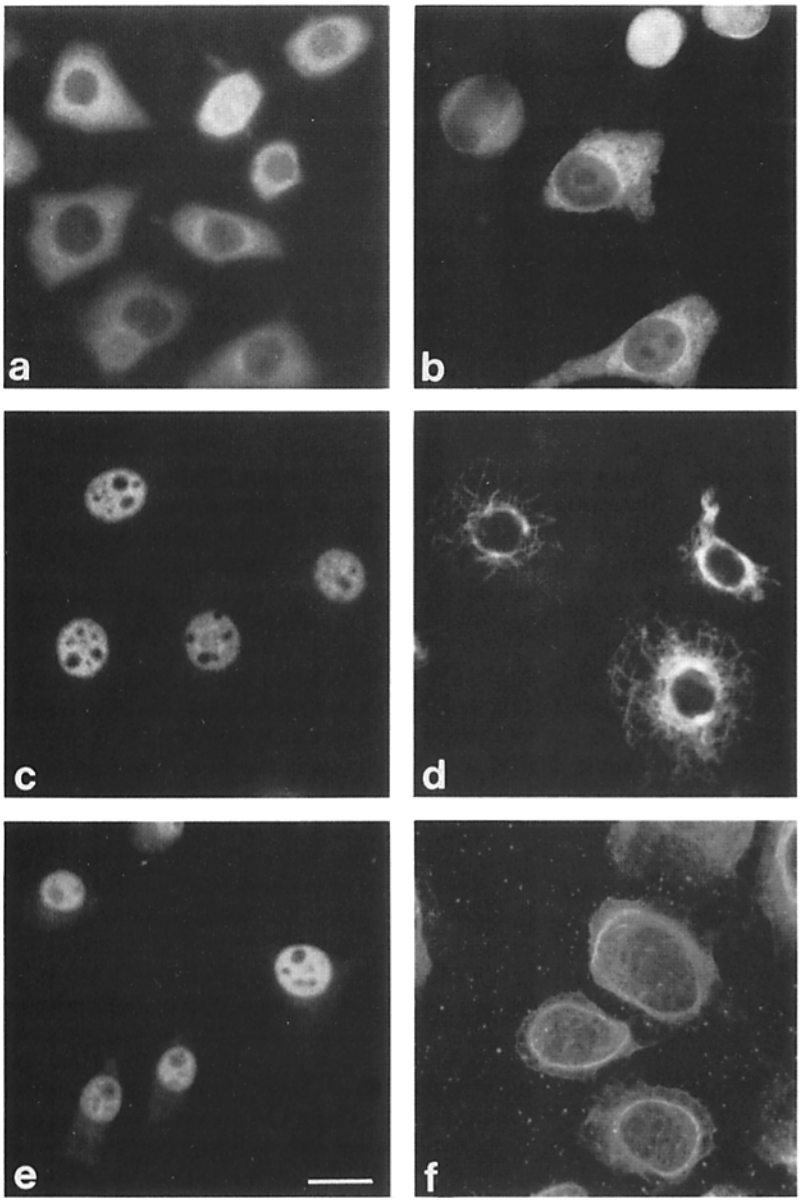

Figure 6. Hormone-induced translocation of Δ638–642 progesterone receptor in the presence of drugs disrupting microtubule or intermediate filament networks. L cells were treated with nocodazole (10 μM, 1 h) or demecolcine (2 μM, 2 h), then incubated for 4 h either with a mixture of progesterone (10^{-6} M) and nocodazole or demecolcine, or with the drug alone (nocodazole or demecolcine). A control experiment was performed in which the cells were returned for 2 h to a medium without nocodazole or demecolcine. (*a* and *c*) Immunofluorescent PR in nocodazole-treated cells either in the absence (*a*) or in the presence (*c*) of hormone. (*b*) Immunofluorescent tubulin in nocodazole-treated cells. (*d*) Immunofluorescent tubulin in cells previously treated by nocodazole and subsequently cultured for 2 h in the absence of the drug. (*e*) Immunofluorescent PR in demecolcine-treated cells in the presence of hormone. (*f*) Immunofluorescent vimentin in demecolcine-treated cells. Bar, 25 μm.

tin microfilaments (6), also did not prevent nuclear translocation of Δ638–642 PR receptor (Fig. 7, *f* and *g*). Treatment with a mixture of both demecolcine and cytochalasin B (2 μM for 6 h and 20 μM for the last hour, respectively) resulted in the disruption of both microtubules and microfilaments and in the disorganization of intermediate filaments. Even the cells lacking all three cytoskeleton networks could efficiently accumulate PR in their nuclei in the presence of hormone (Fig. 8).

L cells were also treated with acrylamide (5 mM, 4 h) (8) to disorganize the intermediate filament network resulting in the formation of coiled bundles and of a perinuclear ring. Again, the Δ638–642 receptor was normally translocated from the cytoplasm into the nucleus of the cells after progesterone treatment (not shown).

Nuclear Accumulation of Wild-type Progesterone Receptor in the Presence of Cytoskeleton-disrupting Drugs

Because it might have been argued that the translocation of Δ638–642 mutant receptor represents a special case, we also examined whether the wild type PR could find its way into the nucleus of cells containing a disrupted cytoskeleton. Cells were transfected with an expression vector encoding the wild type PR. The receptor was nuclear but could be transferred into the cytoplasm using sodium azide, an inhibitor of energy synthesis, as previously described (14). The cells were then incubated in the presence (or absence) of cytochalasin or nocodazole-containing medium, and then returned to azide-free medium. As shown in Fig. 9, the wild type PR which was mainly observed in the cytoplasm in energy-depleted cells, was translocated to the nucleus in cytochalasin or nocodazole-treated cells, as in control cells.

Discussion

The progesterone receptor has been shown to reside in the nucleus irrespective of the presence or absence of hormone (29). Recent studies have shown that this cellular distribution actually reflects a dynamic situation: the receptor con-

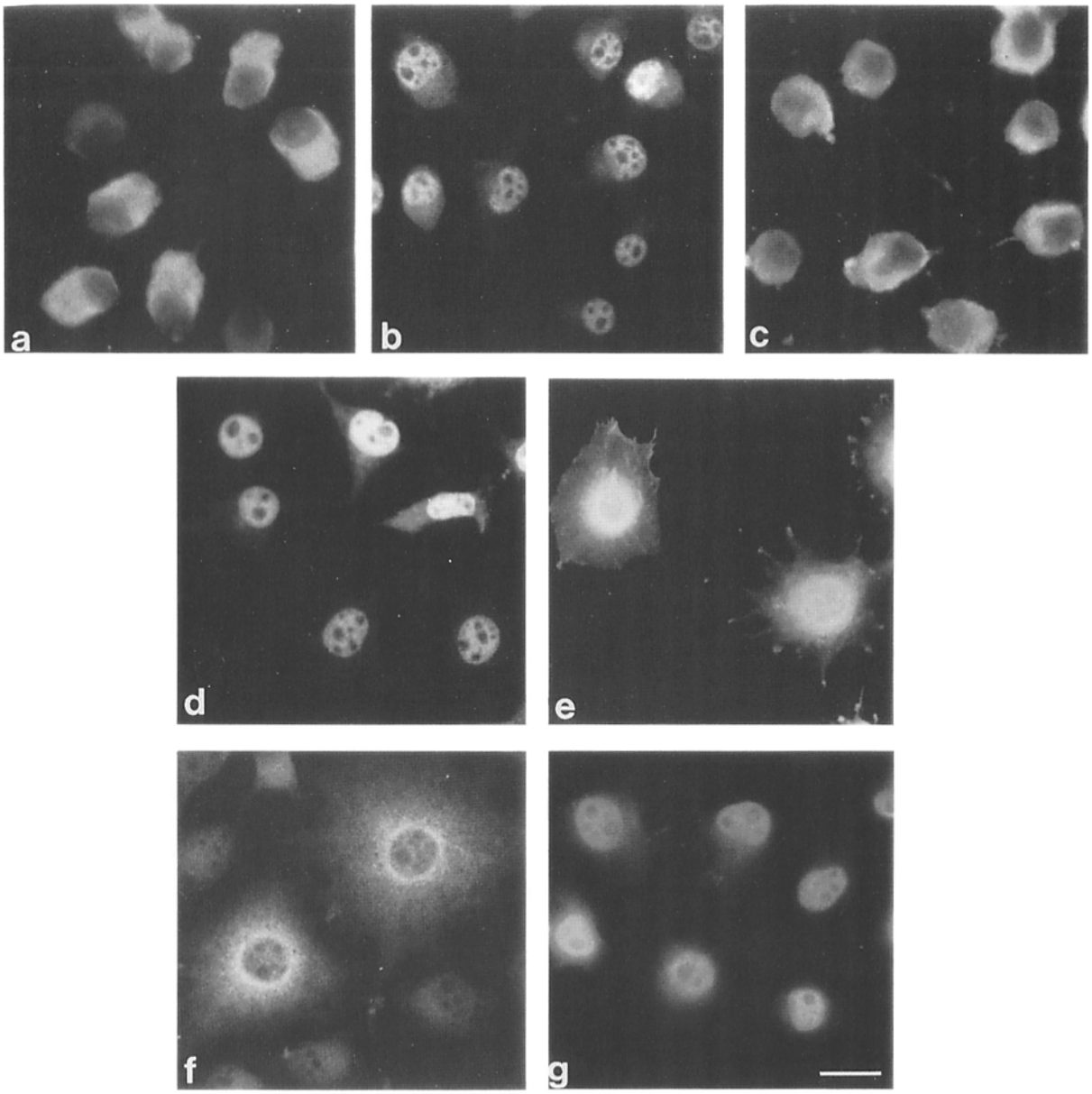

Figure 7. Hormone-induced translocation of the Δ638–642 progesterone receptor in the presence of drugs which disrupt or stabilize the microfilament network. L cells were treated with cytochalasin (20 μM for 1 h) or phalloidin (10 μM for 1 h), then incubated for 4 h either with a mixture of progesterone (10^{-6} M) and the drug (cytochalasin or phalloidin), or with the drug alone. A control experiment was performed in which the cells were returned for 2 h to a medium without cytochalasin or phalloidin. (*a* and *b*) Immunofluorescent PR in cytochalasin-treated cells, either in the absence (*a*) or in the presence (*b*) of hormone. (*c*) Actin pattern in cytochalasin-treated cells. (*d*) Immunofluorescent PR in cells previously treated by cytochalasin and subsequently cultured for 2 h in the absence of the drug. (*e*) Actin staining in the same conditions as in *d*. Note the gradual restoration of the actin network. (*f* and *g*) Immunofluorescent PR in phalloidin-treated cells, either in the absence (*f*) or in the presence (*g*) of hormone. Bar, 25 μm.

stantly diffusing out of the nucleus and being actively reimported into the nucleus (14). However, the cellular route for the translocation of the progesterone receptor from the cytoplasm into the nucleus is not understood, as it is the case for all the other steroid hormone receptors. In the case of the GR, it has been hypothesized that the receptor was interacting with elements of the cytoskeleton and that such interactions were guiding its movements in the cell (2, 27, 33). We have tested this hypothesis using the PR and studying its translocation into the nucleus after disruption of the various cytoskeletal networks.

The studies were performed with L cells permanently expressing a cytoplasmic form of the receptor (mutant Δ638–642) lacking the constitutive NLS (13). This receptor form can be shifted into the nucleus by administration of hormone through the unmasking of a second karyophilic signal located in the region of the second zinc finger. To investigate this translocation in cells containing a disrupted cytoskeleton, we used nocodazole, demecolcine, and cytochalasin, which have well documented deleterious effects on the cytoskeleton networks (7, 16, 23, 39, 42): incubation of cells with nocodazole caused a selective disruption of the microtubules; long treatment (>6 h) with demecolcine resulted in the disappearance of the microtubules and the collapse of the intermediate

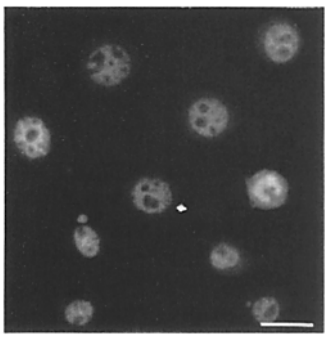

Figure 8. Hormone-induced translocation of the Δ638–642 progesterone receptor in the presence of drugs which disrupt all three cytoskeletal networks. L cells were treated with a mixture of demecolcine (2 μM for 6 h) and cytochalasin B (1 h), then incubated for 4 h either with a mixture of progesterone, demecolcine, and cytochalasin, or with the mixture of cytochalasin and demecolcine. Nuclear PR immunostaining is shown in cytochalasin-demecolcine-treated cells that have been incubated with progesterone. Bar, 25 μm.

filaments. Cytochalasin B treatment resulted in the disruption of the actin-containing microfilaments. Moreover, the combination of demecolcine and cytochalasin resulted in the disruption of all three of the cytoskeleton networks. Whatever the drug used, the penetration of PR into the nucleus after incubation with progesterone was not prevented by these drugs. Control experiments indicated that the drug treatments by themselves did not result in the nuclear translocation of the Δ638–642 receptor. In addition, these effects on the cytoskeleton were reversible since the removal of the drugs and subsequent incubation of the cells in normal culture medium resulted in a gradual restoration of the three cytoskeleton networks, as seen by indirect immunofluorescence.

The possibility that the distribution of Δ638–642 receptor could be different from that of the wild type PR in cytoskeleton-disrupted cells, was also investigated by blockage of the energy-dependent nuclear accumulation of wild type PR by sodium azide which induces an efflux of PR from the nucleus (14). PR reaccumulated within the nucleus when sodium azide was removed, even in the presence of cytochalasin and nocodazole. This indicates that translocation of the wild type receptor from the cytoplasm into the nucleus is not dependent on the integrity of the cytoskeleton.

We have obtained additional evidence against involvement of the cytoskeleton in the cytoplasmic traffic of the receptor using colocalization experiments performed at the light microscope level. Immunofluorescence microscopy showed distinct distributions of the Δ638–642 PR and of microtubule, intermediate filament, and microfilament networks. This result was obtained in various fixation conditions, using both conventional light microscopy and confocal laser mi-

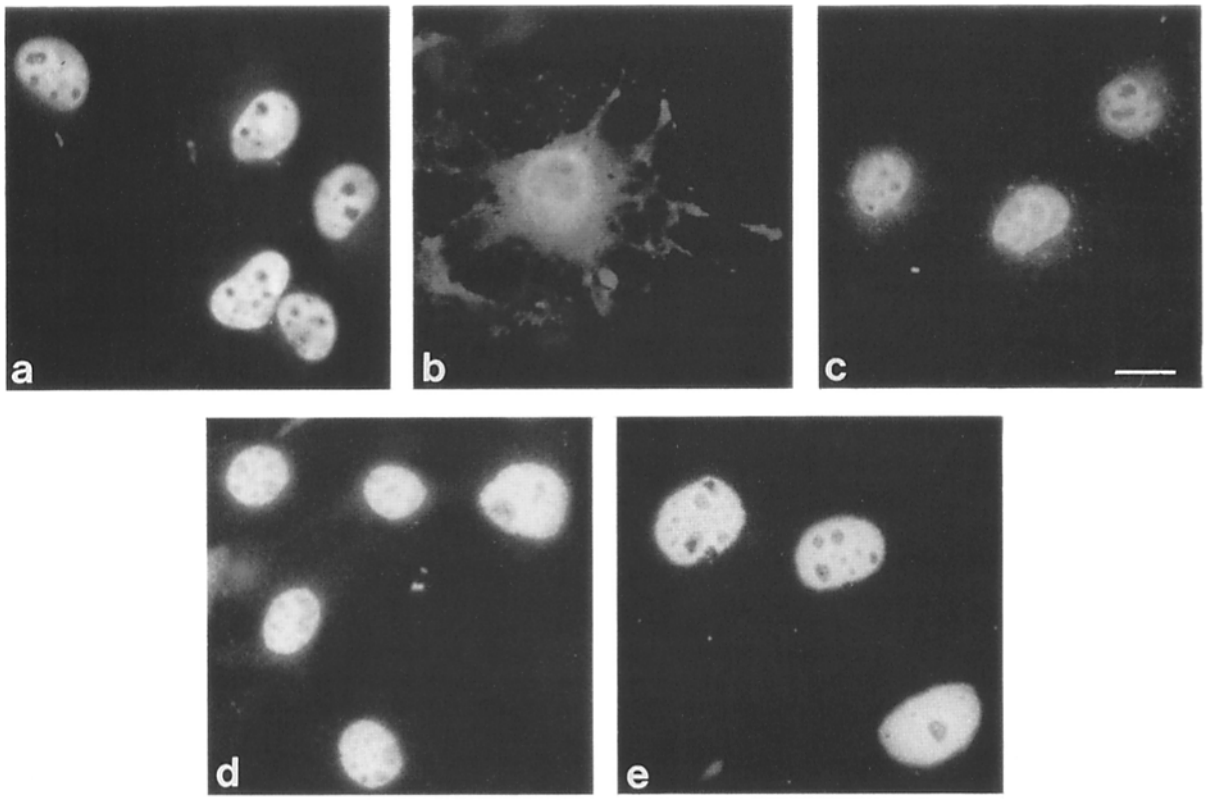

Figure 9. Nuclear accumulation of wild-type PR in the presence of cytoskeleton-disrupting drugs. Cos-7 cells were transfected with wild-type PR as previously described (17). The cells were treated with sodium azide (10 μM) for 3 h. The incubation was continued for 1 h in the presence of sodium azide and in the presence or absence of cytoskeleton-disrupting drugs (nocodazole [20 μM] or cytochalasin [10 μM]). Finally, sodium azide was removed and the cells were further incubated with cytochalasin or nocodazole for 4 h. (*a*) Immunofluorescent PR is nuclear in control Cos-7 cells (nontreated by drugs). (*b*) Immunofluorescent PR has moved into the cytoplasm in Cos-7 cells treated for 4 h by sodium azide. (*c*) PR has been shifted into the cytoplasm by azide treatment for 4 h (see *b*). Then, azide was removed and the cells were cultured for 4 h in DME. The receptor is seen in the nucleus. (*d*) PR has been shifted into the cytoplasm by azide treatment for 4 h (see *b*). Cytochalasin was added for 1 h before removing azide and remained present for 3 h after azide was withdrawn. The receptor has returned into the nucleus. (*e*) The same as in *c*, except that nocodazole was used instead of cytochalasin. Bar, 25 μm.

croscopy. It is noteworthy that there was no overlap of PR and microtubules in both interphasic and mitotic L cells. Kinetic analysis of the hormone-dependent translocation of this receptor towards the nucleus showed that the distribution of PR in the cytoplasm was not changed during this process (our unpublished data). In the case of the glucocorticoid receptor, interactions of receptor with microtubules in human gingival fibroblasts (2) and with actin in hepatoma cells (27) were observed by immunocytochemical methods (2) and by subcellular fractionation (27). The distribution of hsp90 has also been studied, since this protein is associated with steroid hormone receptors (5, 34, 37, 38, 41). Interactions between hsp90 and tubulin (33, 36), or hsp90 and actin (20) have recently been reported. However, at present, no studies with cytoskeleton-disrupting drugs have been used to analyze the cellular traffic of steroid receptors. The diverging observations on the cytoplasmic distribution of GR (2, 44) as compared to PR (see above) may be due to differences of receptor species or of cell types. The experimental conditions and the specificity of the antibodies may also be of major influence. For instance, in the present study the use of a polyclonal antibody (Dakopatts) (2) led to a nonspecific staining which was not observed with highly specific monoclonal antitubulin antibodies. Moreover, mixing of primary (anti-PR and antitubulin) or secondary antibodies was also observed to produce artefacts.

Most PR molecules were released from cells that had been permeabilized with detergent before fixation, indicating that they are not tightly bound to any intracellular structure. The receptor-associated protein hsp90 has also been shown to be extracted with 0.5% NP-40 in chicken embryo fibroblasts (5). After permeabilization of the cell, however, a small but detectable level of PR was retained, especially around the nuclear envelope, suggesting an interaction with an insoluble structure. However, it proved impossible to identify this structure at the light microscopic level, even by confocal laser microscopy. Thus, several pools of PR may coexist in the cells including soluble PR and PR loosely complexed with some cytoplasmic structure(s). Further studies at the electron microscope level using gold-labeled reagents will be necessary to analyze the distribution of these structures in relation to that of the receptor. Finally, the present study cannot exclude a transient association of PR with cytoskeletal proteins. However, it is clear that the intracellular transport of the receptor towards the nucleus appears operable in cells lacking an organized cytoskeleton.

Nuclear protein import is a selective process. Proteins destined for the nucleus contain NLSs (18, 22, 40). Karyophilic signals of steroid receptors (13, 31–32) are similar to those present in SV-40 large T antigen, nucleoplasmin, etc. Experimental evidence for the existence of transport receptors for NLS-containing proteins has been provided by several laboratories (1, 4, reviewed in 40). These proteins, called NLS-binding proteins, are located in the cytoplasm, on the nuclear envelope, and/or at the nuclear pore complex. After binding at the pore complex, proteins are translocated through the pore into the nucleus in an energy requiring step. The interaction of GR with a protein of the nuclear envelope has also recently been identified by cross-linking experiments (21). However, few studies have shed light on the nature of the structures involved in the transport of nuclear proteins and receptors from the cytoplasm into the nucleus. Hypotheses have been proposed that steroid receptor movement towards the nucleus involves cytoskeleton-scaffold supporting protein transfer (2, 33). More generally, few studies have analyzed the role of the cytoskeleton in the process of nuclear import (35, 42). However, it has been shown that the nuclear translocation of the 72-kD hsp and of the adenovirus E1A protein occurs after the disorganization of the three major cytoskeletal systems (42).

In conclusion, we have not been able to observe any colocalization of the progesterone receptor with the various cytoskeletal elements. Disruption of these networks had no effect on the translocation of the receptor into the nucleus. The NLSs and their interaction with the elements of the nuclear pore seem to be the major determinants of receptor traffic in the cells.

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