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MURINE PROGESTERONE RECEPTOR EXISTS PREDOMINANTLY AS THE 83-KILODALTON 'A' FORM

W. SCHNEIDER,[1] C. RAMACHANDRAN,[1] P. G. SATYASWAROOP[2] and G. SHYAMALA[1,3]*

[1]Lady Davis Institute for Medical Research of the Sir Mortimer B. Davis, Jewish General Hospital, Montreal, Quebec, [2]Department of Obstetrics and Gynecology, Hershey Medical Center, Pennsylvania State University, Hershey, Pennsylvania and [3]Division of Cell and Molecular Biology, Lawrence Berkeley Laboratory, University of California, Berkeley, California, U.S.A.

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Summary—Progesterone receptors (PgR) are known to exist in two molecular forms commonly designated as 'A' and 'B' forms, and the relative ratio of these two forms has been shown to vary among species. Although the rodent systems were some of the earliest experimental systems used to examine the regulation of PgR, as yet very little is known concerning the molecular composition of PgR in this species. Accordingly, to define the relative ratio of 'A' and 'B' forms in murine PgR, we have analyzed tissue extracts from normal, ovariectomized, and estradiol treated animals by photoaffinity labeling and immunoblotting techniques using a variety of anti-PgR antibodies. Under all experimental conditions, two forms of PgR with approximate molecular weights of 115 kDa ('B' form) and 83 kDa ('A' form) were found. In all tissues examined, the 83 kDa 'A' form was predominant, and this was independent of the hormonal status of the animal and different buffers used to prepare tissue extracts. In uterus the ratio of 'A' to 'B' was 3:1, in vagina it was 2:1, and in mammary glands it more closely resembled the uterus. This leads us to conclude that murine PgR exists predominantly as the 83-kDa 'A' form which may represent a general characteristic of rodent PgR. In this species there may also be some tissue specificity with regard to the absolute ratio of the two forms of PgR.

INTRODUCTION

The progesterone receptor (PgR) belongs to the superfamily of steroid receptors which function as transcriptional factors and regulate gene expression in their target tissues. Among the various members of this family, PgR is somewhat unique in that it has been shown to exist in two different molecular forms in tissue extracts from various species with the reported molecular weights ranging from 101 to 120 kDa for the large form commonly designated as the 'B' form and 79–94 kDA for the smaller 'A' form [1–9]. The ratio of the two forms has been shown to differ among species: the 'A' form has been shown to exist in equimolar ratio to the 'B' form in avian and human PgR [2, 3], appears to be absent in rabbits [4] and predominant in rodents [6]. Based on the evidence that (a) steroid receptors in tissue extracts may undergo artefactual cleavage and degradation [10]; (b) because only the larger form of PgR corresponding to the 'B' protein has found with rabbit PgR [4]; and (c) *in vitro* translation of cDNA derived mRNA yield only products corresponding to the 'B' but not the 'A' form [11], the argument that the 'A' form of PgR simply represents an *in vitro* proteolytic degradation product of the 'B' form [4] still persists [11].

Although the rodent systems were some of the earliest experimental systems used to examine the regulation of PgR [12], there is very little known concerning the molecular composition of PgR in these species with the exception of one documentation with rat PgR [6]. Since (a) our laboratory is involved in studying the estrogenic and developmental regulation of murine PgR and its functionality [13, 14]; (b) it has been reported that the relative ratio of 'A' and 'B' forms of PgR can change upon estrogen (E_2) withdrawal [15] and during breeding seasons [9, 16]; and (c) the 'A' and 'B' forms may differ in their relative ability to activate specific genes *in vitro* [17, 18], we have performed a detailed investigation on the two molecular forms of murine PgR with respect to its relative distribution in various tissue extracts. These studies reveal that in contrast to avian, human

*Author to whom correspondence should be addressed.

and rabbit PgR, murine PgR exists predominantly as the 'A' form, and also that within this species there may be some differences among the target tissues with regard to the absolute ratio of 'A' to 'B' forms of PgR.

MATERIALS AND METHODS

Tissue, steroids and antibodies

Uteri, mammary glands and vagina were obtained from 2–5-month-old Balb/c mice. Where indicated, ovariectomy has been performed seven days before subcutaneous injection of 1 μg estradiol-17β as a solution in 1% ethanol in saline. [17α-methyl-^{3}H]R5020 (17α,21-dimethyl-19-nor-pregna-4,9-diene-3,20-dione) (SA 87 Ci/mmol), as well as radioinert R5020, were purchased from New England Nuclear Corp. (Boston, Mass). Mouse monoclonal anti-PgR antibodies hPRa-4, 5, and 7 raised against human PgR have been previously described [19]. Mouse monoclonal antibodies αPR6 and αPR13 raised against avian PgR [20] and RAP2, an antiserum prepared against a synthetic peptide (peptide 2) corresponding to the DNA binding region of the human glucocorticoid receptor [21], were provided by Dr D. O. Toft.

Preparation of cytosolic extracts

Unless otherwise indicated, tissues were homogenized in a buffer containing 10 mM Tris, 1.5 mM EDTA, 1 mM dithiothreitol, 10% (vol/vol) glycerol, pH 7.4 (TEDG) to which leupeptin (48 μg/ml), bacitracin (100 μg/ml), aprotinin (77 μg/ml) and pepstatin (1 μg/ml) had been added (TEDG-I). In some experiments, where indicated, the TEDG-I buffer also contained at specified concentrations [8] benzamidine, PMSF and molybdate (TEDG-II). The homogenate was centrifuged for 1 h at 105,000 *g* at 4°C, and the supernatant was immediately frozen in liquid nitrogen and stored at −70°C. Mammary glands were homogenized in TEDG-I buffer and enriched for PgR by phosphocellulose column chromatography and ammonium sulfate precipitation as described previously [14].

Steroid binding assays and photoaffinity labeling of cytosolic extracts

PgR in the cytosol was labeled by incubating for 1 h on ice with 20 nM of [^{3}H]R5020 alone for total binding, or with [^{3}H]R5020 and a 100-fold excess of R5020 for non-specific binding. In both cases a 100-fold excess of unlabeled dexamethasone was added to block glucocorticoid binding sites. For photoaffinity labeling studies, labeled cytosols were irradiated with u.v. light at 300 nm wave length from a Black-Ray lamp model XX-15 (Ultraviolet Products, San Gabriel, Calif.) at a distance of 15 cm for 30 min. All other procedures pertaining to these assays have been described in detail by our laboratory previously [14].

Western blot analyses

Proteins were dissolved in sample buffer and electrophoresed as described by us previously [14]. The antigen-antibody complexes were identified by incubation with rabbit anti-mouse IgG antibodies to amplify the signal (for mouse monoclonals), followed by [^{125}I]protein A (0.1 μCi/ml), or by using a commercially available kit containing biotinylated anti-rabbit IgG antibody and avidin/biotinylated peroxidase according to the manufacturer's instructions (Vectastain kit). Protein molecular weight markers were obtained from different suppliers (Sigma Chemical Co., BRL), and their respective positions are indicated on the figures.

RESULTS AND DISCUSSION

Photoaffinity labeling with [^{3}H]R5020 of uterine cytosols prepared from non-ovariectomized mice, or from ovariectomized mice given either a single injection of estradiol for varying lengths of time or multiple injections of estradiol, followed by fractionation on SDS polyacrylamide gels, revealed in all cases two radioactive bands on the fluorogram (Fig. 1A, C). These bands were not present in parallel samples incubated with a 100-fold excess of unlabeled R5020 prior to u.v. irradiation. Densitometric scanning of representative samples (Fig. 1B) reveals that these two bands, a major one corresponding to 83 kDa and a minor one corresponding to 115 kDa, are present approximately in a ratio of 3:1. It is evident from both Fig. 1B and a summary of all the scanning data presented in Table 1 that the ratio of 3:1 observed between the 83 and 115 kDa form of PgR remains unchanged between non-ovariectomized and ovariectomized uteri, and also in ovariectomized uteri exposed to estradiol for various lengths of time. The non-specific band corresponding to ~60 kDa present in all samples and not eliminated with excess unlabeled R5020 most likely corresponds to serum albumin.

Most studies documenting the ratio of A:B forms of PgR in various species have used photoaffinity labeling experiments to identify the two receptor forms. In this technique the criterion used for receptor identification is its ability to bind the steroid in a saturable manner [2] and therefore reflects its steroid binding capacity. To verify if the observed ratio of the steroid binding capacity of the two forms corresponds to the ratio of the two receptor proteins, we also performed immunological analyses using a variety of anti-PgR antibodies which do not rely on steroid binding as the immunogenic domains of the receptor are generally present towards the amino terminus [22, 23], whereas the steroid binding domain of PgR is represented by the carboxy terminus [22, 24, 25]. In the initial immunoblot experiment using uterine cytosol from non-ovariectomized mice and using hPRa, 4, 5 and 7, two antibody specific bands could be detected in the region of 115 and 83 kDa respectively (Fig. 2A, compare lanes 1

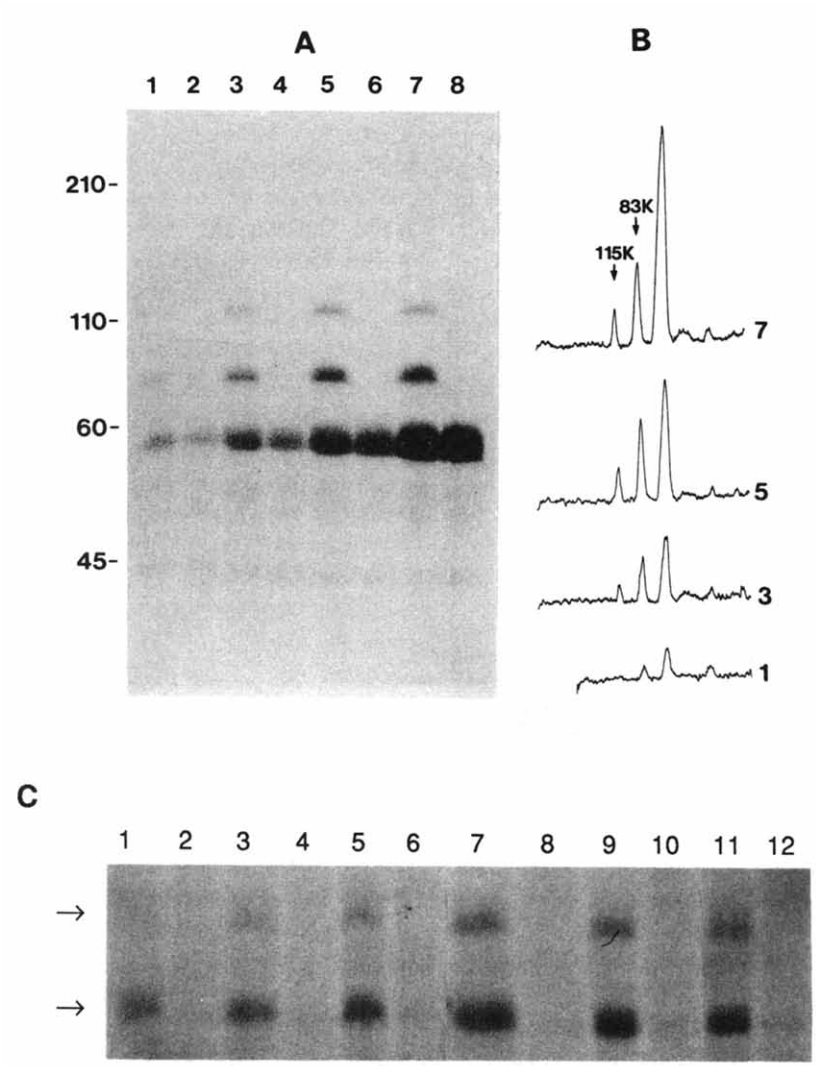

Fig. 1. Photoaffinity labeling of uterine PgR with [^{3}H]R5020. cytosols were incubated with 50 nM [^{3}H]R5020 in the absence (odd lane numbers) or presence (even lane numbers) of a 100-fold excess of unlabeled R5020, u.v.-irradiated and run on SDS–polyacrylamide gels. Gels were soaked in EN^{3}HANCE, dried and exposed to X-ray film. Panel A shows an autoradiogram where lanes 1–6 correspond to animals which had been ovariectomized and injected with either saline (lanes 1, 2; PgR concentration in cytosol = 2.2 nM), or once with estradiol for 24 h (lanes 3, 4; PgR = 5.8 nM), or three times with estradiol at 24 h intervals (lanes 5, 6; PgR = 12.5 nM). Lanes 7, 8 correspond to cytosol from normal nulliparous animals (PgR = 15.0 nM). The position of molecular weight standards is indicated on the left ($\times 10^{-3}$). Panel B represents the densitometric scans of lanes 1, 3, 5, 7 shown in A. Panel C represents ovariectomized animals injected with saline (lanes 1, 2) or estradiol for 4 (lanes 3, 4), 8 (lanes 5, 6), 12 (lanes 7, 8), 18 (lanes 9, 10) and 72 h (lanes 11, 12). Only the region of the autoradiogram corresponding to the 115 kDa and 83 kDa band of PgR (arrows on the left) is shown.

and 5); the 83 kDa band was more prominent. The same profile was also seen with PgR prepared in a buffer containing additional inhibitors previously shown to minimize proteolysis and thus to eliminate the generation of the smaller 'A' form of PgR in quail oviduct [8] (Fig. 2A, compare lanes 1 and 6). Figure 2B shows that there is a linear relationship between the amount of PgR loaded in each lane (as measured by steroid binding) and the immunoreactive PgR corresponding to the 115 and 83 kDa forms. In addition it also demonstrates that the amount of immunoreactive 83 kDa form of PgR is approximately three fold greater than that of the immunoreactive 115 kDa form. Two immunoreactive bands corresponding to 115 and 83 kDa were also apparent when instead of hPRa 4, 5 and 7, the rabbit antiserum RAP2 was used (Fig. 2A, lane 9) which again revealed a ratio of 3:1. The two bands were absent in samples which had been incubated with RAP2 in the presence of synthetic peptide 2, the peptide used for obtaining the rabbit anti-serum. (Fig. 2A, lane 8). Lanes 8 and 9 also show a variety of other non-specific bands, a phenomenon also observed with PgR from other tissues and species when RAP2 is used for the detection of immunoreactive PgR [21].

The profiles of immunoreactive PgR present in uterine cytosols obtained from mice treated with E_2 are shown in Fig. 3. By using hPRa, 4, 5 and 7, we find again a linear relationship between the amount of PgR present in cytosols as estimated by steroid binding and the corresponding levels present as immunoreactive 115 and 83 kDa forms. Figure 3 also shows that the 83 kDa form is approximately three times more prevalent than the 115 kDa form. Experiments similar to those described in Fig. 3 but using antiserum RAP2 (data not shown) also show a constant ratio of 3:1 between the 83 and the 115 kDa form (Table 1).

Finally, to determine if the ratio between the two forms of murine PgR observed with the uterus also holds true for other target tissues, we analyzed by photoaffinity labeling the ratio of the 'A' to the 'B' form of PgR in the mammary gland and vagina. These data, also shown in Table 1, reveal that as with uterus in both mammary glands and vagina, the 'A' form represents the more abundant form. However, as compared to the uterus in which the ratio of 'A' to 'B' was found to be 3:1, it was 2:1 in the vagina. Due to the relatively low levels of PgR in the vagina, a detailed analysis of the ratios of the two forms by immunological techniques

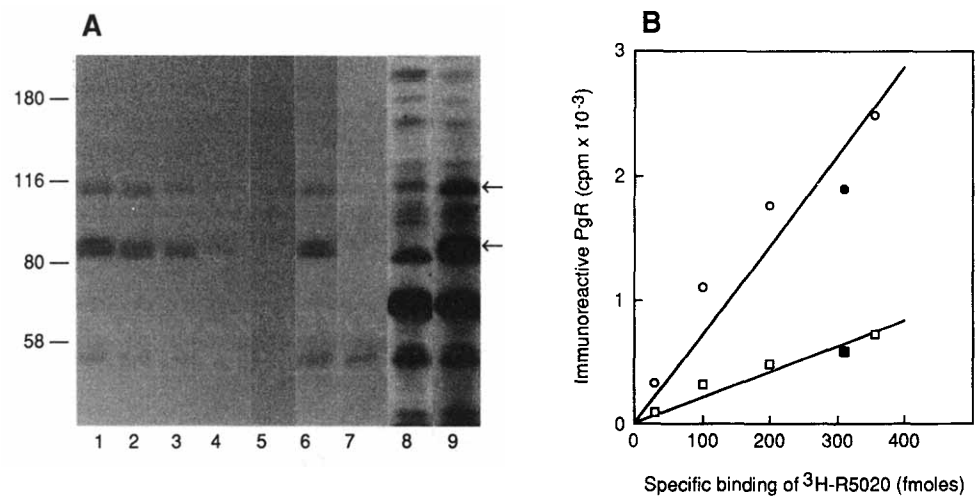

Fig. 2. Immunoblot detection of 115 and 83 kDa forms of PgR from non-ovariectomized animals with antibodies hPRa 4, 5, and 7 and RAP2. Panel A: uterine cytosols from non-ovariectomized animals prepared in TEDG-I buffer were either loaded full strength (lanes 1, 5) or at decreasing concentrations (lanes 2–4) on SDS gels together with aliquots of cytosols prepared in TEDG-II buffer containing benzamidine, PMSF and molybdate (lanes 6, 7), and blotted onto nitrocellulose. Strips were either left as is (lanes 5 and 7) or probed with antibodies hPRa 4, 5 and 7 (lanes 1–4, 6), incubated with [^{125}I]protein A and autoradiographed as described in text. Independent blots of uterine cytosols in TEDG-I were incubated with RAP2 antiserum either in the presence (lane 8) or absence (lane 9) of competing synthetic peptide 2, followed by [^{125}I]protein A as described in text. The arrows indicate the position of the 115 and 83 kDa form of the PgR. The position of molecular weight standards and their sizes ($\times 10^{-3}$) are indicated on the left. Panel B: The 115 kDa bands (squares) and 83 kDa bands (circles) from lanes 1–4 (open symbols) and lane 6 (closed symbols) were cut from the blot, counted and their intensities plotted against the amount of PgR loaded per lane as measured by steroid binding assay.

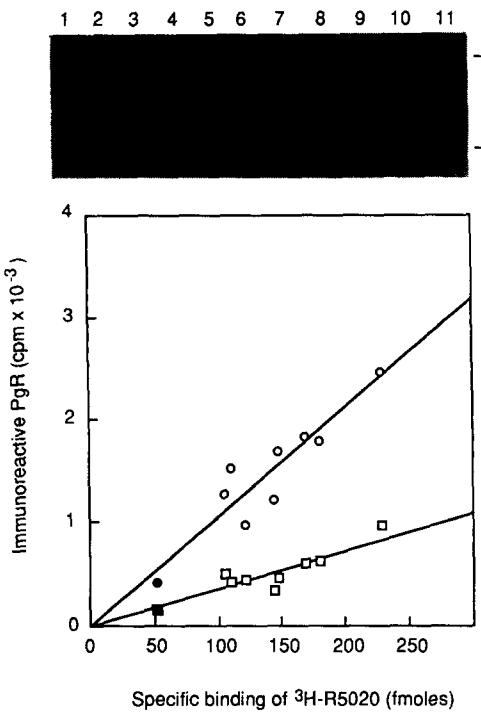

Fig. 3. Immunoblot detection of 115 and 83 kDa forms of PgR from ovariectomized animals injected with estradiol. Cytosols from animals injected with saline (lane 1, closed symbols) or estradiol for 4, 8, 12, 18, 24, 36, 48, 72 h (lanes 2–9, open symbols), or two to three times with estradiol in 24 h intervals (lanes 10, 11) and processed for immunoblot analysis, probed with antibodies hPRa 4, 5 and 7 as indicated in text, and autoradiographed as indicated in legend for Fig. 2 (top panels). The intensity of the 115 kDa (squares) and the 83 kDa bands (circles) was plotted against the amount of PgR loaded on the gel as measured by steroid binding assay (bottom panels).

similar to the uterus was not undertaken. However, based on the observations reported in this paper demonstrating a very good correlation between photoaffinity labeling and immunoblot analyses for uterine PgR, and due to the large number of samples analyzed for both tissues, we believe that the ratio of 2:1 for the 'A' to 'B' form obtained for the vagina from photoaffinity labeling experiments most likely represents the authentic ratio. Although the ratio of 'A' to 'B' forms in mammary glands appeared to more closely resemble the uterus, these experiments were done with partially purified receptor preparations since direct analyses of PgR in cytosols was not possible due to the low levels in this tissue. Similar to the photoaffinity labeling experiments, the immunoblots of partially purified receptor preparations from mammary glands also consistently show a major 'A' and a minor 'B' form with antibodies hPRa 4, 5 and 7 [14] which also remain unaffected as a function of development. Previously, in immunoprecipitation experiments using αPR6, which recognizes only the 'B' form of PgR, we consistently precipated 25% of the total receptor in the native cytosol from both the uterus and the mammary gland [14]. This suggests that the ratio of 'A' to 'B' forms of PgR may indeed be similar between the uterus and the mammary glands.

To summarize, from the combined evidence from photoaffinity labeling and immunoblot analyses, we believe that the 115 and the 83 kDa forms of murine uterine PgR corresponded to the 'B' and 'A' proteins. And since in all the experiments summarized in Table 1, we consistently obtained a ratio of 3:1 for the 'A' and 'B' proteins in the uterus, independent of the technique or antibody used, independent of the hormonal status of the animals and also independent of the buffer used for homogenisation (Fig. 2), we believe that the 'A' form of murine uterine PgR does not represent a product of artefactual proteolysis of the larger 'B' form *in vitro*. The observed ratio of 3:1 for 'A' and 'B' form of murine uterine PgR is similar to that reported for rat uterine PgR [6] and different from human [3], avian [2] and rabbit PgR [4]. As such the existence of PgR predominantly as the 'A' form may reflect a general characteristic of rodent PgR. It also appears that within this species there may be some differences among the various target tissues with regard to the absolute ratio of 'A' to 'B' form. The 'A' and the 'B'

Table 1. A summary of the ratios obtained for the 'A' and 'B' form of murine PgR

		Method used for quantitation of subunit ratio		
Tissue	Hormonal state	Photoaffinity labelling	Immunoblotting using antibodies hPRa 4, 5 and 7	RAP2
Uterus	Non-ovariectomized	3.03 ± 0.22 (11)	3.13 ± 0.22 (7)	3.12 ± 0.35 (3)
Uterus	Ovariectomized	3.37 ± 0.49 (3)	2.78 ± 0.30 (7)	Not done
Uterus	Ovariectomized + estradiol	2.90 ± 0.13 (14)	2.96 ± 0.16 (15)	2.94 ± 0.23 (5)
Vagina	Non-ovariectomized	1.94 ± 0.10 (7)	Not done	
Vagina	Ovariectomized + estradiol	2.03 ± 0.17 (8)	Not done	
Mammary gland	Non-ovariectomized	2.42 ± 0.30 (3)	Not done	

Detailed experimental conditions were as described in text and in legends to Figs 1–3. The data represent mean ± SEM for the number of experiments given in parenthesis.

forms of PgR have been shown to differ in their relative ability to activate target gene(s) [17, 18] which suggests that differences in the ratio of 'A' to 'B' forms of PgR among the various target tissues may be related to differences in their sensitivity to progesterone. Indeed it has been observed that while progesterone can antagonize the E_2 mediated DNA synthesis in the uterine epithelium, it is without such effect in the vaginal epithelium [26].

We have recently cloned and sequenced the cDNA encoding the murine PgR [27] and are in the process of isolating the 5'-flanking sequences. These ongoing studies upon completion should permit a detailed analysis regarding the mechanims responsible for the predominance of the 'A' form of PgR in murine tissues and for the possible existence of additional tissue specific regulations.

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