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MOLECULAR CLONING AND CHARACTERIZATION OF BOVINE PROSTACYCLIN SYNTHASE

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SUMMARY The cDNA encoding prostacyclin synthase (PGIS) was isolated from a bovine arota cDNA library. The cDNA contained an open reading frame of 1500 nucleotides encoding a polypeptide of 500 amino acids with a Mr of 56,675. The predicted amino acid sequence contains four polypeptide sequences determined from purified bovine PGIS. Comparison of the PGIS sequence with other protein sequences in protein data banks indicates that PGIS has considerable sequence similarity with cytochrome P450s; the closest related sequence is that of human cholesterol 7-α-monooxygenase (CYP 7). The PGIS sequence is consistent with several structural elements found in other P450s, including a putative membrane anchoring segement, a helix I which forms an α-helix backbone through the center of the enzyme and a heme-binding pocket. Overall, the PGIS has $\leq$ 31 % identity to other P450s, suggesting that PGIS represents a previously undefined family of cytochrome P450. PGIS shares only 16 % sequence identity with human thromboxane synthase and has a different hydropathy pattern near the amino terminus, suggesting a different membrane anchoring topology. Availability of PGIS cDNA will allow us to elucidate the different catalytic mechanisms between these two enzymes.

Prostacyclin (PGI_2) is a potent inhibitor of platelet secretion and aggregation, smooth muscle cell proliferation and vasoconstriction (1-3). It is considered to play a key role in vasoprotection and its deficiency may lead to thrombosis and other vascular lesions (4, 5). Biosynthesis of PGI_2 is catalyzed serially by (a) phospholipase A_2 which liberates arachidonic acid from the sn-2 position of membrane phospholipids, (b) prostaglandin H synthase, a bifunctional enzyme which catalyzes the conversion of arachidonic acid to prostaglandin G_2 and subsequently to prostaglandin H_2, and (c) prostacyclin synthase (PGIS) which catalyzes the formation of PGI_2 from prostaglandin H_2. PGIS has been shown to be widely distributed, predominantly in vascular endothelial and smooth muscle cells (6, 7). The enzyme is membrane-bound protein located in the the endoplasmic reticulum. It exhibits

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The abbreviations used are: PGIS, prostacyclin synthase; PGI_2, prostacyclin; PCR, polymerase chain reaction; TXA, thromboxane A_2.

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spectral features characteristic of cytochrome P450s but has no mono-oxygenase activity (8, 9). Instead, it catalyzes an isomerization reaction and thus does not require a reductase to initiate the catalytic activity. We have recently reported purification of PGIS from bovine aorta and isolation of an 115-bp cDNA encoding a part of this enzyme (10). Here we report the cloning of the cDNA encoding the entire coding region and characterization of the protein structure from the deduced amino acid sequence.

MATERIALS AND METHODS

Hybridization cloning of bovine PGIS cDNA- Using the "nested primer" PCR technique, we recently obtained an ~100 bp of cDNA encoding the NH_2-terminus of bovine PGIS (10). This fragment of cDNA (Probe A; Fig. 1) was used to screen a bovine aortic endothelial cell 5'-stretch cDNA library constructed in a λgt10 vector (Clontech, Palo Alto, CA). Radiolabeling of the cDNA probe was carried out using a random primer kit (Stratagene, La Jolla,CA) and [α-^{32}P]dCTP. The library was reverse-transcribed by a mixture of oligo(dT) and random primers. Replicate nylon filters containing 2.5 X 10^5 plaques (2.5 X 10^4 plaques/filter) were prehybridized with 6X SSC (1X SSC is 15 mM sodium citrate, pH 7.0 and 150 mM NaCl), 2X Denhardt's reagent (50X Denhardt's reagent contains 1 % each of Ficoll, bovine serum albumin and polyvinylpyrrolidone), 0.1 mg/ml heat-denatured salmon sperm DNA and 40 % formamide at 40 °C for 3 hr. Subsequently, the filters were hybridized with the ^{32}P-labeled probe in hybridization solution containing 6X SSC, 0.5 % SDS, 2X Denhardt's reagent, 10 mM EDTA and 40% formamide at 40 °C for 16 hr. The filters were first washed with 2X SSC/ 0.5 % SDS at room temperature for 30 min, and then with 1X SSC/ 0.5 % SDS at 50 °C for 30 min.

To obtain the full-length sequence, the library was rescreened by hybridization with 3'-end fragments of the PGIS cDNA clones obtained in the earlier round of screening (see "Results and Discussion"). The screening procedures were similar to those described above with the following modifications: (a) the filters were pre-hybridized (3 hr) and hybridized (16 hr) in the solution without formamide at 52 °C; and (b) The filters were first washed in 0.5X SSC/ 0.5 % SDS at 60 °C for 30 min and then washed in 0.2X SSC/ 0.5 %SDS at 60°C for 30 min.

Nucleotide sequencing and protein analysis- Inserts from positive plaques were subcloned into the EcoRI site of pBluescript. Plasmids were isolated using a plasmid mini kit (Qiagen, Chatsworth, CA). The inserts were sequenced by double-strand sequencing using Sequenase 2.0 (United States Biochemical) and [α-^{35}S]dATP (11). T3 primer, T7 primer or PGIS-specific oligonucleotides were used as primers for sequencing. DNA sequence and protein structure analyses were carried out using DNAstar Lasergene software programs (DNASTAR, Madison, WI).

RESULTS AND DISCUSSION

A λgt10 bovine aortic endothelial cell cDNA library was screened with an ~100-bp probe isolated previously (10). The location of this fragment relative to the PGIS cDNA is indicated as Probe A in Fig. 1. Positive clones were plaque-purified and sequences of partial segments were determined by double-stranded DNA cycle sequencing using a PGIS-specific oligonucleotide located at the ATG start site as a primer. The insert of one clone, called bPGIS-1, was 0.85 kb in length

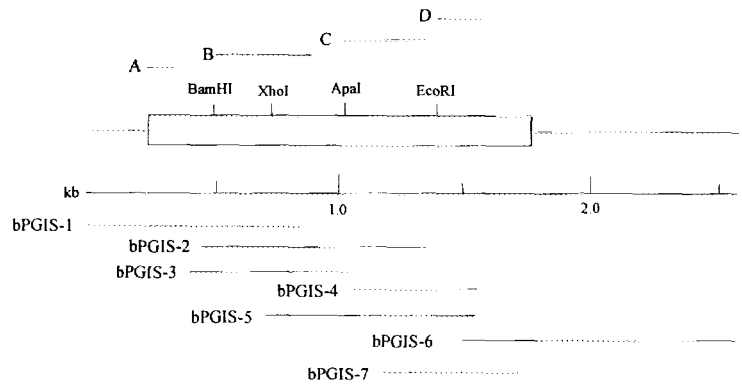

Fig. 1. Schematic representation of bovine PGIS cDNA clones. The box represents the coding region of PGIS. The selected restriction sites are indicated. The probes for screening the cDNA library are shown above the PGIS cDNA and the clones isolated from the cDNA library are shown below the cDNA.

and contained a sequence identical to that of Probe A (Fig. 1). The bPGIS-1 insert was subcloned into pBluescript and the entire insert was sequenced. The nucleotide sequence revealed that bPGIS-1 contained 0.23 kb of 5'-untranslated region and 0.62 kb of coding region. To obtain further downstream sequence, the library was rescreened using the 3'-end of bPGIS-1 as a probe. This second probe (Probe B; Fig. 1) was generated by PCR amplification of bPGIS-1 using the primers located at 244-261 bp and 589-605 bp downstream from the ATG start site. A second round of screening yielded two additional clones, bPGIS-2 (0.86 kb) and bPGIS-3 (0.64 kb).

Two more rounds of screening were carried out to obtain clones covering the entire coding region of bovine PGIS. The probes used for this screening work were generated by: (a) digestion of bPGIS-2 with ApaI (one ApaI site is located in the insert and the other in the pBluescript) to obtain a 0.33-kb DNA fragment, Probe C; and (b) digestion of bPGIS-4 with EcoRI (one EcoRI site in the insert, the other in the vector) to obtain Probe D (0.16 kb). Hybridization cloning using Probe C resulted in the isolation of two additional clones, bPGIS-4 (0.50 kb) and bPGIS-5 (0.80 kb). Screening using probe D yielded two clones, bPGIS-6 (1.1 kb) and bPGIS-7 (0.6 kb). All the inserts of positive clones were subcloned into the pBluescript and the nucleotide sequences were determined by dideoxy chain termination method. Of particular importance is that no sequence differences were found in the overlapping regions of the PGIS clones, indicating that all our clones originated from the same mRNA. The total length of bovine PGIS cDNA thus obtained is 2.6 kb.

The nucleotide sequences of PGIS cDNA predict an open reading frame of 1500 bp encoding 500 amino acids with a molecular weight of 56,675 (Fig. 2). The predicted isoelectric point of PGIS is 7.09, consistent with the pI of the purified enzyme (10). The sequence of the first 15 amino acid

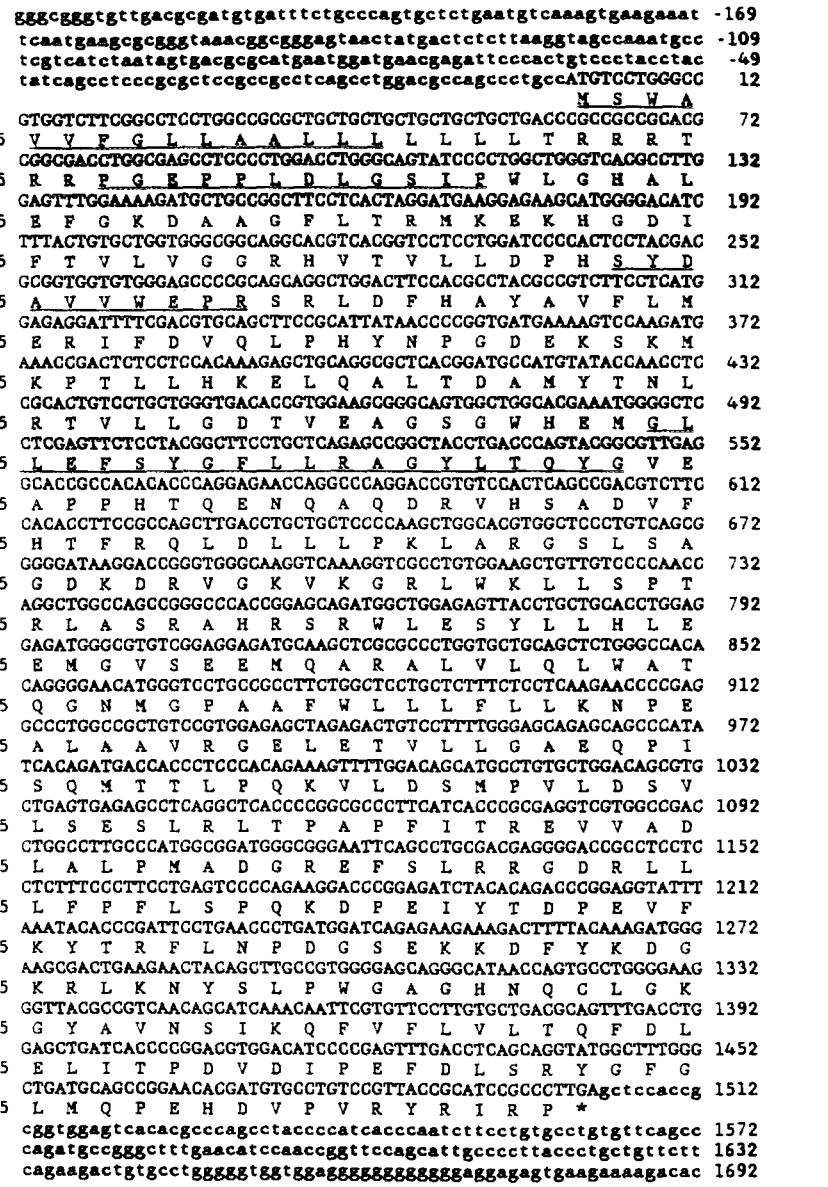

Fig. 2. Nucleotide sequence and deduced amino acid sequence of bovine PGIS. Underlined are those regions correponding to the peptide sequences from the purified bovine PGIS. An asterisk denotes the stop codon. Nucleotide numbers are given at right and amino acid numbers are at left. Not all the sequence of 3'-untranslated region is shown. The nucleotide sequence has been submitted to the GenBank/EMBL Data Bank with accession number L34208.

residues following the translational start site is identical to the NH_2-terminal sequence of the gel-purified bovine PGIS reported previously (10). Furthermore, partial amino acid sequences of a CNBr-cleaved peptide and tryptic peptides reported previously (10, 12) are also consistent with those deduced from the PGIS cDNA (Fig. 2). Re-examining the peptide sequence revealed only one

amino acid residue difference between the deduced cDNA and the CNBr-cleaved peptide (D ->R at position 174), and one residue difference between a tryptic peptide and the immunoaffinity-purified bovine PGIS (K ->R at position 91). These discrepancies may be due to genetic polymorphism or amino acid sequencing errors. These results provide evidence that the PGIS cDNA is authentic. The G residue at the -3 position from the ATG start site is consistent with the Kozak consensus sequence (13). Although the T residue at the +4 position from the ATG start site does not comply with the Kozak sequence, it has been shown that GCCATGT (identical to the ATG start site sequence of bPGIS) functions as efficiently as the Kozak sequence for translational initiation (14).

Comparison of PGIS sequence with known proteins in the GenBank/EMBL libraries revealed that, as predicted from the spectroscopic studies (8), the PGIS had considerable sequence similarity with other cytochrome P450s. The closest member of the P450 family that is related to PGIS is human cholesterol 7-α-monooxygenase (CYP 7; 15, 16), with 31 % sequence identity. However, this level of sequence identity is less than that expected for a member of family 7, thus indicating that PGIS represents a new family in the P450 superfamily. Interestingly, PGIS shares only 16 % sequence identity with human thromboxane (TXA) synthase (17, 18). TXA synthase shares several common features with PGIS. Both enzymes are P450 proteins without the monooxygenation activity; both are located at endoplasmic reticulum; both undergo "suicide" inactivation during catalysis (19, 20) and both utilize the same substrate, i.e. prostaglandin H_2, for catalysis.

The hallmark of cytochrome P450s is the characteristic maximal absorption spectra near 450 nm in the ferrous CO complex caused by heme-iron ligation to a conserved cysteine thiolate of the protein. In other P450s, this cysteine residue is present in a well conserved region near the carboxyl terminus with a consensus sequence of FXXGXXXCXGXXXA (21). This heme-binding sequence is conserved in PGIS except for one amino acid residue: F ->W at position 434 (Fig. 3A). Sequence comparison of PGIS with human CYP 7, TXA synthase, $P450_{cam}$ and P450BM-3 in the heme-binding region is shown in Fig. 3A. $P450_{cam}$ and P450BM-3 are the only two P450s whose three-dimensional structures have been determined by crystallography (22,23). The structure in the heme-binding region of these two proteins is very much alike even though they share less than 15 % sequence identity, suggesting that all P450s may have a similar three-dimensional structure in the heme-binding region. Conservation of the consensus heme-binding sequence in PGIS suggests that the heme-binding region of this enzyme will be similar to other P450 family.

Another highly conserved region among P450s is the helix I, which forms an α-helical backbone through the center of the protein, and contributes part of the heme-binding interactions. This region

A. Heme-binding region

Consensus sequence		F X X G X X X C X G X X X A
PGIS	434	W G A G H N Q C L G K G Y A
CYP 7	437	F G S G A T I C P G R L F A
TXS	473	F G A G P R S C L G V R L G
$P450_{cam}$	350	F G H G S H L C L G Q H L A
P450BM-3	393	F G N G Q R A C I G Q Q F A

B. Helix I region

															*	*									
PGIS	295	L	L	L	K	N	P	E	A	L	A	A	V	R	G	E	L	E	T	V	L	L	G	A	E	Q	P	I	S	Q	M	T	T
TXS	329	V	D	E	I	V	G	Q	A	F	I	F	L	I	A	G	Y	E	I	I	T	N	T	L	S	F	A	T	Y	L	L	A	T
$P450_{cam}$	234	S	D	E	A	K	R	M	C	G	L	L	L	V	G	G	L	D	T	V	V	N	F	L	S	F	S	M	E	F	L	A	K
P450BM-3	251	D	E	N	I	R	Y	Q	I	I	T	F	L	I	A	G	H	E	T	T	S	G	L	L	S	F	A	L	Y	F	L	V	K

C. Membrane anchoring region

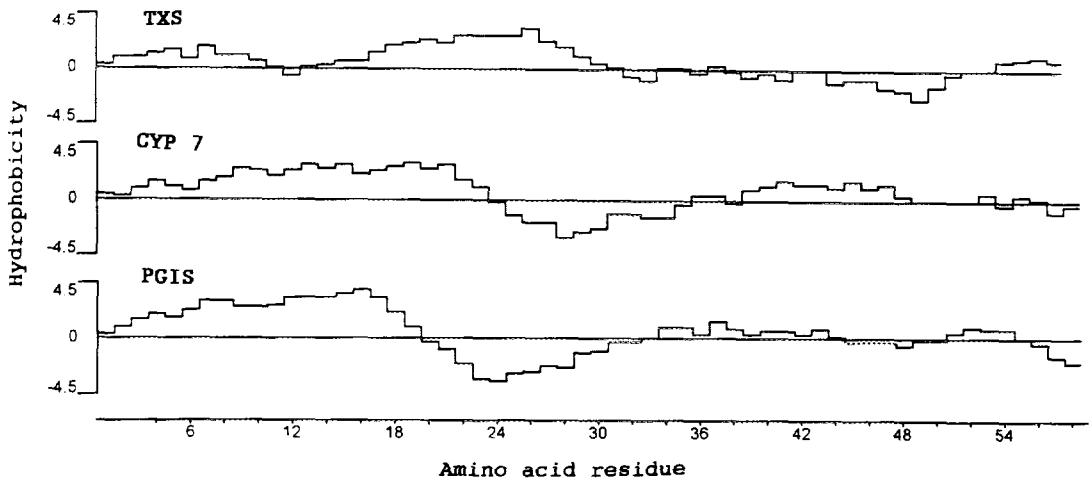

Fig. 3. Comparison of amino acid sequences of PGIS with selected P450. A. Heme-binding region. B. Helix I region. The asterisks denote the D/E T/S motif. TXS denotes TXA synthase. C. The hydrophobicity of the NH_2-terminal regions of thromboxane synthase (top), human CYP 7 (middle) and PGIS (bottom) were analyzed by the method of Kyte and Doolittle (26) using a scan length of 7 amino acids.

is located 120-150 amino acid residues upstream from the thiolate ligand, and is believed to be involved in the oxygen activation. Two amino acid residues have been found to be greatly conserved in helix I: a threonine or serine next to an acidic amino acid residue (aspartic acid or glutamic acid). In $P450_{cam}$, the hydroxyl group of the threonine residue forms hydrogen bonds with both the molecular oxygen and the carboxylate of the aspartate residue via a bridging water molecule. This arrangement together with the proximal cysteine ligand has been proposed to facilitate oxygen-oxygen bond cleavage (24). The glu-thr sequence is found in PGIS at positions 311 and 312, respectively (Fig. 3B). Interestingly, TXA synthase has isoleucine in place of threonine residue (17). It will be interesting to study the difference of mutation of these two amino acid residues on the catalytic differences between PGIS and TXA synthase. It should also be noted that, using the

Protean software in the DNAstar programs, the amino acid residues 301-324 of PGIS is predicted to be an α-helix.

Unlike mitochondrial P450s which use amphipathic NH_2-terminal as targeting sequences, the first twenty amino acid residues of PGIS at the amino terminus are hydrophobic and contain an α-helix. As with most microsomal P450s, this region is predicted to be the membrane anchoring region for endoplasmic reticulum (25). Fig. 3C shows the hydropathy plot of the NH_2-terminal region of PGIS and its comparison with that of TXA synthase and human CYP 7. Surprisingly, the pattern of PGIS hydrophobicity is similar to that of CYP 7 but quite distinct from that of TXA synthase. We have recently shown that TXA synthase anchors to the endoplasmic reticulum membrane via two transmembranous loops and the NH_2-terminal segment as well as bulk of the enzyme is located in the cytoplasm (27). Judging from the different hydropathy plot, in which only a hydrophobic stretch is noted close to NH_2-terminus of PGIS, it may be speculated that PGIS anchors to the membrane via a single transmembranous domain. The different membrane topology between PGIS and TXA synthase may have important impact on the conversion of a common substrate, prostaglandin H_2, into distinct products. The availability of the full-length PGIS and TXA synthase cDNAs will allow for elucidating the differences in catalytic mechanisms between these two enzymes.

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REFERENCES

1. Svensson, J., Hamberg, M. and Samuelsson, B. (1976) Acta Physiol. Scand. 98:285-294.
2. Moncada, S., Gryglewski, R.J., Bunting, S. and Vane, J.R. (1976) Nature 263:663-665.
3. Pober, J.S., Cotran, R.S. (1990) Physiol. Rev. 70:427-455.
4. Bunting, S., Moncada, S. and Vane, J.R. (1983) Brit. Med. Bull. 39:271-276.
5. Oates, J.A., FitzGerald, G.A., Branch, R.A. (1988) New Eng. J. Med. 319:689-717.
6. Ingerman-Wojenski, C., Silver, M.J., Smith, J.B. and Macarak, E. (1981) J. Clin. Invest. 67:1292-1296.
7. Smith, W.L., DeWitt, D.L. and Allen, M.L. (1983) J. Biol. Chem. 258:5922-5926.
8. Ullrich, V., Castle, L. and Weber, P. (1981) Biochem. Pharmacol. 30:2033-2036.
9. Ullrich, V. and Graf, H. (1984) Trends Pharmacol. Sci. 5:352-355.
10. Pereira, B., Wu, K.K. and Wang, L.-H. (1993) Biochem. Biophys. Res. Commun. 197:1041-1048.
11. Mierendorf, R.C. and Pfeffer, D. (1987) Methods Enzymol. 152:556-562.
12. Inoue, M., Smith, W.L. and DeWitt, D.L. (1987) Adv. Prostag. Thromb. Leuk. Res. 17:29-33.
13. Kozak, M. (1989) J. Cell Biol. 108:229-241.
14. Kozak, M. (1989) Mol. Cell. Biol. 9:5073-5080.
15. Noshiro, M. and Okuda, K. (1990) FEBS Lett. 268:137-140.

16. Cohen, J.C., Cali, J.J., Jelinek, D.F., Mehrabain, M., Sparkes, R.S., Lusis, A.J., Russell, D.W. and Hobbs, H.H. (1992) Genomics 14:153-161.
17. Ohashi, K., Ruan, K.-H., Kulmacz, R.J., Wu, K.K. and Wang, L.-H. (1992) J. Biol. Chem. 267:789-793.
18. Yokoyama, C., Miyata, A., Ihara, H., Ulrich, V. and Tanabe, T. (1991) Biochem. Biophys. Res. Commun. 178:1479-1484.
19. Jones, D.A. and Fitzpatrick, F.A. (1990) J. Biol. Chem. 265:20166-20171.
20. DeWitt, D.L. and Smith, W.L. (1983) J. Biol. Chem. 258:3285-3293.
21. Gonzalez, F.J. (1989) Pharmol. Rev. 40:243-288.
22. Poulos, T.L., Finzel, B.C., Gunsalus, I.C., Wagner, G.C. and Kraut, J. (1985) J. Biol. Chem. 260:16122-16130.
23. Ravichandran, K.G., Boddupalli, S.S., Hasemann, C.A., Peterson, J.A. and Deisenhofer, J. (1993) Science 261:731-736.
24. Gerber, N.C. and Sligar, S.G. (1994) J. Biol. Chem. 269:4260-4266.
25. Black, S.D. (1992) FASEB J. 6:680-685.
26. Kyte, J. and Doolittle, R.F. (1982) J. Mol. Chem. 157:105-132.
27. Ruan, K.-H., Wang, L.-H., Wu, K.K. and Kulmacz, R.J. (1993) J. Biol. Chem. 268:19483-19490.