Localization and functions of steroid hormone receptors

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Summary. This review focuses on the subcellular localization of steroid hormone receptors (SHRs), taking into account the technical problems of immunohistochemistry and the characteristics of nuclear localization signals (NLSs) of each receptor, on the interaction between SHRs and cellular components, and on the possible roles of sex SHRs in the reproductive organs. It is concluded that SHRs are basically localized in the nucleus, regardless of hormonal status, and that considerable amounts of unliganded SHRs may be present in the cytoplasm of target cells in exceptional cases. Most immunohistochemical results that demonstrate nuclear translocation of liganded SHRs seem to be responsible for insufficient fixation. Immunoelectron microscopy shows that SHRs associate with the chromatin in absence or presence of hormones and that intranuclear translocation of liganded SHRs from the condensed chromatin to euchromatin which observed in some cell types, may be a passive process caused by a consequence of conformational changes in the chromatin binding receptors. Histochimical data suggest that the nuclear matrix (NM) is not a main binding site of liganded SHRs in the nucleus. The artificial formation of intermolecular disulfide bonds during NM preparation presumably causes the entrapment of liganded SHRs into the fraction. It seems that heat shock protein 90 (hsp90) does not form stable complexes with unliganded receptors in vivo, and it interacts with SHRs transiently cooperating with other heat shock proteins as a chaperone that helps folding of newly synthesized and refolding of denatured receptors. Estrogens transiently induce a number of nuclear protooncogenes, such as c-fos and c-jun family proteins, which act as transcription factors through estrogen receptor (ER) system in the endometrial epithelium of mature and immature rodents. Therefore, it is suggested that the changes in concentrations of these gene products trigger the proliferation and differentiation of uterine epithelium. In addition, ER system, not only in stroma cells but in the epithelial cells appears to participate in the growth response and abnormalities of epithelium elicited by the exogenous estrogen treatment at the neonatal period.

Key words: Steroid hormone receptor, Immunohistochemistry, Heat shock protein, Nuclear matrix, Protooncogene

I. Introduction

Steroid hormones control a variety of cellular activities. Sex steroids induce the development and differentiation of the reproductive system, masculinization and feminization, and control reproduction and reproductive behavior in the adult, while adrenal steroids widely influence body homeostasis and the immune and nervous systems. It has been demonstrated that the action of steroids is mediated by specific intracellular receptors and that hormone-occupied receptors modulate the transcription of target genes by binding to the hormone responsive elements (HREs), cis-acting sequences, of these genes. The receptors consist of similar molecular structures and form the nuclear receptor superfamily (Evans, 1988; Laudet et al., 1992; Tsai and O'Malley, 1994).

A two-step model was initially proposed for the intracellular distribution of steroid hormone receptors (SHRs) based on results obtained by the cell fractionation technique; i.e., unliganded SHRs locating in the cytoplasm translocate into the nucleus after binding with steroids (Gorski et al., 1968; Jensen et al., 1968). However, both liganded and unliganded receptors were demonstrated to be localized exclusively in the nucleus of target cells. Immunohistochemical studies have revealed that unliganded sex SHRs, ERs, progesterone receptors (PRs), and androgen receptors (ARs), are exclusively localized in the nucleus of target cells. However, there are divergent views on the
subcellular localization of glucocorticoid receptors (GRs) and mineral corticoid receptors (MRs) (Perrot-Applanat et al., 1992). It has been reported that ligand-free GRs and MRs are present primarily in the nucleus, or that they are distributed in the cytoplasm and translocate to the nucleus after hormone binding.

The entry of nuclear proteins into the nucleus requires the nuclear localization signals (NLSs) (Feldherr and Akin, 1994). Recent studies have indicated that certain characteristics of NLSs, i.e., the amino acid sequences, numbers, and intramolecular positions of NLSs, are important for effective nuclear import of nuclear receptors (Picard et al., 1990; Ylikomi et al., 1992; Guiochon-Mantel et al., 1996). It has been shown that PRs, ARs, GRs and MRs share homologous NLSs sequences with the NLS of large T antigen, but that ERs have fewer homologous to the NLS. The NLSs seem to be constitutively active in ERs and PRs whether ligands are present or absent but the activity of the NLSs of GRs are masked by the hormone binding domain in the absence of steroids (Picard and Yamamoto, 1987). In addition, it has been shown that the localization of SHRs is not static, but that the receptors are constantly shuttling between the nucleus and cytoplasm independent of hormonal status (Perrot-Applanat et al., 1992).

Biochemical studies have demonstrated that SHRs bind several proteins in vitro (Smith and Toft, 1993). In particular, it has been shown that heat shock protein 90 (hsp90) binds to unliganded SHRs composed of 8S-9S oligomers in vitro, and presumably hsp90 inhibits receptor binding to DNA and binding of steroids to receptors results in the formation of an active 4S form as a result of dissociation of hsp90 (Baulieu, 1987; Pratt et al., 1992; Smith and Toft, 1993). Many investigators have demonstrated the presence of SHRs in nuclear matrix (NM) (Barrack and Coffey, 1982; Lauber et al., 1995), which may participate in DNA organization and important nuclear metabolism, such as DNA replication and RNA synthesis and processing (Berezney, 1991; Razin et al., 1995). However, interaction between SRHs and hsp90 or the NM in vivo is still unclear, and histochemical data do not always support the results obtained by in vitro studies (Yamashita and Korach, 1989a; Tuohimaa et al., 1993).

It is well known that the effects of steroids differ according to target cell types. The amount of receptors may be essential to regulate the responses of cells to steroids. Furthermore, the structure of chromatins and other cell proteins, including transcription factors, which are inducible by steroids or pre-exist in the cells may be involved in differential responses to steroids. Reports in recent studies have claimed that estrogen treatment induces immediate and transient activation of a number of nuclear protooneogenes, which act as transcription factors, in the rodent uterus (Khan et al., 1994; Stancel et al., 1994). Therefore, alteration of expression of these immediate early genes may trigger activation of a cascade in estrogenic responses by modulating other delayed early genes, and even late genes, in uterine cells.

In this article, I will reexamine the subcellular localization of SHRs and their binding sites, taking into account the technical problems of immunohistochemistry, the phenomenon of nucleocytoplasmic shuttling, and the characteristics of nuclear localization signals (NLSs) of each receptor, and will review possible roles of sex SHRs in the development, sexual cycles, and proliferation of reproductive organs.

II. Subcellular localization of SHRs

1) Effect of steroids on receptor localization

Both liganded and unliganded ERs and PRs are claimed to be exclusively localized in the nucleus of cells in the female reproductive organs (McClellan et al., 1984; Press et al., 1988; Okulicz et al., 1989; Yamashita and Korach, 1989a; Isola, 1990; Fig. 1A), other target tissues (Yamashita and Korach, 1989a; Sprangers et al., 1990), including brain (Liposits et al., 1989), and cells transfected with ER or PR genes (Ylikomi et al. 1992) independent of hormonal status, except in a few reports. Blaustein et al. (1992) have reported that unliganded ERs are present in both the nucleus and the cytoplasm of neurons including dendrites and axonal terminals and that the cytoplasmic immunostaining is eliminated 1 h after 17β-estradiol (E2) administration. However, the function of cytoplasmic receptors and whether liganded receptors translocate into the nucleus from axonal terminals within a short time or are degraded in the cytoplasm are unclear, because H 222 anti-ER monoclonal antibody reacts with both liganded and ligand-free ERs.

The effect of hormonal status on AR localization is somewhat controversial, compared to the distribution of ERs and PRs. Exclusive nuclear occupancy of unliganded ARs has been shown in the epithelial cells of the prostate and seminal vesicles of rats (Husmann et al., 1990; Zhuang et al., 1992), the seminal vesicles of monkeys (West et al., 1990), and the epididymis and acinar cells of the submandibular gland in mice (Sawada and Nomura, 1995). In contrast, some investigators have indicated that the intensity of nuclear AR immunostaining decreases and relative cytoplasmic staining increases with time after castration, and that androgen administration elicits the nuclear localization of ARs in male reproductive organs (Prins and Birch, 1993; Paris et al., 1994) and in the brain (Wood and Newman, 1993). Unliganded AR localization appears to be heterogeneous among the cell types. Paris et al. (1994) indicated that epithelial cells of the rat ventral prostate, seminal vesicle and coagulate gland exhibit nuclear and cytoplasmic AR localization after castration, but that the epithelial cells of the epididymis show exclusive nuclear immunostaining. Prins and Birch (1993) demonstrated that androgen withdrawal causes a rapid decrease in nuclear AR immunostaining in the ventral and dorsal lobes, but not in the lateral lobe. It has also been claimed that the
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Fig. 1. Effect of various pretreatments on immunostaining of progesterone receptors (PRs) in unfixed frozen sections. Uteri of adult ovariectomized rat were frozen 1 h after progesterone (10 mg) injection. Frozen sections were fixed with Zamboni's fixative for 10 min at room temperature without pretreatment and immunostained using anti-PR monoclonal antibody and peroxidase-labeled sheep anti-mouse F(ab')2 fragments (a). The sections were incubated with 0.5M NaCl (b) for 15 min, with DNase 1 (10,000 u/ml) for 15 min (c), or with 5 mM sodium tetrazionate (NaTT) for 5 min and subsequently with 0.5M NaCl for 15 min (d), respectively, at room temperature and then fixed and immunostained. Pretreatment with 0.5M NaCl or DNase I results in almost complete disappearance of nuclear staining of PRs (b and c). However, PRs in the sections treated with NaTT are resistant to the extraction with 0.5M NaCl (d). × 1,000. Bar = 10 μm.
distribution pattern of unliganded ARs is different in cell lines transfected with AR genes (Simental et al., 1991; Jenster et al., 1993).

The subcellular distribution of ligand-free GRs has been debated. They are localized exclusively in the nucleus (Gasc et al., 1989; Brink et al., 1992; Pekki et al., 1992), or principally in the cytoplasm and translocated into the nucleus after binding with ligand (Govindan, 1980; Papamichael et al., 1980; Antakly and Eisen, 1984; Wikstrom et al., 1987; McGimsey et al., 1991). In cells transfected with GR genes, overexpressed ligand-free GRs were found to reside in the nucleus (Martins et al., 1991) or the cytoplasm (Cidlowsky et al., 1990; DeFranco et al., 1991). Furthermore, several investigators have described that they distribute in both the nucleus and cytoplasm independently of hormonal status (Antakly et al., 1990; Farman et al., 1991). Cell-type-specific subcellular localization of GRs has also been reported as a result of the nucleation technique or nucleation followed by immunocytochemistry: liganded GRs were mainly present in the nucleus in GH3 rat pituitary tumor cells (Welshons et al., 1985; LaFond et al., 1988), but were found in both the cytoplasm and the nucleus in mouse fibroblast L-cells (LaFond et al., 1988).

The localization of MRs has not been investigated as extensively as that of other SHRs. Both nuclear and cytoplasmic MR distribution has been reported in the kidney, independent of hormonal status (Krozowsky et al., 1989; Lomber et al., 1990; Farman et al., 1991).

2) Considerations based on technical immunohistochemistry problems

Immunohistochemistry requires both accessibility of antibodies to antigens and good morphological preservation of tissues, i.e., minimal dislocation of antigens. However, these requirements conflict because fixatives good for immobilizing proteins usually cause denaturation or masking of antigenicity and poor penetration of antibodies into tissues. Since proteins and nucleic acids are tightly packed in the nucleus, and large molecules are not freely accessible to nuclear SHRs, the molecular mass of probes for immunohistochemistry is also important (McClellan et al., 1984; Yamashita and Korach, 1989a, Yamashita, 1995a). Whether immunostaining of unliganded GRs in the cytoplasm is a diffusion artifact has been discussed extensively, because several investigators have demonstrated that unliganded sex SHRs are more liable to extraction during fixation than liganded receptors and that low receptor levels are hard to detect in the absence of ligand (McClellan et al., 1984; Sar and Parikh, 1986; Yamashita and Korach, 1989a). Gasc et al. (1989) showed that when unfixed frozen sections of liver are preincubated in PBS-sucrose for a short time before fixation, all nuclear GR immunostaining is lost in the liver cells of adrenalectomized rats but that there is no significant reduction of staining after dexamethasone treatment. However, they did not observe any clear change in the nuclear PR immunoreactivity of chick oviduct under the same tissue processing conditions. Pekki et al. (1992) demonstrated the nuclear distribution of ligand-free GRs by using the freeze-drying and vapor-fixation method to minimize the antigen diffusion that can be caused by the conventional fixation procedure in an aqueous solution. Brink et al. (1992) concluded that unliganded GRs are localized in the nucleus, but not in the cytoplasm, of rat hepatoma cell line FTO-2B after systematic immunohistochemical studies on the effect of fixation and permeabilization procedures in cultured cells. Wikstrom et al. (1987) clearly showed that different fixatives produce a variety of GR immunostaining patterns and that Bouin’s fixative and precipitation fixation with organic solvent are unsuitable for the fixation of ligand-free GRs.

Careful examination of the studies cited above that demonstrate nuclear translocation of liganded GRs or ARs reveals that surprisingly few report that unliganded GRs and ARs reside in the cytoplasm and translocate into the nucleus after hormonal stimulation. Furthermore, the following findings strongly suggest that the diffusion of unliganded GRs and ARs takes place during fixation: 1) the GR immunoreaction in cultured cells changes from very faint nuclear and cytoplasmic staining to intense nuclear staining within a short time after dexamethasone treatment (Wikstrom et al., 1987); 2) administration of high doses of corticosterone elicits an intense immunoreaction in the nucleus of brain cells, whereas low doses of the ligand yield very faint immunostaining in both the cytoplasm and nucleus (Martins et al., 1991; McGimsey et al., 1991); and 3) AR immunostaining is undetectable in rat epididymis and ventral prostate after castration, however, nuclear staining in the epithelium is evident 15 min after dihydrotestosterone (DHT) injection (Sar et al., 1990). Therefore, the following tissue preparation methods may be effective in immobilizing ARs, GRs and MRs without diffusion artifacts: 1) rapid-freezing and vapor fixation (Pekki and Tuohimaa, 1989); 2) rapid-freezing and substitution fixation (Yamashita and Yasuda, 1992); 3) rapid fixation with microwaves (Brenner et al., 1996); and 4) use of fixatives containing the sulphydryl-cross-linking reagent sodium tetrathionate (NaTT) (the effect of NaTT is discussed below). If the antigenic determinants are denatured or masked after fixation, application of “antigen retrieval methods” should be useful in obtaining a strong immunoreaction for some receptors (Cheng et al., 1988; Shi et al., 1993).

Antibody specificity is essential to immunohistochemical studies. Husmann et al. (1990) indicated that immunoreaction with antibody to the C-terminal of ARs is influenced by the presence of DHT, whereas antibody to N-terminal is unaffected by hormonal status in prostatic cells. Some antibodies to DNA binding domains of SHRs have been found to be reactive to 4S-transformed receptors in vitro but not to 8S-nontransformed receptors (Smith et al., 1988; Wilson et al., 1988). Thus, they may be unable to recognize un-
liganded receptors in tissue sections.

3) NLSs and nucleocytoplasmic shuttling of SHRs

The activity of the NLSs of each receptor may play an important role when subcellular localization of receptors is cell-type-specific or is influenced by cell culture conditions, because the tissues or cells are treated with the same fixatives in each examination. Less effective NLSs or masking of constitutive NLSs would lead to an increased duration of receptor presence in the cytoplasm and to differential nuclear and cytoplasmic immunostaining (Picard et al., 1990; Guiochon-Mantel et al., 1991; Petrot-Applanat et al., 1992; Ylikomi et al., 1992). Cell-type-specific proteins including heat shock proteins presumably mask the NLSs of unliganded GRs and ARs in certain cell types, and reduce nuclear transport (Picard et al., 1990). The concentration and groups of nuclear transport factors which bind NLSs and mediate nuclear translocation, may also vary according to cell type (Yoneda, 1996). Cell culture conditions seem to affect the subcellular localization of ligand-free GRs. Picard and Yamamoto (1987) stated that unliganded GRs are localized in the nuclei of cells cultured in medium containing bovine serum but they are present in the cytoplasm of cells maintained in serum-free medium. Van den Berg et al. (1996) demonstrated that hormone-free GRs are found in the nucleus in the aggregated human lymphoma cells, although they are localized in the cytoplasm in the non-aggregated cells. The serum-dependent nuclear localization was shown for other nuclear proteins such as c-fos protein and adenovirus Ela protein (Roux et al., 1990; Lyons, 1991). Presumably the presence of serum in the medium greatly influences proliferating activity of cells, and subsequently may change the properties of nuclear pore complexes and cytoplasmic NLSs binding proteins (Feldherr and Akin, 1994).

Nucleocytoplasmic shuttling of PRs and ERs was directly demonstrated in heterokaryons prepared by the cell fusion technique (Guiochon-Mantel et al., 1991; Chandran and DeFranco, 1992; Petrot-Applanat et al., 1992). The steady-state nucleocytoplasmic distribution of a shuttling protein seems to be controlled by the relative rates of nuclear import and export, but also by its relative affinities for cytoplasmic and nuclear binding partners (Laskey and Dingwall, 1993; Schmidt-Zachmann et al., 1993). Without selective binding, proteins may escape more rapidly from the nucleus and shuttle repeatedly between nucleus and cytoplasm. The immunohistochemical findings discussed above suggest that the binding affinity of unliganded ERs and PRs to nuclear components is higher than that of GRs, and that ARs probably have intermediate affinity.

III. Immunoelectron microscopy of SHRs

Immunoelectron microscopy of SHRs, particularly sex SHRs, has been performed in several cell types to elucidate the nuclear binding sites of receptors and to investigate whether liganded receptors translocate within the nucleus for transcriptional regulation of target genes. Pre-embedding methods using immunoperoxidase or immunogold-silver enhancement were employed initially. Later, post-embedding methods and immunocytoultramicrotomy with gold-labeled probes were applied to minimize false negative immunoreactions caused by insufficient penetration of labeled antibodies into tissues and to obtain more detailed intranuclear distribution of receptors.

Press et al. (1985) localized ERs in the euchromatin, but not in the heterochromatin associated with the nuclear envelopes and nucleoli in the epithelial and stromal cells of human endometrium by employing a pre-embedding method with the peroxidase-anti-peroxidase (PAP) procedure. Almost identical intranuclear localization of ERs was reported in human breast cancer cells as a result of employing the same immunostaining procedure (Charpin et al., 1986; Fukushima et al., 1995). Liposits et al. (1989) demonstrated that ERs associate with chromatin in rat brain cells regardless of hormone administration by using the pre-embedding method followed by silver enhancement of diaminobenzidine (DAB) reaction products. Post-embedding methods and labeling on ultrathin cryosections have also been applied to detect ERs without the diffusion artifact of DAB reaction products. Yamashita (1995a) employed immunocytoultramicrotomy and demonstrated that neither ER redistribution nor structural changes in nuclei are observed in the uterine epithelium of ovariectomized adult mice 1 h after E2 stimulation. ERs were localized in the dispersed chromatin and slightly condensed chromatin and the margins of highly condensed chromatin located at the periphery of nuclear envelopes, but not in the nucleolus (Figs. 2a, b, d). Kudo et al. (1996), using immunocytoultramicrotomy, showed that ERs were localized in the chromatin of human breast cancer cells. In contrast, Vázquez-Nin et al. (1991), using the post-embedding method with protein A-gold, showed that ERs localize mainly in the interchromatin space, probably on ribonucleoprotein (RNP) fibrils or particles, and nucleoli in major uterine cell types of ovariectomized immature rats, and that there were no clear differences in ER distribution in the presence or absence of E2. This discrepancy in intranuclear ER distribution is very likely attributable to the specificity and affinity of antibodies to ERs. They stated that the antibody had low affinity for rat ERs. Since the antibody used in their study was produced to the DNA-binding region of human ERs and 5 of its 15 amino acids were lysine and arginine, the antibody may cross-react with other SHRs family proteins and the epitope may be easily modified by aldehyde during fixation. Sierralta and Thole (1992) showed that unoccupied ERs are present in both the nucleus and cytoplasm of porcine endometrium by using the post-embedding method with immunogold or protein G-gold with the nucleus exhibiting lower gold labeling density than the cytoplasm. Blaustein et al.
Fig. 2. Intranuclear localization of estrogen receptors (ERs) in the endometrial epithelium of mice. Ovariectomized adult mice were killed at 1 h after saline (a, b and c) or 17β-estradiol (20 μg/Kg b.w.) (d) injections. ERs were localized on ultrathin frozen sections using anti-ER monoclonal antibody and 1 nm immunogold. The gold particles were then visualized with the silver enhancement procedure (a, b and d). For the control, the section was incubated with normal rat IgG in place of anti-ER antibody (c). Unliganded ERs are present in the dispersed and slightly condensed chromatin in the glandular (a) and luminal (b) epithelium. Highly condensed chromatin associating with nuclear envelopes and nucleolus (*) show no reaction. Liganded ERs in the luminal epithelium display the same intranuclear distribution pattern as unliganded ERs (d). a, x 32,900; b, x 21,900; c, x 25,300; d, x 23,000; Bars: 1 μm. (Yamashita, 1995, vol. 44, J. Electron Microsc.).
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(1992) claimed that unliganded ERs localized in both the nucleus and cytoplasm, particularly around the rough-surfaced endoplasmic reticulum, in the dendrites and axons of guinea pig hypothalamus neurons but that the ERs in the cytoplasm disappeared after E2 administration.

Perrot-Applaat et al. (1986), employing the post-embedding method with protein A-gold, demonstrated that progesterone injection caused dispersion of chromatin and redistribution of PRs from condensed chromatin to the periphery of the condensed chromatin in the nucleus of the uterine stromal cells of immature estrogen-primed rabbits, but that it did not produce clear changes in chromatin structure and PR distribution in the nucleus of myometrial cells. They confirmed localization of PRs in the chromatin, but not in the nucleolus, by means of the progressive EDTA technique. Isola (1987) showed almost the same results with regard to changes in PR labeling pattern in the nucleus and rearrangement of chromatin in the epithelial cells of chick oviduct 1 h after progesterone administration by applying the pre-embedding method with immunogold-silver enhancement technique. However, the distribution of hormone-occupied and -unoccupied PRs appeared heterogeneous among the epithelial cells; strong immunoreaction was observed in the heterochromatin in some cells and in the euchromatin in others (Isola et al., 1987).

Zuhang et al. (1992) reported that ARs are present mainly in the heterochromatin of prostatic acinar cells of castrated rats, and that the heterochromatin becomes less condensed and more AR immunoreaction products are seen in the euchromatin than in non-treated animals 6 h after DHT. Zhou et al. (1996) indicated that ARs localize in the euchromatin and nucleoli of spermatogonia, Sertoli cells and Leydig cells of young mouse testis, but not in the highly condensed chromatin by using pre-embedding method. The GR localization was seen in euchromatin but not in heterochromatin or nucleoli of Leydig cells of intact rats by the pre-embedding method with the avidin-biotin-complex (ABC) procedure (Schultz et al., 1993).

Immunoelectron microscopic findings have indicated that SHRs are present exclusively in chromatin, but that the time course of structural changes caused by hormone treatments in the nucleus differs according to species, target cells, dose of hormones and age of the animals. In addition, the following findings at the light microscopic level indicate that ERs and PRs are associated with the chromatin independence of hormonal status. When fresh frozen sections were fixed after incubation with DNase I and subjected to immunohistochemistry, nuclear ER and PR immunostaining in rodent uterine cells was diminished (Yamashita and Korach, 1989b; Fig. 1c). Administration of steroids elicits transcriptional activation of a variety of target genes within a short time (Khan et al., 1994; Stancel et al., 1994; Yamashita, 1995b; Yamashita et al., 1996). Taken together, these data strongly suggest that a marked change in the distribution of receptors in some cells takes place as a consequence of conformational changes in the chromatin binding receptors, i.e., the clear intranuclear translocation of liganded receptors may be a passive process, rather than receptors translocating within the nucleus after hormone binding (Perrot-Applaat et al., 1986; Isola, 1987; Yamashita, 1995b). The majority of SHRs may not change their intranuclear localization significantly after binding to steroids and bind to HREs of target genes locating nearby. However, the possibility that a small number of receptors translocate the relatively long distance to reach HREs of target genes cannot be ruled out. Nevertheless, this translocation cannot be visualized by immunoelectron microscopy, because the number of target genes is much smaller than that of receptors in the nucleus; the former is assumed to be less than 100 and the latter greater than 10,000 molecules in each nucleus (Evans, 1988).

IV. Interactions between SHRs and cellular components

Immunoelectron microscopic studies have demonstrated that SHRs associate with the chromatin of target cells independent of hormonal status. However, biochemical data have suggested that unliganded receptors bind with hsp90 both in vitro and in vivo that a high percentage of liganded receptors are tightly bound to nuclear matrix (NM).

1) Heat shock proteins (hsp) and unliganded receptors

It has been demonstrated that SHRs form oligometric complexes with hsp90, hsp70, hsp56, and a few other proteins in the absence of steroids in vitro, although unliganded receptors for thyroid hormone, retinoic acid and vitamin D3 appear to be unassociated with hsp90 (Dalman et al., 1990). Recent studies by gene transfection techniques have shown that GRs and MRs require hsp90 for high affinity hormone binding, but that ERs, PRs and ARs are able to bind to ligands with high affinity in the absence of hsp90 (Bresnick et al., 1989; Schulman et al., 1992; Bohen and Yamamoto, 1993). In contrast, most immunohistochemical data have indicated exclusive cytoplasmic localization of hsp90 in several cell types (Ito et al., 1990; Matsubara et al., 1990; Pekki, 1991; Osako et al., 1995), with few reports showing cytoplasmic and nuclear distribution of hsp90 (Gasc et al., 1990, 1994; Bornman et al., 1996). It seemed difficult to verify whether hsp90 is present exclusively in the cytoplasm and artificially complexes with SHRs during tissue homogenization or whether a small amount of hsp90 is present in the nucleus. Since hsp90 is one of the major components of soluble cellular proteins, the presence of only a small percentage of hsp90 in the nucleus may be sufficient for binding to SHRs. To resolve this problem directly, Tuohimaa et al. (1993) transfected chimeric DNA of hsp90 and PR into HeLa cells and immunostained transiently-expressed chimeric
protein with antibodies to hsp90 and PRs, respectively. They confirmed that the sensitivity of immunostaining for hsp90 and PR is almost the same and that the chimeric protein is exclusively present in the nucleus regardless of hormonal status. Furthermore, PRs were localized in the nucleus and hsp90 was detected only in the cytoplasm in the oviductal epithelium of estrogen-primed immature chickens. These results clearly indicate that at least unliganded PRs do not associate with hsp90 in the nucleus in situ.

Taking these data together, hsp90 may not form stable complexes with unliganded receptors in vivo and formation of stable complexes may be exceptional. Rather, hsp90 seems to interact with SHRs transiently cooperating with hsp70, hsp56 and other proteins as a chaperone that helps folding of synthesized receptors and reactivates denatured receptors under physiological conditions (Smith, 1993; Holley and Yamamoto, 1995), because SHRs appear to be unstable and rapidly lose hormonal binding activity at 37°C (Bresnick et al., 1983; Rennie et al., 1983).

2) Nuclear matrix (NM) and liganded SHR

Since NM is not observed in the nucleus of intact cells on electron microscopy, it is impossible to determine whether SHRs localize in the NM by immunoelectron microscopy in situ. Studies concerning the localization of SHRs in the NM may be classified into three categories. First, using [3H]steroids: saturable high-affinity and tissue-specific binding sites for steroids thought to be SHRs have been shown in the NM obtained from the target tissues stimulated with steroids. Second, SHR proteins are directly detected in NM preparations by using the immunoblot or sucrose gradient centrifugation methods. Third, saturable and tissue-specific binding or acceptor sites of receptors are found in the NM by employing the cell-free binding assay between the NM and [3H]steroid-bound receptors.

[3H]Estrogen-binding sites in the NM have been shown in rat uterus (Barrack and Coffey, 1980, 1982; Buttyan et al., 1983), rat liver (Alexander et al., 1987), and chicken liver (Barrack and Coffey, 1982; Simmen et al., 1984). The presence of androgen-binding sites have been reported in the NM of rat prostate (Barrack, 1983; Buttyan et al., 1983; Rennie et al., 1983), guinea pig seminal vesicle (Epperly et al., 1984), and human prostate cells (Donnelly et al., 1984). Binding sites for glucocorticoid have been demonstrated in the NM fraction of rat liver cells (Kaufmann et al., 1986) and COS-1 cells transfected with GR plasmid (Van Steensel et al., 1995). These studies revealed that more than 50% of receptors contained in the nucleus are associated with the NM. However, it is important to keep in mind that the structures and components of the NM, the internal fibrogranular network, depend on the methods and conditions of NM isolation. In these experiments, NM is prepared by the original or slightly modified methods of Berezney and Coffey (1977), in which nuclei are isolated and subjected to serial treatment with detergents, DNase I digestion, and high-salt solution: NM preparation requires several hours.

Few examinations categorizing the direct detection of SHR proteins in the NM have been performed. Alexander et al. (1987) demonstrated that about 60% of the total nuclear [3H]estrogen binding sites are recovered in the NM preparations obtained from liver cells of estrogen-stimulated rats, and that ERs are present in the NM by using Western blot with specific antibody. Very low concentration of ERs was shown in the NM of liver cells of untreated animals. GRs were detected in the NM purified rat liver nuclei by using Western blot analysis (Kaufmann et al., 1986). Van Steensel et al. (1995), by using Western blot technique, showed that ARs and GRs are present in the NM of COS-1 cells transfected with AR and GR expression plasmids, respectively. Rennie et al. (1983) reported that mild trypsinization releases 3S ARs from the NM of prostatic cells of rat treated with testosterone.

On the other hand, many investigators have challenged not only the existence of SHRs in NM, but the presence of nuclear receptors that are resistant to extraction with high-salt solution. Traish et al. (1977) suggested that the presence of salt-resistant SHRs in the nucleus is an artificial phenomenon as a result of their simply being entrapped in the viscous and gelatin-like nuclear pellet, because more than 90% of the ERs in rat uterus could be extracted after repeated extraction with 0.6M KCl or brief sonication. Our immunohistochemical findings also indicate that NM is not the main binding site of ERs in all uterine cell types. When frozen sections of mouse uterus were treated with RNase A before fixation, no clear changes in the intensity of ER immunostaining were observed in the nucleus (Yamashita and Korach, 1989b), although the fibrogranular network structures of the NM were reported to have been almost completely destroyed by such treatment (Berezney, 1991). Furthermore, the salt-resistant ERs and PRs are hardly detectable in unfixed frozen sections (Yamashita and Korach, 1989a; Fig. 1b). Kaufmann et al. (1981) demonstrated that the formation of intra- and inter-molecular disulfide bonds causes significant changes in the ultrastructure, protein composition, and SHR content of the NM. The following findings strongly suggest that SHRs in the NM, which are classified into first and second categories, are artificially produced by oxidative cross-linking of sulphydryl groups during NM preparation. 1) Approximately 60% of liganded GRs were recovered from the NM prepared from rat liver cells by a conventional procedure, and the GRs exhibited high molecular weight disulfide-cross-linked complexes on SDS-PAGE (Kaufmann et al., 1986). Moreover, GRs were hardly recognizable in the NM when the nuclear fraction was isolated in the presence of sulphydryl-blocking reagent (iodoacetamide), but more than 95% of nuclear GRs were associated with the NM when prepared in the presence of the sulphydryl-cross-linking reagent NaN3T.
Barrack and Coffey (1982) also pointed out that only small amounts of ARs and ERs are detectable in the NM obtained from the nuclei of rat prostate and uterus in the presence of the disulfide reducing reagent dithiothreitol (DTT). 2) When the COS-1 cells transfected AR and GR genes were permeabilized with detergent in the absence of NaTT and subsequently treated with DNase I and ammonium sulfate solution according to the methods of modified procedure of Penman and associates (He et al., 1990), the NM contained liganded ARs but not GRs. However, both receptors were detectable in the NM when the cells were permeabilized with the detergent in the presence of NaTT (Van Steesel et al., 1995). 3) Strong PR immunostaining was present in the nucleus of rat uterine sections pretreated with NaIT for 5 min and subsequently with high-salt solution before fixation, but no reaction was found in the specimens untreated with NaTT (Fig. 1a,d).

The third type of experiments may be valuable for investigating the interaction between SHRs and components of the NM. Furthermore, these studies seem to minimize simple artificial entrapment of receptors in the NM during the experiments. NM binding or acceptor sites were reported for ARs in the rat prostate (Barrack, 1983, 1987; Buttyan et al., 1983), for ERs in rat liver and mouse uterus (Barrack, 1987; Metzger and Korach, 1990), and for PRs in chicken oviduct (Schuchard et al., 1991). Most reports did not describe whether the receptors and NM complexes were resistant to high-salt solution, whereas Metzger and Korach (1990) reported that about 40% of hormone-occupied ERs bound to NM in a cell-free system are resistant to extraction with 0.6M KCl. However, even in this type of study, incubation of NM and liganded receptors should be performed in the presence of DTT in order to rule out the possible formation of artificial disulfide bonds, and more systematic examination of dissociation conditions may be necessary to characterize the putative acceptor sites for receptors in the NM. Spersberg and co-workers have demonstrated that PR binding non-histone nuclear protein RBF-1, which shows high binding affinity to PRs, are present in the chromatin or NM of avian oviductal cells (Lauber et al., 1995).

V. SHRs and cell-type-specific responses to steroids

1) Ontogeny of steroid hormone receptors

The ontogeny of SHRs has been of interest in connection with the molecular basis of steroid hormone-dependent interaction between mesenchymal and epithelial cells during the development of the genital tract (Cunha et al., 1981, 1983; Bigsby and Cunha, 1986), and of abnormalities of sex organs which are induced by estrogen treatment during prenatal and neonatal periods (Korach et al., 1988; Newbold et al., 1989; Sato et al., 1996). It was initially investigated by using autoradiography with [3H]steroids. ERs and ARs were localized exclusively in the mesenchymal cells, and not in the epithelial cells, of the genital tract of prenatal and neonatal mice (Stumpf et al., 1980; Cunha et al., 1982; Bigsby and Cunha, 1986; Holderegger and Keefer, 1986), while epithelial cells were capable of responding to exogenous sex steroids and underwent proliferation and cytodifferentiation (Cunha et al., 1983; Bigsby and Cunha, 1986). ERs were detected in the epithelial cells of mouse uterus about 2 weeks after birth (Cunha et al., 1982). ARs were not recognized in the acinar cells of the mouse prostate on approximately postnatal day 6 (Cunha et al., 1983). In addition, recombination experiments between stroma and epithelium from the female and male genital tract, revealed that mesenchymal cells are highly responsible for the epithelial morphogenesis during the embryonic and neonatal period. Thus, it was postulated that the mesenchymal cells are the mediators of hormonal action on the epithelial cells of the genital tract in neonatal animals, i.e., factors secreted by the mesenchyme may activate epithelial cells after hormonal stimulation (Cunha et al., 1981, 1983).

In contrast, Korach et al. (1988) demonstrated that ERs are present in the uterine epithelium of 5-day-old mice based on the results of Western blot analysis of isolated epithelium and immunohistochemistry. The presence of ERs in the neonatal mouse uterus has been confirmed by using immunohistochemical and sensitive autoradiographic techniques, and the concentration of ERs has been found to gradually increase with neonatal age, even though the ontogeny of ERs differs slightly among strains of mice (Yamashita et al., 1989; Bigsby et al., 1990; Greco et al., 1991; Sato et al., 1992). ERs were demonstrated as early as fetal day 15 in the epithelium of mouse oviduct and cervix (Cunha et al., 1982; Yamashita et al., 1989; Bigsby et al., 1990). Therefore, we can conclude that the absence of immunohistochemical and autoradiographic reactions of sex SHRs in the fetal and early neonatal period does not necessarily mean the complete absence of receptors in the cells, but it may indicate that their concentration is below the detection limit of these techniques.

The physicochemical nature, i.e., the molecular weight, nuclear binding sites, and binding force to nuclear components of ERs detected in neonatal mice appears to be essentially the same as in mature animals (Korach et al., 1988; Yamashita et al., 1989). Estrogen treatment has been demonstrated to up-regulate ER expression in the uterine epithelium of prenatal and neonatal mice (Yamashita et al., 1990). A single injection of diethylstilbestrol (DES) increased the ER level within 6 h in the uterine epithelium of newborn and 4-day-old mice, and a significant increase in ER mRNA level was observed within 4 h in uterine epithelial cells and uterine stromal cells of newborn mice (Yamashita et al., 1990; Sato et al., 1996). ERs could be induced in the uterine epithelium of day 15 fetal mice by exposure to DES (Yamashita et al., 1989). It is still unclear whether the stromal and epithelial cells of female genital tract tissue are under the influence of endogenous ovarian steroids during early development (Ogasawara et al.,
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1983). However, the findings described above strongly suggest that exogenous estrogen treatment elicits cell proliferation, cytodifferentiation, and abnormalities through an ER system, not only in stroma cells but in epithelial cells, in both the fetal and the neonatal genital tract.

2) Localization of ERs and PRs during the sexual cycle

It is well known that the menstrual cycle and estrous cycle are controlled by ovarian steroids and that each uterine cell type responds uniquely to steroids. Immunohistochemical studies have been carried out to elucidate the relationship between the expression of ERs and PRs and cellular activity in each target cell during the cycles. ERs and PRs are localized in the nucleus of uterine major cell types (epithelial cells, stromal cells and myometrial muscle cells), and smooth muscle cells of blood vessels in humans and animals (King and Greene, 1984; Press et al., 1988; Pekki and Tuohimaa, 1989; Yamashita and Korach, 1989a; Koji and Brenner, 1993). Most of the major cell types in the uterus seem to exhibit almost the same cyclic pattern of ER and PR expression during the human menstrual cycle (Press et al., 1984; Lessey et al., 1988; Ben-Hur et al., 1995), whereas the endometrial epithelium in the functionalis displays the most significant changes in ER and PR levels (Press et al., 1984, 1988). ER immunoreactivity in the endometrial epithelium of the functionalis has been shown to be negative in the menstrual phase and to increase during the proliferative phase, peaking in the late proliferative and early secretory phases, and then decreasing in the middle and late secretory phases. In contrast, the glandular epithelium of the basalis displays essentially steady and strong ER immunoreactivity throughout the menstrual cycle (Press et al., 1984; Bergeron et al., 1988a; Snijders et al., 1992). PR levels in the epithelium of the functionalis increase during the proliferative phase, reach a maximum in the early secretory phase, and sharply decrease in the mid secretory phase, displaying almost the same pattern as ERs with a lag time of a few days (Bergeron et al., 1988a,b; Press et al., 1988; Snijders et al., 1992). A small portion of the uterine glandular cells of the basalis have been reported to contain relatively high concentrations of PRs in the mid and late secretory phase (Press et al., 1988). PR immunostaining is slightly decreased in stroma and myometrial muscle cells during the mid and late secretory phases; however, it still maintains a strong to moderate level (Bergeron et al., 1988b; Press et al., 1988; Snijders, 1992). The cyclic changes in ER and PR expression in each type of endometrial cells during normal or experimentally-induced menstrual cycles in primates, are almost identical to those in humans (McClellan et al., 1986; Okulicz et al., 1989; Brenner et al., 1990; Koji and Brenner, 1993). There have been a few reports regarding ER and PR distribution in laboratory rodents during the estrous cycle. The highest PR level in the uterine epithelial and stromal cells of the guinea pigs occurs around the time of ovulation (Alkhalaf et al., 1992). In the rat uterus, maximal PR expression is observed in the epithelial cells in the diestrus phase, and in stromal and myometrial muscle cells in the proestrus phase (Ohta et al., 1993).

In general, all of these immunohistochemical studies support the biochemical evidence that estrogen stimulates ER and PR expression and that progesterone decreases the levels of both receptors, and that PR expression may be controlled by ERs (Levy et al., 1980; Ekka et al., 1987). It is unclear whether estrogen directly regulates the ER gene via the ERs themselves, but Drean et al. (1995) have demonstrated that the ER gene of rainbow trout contains an estrogen-responsive element (ERE) that is modulated by liganded ERs. The regulation of PR expression by ovarian steroids should be involved in the ERE of PR gene; ERs binding to the ERE activate PR gene expression, and the ERE also may participate in down-regulation of PR gene transcription by progesterone in the presence of PRs (Savouret et al., 1991). The extent of activation and suppression of ER and PR expression by ovarian steroids may be specific for each cell type, probably depending on the concentration of receptors and variations of other cellular proteins, including nuclear transcription factors.

Mice lacking ERs or PRs have been produced by using gene targeting techniques (Lubahn et al., 1993; Lydon et al., 1995; Korach et al., 1996). In these mice, both sexes survive to adulthood with almost normal gross external genitalia. These findings indicate that sex steroid receptors are not essential for survival but play an important role in maturation of reproductive organs, sexual behavior, and fertility.

3) ERs and nuclear protooncogenes

Immunohistochemical and in situ hybridization techniques have been applied to study the expression of estrogen-induced immediate early genes acting as transcription factors in the uterus of rodents. Estrogen injection provokes rapid and transient activation of the c-fos gene exclusively in the epithelial cells of immature and ovariectomized adult rats and mice, with expression of the c-fos transcript and protein reaching a peak at 2 h (Papa et al., 1991; Bigsby and Li, 1994; Nephew et al., 1995; Yamashita et al., 1996). Rapid increases in jun-B and jun-D mRNA levels have also been shown in the uterine epithelium of estrogen-stimulated rats, but not in stromal or myometrial muscle cells (Webb et al., 1993; Nephew et al. 1996). Since the increased expression of these nuclear protooncogenes does not require de novo protein synthesis, it is probably a direct effect of estrogen through the ER system (Loose-Mitchell et al., 1988; Webb et al., 1993). In contrast, expression of c-jun has been demonstrated to be suppressed in the epithelium and activated in the stroma and myometrium (Bigsby and Li, 1994; Nephew et al., 1994; Yamashita et al., 1996). However, the changes in c-fos and c-jun
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protein concentrations in the early phase appear to be insufficient to achieve epithelial proliferation in the rat uterus. Persico et al. (1990) reported that short-acting estrogens, estradiol and 16α-estradiol, rapidly induce c-fos mRNA without subsequent cell proliferation in the uterus of ovariectomized rats. Bigsby and Li (1994) found that E2 injection rapidly activates c-fos mRNA expression but not epithelial proliferation, in the uterus of immature rats pretreated with progesterone for 2 days.

Since it has been found that c-Jun is capable of forming dimers, AP-1, with other jun family proteins, Jun-B and Jun-D, and with fos family proteins, c-Fos, Fos-B, Fra-1 and Fra-2, that AP-1 regulates the transcription of a variety of genes, both positively and negatively, by binding AP-1 sites and cAMP-responsive elements, and that a complex of ERs and c-Jun/c-Fos cooperatively activates the AP-1 sites of some genes (Hai and Curran, 1991; Ryseck and Bravo, 1991; Webb et al., 1995), it would seem that changes in the balance of the AP-1 dimer comprising the jun and fos oncprotein families may play an important role in epithelial proliferation. Since growth factors such as epidermal growth factor and insulin-like growth factor are known to be induced by E2 stimulation and to elicit growth of mouse uterine epithelium in vivo and in vitro (Tomooka et al., 1986; Murphy and Ghahary, 1990; Nelson et al., 1991; Ignar-Trowbridge et al., 1993; O'Malley et al., 1995), cross-talk between protooncogene expression and growth factor signaling pathways may also be involved in the growth and cytodifferentiation of uterine cells after estrogenic stimulation.

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