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Purification and Immunochemical Characterization of the Cytoplasmic Androgen-Binding Protein of Rat Liver†

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ABSTRACT: The cytoplasmic androgen-binding (CAB) protein of the male rat liver has been implicated to play a role in the androgen-dependent regulation of α_{2u}-globulin synthesis. The liver of the adult male rat contains about 50 fmol of specific high-affinity androgen-binding activity per milligram of total cytosolic protein. Photoaffinity labeling with [^{3}H]R-1881 followed by SDS–polyacrylamide gel electrophoresis and autoradiography shows that the CAB is a 31-kilodalton protein. By means of DEAE-cellulose chromatography and preparative SDS–polyacrylamide gel electrophoresis, we have purified the CAB protein to electrophoretic homogeneity and have raised polyclonal rabbit antiserum that is monospecific to this protein. In the sucrose density gradient, the antiserum reacted with the androgen-binding component of the male liver cytosol prelabeled with tritiated dihydrotestosterone. Western blot analysis of the liver cytosol showed that the antiserum recognizes only the 31-kDa androgen-binding component. Such immunoblotting also showed that unlike the young adult, the androgen-insensitive states during prepuberty and senescence are associated with a marked reduction in the hepatic concentration of the immunoreactive CAB protein. No immunochemical cross-reactivity between CAB and another androgen-binding component of M_r 29K (which is associated with androgen insensitivity during prepuberty and senescence) was observed. The latter finding favors the possibility that 31- and 29-kDa androgen-binding components may have distinct sequence structure.

A number of hepatic genes are differentially expressed in the male and female liver (Roy & Chatterjee, 1983; Gustafsson et al., 1983). In addition, synthesis of these sex-dependent proteins undergoes changes during maturation and aging (Roy et al., 1983a; Richardson et al., 1987). In the rat, the male-specific urinary protein α_{2u}-globulin is one of the major sexually dimorphic secretory proteins of hepatic origin (Roy et al., 1983a; Dolan et al., 1983). α_{2u}-Globulin is normally absent in the liver of female rats, and, in the case of males, its hepatic synthesis begins at puberty (~40 days) and reaches a peak level at about 75–85 days; the protein level

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starts to decline after 150 days of age. Beyond 750–800 days, α_{2u}-globulin in the liver becomes almost undetectable (Roy et al., 1983a,b). Both in the whole animal and in the in vitro liver perfusion system, hepatic synthesis of α_{2u}-globulin can be induced by the androgen (Roy & Neuhaus, 1967; Murty et al., 1987). Androgen-mediated synthesis of α_{2u}-globulin in the rat liver is correlated with the presence of a low-capacity, moderate-affinity ($K_d = 10^{-8}$ M) cytoplasmic androgen-binding (CAB) protein that displays a high degree of androgen specificity (Roy et al., 1974). Androgen administration to prepubertal (<40 day) and senescent (>750–800 day) male rats fails to induce α_{2u}-globulin (Roy et al., 1983a). The androgen-insensitive state during this life period is correlated with an absence of the CAB protein. Similar correlative data under different endocrine conditions and colocalization of CAB in the specific α_{2u}-globulin-producing hepatocytes have indicated a regulatory role for this androgen-binding protein in the androgen-dependent synthesis of α_{2u}-globulin.

Photoaffinity labeling of the liver cytosol with [^{3}H]-methyltrienolone ([^{3}H]R-1881), a synthetic androgen, followed by SDS–polyacrylamide gel electrophoresis has aided in the molecular characterization of the CAB protein (Sarkar et al., 1987). This male-specific androgen binder has a molecular weight of 31 000. The photoaffinity labeling data also showed that disappearance of the 31-kDa androgen biinder from the liver of androgen-insensitive animals, such as prepubertal and senescent males and testicular feminized male (Tfm), is associated with the concomitant appearance of another androgen-binding component of 29 000 daltons. These results suggested a molecular conversion of the 31-kDa CAB to a 29-kDa form associated with the transition from androgen-sensitive to androgen-insensitive states. In order to understand the biochemical role of CAB in androgen action and to examine its relationship with the 29-kDa androgen-binding protein, we have undertaken a study for further characterization of the CAB protein. This report describes purification and immunological characterization of the 31-kDa androgen-binding protein.

Experimental Procedures

Preparation of Liver Cytosol and Photoaffinity Labeling. Liver cytosol was prepared according to the procedure described previously (Sarkar et al., 1987). Briefly, the liver was flushed with 0.15 M NaCl at 4 °C to remove the blood and was homogenized in a buffer containing 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.4, 2 mM ethylenediaminetetraacetic acid (EDTA), 3 mM dithiothreitol (DTT), 20 mM sodium molybdate, 2 mM phenylmethanesulfonyl fluoride, 0.5 mM leupeptin, and 20% glycerol. The cytosolic fraction was obtained by centrifuging the homogenate at 140000*g* for 120 min at 4 °C. Cytosol was diluted in the homogenizing buffer to a protein concentration of 5 mg/mL and incubated with 58 nM [^{3}H]R-1881 (New England Nuclear, Boston, MA) at 0 °C for 60 min. The incubation mixture was then exposed to the ultraviolet light for 15 min via the photolysis setup described by Katzenellenbogen et al. (1974) with modifications (Sarkar et al., 1987). The labeling efficiency under this condition varied from 10% to 15% (Sarkar et al., 1987). The protein-bound radioactivity was separated from the free [^{3}H]R-1881 by gel filtration on a Sephadex column and the labeled cytosol used for further purification of CAB.

Polyacrylamide Slab Gel Electrophoresis and Western Transfer. The protein samples were dissolved in a buffer containing SDS and subjected to SDS–PAGE on 12% gels as described by Laemmli (1970). The electrophoretically separated proteins were transferred to nitrocellulose filter papers using the Genie electroblotting apparatus (Idea Scientific Inc., Corvallis, OR). In the case of cytosolic proteins that are covalently labeled with [^{3}H]R-1881, the filter was dried, sprayed with EN^{3}HANCE (New England Nuclear, Boston, MA), and exposed to Kodak XAR-5 X-ray film. The autoradiograms were aligned with the nitrocellulose filter, and the portion of the filter corresponding to the labeled CAB band was excised for further processing.

In experiments involving antibody binding after transfer of proteins onto the nitrocellulose paper, immunoreaction was carried out according to the procedure of Burnette (1981). Briefly, the nitrocellulose filter was incubated at 37 °C for 30 min with a buffer containing bovine serum albumin, followed by incubation with rabbit antiserum against CAB for 2 h at room temperature. At the end of this period, the paper was washed extensively and treated with ^{125}I-labeled protein A for 30 min. The free radioactivity was washed and the filter dried, covered with plastic film, and autoradiographed at −70 °C.

Preparation of Antibody. Each rabbit received 20 μg of purified CAB protein, which was first dissolved in 1.0 mL of phosphate-buffered saline and subsequently emulsified with an equal volume of Freund's complete adjuvant. The mixture was divided into 0.5-mL portions and injected once every 2 weeks subcutaneously into four different areas of the back. Two weeks after the last of four such injections, a booster dose of the protein in incomplete Freund's adjuvant was administered. One week after the final injection, blood was collected by arterial puncture.

Sucrose Density Gradient Analysis. Aliquots of liver cytosol (100 μL) were incubated for 60 min at 0 °C with 50 nM [^{3}H]dihydrotestosterone (DHT) according to the procedure previously described (Roy et al., 1974). Cytosol samples prelabeled with DHT were incubated further for 4 h at 0 °C with either 100 μL of anti-CAB rabbit antiserum or 100 μL of preimmune rabbit serum. Samples (200 μL) were then loaded on a 5–15% sucrose gradient (in 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, and 6 mM DTT) and centrifuged for 20 h in a Beckman SW41 rotor at 40 000 rpm, 4 °C.

Results

Purification of CAB. Radioactively labeled R-1881 covalently attached to the 31-kDa CAB protein was used to monitor the different steps in its purification. Cytosol prepared from adult (100–150 days) male rat livers was used as the starting material. All operations were carried out at 4 °C unless otherwise specified. For each batch of preparation, 100 mL of cytosol (5 mg/mL protein, 500 mg total; 5 mL per reaction tube) was labeled with [^{3}H]R-1881 by UV irradiation and separated from unbound hormone by gel filtration on Sephadex G-25. Labeled cytosol samples containing approximately 5×10^5 dpm/mg of bound radioactivity were diluted with an equal volume of unlabeled cytosol (25 mg/mL protein). The total mixture was loaded on a DEAE-cellulose (DE-52) column (2-cm diameter, 55 cm long) preequilibrated with 3 volumes of TED buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, and 6 mM DTT).

After the column was loaded, it was washed with 200 mL of TED buffer and the eluant discarded. Proteins bound to the DE-52 column were eluted with a linear gradient of KCl (0–1.0 M in TED buffer) and 2.5-mL fractions collected for further analysis. Fifty-microliter aliquots from each fraction were routinely assayed for radioactivity by liquid scintillation spectrometry and for protein content by the optical density at 280 nm after appropriate dilution. In the initial phase of

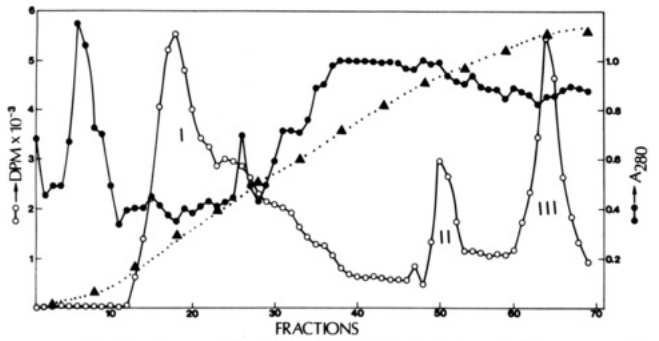

FIGURE 1: Elution profile of the R-1881-labeled male liver cytosolic proteins from a DEAE-cellulose column. Male liver cytosol was photolabeled with tritiated R-1881. After removal of uncoupled steroid by gel filtration, the cytosol was loaded on a DE-52 column. The pattern shows elution of the DE-52-bound material with a linear 0–1.0 M KCl gradient [(▲) full range 0–1.0 M]. Each fraction contains 2.5 mL of the eluant. Radioactivity (○); absorbance at 280 nm (●). The major radioactivity peaks are marked with Roman numerals I, II, and III.

procedural standardization, 50-μL aliquots from each fraction were also used for monitoring the CAB protein by SDS–polyacrylamide slab gel electrophoresis and autoradiography. For this assay, aliquots of the gradient fractions were precipitated with 5 volumes of acetone at 0 °C, and the precipitate was dissolved in 3% sodium dodecyl sulfate, 5% β-mercaptoethanol, and 10% sucrose and loaded on a 12% polyacrylamide slab gel as described by Laemmli (1970). After electrophoresis, the gel was treated with EN^{3}HANCE, dried, and autoradiographed. Figure 1 shows the elution profile of the total protein and the [^{3}H]R-1881-labeled radioactivity. These profiles reveal that the protein-bound radioactivity can be resolved into three major peaks. Electrophoretic analysis of the bound radioactivity in different fractions eluted by the salt gradient showed that the 31-kDa male-specific CAB protein can be resolved from the other R-1881-binding component and is largely confined within the material eluting at the radioactive peak II (fractions 48–53 in Figure 1). Figure 2 shows a typical electrophoretic autoradiogram of the protein fractions within radioactivity peaks II and III. Although the material eluting with peak II contained only one (31 kDa) radioactive band, staining of the gel for total proteins with silver reagent (Merril et al., 1981) showed a number of other contaminating protein bands belonging to a broad range of molecular weights. Despite the presence of such contaminating proteins, the DE-52 step is effective in resolving all other R-1881-binding proteins from the 31-kDa CAB protein.

For the next step of purification, we took advantage of the size differences among the proteins eluting within the second peak. Peak II fractions were pooled and dialyzed against 25 volumes of TED buffer with four changes of the buffer over a period of 22 h. The dialyzed sample was freeze-dried, dissolved in 35 mL of Laemmli's sample buffer, and subjected to SDS–polyacrylamide slab gel electrophoresis (12% gel). The electrophoretically separated proteins were transferred to nitrocellulose paper by electroblotting. The nitrocellulose paper was then sprayed with EN^{3}HANCE, dried, and exposed to Kodak XAR-5 X-ray film for 7 days. After the X-ray film was developed, the 31-kDa radioactive band was carefully matched with the nitrocellulose paper and the corresponding area of the paper excised. Excised nitrocellulose strips (DE-52 fraction II) were extracted for 1 h with 70% formic acid at 4 °C with vigorous shaking (Anderson, 1985). Formic acid was removed from the CAB protein by freeze-drying, and this procedure yielded approximately 5 μg of purified CAB from 3 g of total liver cytosolic protein. Labeling of the protein

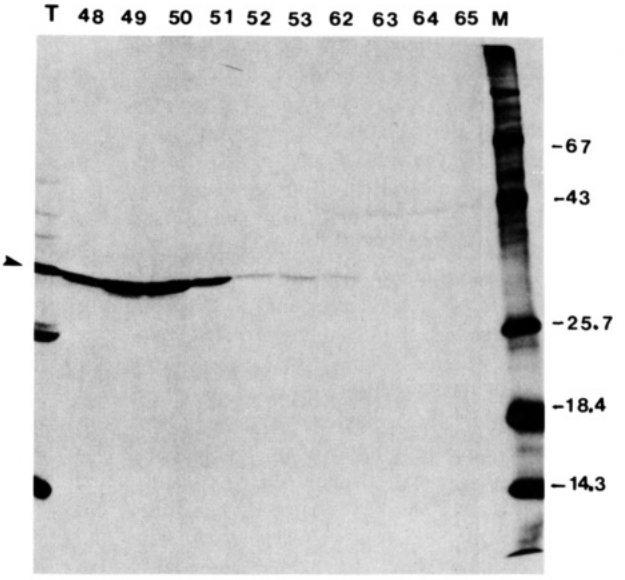

FIGURE 2: Electrophoretic distribution of R-1881-labeled proteins in fractions eluted from the DE-52 column. The picture shows the autoradiographic pattern of a polyacrylamide slab gel of DEAE fractions 48–65 as indicated in Figure 1 (radioactivity peaks II and III). Lanes are labeled as follows: T, total unfractionated cytosol; M, molecular weight markers (specific bands are labeled × 10^{-3}). The numbered lanes correspond to fraction numbers shown in Figure 1. The arrowhead points to the 31-kDa CAB band.

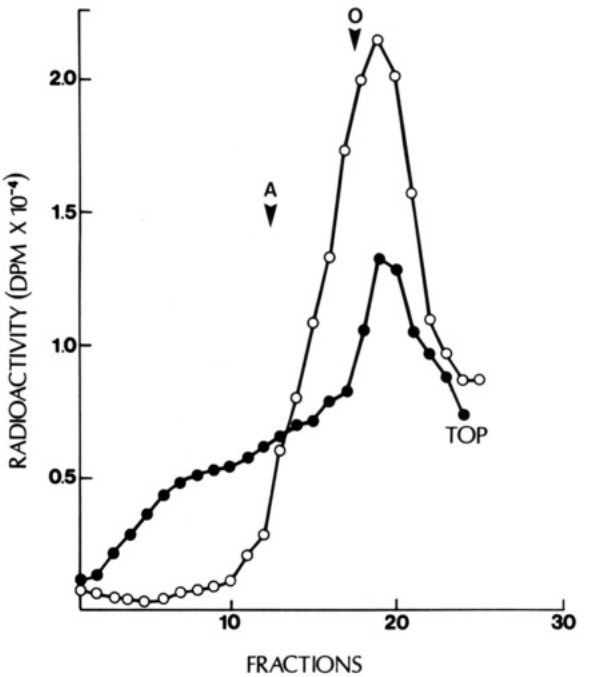

FIGURE 3: Antibody-mediated displacement of the cytoplasmic androgen-binding protein on a sucrose density gradient. Male rat liver cytosol was prelabeled with 50 nM [^{3}H]DHT and subsequently treated with either preimmune (○) or immune (●) serum. Samples were layered on a 5–15% sucrose gradient (in 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, and 6 mM DTT) and centrifuged for 20 h in a Beckman SW41 rotor (4 °C, 198000*g*). Arrowheads show positions of ovalbumin (O) and bovine serum albumin (A) marker proteins.

bound to the nitrocellulose strip with ^{125}I through chloramine-T oxidation (Plagens & Traub, 1986), followed by formic acid extraction, showed that the method of extraction is 80–90% efficient.

Determination of Antibody Activity against the CAB Protein in the Sucrose Density Gradient. After the fourth injection of the antigen, as described under Experimental Pro-

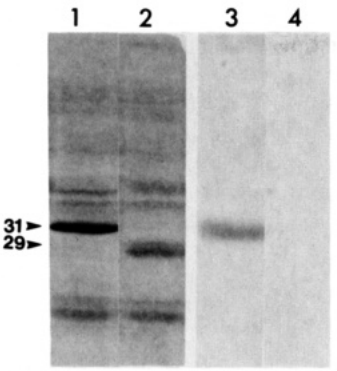

FIGURE 4: Immunoidentification of the male-specific 31-kDa androgen-binding component as the CAB protein. The picture shows segments of the autoradiogram of the liver cytosol after [^{3}H]R-1881 labeling or immunoreaction with rabbit antiserum to the cytoplasmic androgen-binding protein. Lanes 1 and 2 represent young adult male and female liver cytosol photoaffinity labeled with [^{3}H]R-1881 and autoradiographed after EN^{3}HANCE treatment. Lanes 3 and 4 represent Western-blotted cytosolic proteins from young adult male and female rat liver immunoreacted with anti-CAB antiserum and subsequently treated with ^{125}I-labeled protein A. The 31-kDa band in the male is seen to react with the antibody. Thirty micrograms of total cytosolic proteins was applied to each lane. Exposure time: lanes 1 and 2, 7 days; lanes 3 and 4, 12 h.

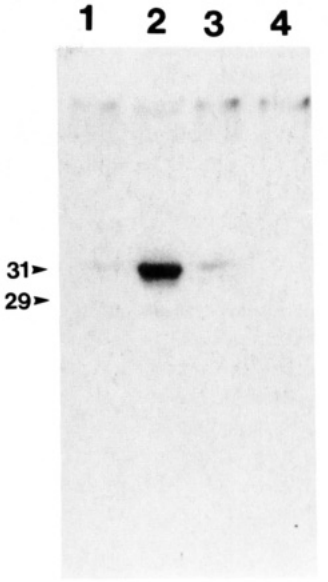

FIGURE 5: Immunoblot of the liver cytosol of rats of different age and sex. Lanes are marked as follows: 1, prepubertal male (30 day old); 2, young adult male (100 day old); 3, senescent males (850 day old); 4, adult female (100 day old). Thirty micrograms of total cytosolic proteins was applied to each lane. The positions of 31- and 29-kDa bands are marked with arrowheads. Apparent immunoreactivity near the sample slot is an experimental artifact which is also observed in lanes with sample buffer alone (no protein lane).

cedures, immunoreactivity of the antiserum to CAB was tested in a sucrose density gradient "peak displacement assay". As illustrated in Figure 3, in contrast to the preimmune serum, the immune serum caused a large displacement of the radioactively labeled R-1881 from the male-specific 3.5S binding peak to heavier aggregates. This result indicates that the purified CAB protein has been able to elicit immunogenic response in the rabbit-producing antibody which can bind to the CAB protein without abolishing its ability to retain the bound hormone. Because of the polyclonal nature of the antibody, the antibody–CAB complex shows a broad distribution within the heavier part of the gradient.

Examination of Antibody Specificity by Immunoblot Analysis. charcoal-binding assay with labeled R-1881 has shown that, unlike female liver cytosol, adult male liver cytosol contains a high concentration of specific androgen-binding activity (Sarkar et al., 1987). Furthermore, the female cytosol did not show any definite 31-kDa-binding band after photoaffinity labeling with R-1881 nor did it produce the 3.5S CAB peak in the sucrose density gradient analysis (Roy et al., 1974; Sarkar et al., 1987). Photoaffinity labeling studies have also shown that disappearance or absence of the 31-kDa androgen-binding band is always associated with the concomitant appearance of a 29-kDa-binding component. It was therefore of interest to perform immunoblot analysis of the liver cytosol with the ^{125}I-labeled antibody, not only to monitor the specificity of the antiserum but also to test the possible immunological relationship between the 31-kDa and the 29-kDa androgen-binding proteins.

Figure 4 shows the composite of two sets of autoradiograms prepared with the same cytosol samples from a young adult male and female rat liver autoradiographed following either photoaffinity labeling with [^{3}H]R-1881 or immunolabeling with the antibody and ^{125}I-labeled protein A. In the photoaffinity-labeled samples, the dominant 31-kDa androgen-binding band in the male and its absence in the female are seen with the concomitant appearance of the 29-kDa band. However, the immunoblot pattern developed with the ^{125}I-labeled protein A reveals that the antibody recognizes only the 31-kDa male-specific binding component, without any evidence for cross-reactivity with the female-specific 29-kDa band. Thus, these results show that the antiserum raised against the purified protein is monospecific to CAB and does not react to the 29-kDa androgen-binding band.

CAB Protein and Hepatic Androgen Sensitivity. Prelabeling of the cytosolic protein with labeled androgen followed by either sucrose density gradient analysis or photoaffinity labeling has shown that the states of hepatic androgen insensitivity as found in prepubertal, senescent, and testicular feminized males and in normal female rats are correlated with either a marked reduction or a total absence of the biologically active CAB protein (Roy et al., 1974; Sarkar et al., 1987). Availability of the monospecific antiserum to CAB provided the opportunity to examine the possible absence of the CAB antigen in hepatic cytosols derived from these animals. Figure 5 shows the immunoblot pattern of hepatic cytosols from a female rat and male rats of different ages. These results show that as compared to young adult male, livers of both prepubertal and senescent males containing greatly reduced levels of immunoreactive CAB. Furthermore, in the case of female liver, no immunoreactive CAB can be detected. These findings extend our earlier observations based on the androgen-binding assay of the CAB activity and suggest that the decreased androgen sensitivity during prepuberty and senescence is due to a marked reduction of the immunoreactive CAB protein.

Discussion

Assuming that the androgen is bound to the 31-kDa CAB protein in equimolar proportion and about 50 fmol of CAB is present per milligram of cytosolic protein (Sarkar et al., 1987), one can estimate that CAB constitutes only 0.000 155% of the total cytosolic proteins. Although this value can be a gross underestimation due to the possible presence of biologically inactive (i.e., unable to bind the ligand) CAB, such a calculation points to the rarity of the molecule within the cytosolic proteins. Furthermore, this approximation places the cellular concentration of CAB within the range of putative steroid receptor molecules (Evans, 1988). Results presented in this paper show that we have been able to develop a pro-

cedure to purify such a trace protein from hepatic cytosol to a point which allowed us to raise monospecific rabbit antiserum to this androgen-binding protein. The purified CAB protein was able, in the rabbit, to induce antibody which on sucrose density gradient shifted sedimentation of the binding protein to higher aggregates without interfering with androgen-binding activity. Since this is a polyclonal antibody, the reaction product is diffusely distributed toward the bottom of the tube, indicating a soluble antigen–antibody complex containing multiple antigen aggregates. Similar results with polyclonal antibodies against other steroid-binding proteins have also been reported (Birnbaumer et al., 1987).

Western blot data show that the antibody reacts with only a 31-kDa band, which indicates the monospecificity of the antiserum. A gradual decline and ultimate loss of the CAB activity during aging, as determined previously both by sucrose density gradient analyses and by photoaffinity labeling (Roy et al., 1974; Roy 1977), have now been confirmed by the age-dependent loss of the immunoreactive 31-kDa androgen-binding protein. Androgen treatment of prepubertal, senescent, and testicular feminized male rats fails to induce α_{2u}-globulin. In addition, sucrose gradient analysis failed to show any CAB activity in these animals after androgen treatment (Roy et al., 1983a). Androgen treatment of ovariectomized females, on the other hand, first results in the appearance of the CAB protein and, subsequently, androgen sensitivity with the concomitant induction of α_{2u}-globulin (Roy et al., 1974; Roy, 1977).

In the case of female, prepubertal, senescent, and testicular feminized males, photoaffinity labeling with [^{3}H]R-1881 has shown that the 31-kDa CAB is replaced by a 29-kDa androgen binder (Sarkar et al., 1987). We have previously hypothesized that molecular conversion of the 29-kDa androgen binder to the 31-kDa active form (CAB) by posttranslational modification may be involved in imparting androgen sensitivity during maturation and in its reversal during aging (Sarkar et al., 1987). Inability of the polyclonal antiserum to 31-kDa CAB to react with the 29-kDa band does not seem to support this hypothesis. However, it may be mentioned that CAB is weakly immunogenic and, of four rabbits which were injected with the CAB antigen, only one produced the immune response. Thus, there is a distant possibility that the antibody is directed only to a modified domain of the CAB which is different in the 29-kDa-binding protein. Further studies are needed to resolve this issue.

Recently, two groups of investigators have reported molecular cloning and structural elucidation of rat and human androgen receptor genes (Chang et al., 1988; Lubahn et al., 1988). In addition, the amino acid sequence of the androgen receptor deduced from the cDNA sequence indicates three distinct domains. Beginning from the amino-terminal end, these domains include a hypervariable region and a DNA-binding region followed by the androgen-binding segment at the -COOH-terminal end. Presently, we cannot rule out the possibility that hepatic CAB may be related to the androgen-binding domain of the receptor molecule. It is also of interest to note that androgen receptor mRNA is present in the liver of normal but not Tfm mice and that in vitro transcription of the cloned cDNA, followed by translation in the reticulocyte lysate system, produced several discrete protein bands, including a 30–40-kDa androgen-binding protein (Lubahn et al., 1988). In the context of the above findings, it will be of great interest to establish the structural relationship of the CAB with the putative androgen receptor. Such studies are currently in progress.

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