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MOLECULAR CLONING OF RAT LIVER 3α-HYDROXYSTEROID DEHYDROGENASE AND IDENTIFICATION OF STRUCTURALLY RELATED PROTEINS FROM RAT LUNG AND KIDNEY

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Summary—3α-Hydroxysteroid dehydrogenase and related enzymes play important roles in the metabolism of endogenous compounds including androgens, corticosteroid, prostaglandins and bile acids, as well as drugs and xenobiotics such as benzo(*a*)pyrene. Complementary DNA clones encoding 3α-hydroxysteroid dehydrogenase were isolated from a rat liver cDNA lambda gt11 expression library using monoclonal antibodies as probes. A full-length cDNA clone of 1286 base pairs contained an open reading frame encoding a protein of 322 amino acids with an estimated M_w of 37 kD. When expressed in *E. coli*, the encoded protein migrated to the same position on SDS–polyacrylamide gels as the enzyme in rat liver cytosols. The protein expressed in bacteria was highly active in androsterone oxidation in the presence of NAD as cofactor and this activity was inhibited by indomethacin, a potent inhibitor of 3α-hydroxysteroid dehydrogenase. The predicted amino acid sequence of 3α-hydroxysteroid dehydrogenase was related to sequences of several other aldo–keto reductases such as bovine prostaglandin F synthase, human chlordecone reductase, human aldose reductase, human aldehyde reductase and frog lens epsilon-crystallin, suggesting that these proteins belong to the same gene family. Recently, we have found that monoclonal antibodies against 3α-hydroxysteroid dehydrogenase also recognized multiple antigenically related proteins in rat lung, kidney and testis. Further screening of liver, lung and kidney cDNA libraries using these monoclonal antibodies as probes resulted in the isolation of additional five different cDNAs encoding proteins with high degree of structural homology to rat liver 3α-hydroxysteroid dehydrogenase.

INTRODUCTION

3α-Hydroxysteroid dehydrogenase (3α-HSD) was first identified by its activity in converting dihydrocortisone to 3α-tetrahydrocortisone [1]. It was subsequently found to reduce dihydrotestosterone and dihydroprogesterone to their respective 3α-reduced metabolites [2]. A rat liver cytosolic protein of 34 kD exhibiting both 3α-HSD and dihydrodiol dehydrogenase activities has been purified to apparent homogeneity [3]; this protein comprised approx. 1% of the total liver cytosolic proteins in rats.

In addition to its role in the metaboism of steroid hormones, 3α-HSD has been shown to metabolize a wide range of other endogenous substrates. For example, 3α-HSD converts both 7α-hydroxy-5β-cholestane-3-one and 7α,12α-dihydroxy-5β-cholestan-3-one to bile acids and it is potentially involved in the transportation of bile acids in liver [4]. 3α-HSD also catalyzes the dehydrogenation of prostaglandin F2α [5]; the products, prostaglandin E2 and prostaglandin B2, are potential causative agents of inflammatory reaction. Many steroidal and non-steroidal anti-inflammatory reagents, such as indomethacin and aspirin, inhibit 3α-HSD in concentrations similar to those that inhibit cyclooxygenase and phospholipase A2 [6]. 3α-HSD is also involved in the metabolism of many xenobiotics. The intrinsic dihydrodiol dehydrogenase activity in 3α-HSD may contribute to its ability to reduce the mutagenic effects of benzo(*a*)pyrene metabolites in the Ames test [7].

There is conflicting evidence concerning the existence of multiple forms of 3α-HSD in rat liver. Stolz *et al.* [8] separated three bile acid binding proteins which all exhibited 3α-HSD activity. Worner and Oesch [9] separated a purified 3α-HSD into multiple protein bands by isoelectrofocusing. In contrast, Smithgall and Penning [10] detected a single major species of 3α-HSD by two-dimensional gel electrophoresis.

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As an initial step in understanding the relationship between structure and function of 3α-HSD and determining whether multiple isozymes exist, we prepared monoclonal antibodies against 3α-HSD purified from rat liver cytosols and have demonstrated that these monoclonal antibodies recognize multiple structurally related proteins in rat liver, lung, kidney and testis [11]. We have also reported previously the sequence of rat 3α-HSD cDNA and its structural similarity to other aldo–keto reductases [12]. In addition, we have recently isolated five cDNAs encoding proteins similar to 3α-HSD.

MATERIALS AND METHODS

Library screening

Rat liver cDNA libraries derived from liver, lung and kidney in the expression vector, lambda gt11, were screened using supernatants from two hybridomas secreting monoclonal antibodies against 3α-HSD [13]. Fusion proteins adsorbed to nitrocellulose filters were detected by sequential incubation with primary monoclonal antibodies goat anti-mouse IgG–peroxidase and 4-chloronaphthol plus hydrogen peroxide.

Positive plaques were replated at lower titers and rescreened with monoclonal antibodies until all plaques reacted positively. Phage was isolated by the plate lysate method [14] and DNA was extracted after treatment with phenol/chloroform.

Subcloning and sequence analysis

Phage DNA was digested with *Eco* RI, subjected to electrophoresis in a low-melting agarose gel and isolated by binding to glass beads. Recovered DNA fragments were subcloned into the *Eco* RI site of pUC19. Chain termination sequencing was performed on denatured supercoiled plasmid DNA using T7 DNA polymerase [15].

Immunoblotting

Proteins were subjected to electrophoresis on a 8% polyacrylamide–0.1% SDS gel and then electrotransferred to a nitrocellulose filter [16]. The filter was sequentially treated with 3% bovine serum albumin (BSA) in PBS, hybridoma supernatant and goat anti-mouse IgG–horseradish peroxidase conjugate. The proteins interacted with monoclonal antibody were visualized by incubating the filter with a solution containing 4-chloronaphthol and hydrogen peroxide.

Enzymatic assay

Androsterone oxidation was performed as described previously [6]. The assay mixture contained 20 μM androsterone and 1 mM NAD in 100 mM sodium phosphate buffer, pH 7.2. NAD reduction was monitored by the increment of absorption at 340 nm using a Beckman UV/Vis spectrophotometer.

RESULTS

Isolation and characterization of clones encoding 3α-HSD

We previously prepared several monoclonal antibodies against 3α-HSD purified from rat liver cytosols. Using two monoclonal antibodies, 3G6 and 7D3, as probes to screen a female rat liver cDNA library constructed in lambda gt11, we isolated nine clones from approx. 300,000 recombinant phage. The insert sizes ranged from 1.3 to 2.3 kilobases (kb). The size of the corresponding β-galactosidase fusion proteins that were produced varied only slightly when analyzed by SDS–polyacrylamide gel electrophoresis. Digestion of the phage DNA with *Eco* RI yielded two small DNA inserts from most of the clones, indicating the presence of an internal *Eco* RI restriction site. Each clone had one fragment of approx. 850 bp, whereas the other fragment varied in length from 400 bp to 1.3 kb. The complete sequence of one clone, termed pHSD8, consisted of a 966 bp open reading frame, 1 bp of 5′-non-coding region and 320 bp of 3′-non-coding region (Fig. 1). The open reading frame predicts a protein of 322 amino acids with M_w of approx. 37 kD. This is consistent with the M_w (34 kD) estimated by SDS–polyacrylamide gel electrophoresis of the enzyme purified from rat liver. Sequence analysis of the other clones indicated that they contained overlapping 5′-sequences but had varying lengths of 3′-non-coding region. Because poly-(A) tails were not found in any of the clones, it is not clear whether the heterogeneity in the 3′-sequences is due to an artifact created during the generation of the cDNA library or reflects the presence of multiple polyadenylation sites.

A search of GenBank using FASTP and FASTN [17] programs revealed several cDNA sequences encoding aldo–keto reductases that

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A    1-MDSISLRVALNDGNFIPVLGFGTTVPEKVAKDEVIKATKIAIDNGFRHFDSAYLYEVEEEVGQAI-65
B      M PKYQ  E    H M       YA PE PRNRAVEV  L   EA   I      NN  Q  L
C      M PK Q  K    H         RA  E P S ALE   F  EV    V   H  QN  Q
D       MAASC L HT QKM L       WEPGQV AA ---- Y LSV Y  I C AI GN P I E L
E        MAS LL  NCAKM I  L  WKSPPGQVT AV--- V   V Y  I C HV QN N   V
F

A   66-RSKIEDG-TVKREDIFYTSKLWSTFHRPELVRTCLEKTLKSTQQDYVDLYIIHFPMALQPGDIFF-129
B         A  -S              C  FQ QM QPA  SS  KL L       LL       K  ETPL
C         AE -               CNSLQ      PA   S QNL L          S VS K  NK V
D      KEDVPG KA P  EL V     N K H  D EPA R   ADL LE L   LM W Y FER  NP
E      QE LREQ-V    EL IV     C Y EKG  KGACQ   SDLKL  L   L  W TGFK  KE
F                                    L RS RDVGM  L  FLM W VS K S ASD

A  130-PRDEHGKLLFETVDICDTWEAMEKCKDAGLAKSIGVSNFNCRQLERILNKPGLKYKPVCNQVECH-194
B       K  N  VI D   LSA   V                          M
C       K  S   I DS  L H    L          T          HK   K
D       KNAD TICYDSTHYKE  K L ALVAK  VQAL L    S  IDD  SVASV--R AV
E       L  S NVVPSDTN L   A   ELV E  V A  I    HL V M           AV  I
F       S KDKPFIYDNV L A    L AR     VR L      R

A  195-LYLNQSKMLDYCKSKDIILVSYCTLGSSRDKTWVDQKSPVLLDDPVLCAIAKKYKQTPALVALRY-259
B       P       L  F       V  AHSA  TQ H L   PN      E       L   H R    I
C       P       L EF    HE V  A AA  AQLLSE  NSNN     E           H
D       P  A NELIAH QARGLEVTA -P     - RA RDPDE     EE  VL L E  GRS  QIL  W
E       P  T E LIQ  Q  G VVTA SP    P- RP AKPED S   E  RIK   A HNK T Q LI F
F       V    N LHS        V  T SV   H  RN   LSL        ILNKV A  NR S EI M F

A  260-QLQRGVVPLIRSFKPKRIKEPTQVFEFQLASEDMKALDGLNRNFRYNNAKYFDDHPNHPFTDE  -322
B              V AK YNEQ  R NI        T      V        Y  VVMDFLM    DY  S  Y
C        V     V AK  NK     NM    D E TP      I        I  YDFQKGIG   EY  SE Y
D        V  K ICIPK IT S  LQNIK  D TFSP E   Q NA  KNW   IVPMLTV GKRV RDAGHP
E       PM  NL VIPK VT E  A NFK  D E S Q   TT LSY    W VCALLSCTS KDY  HE F
F       I  K I V AK  T A   QNLG     E KP     S ES D   LH GPFREVKQ  EY  H  Y
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Fig. 1. Comparison of deduced amino acid sequences for 3α-HSD (A), human chlordecone reductase (B), bovine prostaglandin F synthase (C), human aldehyde reductase (D), human aldose reductase (E) and frog eye lens epsilon-crystallin (F). Only differences from the 3α-HSD sequence are displayed. **Boldface** one-letter codes denote the beginning of the full sequences of aldose reductase and prostaglandin F synthase and of the partial sequences of aldehyde reductase and frog epsilon-crystallin. The dashed lines represent gaps necessary for optimal alignment of different sequences. The amino acid residues are numbered in **boldface** letters in the amino terminus to carboxyl terminus direction.

were similar to pHSD8 (Fig. 2). These proteins included bovine lung prostaglandin F synthase [18] and human chlordecone reductase [19] (each about 70% identical to the predicted sequence of 3α-HSD), human aldose reductase (49% identical) [20], human aldehyde reductase (39%) [21], and, interestingly, frog lens epsilon-crystallin (48%) [22].

Expression of 3α-HSD in bacteria

In order to confirm that the sequence we isolated actually encoded 3α-HSD, we expressed the protein in bacteria by transforming protease-deficient *E. coli* strain 1899 cells [23] with the PCR-amplified cDNA insert ligated in the expression vector pKK2.7. Lysates from the transformed bacteria were able to oxidize androsterone in the presence of NAD cofactor. Furthermore, the enzyme activity was inhibited by indomethacin, which also inhibits the 3α-HSD purified from rat liver cytosols. As detected by immunoblotting, the expressed protein had the same mobility on SDS–polyacrylamide gel as the enzyme in rat liver.

We have recently purified a large quantity of 3α-HSD expressed in *E. coli* and characterized the purified enzyme with respect to its substrate specificity. The 3α-HSD expressed in *E. coli* appears to be very similar to the enzyme purified

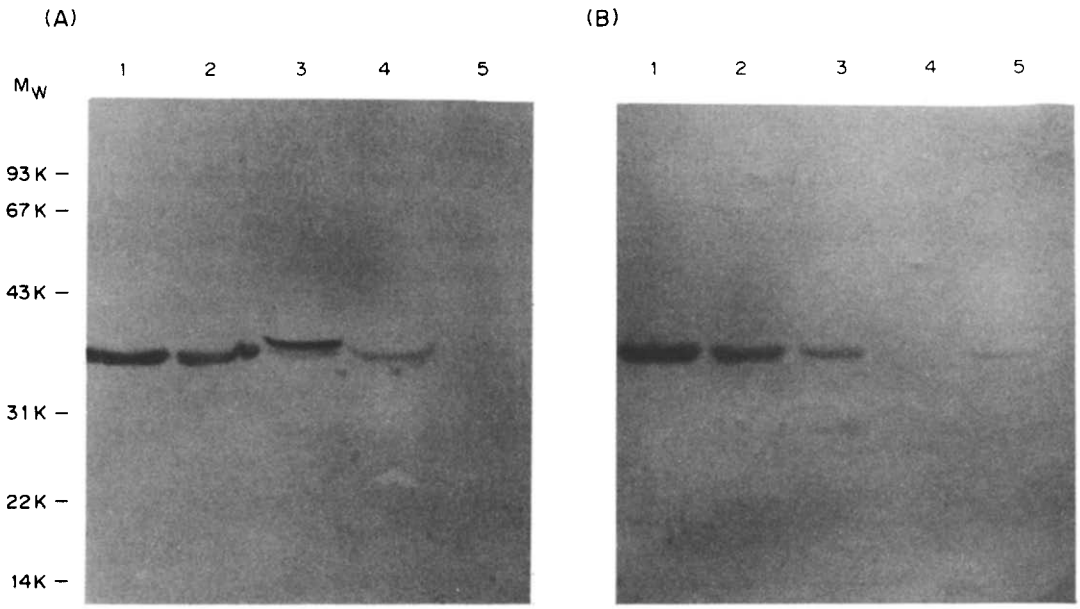

Fig. 2. Immunodetection of 3α-HSD and related cytosolic proteins in different rat tissues with monoclonal antibodies 3G6 (A) and monoclonal antibodies 7D3 (B). Lane 1 contains 25 μg of liver proteins from female rats; lane 2 contains 25 μg of liver proteins from male rats; lane 3 contains 100 μg of lung proteins from male rats; lane 4 contains 200 μg of kidney proteins from male rats; and lane 5 contains 200 μg of testicular proteins. Protein size markers are shown on the left side of the blots.

from rat liver in their substrate specificities and in their specific activities.

Detection of multiple structurally related proteins in different tissues

In the following immunoblotting experiment, we used both monoclonal antibodies 3G6 and 7D3 to survey the expression of 3α-HSD and possible related enzyme(s) in various rat tissues [Fig. 2(A and B)]. Lane 1 of Fig. 2(A and B) shows a 34 kD protein from the liver cytosols of female rats that was recognized by both monoclonal antibodies and the intensities of the bands, as visualized by the horseradish peroxidase, are approximately the same. As shown in lane 2 of Fig. 2(A and B), liver cytosols from male rats also contain a 34 kD protein that is recognized by both monoclonal antibodies, but the intensity of the band is weaker than that observed in female rat liver. Lane 3 in Fig. 2(A and B) shows the protein detected in the lung of male rats by each of the monoclonal antibodies.

Table 1. Amino acid sequence homology between various aldo–keto reductases and 3α-HSD

Aldo–keto reductase	% Homology to rat 3α-HSD
RAKa (3α-HSD)	100
RAKb	62
RAKc	60
RAKd	60
RAKe	37
RAKf	60

Monoclonal antibody 7D3 recognizes a 34 kD protein, whereas 3G6 binds to two proteins of 34 and 36 kD. The intensity of the 36 kD band recognized by monoclonal antibody 3G6, however, appears to be much stronger than that of the 34 kD band. The band intensities in livers of female and male rats seems to be consistent with the fact that female livers contain 2- to 3-fold higher 3α-HSD activities than that of male livers. However, there appears to be a lack of correlation between the levels of the lung 36 and 34 kD proteins and the liver protein and their corresponding 3α-HSD activities. The lung has approx. 20-fold less 3α-HSD activity than that

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     1                              30
a    MDSISLRVALNDGNFIPVLGFGTTVPEKVA
b          HC K  H H   A      YK    P
c    MDLKH RS K   P LM         FASKEIP
d     MS KHHC K    H   A       SI NE P
e       MTASS L HT QKM LI L    WKS PGQ
f     MS KLHC K  N H   A       YK KE P
g     M PKYQ  E    H M         YA PE P
h     M PK Q  K    H           RA  E P
i       MAASC L HT QKM L  L    WKS PGQ
j        MAS LL  NCAKM I  L    WKSPPGQ
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Fig. 3. Comparison of partial deduced amino acid sequences of RAKa (3α-HSD) (a), RAKb, (b), RAKc (c), RAKd (d), RAKe (e), RAKf (f), human chlordecone reductase (g), bovine prostaglandin F synthase (h), human aldehyde reductase (i), human aldose reductase (j) and frog eye lens epsilon-crystallin (k). Only differences from the 3α-HSD sequence are displayed. **Boldface** one-letter codes denote the beginning of the sequences derived from the cDNA sequences. The amino acid residues are numbered in **boldface** letters in the amino terminus to carboxyl terminus direction.

of liver, whereas the total intensity of the 34 plus 36 kD proteins is only 3- to 4-fold less than that of the liver 34 kD proteins. Lane 4 of Fig. 2(A) shows a 34 kD protein in kidney cytosols that was recognized by monoclonal antibody 3G6. Rather surprisingly, this kidney 34 kD protein was not recognized by monoclonal antibody 7D3 [lane 4, Fig. 2(B)]. Conversely, the 34 kD protein in testis was recognized by monoclonal antibody 7D3, but not by 3G6 [lane 5, Fig. 2(A and B)]. We have also surveyed several other rat tissues such as brain, prostate, skeletal muscle and eye, the band patterns of these tissues have been found to be similar to that of testis.

Isolation of multiple cDNAs

Screening of several cDNA libraries derived from male rat liver, kidney and lung resulted in the isolation of another five cDNA clones. Each clone was assigned a small letter in an alphabetic order according to their chronological order of isolation. As shown in Table 1 and in Fig. 3, the homology between the proteins encoded by these new cDNAs and 3α-HSD range from 40 to 71%. Without an understanding of the enzymatic characteristics of these proteins it is only speculation that these enzymes may be functionally related to aldo–keto reductase, and therefore, they are tentatively named aldo–keto reductases.

DISCUSSION

3α-HSD is an important enzyme involved in the metabolism of endogenous substrates, such as steroid hormones, prostaglandins and bile acids, as well as in the detoxification of many xenobiotics, such as benzo(*a*)pyrene. Penning's group, using a polyclonal antibody, detected only a single species of 3α-HSD in several tissues and in livers of different strains of rats [7]. Whereas several other groups have found evidence for the existence of multiple 3α-HSD [4–6].

One approach to further resolving the multiplicity issue involves generating a panel of highly specific monoclonal antibodies and using these reagents in analyzing the existence of structurally related proteins. To this end we have produced several monoclonal antibodies, two of which react with denatured enzyme. These two monoclonal antibodies did not detect multiple species of 3α-HSD in liver by immunoblotting; however, they did reveal the existence of multiple protein species antigenically distinct from the liver protein in other tissues. Several possibilities could potentially cause the differences in the band patterns. First, post-translational modifications, such as proteolytic digestion or glycosylation, could modify the antigenic site and prevent monoclonal antibody binding. These processes may change the size of the proteins and result in multiple band sizes. However, neither process appears to apply to the observations described here since all tissues exhibit a 34 kD polypeptide except the lung where a 36 kD polypeptide was also found. Secondly, there may be different structurally related enzymes distributed in various tissues. In view of our cloning and identification of 3α-HSD as a member of the aldo–keto reductase superfamily [12] and our recent study in the isolation of a total of six different cDNAs encoding proteins with high degrees of homology to 3α-HSD from liver, lung and kidney cDNA libraries using monoclonal antibodies as probes, it is most likely that the multiple band patterns are caused by the existence of multiple structurally related proteins in various tissues. Thus far, several enzymes possessing either reductase or dehydrogenase activities have been identified to be related to 3α-HSD. These enzymes include aldose reductase, human aldehyde reductase, human chlordecone reductase, bovine lung prostaglandin F synthase and frog eye lens epsilon-crystallin. Despite their phylogenic distance and various substrate specificities, this group of enzymes displays strong sequence homology (45–71%) with the rat 3α-HSD.

In contrast to early biochemical studies which suggested that several forms of aldo–keto reductases (low K_m aldehyde reductase, high K_m aldehyde reductase) exist ubiquitously in various tissues, our cloning and Northern blotting studies indicate that the expression of different forms of aldo–keto reductases is tissue specific and/or sexually dimorphic. We hypothesize that each isozyme may carry out a specific function in a given tissue. For example, aldo–keto reductases in the liver function as detoxification enzymes, enzymes in the lung may be involved in asthma by regulating the catabolism and interconversion of prostaglandins and in the detoxification of chemical carcinogens, enzymes in the intestine may be utilized for the transportation of bile acids and other xenobiotics, and enzymes in the kidney may regulate the excretion of solutes by modulating the metabolism of corticosteroids. These questions regarding

the functional role(s) of multiple isozyme will be answered when we have expressed the enzymes and studied their substrate specificities.

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