Tissue-specific Expression of an 11β-Hydroxysteroid Dehydrogenase with a Truncated N-terminal Domain

A POTENTIAL MECHANISM FOR DIFFERENTIAL INTRACELLULAR LOCALIZATION WITHIN MINERALOCORTICOID TARGET CELLS*

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Zygmunt Krozowski, Varuni Obeyesekere, Robin Smith, and Wendy Mercer
From the Laboratory of Molecular Hypertension, Baker Institute of Medical Research, P. O. Box 348, Prahran 3181, Australia

The enzyme 11β-hydroxysteroid dehydrogenase (11-HSD) is thought to confer specificity on the nonselective Type I adrenocorticotropin receptor by converting glucocorticoids to receptor-inactive metabolites in mineralocorticoid target tissues. S1 nuclease analyses using a rat liver 11-HSD probe demonstrated tissue-specific expression of the 5' region of the 11-HSD gene in the liver, lung, and kidney not evident in previous studies. Renal tissue contained a unique protected species which mapped to a position within the coding region, consistent with a divergence in liver and kidney protein sequences. Screening of a rat kidney cDNA library resulted in the isolation of several clones (11-HSD1B) noncoincident in their 5' regions with the liver sequence (11-HSD1A). Nucleic acid sequence analysis showed that the divergent clones code for a protein lacking a 26-amino acid NHz-terminal putative membrane-spanning signal peptide. The deletion of the leader sequence from the microsomal 11-HSD1A protein may result in a nuclear localization of the 11-HSD1B isoform. The renal 11-HSD1A and 11-HSD1B species increased coordinately during ontogeny and in parallel with the developmental surge in glucocorticoids. At least three alternate sites of polyadenylation were found to be utilized by the 11-HSD gene. Southern blot analysis showed the presence of a single gene in the rat. This study shows the expression of a kidney-specific 11-HSD isoform which may protect the Type I adrenocorticotropin receptor from occupation by glucocorticoids in the nucleus of a mineralocorticoid target cell.

The mineralocorticoid (or Type I adrenocorticotropin) receptor exhibits equal affinity for mineralocorticoid and glucocorticoid hormones (Krozowski and Funder, 1983; Beaumont and Fanestil, 1983). It has been proposed that the enzyme 11β-hydroxysteroid dehydrogenase (11-HSD)1 confers mineralocorticoid specificity on the Type I receptor by converting the much higher levels of circulating glucocorticoids to receptor-inactive metabolites. The action of 11-HSD on corticosterone produces an 11-keto metabolite which has about 0.3% of the affinity of the parent compound for Type I receptors and a correspondingly lower affinity for the Type II or classical glucocorticoid receptor (Funder et al., 1988). The mineralocorticoid aldosterone is not metabolized by 11-HSD due to the presence of a highly reactive aldehyde group at C18, which in solution cyclizes to the 11,18 hemiketal or the 11,18,20 hemiacetal.

In vivo studies have shown that inhibition of 11-HSD activity by administration of carbonoxolone or glycyrrehitin acid abolishes mineralocorticoid specificity of the Type I receptor (Edwards et al., 1988, Funder et al., 1988, Brem et al., 1989); in man a congenital deficiency in 11-HSD activity results in the syndrome of apparent mineralocorticoid excess which is characterized by salt retention and elevated blood pressure (Ulick et al., 1979). Although these effects may be due to the inappropriate occupation of the Type I receptor by glucocorticoids, there is now increasing evidence that glucocorticoids acting through the Type II receptor can also affect mineralocorticoid responses in whole cell2 and in vivo systems (Funder et al., 1990). The Type II adrenocorticotropin receptor seemingly activates the mineralocorticoid response element, exacerbating the nonselectivity of glucocorticoid hormones. 11-HSD, and probably of other enzymes, thus endow mineralocorticoid specificity both to the receptor and to the mineralocorticoid response element.

Recently, an NADP+-dependent 11-HSD has been purified from rat liver microsomes and cloned from a rat liver expression library (Lakshmi and Monder, 1988; Agarwal et al., 1989). Northern blot analysis has revealed the presence of a 1700-nucleotide (nt) mRNA in all tissues examined with the exception of the kidney where we have recently identified multiple unique species of the message (Krozowski et al., 1990). The pattern of 11-HSD gene expression in the renal papilla also differs from that observed in the medulla and cortex where mineralocorticoids are known to modulate sodium retention.

Although it has been proposed that in mineralocorticoid target cells the Type I receptor is protected by abundant 11-HSD activity, antibodies raised against the hepatic 11-HSD enzyme have failed to colocalize 11-HSD and Type I receptor immunoreactivity (Rundle et al., 1989). Northern blot analyses have readily detected 11-HSD mRNA in those regions of the kidney with high concentrations of Type I receptors (Krozowski et al., 1990). Together these results suggest that the kidney may contain an 11-HSD enzyme(s) which differs from that found in hepatocytes. Indeed, Western blot analysis has revealed that the kidney contains immunoreactive species...
which are absent from tissue homogenates of the liver (Monder and Lakshmi, 1990).

Southern blot analyses indicate that there may be more than one 11-HSD gene in the rat and human genomes (Agarwal et al., 1989). There is also increasing evidence that there may be several unrelated 11-HSD enzymes present in the rat kidney. Studies on immunopurified cortical-collecting cells have demonstrated an 11-HSD with a $K_c$, 2 orders of magnitude lower than that of the hepatic species, whereas histochemical studies in the rat kidney have identified an NAD$^+$-dependent enzyme with a clearly different distribution to that of the NADP$^+$-requiring species (Mercer and Krozowski, 1991). Further studies are needed to characterize 11-HSD gene expression in the kidney in order to determine the functional significance of the unique mRNA species observed in this tissue.

In the present study we have investigated the heterogeneity of 11-HSD mRNA expression in the rat kidney and have identified 11-HSD cDNAs containing an alternate 5' UT region and a conserved reading frame which codes for an NAD$^+$-dependent enzyme with a deleted signal peptide. Recent studies (Mercer and Krozowski, 1991) from our laboratory have also identified an NAD$^+$-dependent renal 11-HSD which we have named 11-HSD2. In the present study we refer to the NADP$^+$-dependent enzyme as 11-HSD1 (Lakshmi and Monder, 1988; Agarwal et al., 1990).

MATERIALS AND METHODS

Isolation of RNA—Total RNA was isolated from the liver, lung, and kidney of male Sprague-Dawley rats by the guanidinium isothiocyanate method as described previously (Krozowski et al., 1990).

S1 Nuclease Mapping—Antisense RNA probes were used to perform in vivo hybridization analyses of tissue RNA. Plasmid constructs used for the generation of riboprobes consisted of fragments of the pl1DH-1 insert (Agarwal et al., 1989) ligated into either the pGEM-3Z or Bluescript KS vector. Plasmid pRPA consisted of the complete BamHI or EcoRI. Probe A was transcribed from the T7 promoter pGEM-3Z or Bluescript KS vector. Plasmid pRPB consisted of the 439-bp AccI/EcoRI fragment into pGEM-3Z. Probe B was then transcribed from the T3 promoter after linearizing the pRPB plasmid with XbaI. The plasmid pRPC was linearized with HindIII and transcribed from the T7 promoter after linearizing the pRPB plasmid with XbaI. The plasmid pRPC was digested with EcoRI, BglII, XbaI, HindIII, and HincII, fractionated on a 0.8% agarose gel, and transferred to a nylon membrane. The blots were probed with an oligo-labeled (10$^6$ cpmp/ug) kidney cDNA probe (clone c8) and washed under the same conditions as used for the cDNA probe (see Fig. 2a). Filters were stripped by briefly boiling in deionized water before reprobing with the insert from pl1DH-1 as described previously (Krozowski et al., 1990).

Southern Blot Analysis—Genomic DNA was isolated from the livers of Sprague-Dawley rats (Maniatis et al., 1982). Aliquots (10 µg) were digested with EcoRI, BglII, XbaI, HindIII, and HincII, fractionated on a 0.8% agarose gel, and transferred to a nylon membrane. The blots were probed with an oligo-labeled (10$^6$ cpmp/µg) kidney cDNA probe (clone c8) and washed under the same conditions as used for the cDNA probe (see Fig. 2b). Filters were stripped by briefly boiling in deionized water before reprobing with the insert from pl1DH-1 as described previously (Krozowski et al., 1990).

RESULTS

S1 Nuclease Mapping of Liver, Lung, and Kidney RNA—Total RNA was prepared from liver, lung, and kidney tissue and subjected to S1 nuclease analysis using antisense RNA probes. Probes A, C, and D (Fig. 1a) showed no discernable differences in probe protection between the three tissues examined (results not shown), but probe B gave a distinctly different pattern between liver, lung, and kidney RNA (Fig. 1b). In all three tissues a band was observed at 466 nt corresponding to the fully protected probe B minus polylinker sequences. In the liver protected RNA species were present at 420 nt and 369 nt, whereas in the lung and kidney bands were seen at 410 nt and 369 nt. Furthermore, the kidney alone contained a protected species at 307 bp. The presence of identical truncated species in all tissues may be due to a divergence in sequence between probe and message or it may be due to RNA secondary structure. However, protected bands not present in all three preparations of RNA are more likely to be the result of nonlinearity, and an unequivocal resolution of this issue can only come from the isolation of cDNAs containing sequences which show dishomology with the probe.

Isolation of Rat Kidney cDNA Clones—A rat kidney cDNA library was screened with the insert from pl1DH-1 in order to identify clones which may contain 5′ sequences not colinear with the hepatic cDNA. Approximately 450,000 recombinants were screened and fourteen clones isolated (Fig. 2a). Restriction mapping and sequence analysis indicated that two clones, c13 and c18 (Fig. 2a) contained 5′ sequences noncolinear with pl1DH-1. An oligonucleotide probe (oligo 1301) corresponding to part of the noncolinear region (Fig. 2b) was then used to screen another 300,000 clones. A single clone was obtained (c20) which extended upstream of clone c13 (Fig. 2a). All clones noncolinear with pl1DH-1 were found to be colinear with each other. The sequence colinear with pl1DH-1 was named 11-HSD1A and the sequence containing the noncolinear region was termed 11-HSD1B.

A comparison of the 11-HSD1A and 11-HSD1B sequences is presented in Fig. 2b. The sequences of the longest 5′ extended kidney clones corresponding to 11-HSD1A (clone...
c12) and 11-HSD1B (clone c20) are compared with the liver
11-HSD1A sequence (p11DH-1). The 5' region of 11-HSD1B
diverges from 11-HSD1A at a position corresponding to 159
nt in the liver 11-HSD1A sequence (Fig. 2b), consistent
with the band obtained at 307 nt during S1 nuclease studies. Sequence
analysis of clone c13 also showed that the TAC
codon coding for Tyr in p11DH-1 was replaced by a TAT,
resulting in a silent mutation. This single base
change was not unique to the 11-HSD1B cDNAs but was also found
in all 11-HSD1A clones, suggesting the mutation is due to allelic
polymorphism.

The noncontiguous region does not contain translation
initiation codons (Fig. 2b). Instead Met in the 11-HSD1B
protein is translated from an ATG equivalent to a codon
resulting in a 1.5-kilobase species (Fig. 3b). When oligo
1301 was used as a probe Northern blot analysis of kidney RNA showed that the 11-HSD1B
message migrated as a 1.5-kilobase species (Fig. 3b).

Hydropathy Analysis of the 11-HSD1A Protein—An
analysis of the hydrophilicity profile of the 11-HSD1A protein
showed the presence of a strongly hydrophobic NH2 terminus
characteristic of a signal peptide (Fig. 4). Alignment of the
11-HSD1A and 11-HSD1B proteins revealed that the latter
molecule is a truncated protein with a deleted signal sequence.
However, the truncation stops short of the NADP+ cofactor
binding domain. It is likely the shortened protein has retained
enzyme activity given the high homology of the NH2 terminus
of 11-HSD1B with the NH2 termini of other dehydrogenases
(The et al., 1989, Debelle and Sharma, 1986).
extended using reverse transcriptase in the presence of all four primer extension products evident in and kidney using oligo 1301. Twenty pg of total liver run in parallel on a 6% polyacrylamide sequencing gel. The two to autoradiography for two days with an intensifying screen as de-
unlabeled deoxyribonucleotides as described under "Materials and
methods." To obtain size markers a dideoxy sequencing reaction was performed on clone c13 using oligo 1301 as a primer and the reactions run in parallel on a 6% polyacrylamide sequencing gel. The two primer extension products evident in lane 1 correspond to positions 11 and 15 nt upstream of clone c20. b, Northern blot analysis of liver and kidney using oligo 1301. Twenty µg of total liver (lane 1) or kidney (lane 2) RNA were denatured with glyoxyl and electrophoresed on a 1.2% agarose gel. After transfer to Hybond membrane the RNA was hybridized with 32P-end-labeled oligo 1301, washed, and subjected to autoradiography for two days with an intensifying screen as de-
scribed under "Materials and Methods." The results are depicted in a. The filter was then stripped and reprobed with the 32P-labeled p11HD-1 cDNA probe as described previously (Krozowski et al., 1990). The results obtained with the cDNA probe are depicted in b. Size markers shown on the left are in kilobases.

Otogeny of 11-HSD1A and 11-HSD1B mRNA Expression in the Kidney—Using Northern blot analysis we have previously shown that the 11-HSD1 gene is differentially expressed during development (Krozowski et al., 1990). Modest levels of message are present in the liver and lung of the neonate, whereas renal 11-HSD1 mRNA remains undetectable until three weeks of age. In the present study we used the more sensitive technique of S1 nuclease analysis to determine the developmental expression of renal 11-HSD1 mRNA. An autoradiogram showing the protected 11-HSD1A and 11-
HSD1B mRNA species is shown in Fig. 5. Equal amounts of both species of mRNA were found to be present in kidney tissue from 1-week old rats. Between 1 and 2 weeks of age the 11-HSD1A and 11-HSD1B messages were found to be coordinate expressed and increase in abundance in an exponen-
tial fashion. There was a further coordinate increase in messages up to 4 weeks of age but after this time there were no further increases up to the age of 16 weeks.

Southern Blot Analysis—Expression of the two 11-HSD1 messages may arise as a result of transcription from a single gene or they may be the product of two separate but closely related genes. It has previously been suggested that there are multiple 11-HSD1 genes in the rat (Agarwal et al., 1989), and we have also obtained multiple bands on Southern blots when using the full-length p11DH-1 clone as a probe (results not shown). However, these data are also consistent with the presence of a single large gene containing internal restriction sites recognised by enzymes used in the Southern blot analy-
sis. In order to resolve this issue we probed a rat genomic blot with clone c8 (see Fig. 2a), a cDNA which extends 3' of 772 bp in p11DH-1. If there are multiple genes coding for 11-
HSD1, one would still expect to see more than one band, in the majority of restriction enzyme digests, when using the truncated probe. However, EcoRI, BglII, HindIII, and HincII digests gave a single band while only Xbal appeared to give two bands (Fig. 6). These results are consistent with the existence of a single 11-HSD1 gene in the rat genome.

DISCUSSION

In the present study we have demonstrated that the liver, lung, and kidney express the 11-HSD1 gene in a tissue-specific manner and that the kidney expresses an mRNA which codes for a truncated form of the enzyme. It has been proposed that

FIG. 5. Ontogeny of 11-HSD1A and 11-HSD1B gene expression in the rat kidney. Total RNA was extracted from whole rat kidneys and subjected to S1 nuclease analysis using Probe B as described in Fig. 1b. Autoradiography was performed for 3 days with an intensifying screen. The equivalence of RNA added to each sample was checked by subjecting an aliquot to Northern blot analysis and probing for 18 S ribosomal RNA as described previously (Krozowski et al., 1990).
the 11-HSD1 enzyme is involved in mediating aldosterone specificity in mineralocorticoid target tissues where high levels of circulating glucocorticoids would otherwise occupy the nonselective Type I adrenocorticoid receptor (Edwards et al., 1988; Funder et al., 1988). The widespread distribution of the enzyme suggests that it may also modulate occupancy of the Type II adrenocorticoid or classical glucocorticoid receptor, although it is not known whether the same form of 11-HSD1 is present in all tissues. Indeed, the unique species of 11-HSD1 RNA expressed in the kidney suggest that some renal cells may produce tissue specific isoforms.

S1 nuclease analysis revealed a complexity of tissue specific 11-HSD1 gene expression not evident in previous studies. The heterogeneity of renal RNAs observed on Northern blot analysis (Krozowski et al., 1990) is mainly due to the existence of alternate 5’ sequences. The presence of multiple sites of poly(A) addition also make a small contribution to the differences in message size. We originally observed five species of 11-HSD1 RNA in the kidney by Northern blot analysis (Krozowski et al., 1990), whereas in the present studies only four RNAs were found with differing 5’ regions. This apparent inconsistency can be reconciled by the presence of RNA species which extend 5’ of the probe, consistent with the identification of 1900 nt RNAs in renal medulla and cortex (Krozowski et al., 1990).

Sequence analysis of the 3’ end of renal 11-HSD1 clones showed the presence of three alternate sites of poly(A) addition. Alternate polyadenylation may be due to the presence of the two overlapping poly(A) addition signals present in the sequence AATAAATAAA. The insertion of an AU-rich sequence into the 3’ noncoding region of genes has been shown to destabilize transcripts (Shaw and Kamen, 1986); the presence of the ATAAATT sequence in the 3’-extended 11-HSD1 cDNAs indicates that the corresponding messages may also be less stable. Since no correlation was apparent between the site of poly(A) addition and 11-HSD1A or 11-HSD1B clones, it is likely that similar mechanisms are used to modulate the stability of both messages. These observations suggest that 11-HSD1 messages can be regulated at the level of mRNA turnover by the selection of different 3’ UT sequences.

The heterogeneity of mRNA species observed in the kidney suggests a complex scenario of enzyme expression. Several eucaryotic genes are known to display 5’ heterogeneity in their mRNAs by the use of alternative promoters with or without alternate splicing (Chobert et al., 1990, Mukai et al., 1986, Frunzio et al., 1986). The noncolinearity near the start of the 11-HSD1A and 11-HSD1B mRNAs suggests that the 11-HSD1 gene uses alternative promoters to express the two forms of the message. Alternatively, the two 11-HSD1 isoforms may be expressed from different genes. However, our study indicates the presence of a single gene in the rat. Although early studies (Agarwal et al., 1989) in the human showed the presence of multiple bands on Southern blot analysis and suggested the presence of several 11-HSD1 genes, a single copy 11-HSD1 gene consisting of six exons has recently been isolated from a human library (Tannin et al., 1991); the fourth intron was of indeterminate size, reminiscent of the large configuration proposed for the rat gene in the present study.

Some genes have been shown to effectively turn off protein production by expression of different forms of the message (Laski et al., 1986). If this were the case for the 11-HSD1 gene a reciprocal relationship may be expected between 11-HSD1A expression and that of 11-HSD1B mRNA. However, the coordinate increase in the 11-HSD1A and 11-HSD1B species in the kidney during development suggests that expression of the 11-HSD1B mRNA is not a means of terminating production of the enzyme.

Other evidence also indicates that 11-HSD1B represents a fully functional enzyme. The NADP+ binding domain of the 11-HSD1B protein shows a high degree of similarity to the NH2-terminal region of the nodG dehydrogenase (Debelle and Sharma, 1996) and 17β-hydroxysteroid dehydrogenase (The et al., 1989), implying that upstream coding sequences are not critical for cofactor binding or enzyme activity. Alternate splicing, directly adjacent to the NADPH binding site, has been previously reported (Lin et al., 1990) in the human adrenodoxin reductase gene and does not appear to influence the activity of the enzyme.

An analysis of the ontogeny of 11-HSD1 gene expression in the kidney showed a tissue specific pattern of mRNA induction. The large increase in renal 11-HSD1 messages during the second week of life in the neonatal rat is in marked contrast to the more gradual increases observed in the liver and lung over the same period (Krozowski et al., 1990). The exponential rise in renal message parallels the developmental surge in 11-HSD enzyme activity (Ghraf et al., 1975), corticosteroid-binding globulin, and glucocorticoids at this time (Henning, 1978). This suggests that the kidney, but neither the liver nor lung, is dependent on adrenal steroids for the increase in 11-HSD1 gene expression during ontogenesis.

The strongly hydrophobic NH2-terminal domain in 11-HSD1A is strongly suggestive of the presence of a signal peptide. Although there is no consensus sequence as such, the 11-HSD1A leader has all the characteristics of a signal peptide including a charged amino terminus, a hydrophobic core and a more polar carboxyl end (von Heijne, 1986). The length of the hydrophobic leader indicates that it may be sufficient to form a membrane spanning domain (Wickner and Lodish, 1985). Inspection of the sequence shows no obvious signal peptidase cleavage sites, consistent with the finding of an intact signal peptide in the purified microsomal protein (Agarwal et al., 1989). These data indicate that the hydrophobic NH2-terminal domain of 11-HSD1A forms an uncleaved signal sequence which may also function as a membrane binding domain.

The absence of a signal peptide in 11-HSD1B suggests that the truncated enzyme may be located in a different subcellular compartment to the 11-HSD1A protein. There are a number of examples where alternative forms of the message generate proteins with different intracellular routing (Caras et al., 1987; Gower et al., 1988; Hynes, 1985). Renal subcellular fractionation studies have shown the presence of 11-HSD1 enzyme
activity in both microsomes and nuclei (Kobayashi et al., 1987). Since 11-HSD1A is present in microsomal membranes, it is possible that the 11-HSD1B protein is localized in the nucleus. The nuclear localization of the 11-HSD1B enzyme in the kidney would be consistent with its role as an autocrine protector of the mineralocorticoid receptor.

It is also likely that the distribution of the alternate forms of the enzyme is cell-specific. The cell-specific expression of an immunologically nonreactive 11-HSD1B enzyme may explain the absence of immunoreactivity in the outer regions of the kidney where the Type I receptor is localized (Rundle et al., 1989). The renal papilla, which displayed a similar pattern of mRNA expression to the liver (Krozowski et al., 1990), may predominantly express the 11-HSD1A protein, whereas the cortex and medulla could contain the majority of renal 11-HSD1B enzyme.

The identification of a kidney-specific 11-HSD1 isoform should help elucidate the mechanism by which this enzyme endows specificity on the Type I and Type II adrenocorticoid receptors in mineralocorticoid target tissues. Cell-specific expression and organelle-specific targeting of isoenzymes has the potential to endow renal cells with a complex system for modulating the biological actions of both glucocorticoid and mineralocorticoid hormones.

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