Cloning and characterization of two novel aldo-keto reductases (AKR1C12 and AKR1C13) from mouse stomach

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Received 15 August 1999; received in revised form 3 September 1999

Abstract In contrast to hepatic hydrosteroid dehydrogenases (HSDs) of the aldo-keto reductase family (AKR1C), little is known about a stomach one. From a mouse stomach cDNA library, we isolated two clones encoding proteins of 323 amino acid residues. They exhibited 93.2% amino acid sequence identity and 64–68% with any known HSDs. Recombinant proteins expressed in *Escherichia coli* reduced 9,10-phenanthraquinone with NAD(P)H as cofactor. The mRNAs were exclusively expressed in stomach, liver and ileum. The present study demonstrates that these proteins are new members of the HSD subfamily and they are named AKR1C12 and AKR1C13. Immunohistochemical analysis suggests that they are involved in detoxification of xenobiotics in the stomach.

Key words: cDNA cloning; Hydroxysteroid dehydrogenase; Phylogeny; Stomach; Immunohistochemistry

1. Introduction

The aldo-keto reductases are monomeric proteins, about 320 residues in size, that share a common $(\alpha/\beta)_8$-barrel scaffold [1]. These proteins catalyze reactions with a diverse range of substrates including prostaglandins (PGs), monosaccharides, steroids and xenobiotic aldehydes and ketones using nicotinamide dinucleotide as cofactor. With the advent of recombinant DNA techniques, the primary sequences of enzymes belonging to the aldo-keto reductase superfamily have been recently delineated [1]. These enzymes include aldehyde reductase (EC 1.1.1.2) [2], aldose reductases (EC 1.1.1.21) [2–4], bovine lung PGF synthase (PGFS) (EC 1.1.1.188) [5], chlordecone reductase (CDR) (EC 1.1.1.225) [6], 3α-hydroxysteroid dehydrogenase (3α-HSD) (EC 1.1.1.213)/dihydrodiol dehydrogenase (DD) (EC 1.3.1.20) [7,8], estradiol 17β-HSD (EC 1.1.1.62) [9] and 20α-HSD (EC 1.1.1.149) [10,11]. These proteins belong to the AKR1 family according to a new nomenclature of the aldo-keto reductase superfamily proposed by Jez et al. [12].

PGFS was purified to apparent homogeneity from bovine lung and its properties have been characterized in detail [13–15]. The enzyme exhibits a broad substrate specificity and catalyzes the conversion of PGD_2 to 9α,11β-PGF_2 (11-ketoreductase activity) and PGH_2 to $PGF_{2\alpha}$ (9,11-endoperoxide reductase activity) as well as the reduction of various carbonyl compounds. We previously demonstrated that expression of PGFS-like immunoreactivity increased in mouse uterus at the term of pregnancy accompanied with the increase in $PGF_{2\alpha}$ production [16]. In the course of the studies in the reproductive system, we found that a PGFS-immunoreactive protein(s) was abundantly present in mouse stomach and liver. Since the stomach is the site which is directly exposed to food, drugs and microorganisms, it is likely that aldo-keto reductases play an important role in this tissue. Nevertheless, little information is available on a stomach aldo-keto reductase(s) in contrast to the hepatic ones which have been studied extensively in many laboratories [1,12,17,18]. To investigate the structure and function of the antigenetically related protein, we constructed a cDNA library from mouse stomach and screened using PGFS cDNA as probe. In the present study, we report the isolation and characterization of two novel enzymes belonging to the HSD subfamily of aldo-keto reductases.

2. Materials and methods

2.1. Isolation of cDNA clones and DNA sequencing

A mouse stomach cDNA library was constructed in λgt11 by the method of Huynh et al. [19]. The 1×10^6 phages of cDNA library were hybridized with PGFS cDNA [5]. Hybridization was carried out at 37°C in 5×SSPE (1×SSPC: 0.18 M NaCl, 10 mM sodium phosphate, pH 7.7, and 1 mM EDTA), 0.1% sodium dodecyl sulfate (SDS), 1×Denhardt's solution, 50% (w/v) dextran sulfate, 50% formamide and 0.1 mg/ml denatured salmon sperm DNA and filters were washed with 2×SSC (1×SSC: 0.15 M NaCl and 15 mM sodium citrate, pH 7.0) containing 0.1% SDS at 37°C. Eight positive phage clones were isolated and purified. The inserts were digested with *Bam*HI and subcloned into the pBluescript SK(+) vector for DNA sequencing. Two plasmid clones with a high homology to PGFS cDNA were designated as AKR1C12 and AKR1C13 and analyzed in subsequent experiments.

2.2. Expression and purification of AKR1C12 and AKR1C13 proteins in Escherichia coli

To express AKR1C12 and AKR1C13 proteins, the cDNAs were subcloned into the pBluescript SK(+) plasmids in such a way that the translation initiation signal of the β-galactosidase gene was used to overexpress the proteins. These expression plasmids were transfected into the host strain *E. coli* DH5α. Recombinant AKR1C12 and AKR1C13 proteins were purified to apparent homogeneity from the crude extract of transfected cells by three steps consisting of 35–75% ammonium sulfate precipitation and Ultrogel AcA 54 (BioSepra, France) column chromatography followed by Mono S HR5/5 column chromatography for AKR1C12 or DEAE-cosmogel (Nacalai Tesque, Kyoto, Japan) column chromatography for AKR1C13. The yields of AKR1C12 and AKR1C13 were 9.8 and 66.7% and specific activities

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Abbreviations: PG, prostaglandin; PGFS, bovine lung PGF synthase; HSD, hydroxysteroid dehydrogenase; CDR, chlordecone reductase; DD, dihydrodiol dehydrogenase

0014-5793/99/$20.00
PII: S0014-5793(99)01243-0

of the purified enzymes were 7.49 and 3.36 U/mg protein using 9,10-phenanthraquinone as substrate, respectively.

2.3. Enzyme assay

The reductase activities for 9,10-phenanthraquinone, 4-nitrobenzaldehyde, 4-nitroacetophenone, menadione, progesterone, cortisone, corticosterone, esterone and D-glucose were determined spectrophotometrically by the oxidation of NADPH at pH 6.5. The oxidase activities for testosterone, androsterone and estradiol were determined spectrophotometrically by the reduction of $NADP^+$ at pH 8.0. PGD_2 11-ketoreductase, PGE_2 9-ketoreductase and PGH_2 9,11-endoperoxide reductase activities were determined as described previously [13]. One unit of enzyme activity was defined as the amount that oxidized 1 μmol of NADPH, reduced 1 μmol of $NADP^+$ or formed 1 μmol of PGF_2 per min at 37°C.

2.4. Miscellaneous

For immunoblotting, the membrane was immunostained with the rabbit anti-PGFS antiserum at 1:2500 dilution, biotinylated anti-rabbit IgG goat serum at 1:3000 dilution and a Vectastain ABC kit (Vector Laboratories) as described previously [15]. Protein bands were visualized with an ECL kit (Amersham Pharmacia Biotech, UK). For immunohistochemistry, transverse frozen sections (10 μm thickness) were immunostained with anti-PGFS antiserum at a 1:1000 dilution as the first antibody and immunoreactive structures were revealed with a goat anti-rabbit IgG-Cy3 conjugate by a fluorescence microscope (LSM-GB2000, Olympus, Tokyo, Japan). For Northern blot analysis, total RNAs (10 μg) isolated from various mouse tissues were analyzed according to standard procedures [20]. The full-length cDNA of AKR1C12 was labelled with [α-^{32}P]dCTP by random priming and used as probe.

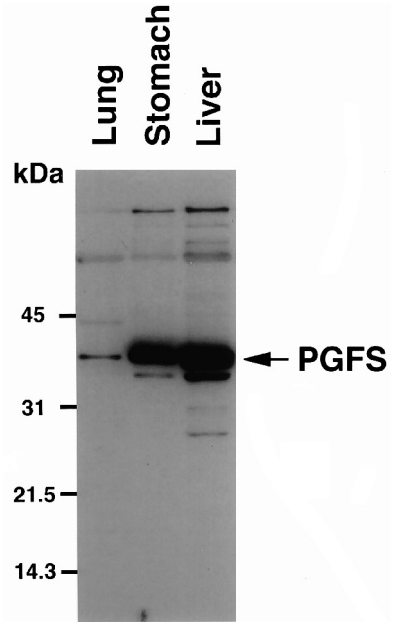

Fig. 1. PGFS-like immunoreactivity in mouse tissues. Crude extracts from lung, stomach and liver were subjected to 12.5% SDS-polyacrylamide gel electrophoresis, transferred to a PVDF membrane and immunostained with anti-PGFS antiserum. The arrow on the right indicates the position of recombinant PGFS.

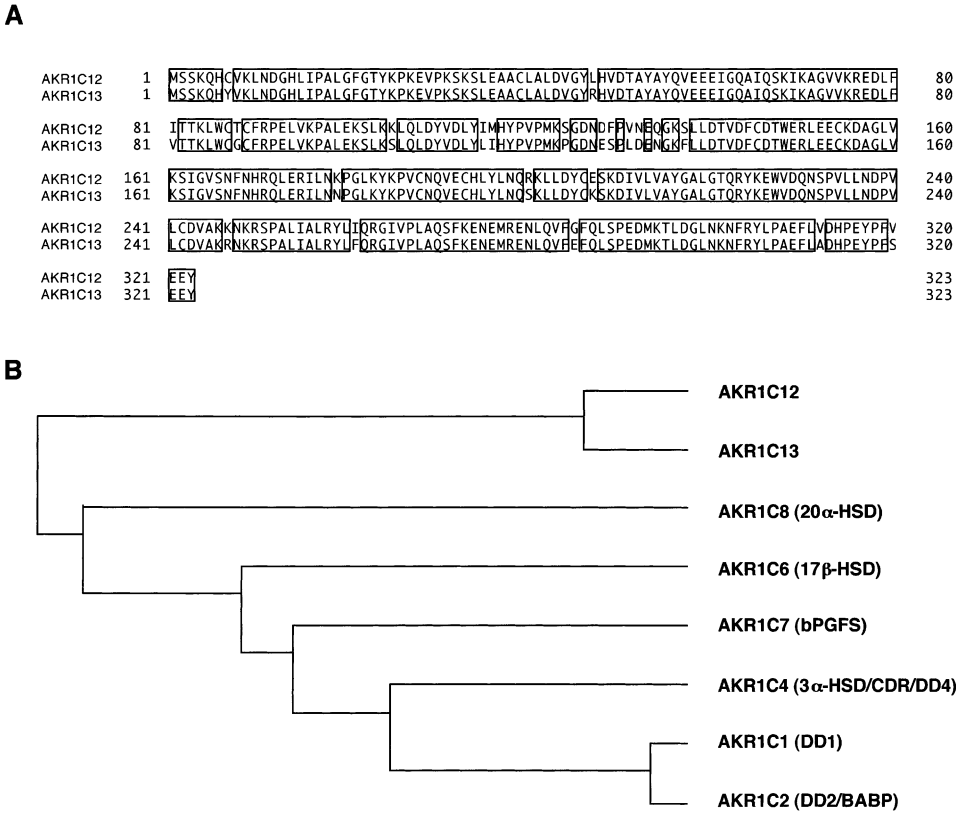

Fig. 2. (A) Alignment of the deduced amino acid sequences of AKR1C12 and AKR1C13. Conserved amino acids are boxed. The nucleotide sequences of AKR1C12 and AKR1C13 have been submitted to the DDBJ/EMBL/GenBank database and are available under accession numbers AB027237 and AB027125, respectively. (B) Phylogenic tree of AKR1C12, AKR1C13 and HSD subfamily. Analysis of sequence homologies among AKR1C12, AKR1C13 and entries in the Swiss Prot and PIR data banks and cluster analysis of HSDs were carried out using the GENETEX-MAC program (9.0). On the basis of the distance matrix between aligned amino acid sequences, the tree was inferred by the UPGMA method [23].

3. Results and discussion

3.1. Cloning and cDNA sequences of PGFS homologues

When polyclonal antibodies against PGFS were used, an immunoreactive band was intensely detected in crude extracts of mouse stomach and liver and weakly in lung (Fig. 1) at the same position as that of recombinant PGFS (M_r = 36 666). A cDNA library from mouse stomach was screened by plaque hybridization with PGFS cDNA as probe under low stringency conditions and the two PGFS homologs named AKR1C12 and AKR1C13 were analyzed in subsequent experiments.

The full-length cDNA of AKR1C12 was 1217 bp long, of which a 19 bp 5′-flanking region and a 969 bp open reading frame encoding a 323 amino acid polypeptide with a calculated M_r of 37 035 followed by a 229 bp 3′-untranslated region including a poly(A) tail. The cDNA insert in AKR1C13 contained 1207 bp with a 969 bp open reading frame, specifying a 323 amino acid polypeptide with a M_r of 36 990 and a 226 bp 3′-untranslated region including a poly(A) tail. When the deduced amino acid sequences of AKR1C12 and AKR1C13 were compared, the identity between them was 93.2% without amino acid deletion/insertion (Fig. 2A). The sequence comparison analysis revealed the significant homology of AKR1C12 and AKR1C13 with known enzymes belonging to the HSD subfamily of aldo-keto reductases. The amino acid sequence of AKR1C12 was 65, 66, 67, 67, 68 and 68% identical with those of rat corpus luteum 20α-HSD (AKR1C8) [11], mouse liver 17β-HSD (AKR1C6) [9], human liver 3α-HSD type 1 [8]/CDR [6,21]/DD4 (AKR1C4) [17], PGFS (AKR1C7) [5], human liver DD2/bile acid-binding protein (AKR1C2) [17,18] and human liver DD1 (AKR1C1) [22], respectively. The amino acid sequence of AKR1C13 was 64, 65, 65, 65, 66 and 67% identical with them in the same order. To establish evolutionary relationships among these aldo-keto reductases, a phylogenetic tree was constructed by the UPGMA method [23] (Fig. 2B). AKR1C12 and AKR1C13 proteins belong to the mammalian HSD group and form a single cluster which is separated from the other HSDs, indicating that they constitute a closed group within the HSD subfamily.

3.2. Enzymatic activity of expressed AKR1C12 and AKR1C13

To characterize properties of AKR1C12 and AKR1C13, these proteins were expressed in *E. coli*. Table 1 summarizes the substrate specificity of purified recombinant AKR1C12, AKR1C13 and PGFS. While PGFS catalyzed a reaction with a broad spectrum of substrates including synthetic compounds, PGs and testosterone, typical of aldo-keto reductases [1], both AKR1C12 and AKR1C13 exhibited a rather selective substrate specificity for 9,10-phenanthraquinone. The specific activities of AKR1C12 and AKR1C13 for 9,10-phenanthraquinone were 20- and 10-fold higher than that of PGFS. The purified AKR1C12 reduced 4-nitrobenzaldehyde with a specific activity (0.32 U/mg) comparable to that (0.38 U/mg) of PGFS. PGH_2, progesterone, cortisone and testosterone served as substrates for AKR1C12, but their reaction rates were less than 0.5% of that observed with 9,10-phenanthraquinone. AKR1C13 almost exclusively reduced 9,10-phenanthraquinone among substrates examined. The K_m values for 9,10-phenanthraquinone were 1.4 and 2.1 μM with AKR1C12 and AKR1C13, respectively, comparable to that (0.7 μM) of PGFS. When NADH and NADPH were examined for their capacity to serve as a cofactor using 9,10-phenanthraquinone as substrate, both NADH and NADPH were similarly used as cofactors by AKR1C12 and AKR1C13 with K_m values of 20–25 μM. When the 9,10-phenanthraquinone reductase activity was compared between NADPH and NADH at 80 μM under standard assay conditions, the specific activity of AKR1C13 was 6-fold higher with NADPH than with NADH. In contrast, the specific activity of AKR1C12 with NADPH was

Table 1
Comparison of substrate specificity among recombinant AKR1C12, AKR1C13 and PGFS

Substrate/cofactor	Concentration (mM)	Specific activity (U/mg protein)		
		AKR1C12	AKR1C13	PGFS[a]
Synthetic				
Nitrobenzaldehyde	0.5	0.32	0.03	0.38
Phenanthraquinone	0.1	7.49	3.36	0.38
Nitroacetophenone	0.5	ND	ND	0.43
Menadione	0.25	ND	ND	0.42
PGs				
PGH_2	0.08	0.012	0.0005	0.057
PGD_2	1.00	0.0006	0.0002	0.13
PGE_2	1.00	0.0018	ND	0.0076
Steroids				
Testosterone	0.05	0.009	ND	0.004
Androsterone	0.12	ND	ND	–
Progesterone	0.25	0.024	ND	–
Cortisone	0.25	0.015	ND	–
Corticosterone	0.25	ND	ND	–
Esterone	0.05	ND	ND	–
Estradiol	0.05	ND	ND	–
D-Glucose	80	ND	ND	–
Cofactor				
NADPH	0.08	7.47	3.36	–
NADH	0.08	10.2	0.55	–

Substrate specificity was measured in the presence of 80 μM NADPH as cofactor and cofactor selectivity was measured with 10 μM 9,10-phenanthraquinone as substrate. ND, not detected. –, not examined.
[a]Reported by Watanabe et al. [5].

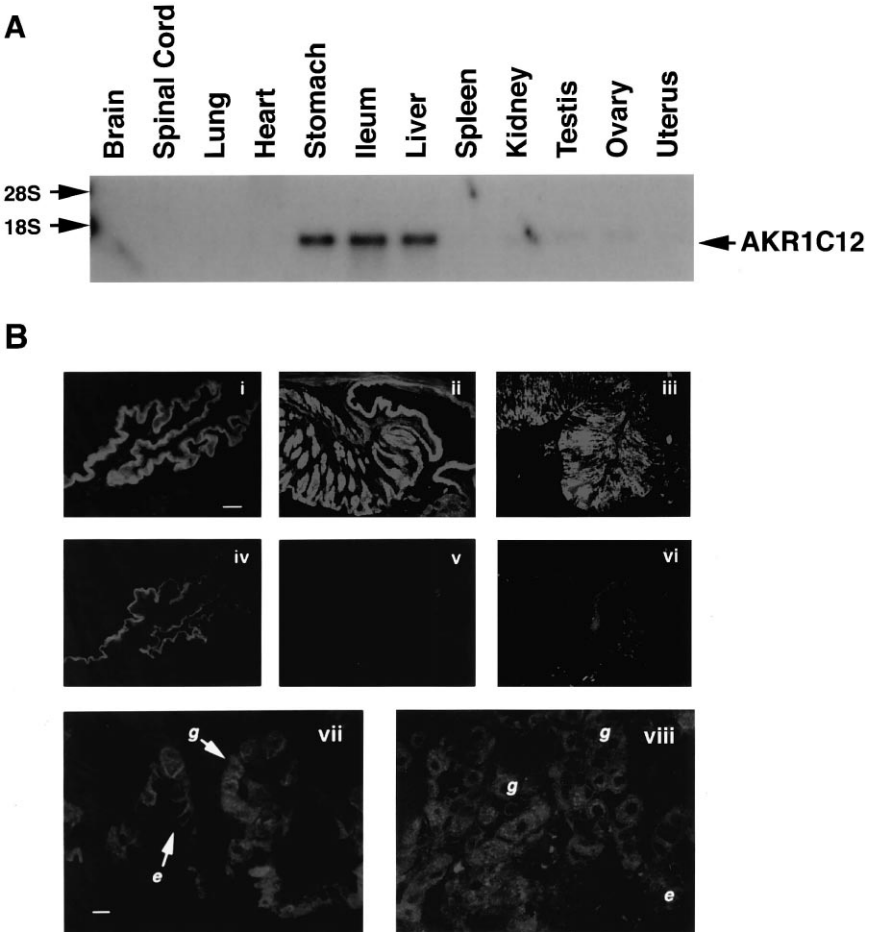

Fig. 3. (A) Northern blot of total RNAs (10 μg of each) from various mouse tissues. The membrane was hybridized with a ^{32}P-labelled AKR1C12 cDNA. The positions of rRNAs are indicated on the left. (B) AKR1C12-like immunoreactivity in mouse stomach. Cryosections (10 μm thickness) of forestomach (i, iv) and fundus (ii, v, vii) and pylorus (iii, vi, viii) of glandular stomach were immunostained with anti-PGFS antibody (i–iii, vii, viii) and the antibody pre-absorbed with an excess (1 mg/ml) of purified AKR1C12 (iv–vi). e and g represent surface epithelium and gastric glands. Bars, 100 μm (i–vi), 10 μm (vii, viii).

rather low as compared with NADH (Table 1). These results demonstrate that both AKR1C12 and AKR1C13 are members of aldo-keto reductases with different catalytic properties.

3.3. Distribution of AKR1C12 and AKR1C13 in mouse tissues

As shown in Fig. 3A, a single mRNA species with a size of approximately 1.2 kb was detected with AKR1C12 cDNA as probe. Similar results were obtained with AKR1C13 cDNA as probe (data not shown). Expression of AKR1C12 and AKR1C13 transcripts was restricted to stomach, ileum and liver. None of the other tissues such as brain, lung, spleen, kidney and the reproductive system expressed any detectable level of the transcript.

Anti-PGFS antibodies recognized the expressed AKR1C12 intensely as did PGFS, but weakly the expressed AKR1C13. When cryosections of mouse stomach were immunostained with anti-PGFS antiserum, specific staining was observed in the mucosal layer of the fundic portion and pylorus of glandular stomach, but not in the muscular layer (Fig. 3B, ii and iii). Under higher magnification, it was observed that the PGFS immunoreactivity was localized to the glandular epithelium composed of the surface cells arranged in ridges and shallow pits and gastric glands (Fig. 3B, vii and viii). The positive stains in the glandular epithelium were inhibited by pre-absorption of the antiserum with purified AKR1C12 (Fig. 3B, v and vi). Forestomach epithelium is keratinized, stratified squamous. While staining of the keratinized layer was not absorbed with the purified AKR1C12, the staining of the epithelium was inhibited (Fig. 3B, i and iv), suggesting that the epithelium was also positively stained in the forestomach. They were not detected without anti-PGFS antiserum or by pre-absorption of the antiserum with AKR1C13. These studies demonstrate the restriction of AKR1C12 and AKR1C13 to the epithelium of stomach.

In the present study, we cloned two cDNAs from mouse stomach with 64–68% amino acid identity to any known enzymes belonging to the HSD subfamily (AKR1C) of aldo-keto reductases. Recombinant proteins expressed in *E. coli* showed a high 9,10-phenanthraquinone reductase activity and they are designated as AKR1C12 and AKR1C13. Unlike the seemingly ubiquitous distribution of known aldo-keto reductases, Northern blot analysis of various mouse tissues shows that AKR1C12 and AKR1C13 are confined to the digestive system. *Helicobacter pylori* plays a central role in the etiology of chronic superficial gastritis and peptic ulcer and seems to increase the risk for development of adenocarcinoma in humans [24]. Constitutive and abundant expression of AKR1C12 and AKR1C13 in the mucosal layer of stom-

ach suggests that the enzymes serve as detoxification of polycyclic aromatic hydrocarbon carcinogens. AKR1C12 and AKR1C13 are the first HSDs highly expressed in the stomach and these novel enzymes may have an important clinical implication in organs exposed to microorganisms and xenobiotics. Cloning of AKR1C12 and AKR1C13 provides the tools required for in vivo and in vitro studies to further understand pathophysiological roles of aldo-keto reductases in the digestive system, especially stomach.

Acknowledgements: We are grateful to Drs Ken Hirotsu, Ikuko Miyahara and Airo Tsubura for helpful discussion and Fuso Pharmaceutical Industries for oligonucleotides. This work was supported in part by Grants-in-Aids for Scientific Research on Priority Areas, Scientific Research (B) (11558093) and (C) (11670135) from the Ministry of Education, Science, Sports and Culture of Japan and by a grant from the Science Research Promotion Fund of the Japan Private School Promotion Foundation.

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