Molecular characterization of a mouse prostaglandin D receptor and functional expression of the cloned gene

(prostaglandin D₂/G protein-coupled receptor/gene cloning/mRNA distribution)

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ABSTRACT Prostanoid receptors belong to the family of G protein-coupled receptors with seven transmembrane domains. By taking advantage of nucleotide sequence homology among the prostanoid receptors, we have isolated and identified a cDNA fragment and its gene encoding a mouse prostaglandin (PG) D receptor by reverse transcription polymerase chain reaction and gene cloning. This gene codes for a polypeptide of 357 amino acids, with a calculated molecular weight of 40,012. The deduced amino acid sequence has a high degree of similarity with the mouse PGI receptor and the EP₂ subtype of the PGE receptor, which together form a subgroup of the prostanoid receptors. Chinese hamster ovary cells stably expressing the gene showed a single class of binding sites for $[^{3}H]PGD_{2}$ with a K_{d} of 40 nM. This binding was displaced by unlabeled ligands in the following order: PGD₂ > BW 245C (a PGD agonist) > BW A868C (a PGD antagonist) > STA₂ (a thromboxane A_2 agonist). PGE₂, PGF_{2 α}, and iloprost showed little displacement activity at concentrations up to 10 μ M. PGD₂ and BW 245C also increased cAMP levels in Chinese hamster ovary cells expressing the receptor, in a concentrationdependent manner. BW A868C showed a partial agonist activity in the cAMP assay. Northern blotting analysis with mouse poly (A)⁺ RNA identified a major mRNA species of 3.5 kb that was most abundantly expressed in the ileum, followed by lung, stomach, and uterus.

Prostanoids such as the prostaglandins (PGs) and thromboxane (TX) are cyclooxygenase metabolites of arachidonic acid (1) and exert a variety of biological activities to maintain local homeostasis in the body (2). Each of these compounds acts through its own specific receptor, which is coupled to the regulatory G protein(s) (3). At least seven pharmacologically distinct prostanoid receptors have been identified (4): the TXA₂ receptor, the EP₁, EP₂, and EP₃ subtypes of the PGE receptor, the PGF receptor, the PGI receptor, and the PGD receptor. Of these, the EP₂ subtype of the PGE receptor, the PGI receptor, and the PGD receptor have been classified as relaxant prostanoid receptors (4); they preferentially activate adenylate cyclase in their second messenger pathways and produce relaxation in smooth muscle preparations. PGD₂ is formed in a number of tissues and cells and produces a variety of biological responses (4, 5). For example, PGD₂ acts during platelet aggregation as a negative-feedback regulator (5), produces relaxation of vascular and nonvascular smooth muscle (6-8), and modifies autonomic as well as sensory nerve functions (9, 10). It is also released by mast cells and cells of the macrophage lineage to play a role during immunologic reactions (11-13). Finally, PGD₂, synthesized in the brain, has been proposed to be a physiological regulator of sleep (14).

Recently, we isolated cDNAs for the TXA₂ receptor (15, 16) and the EP_1 (17), the EP_2 (18), and the EP_3 (19, 20) subtypes of the PGE receptors by homology screening. These studies revealed that prostanoid receptors constitute a subfamily within the rhodopsin-type, G protein-coupled receptor superfamily (21), and they enabled us to identify highly conserved amino acid sequences in these receptors. To clone the cDNAs for other prostanoid receptors, we have carried out reverse transcription polymerase chain reaction (RT-PCR) studies based on such motifs and have successfully amplified three cDNAs encoding sequences homologous to prostanoid receptors. Of these three, one has been identified as being part of the PGF receptor cDNA (22) and another as being part of the PGI receptor cDNA (23). The third, unidentified, cDNA showed a high degree of sequence similarity to the PGI receptor (23) and to the EP_2 subtype of the PGE receptor (18). We postulated that the cDNA may code for a member of the relaxant prostanoid receptor family. Here, we report the primary structure of this mouse cDNA and its gene.[†] We also describe the identification of the gene product as a mouse PGD receptor, based on its ligand binding characteristics and second messenger responses. The tissue distribution of its mRNA expression is also shown.

MATERIALS AND METHODS

Ligands. PGD₂, PGE₂, PGF₂, and STA₂ (9,11-epithio-11,12-methano-TXA₂) were kindly provided by Ono Pharmaceuticals (Osaka). BW 245C [5-(6-carboxyhexyl)-1-(3cyclohexyl-3-hydroxypropyl)hydantoin] and BW A868C [3-benzyl-5-(6-carboxyhexyl)-1-(2-cyclohexyl-2-hydroxyethylamino)hydantoin] were generous gifts from Wellcome. Iloprost was purchased from Amersham, and [5,6,8,9,12, 14,15(N)-³H]PGD₂ (4255 GBq/mmol) was obtained from New England Nuclear.

RT-PCR Screening. A pair of degenerate oligonucleotides (5'-GG(N)AC(N)TGGTG(C/T)TT(C/T)(A/T)T-3' and 5'-A(T/G)(A/G)(A/T)A(A/G/T)ATCCA(N)GG(A/G)TC-3') were synthesized which corresponded to the peptides GT-WCF (I/L) in the second extracellular loop and DPWI (Y/F)(I/L) in the seventh transmembrane domain, respectively. DNA amplification of first strand cDNA from mouse thymus was performed with a cycling profile of 95°C for 60 sec, 38°C for 60 sec, and 70°C for 60 sec, repeated over 35 cycles. One of the amplified fragments, PG25, contained a sequence highly homologous to the fifth and sixth transmembrane domains of the prostanoid receptors.

Mouse Genomic Library Screening. A mouse (strain 129Sv) genomic library in Lambda FIX II (Stratagene) was screened

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Abbreviations: PG, prostaglandin; TX, thromboxane; RT-PCR, reverse transcription polymerase chain reaction; CHO, Chinese hamster ovary; dhfr, dihydrofolate reductase.

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[†]The sequences reported in this paper have been deposited in the GenBank data base (accession nos. D29764 and D29765).

with the ³²P-labeled PG25 fragment. Four positive, overlapping clones were isolated from 2×10^5 recombinant plaques originally screened. One of these clones contained a 13-kb insert. Southern hybridization and restriction analysis of the insert identified two Xba I fragments (3.3 kb and 6 kb) that hybridized to the probe. These Xba I restriction fragments were subcloned and further analyzed.

Construction of an Open Reading Frame and Its Expression in Chinese Hamster Ovary (CHO) Cells. PGc1, an 824-bp cDNA fragment which spans both exons of the PG25 gene, was amplified by PCR from mouse lung cDNA using a pair of oligonucleotides (5'-GGAGTGCTGGCTGTCTCT-3' and 5'-CTTCAGTGCTGATCCCTC-3'). The nucleotide sequence of PGc1 was confirmed to be identical to the corresponding exons from the genomic clone. PGc1 was digested by Nar I, and the 3' restriction fragment was then ligated to the Pst I-Nar I fragment of the genomic clone at the Nar I site. This resulted in a 1.2-kb fragment, PGc9, which contains the putative open reading frame. PGc9 was subcloned into the eukaryotic expression vector pdKCR-dhfr, which contains a mouse dihydrofolate reductase (dhfr) gene (24). The resulting plasmid, pdKCR-dhfr-PGc9, was transfected into dhfrdeficient CHO (CHO-dhfr⁻) cells (25) as previously described (26). Several transformant CHO cell clones were isolated by selecting cultures with nucleoside-depleted medium. One of these clones, named CHO-J, was used in subsequent experiments.

[³H]PGD₂ Binding Analysis. CHO-J cells were maintained in α -MEM(-) (MEM lacking ribo- and deoxyribonucleosides; Sigma) supplemented with 10% fetal calf serum and penicillin (100 units/ml). The cells were washed once with phosphate buffered saline (137 mM NaCl/2.7 mM KCl/4.3 mM $Na_2HPO_4/1.4$ mM KH_2PO_4), scraped from the culture plates, and collected by centrifugation for 15 min at $800 \times g$. The cell pellet was suspended in 10 vol of homogenization buffer (25 mM Hepes-NaOH, pH 7.4 at 20°C/1 mM EDTA/5 mM MgCl₂/250 mM D-mannitol/2 mM 2-mercaptoethanol/1 mM phenylmethylsulfonyl fluoride/1 mM benzamidine·HCl) and homogenized by sonication. The homogenate was then incubated in the presence of 10 nM [³H]PGD₂, with or without unlabeled ligands, at 4°C for 60 min. After incubation, the reaction was terminated by the addition of ice-cold washing buffer (25 mM Hepes-NaOH, pH 7.4/1 mM EDTA/5 mM MgCl₂/140 mM NaCl/5 mM KCl) and the mixture was passed through a GF/C glass fiber filter (Whatman). The filter was then washed four times with ice-cold washing buffer and the bound radioactivity was measured by liquid scintillation counting. Nonspecific binding was defined as the radioactivity bound to the filter in the presence of a 200-fold excess of unlabeled PGD₂. Incubation of the reaction mixture at 37°C resulted in a high nonspecific binding as noted previously (27).

cAMP Assay. CHO-J cells were seeded at 5×10^4 cells per well in 24-well plates and were cultured in $\alpha MEM(-)$ with 10% fetal calf serum for 48 h. The cellular cAMP levels were measured by radioimmunoassay as previously described (23), using a cAMP radioimmunoassay kit (Amersham) after stimulation with PGD_2 , BW 245C, or BW A868C in the presence of 1 mM 3-isobutyl-1-methylxanthine.

Northern Blotting. Total RNA was isolated from several organs of 5-week-old mice, and enriched for poly(A)⁺ RNA with OligoTex dT30 (Takara, Kyoto). After electrophoresis and transfer of the RNA to a nylon membrane, the membrane was prehybridized in $5 \times$ SSPE ($1 \times$ SSPE = 0.15 M sodium chloride/10 mM sodium phosphate/1 mM EDTA, pH 7.4), 5× Denhardt's solution (1× Denhardt's solution = 0.02%Ficoll 400/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin), 1% SDS, yeast RNA at 100 μ g/ml, then hybridized to the ³²P-labeled PG25 fragment in the same solution for 14 h at 65°C. The membrane was finally washed in $0.2 \times SSC(1 \times$

SSC = 0.15M sodium chloride/0.015 M sodium citrate, pH 7), 0.1% SDS at 65°C and exposed to x-ray film for 7 days.

RESULTS

RT-PCR based on the highly conserved residues in the prostanoid receptors amplified a cDNA fragment from mouse thymus. This fragment, called PG25, is 425 bp long and codes for an open reading frame that shows sequence homology to the fifth (45%), sixth (35%), and seventh (76%) transmembrane domains of the EP2 subtype of the mouse PGE receptor (18). Initial attempts to identify the cells or tissues expressing PG25 by RNA blotting analysis were unsuccessful, as was using PG25 as a probe to screen cDNA libraries generated



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CTCC L ATG D GACC D GACC D CAG C GACC D ATC L L CAG D ATC L L TAC	TAC Y TAC R CGC R CAC H GT TAT Y CTC L TTC. F TC. F	TCC S AACC N GCCC A TTTT GCCC A TTTT GCCC R CAAA CAAA C AAGG, R AAAC	AGCC S CTC Q Q CAG Q Q T T T G G G G C A C T T T G G C A C T T T G G C T T T G C T C T C T C T C	TATE TATE TCA S CTG CTG CTG TTG TTG I I I I I I S AGC	ATG GAC GAC G G G G G G G G C TG C G G C TG C C G G C C G G C C G G C C G G C C G G C C G G C C G G C C G C C G C C G C C G C C G C C G C C G C C G C C G C C G C C G C C G C C G C C G C C G C C G C C G C C G C C G C C G C C G C C G C C G C C G C C G C C G C C C G C C C G C C C C G C C C C C G C C C C C C C C C C C C C C C C C C C C	ATCA ATCA M TCA S GCT A S GCT CAA	A CAC H GAC D CTC L CTC L CTC L CTC C C C C C C C C C	AGG R TAC Y ATG M  F TCT F TCT S ATG M CAG	GTC V R AGG R AGG R ACA T T AAAA K GTG V TTA L CAA	CAC CAC Q CAC H GTG V  CAC L CAC T T T T T T T T T T T T T T T T	A CCCC CCCC CCCC CCCC CCCC CCCCC CCCCC H CCCCCC H CCCCCC CCCCC H	T CAC T T T T T T T T T T T T T T T T T	GTG V TATT Y CTG L CAA2 K GTG CAA2 K GTG V GTT V GAA	GTG V CCT P CAT H ATG A GAC D TTC F TCC	TGC CAC T P TGT P TGT C C C C C C C C C T C C C T C T C T	CCCC R TTC TTC S TTC G G TTC G G TTC R N N N N N N N N N N N N N N N N N N	I CTC L C C C C C C C C C C C C C C C C C	GCC G G C C C C C C C C C C C C C C C C	AGAA AGAA AGAA AGAA E AGAA E AGAA E AGAA E I I I I I I I	200 660 220 720 240 780 260 840 280 900 300 960 320 1020 340
CTCC L ATC D GACC D GACC D CAC D CAC D CAC D CAC D CAC D CAC D CAC D CAC D CAC D CAC C D C CAC D C CAC C D C C C C	TAC Y TAC R CGC R CAC H I GT TAT Y CTC L F TAC AGA	TCC S AACC N GCCC A TTT F V GAG R CAAA CAAA R CAAA CAAA C AAGG, R	AGC S CTC Q CTC Q GTG V I TTTG A GCC A V A CT T T GC C A C V I TTTG A C C C C C C C C C C C C C C C C C C	CTC L TATF Y S CTG CTG CTG CTG TTG TTG TTG L I I S S S S S S	ATG GAC GGC G G CTG G G G C TAI Y Y C GTA R GTA V AGC	ATCA ATCA M TCA S GCT S CAA TTCC F TTCC F TTCC F	GAC D CTC D CTC L CTC L CTC C C C C C C C C C C C C	AGG R TAC Y ATG M  F TCT F TCT S ATG M CAG	GTC V CGC R ACG R ACA T ACA K GTG V TTA L CAA	CTCL CAG Q CAC H GTC V  CTL L TTT F AGI	A A A A C C C C C C C C C C C C C C C C	T CAC T T T T T T T T T T T T T T T T T	GTG V TATT Y CTG L CAA2 K GTG V GTT V GTT V GAA	GTG V CCT P CAT H ATG A GAC D TTC F TCC	TGC CAC H CCT P TGT C CCT P TGT C CCT P CCC P ACA T	TTC R TTC TTC GG G G G G G G G G G G G G G G	L CTCC C C C C C C C C C C C C C C C C C	S G G T C C C C C C C C C C C C C C C C C	AGAA E CCAA CCAA CCAA CCAA E CCAA CCAA E CCAA I CCAA I CCAA I I CCAA	200 660 220 720 240 780 260 840 280 900 300 900 320 1020 340
CTCC L ATG GACC D GACC D CAG C CAG C CAG C CAG C C CAG C C C C C	TAC Y TAC R CGC R TAC H GT TAC H CTC L I TCC F AGA R TCC SCC	TTCC S AACC N GCCC A GCCC A CCAA CCAA CCAA CCA	AGCC S CACC Q GTC Q GTC Q GTC Q Q TTTC GCC A TTTC GCC A C TTC GCC A C TTC GCC A C C C C C C C C C C C C C C C C	CTC L TAT Y TCA S CTG L CTG C CTG C CTG L CTG L CTG L S CTG L S CTG L S CTG L S CTG L S CTG L S CTG L S CTG S S CTG S CTG S CTG S S S S S S S S S S S S S S S S S S S	ATG M GAC G G G G G G G G G G G G C TG C TG C	ATCA ATCA M TCA S GCT A S GCT A S GCT A S CAA TTCC F TTCC F CAT H TTAA TCC	A CTC CAC H CTC CTC L CTC L CTC CTC CTC CT	A CTG L AGG R TACC Y ATG M TTTT F TCT S ATG M CAG Q GAA	GTC V CGC R AGCG R ACA R ACA T T T T T T T T T T C AAAA R C C C C C R R T C C C C R R T C C C R R T C C C R R T C C C C	CAC CAC CAC CAC CAC CAC CAC H GTC V  CAC H CAC CAC CAC CAC CAC CAC CAC CAC C	A A A A A A C C C C C C C C C C C C C C	ACC T T CAC H T T C C T T C T T C T T C T T C T T C T T C T T C T T T C T T T C T T T C T T T T C T T T T T T T T C T T T T T T T T C T T T T T T T C C T T T T T C C T T T T C C T T T T C C T T T T C C T T T T C C T T T C C T T T C C T T T C C T T T C C T T T C C T T T C C T T T C C T T C C T T C C T T C C T T C C T T C C C T T C C C T T C C C T T C C C T T C C C T T C C C T C C C T C C C C C C C C C C C C C C C C C C C C	GTG GTG L ACC T ACC T ACC T C ACC T C ACC T C ACC C T C C C C	CAT CAT H ATG M GAC D TTCC S CAA GAC C CAA GAC C C C CAA C C C C C C	TGC CAC H P TGT C TGT C C C C C C C C C C C C C C C	TTCCCCR R TTCCCR TTCCCR TTCCCR TTCCCR TTCCCCR TTCCCCCR TTCCCCCR TTCCCCCCR TTCCCCCCR TTCCCCCCR TTCCCCCCCR TTCCCCCCR TTCCCCCCR TTCCCCCCR TTCCCCCCR TTCCCCCCR TTCCCCCCR TTCCCCCCR TTCCCCCCR TTCCCCCCR TTCCCCCCR TTCCCCCCR TTCCCCCCR TTCCCCCCR TTCCCCCCR TTCCCCCCR TTCCCCCCR TTCCCCCCCR TTCCCCCCCR TTCCCCCCCR TTCCCCCCCR TTCCCCCCCR TTCCCCCCCR TTCCCCCCCR TTCCCCCCCR TTCCCCCCCR TTCCCCCCCR TTCCCCCCCR TTCCCCCCCC	I CCTC C C C C C C C C C C C C C C C C C	S GAGE G G C C C C C C C C C C C C C C C C C C C	TTA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA CCAA	200 660 220 720 240 260 280 280 300 900 320 1020 340 1024 357 1134

FIG. 1. Nucleotide and deduced amino acid sequences of the mouse PGD receptor gene. (A) The relationship between the genome, the RT-PCR products PGc1 and PG25, and the synthetic cDNA PGc9 is shown. The exons in the genomic clone are indicated by open boxes, and the proposed open reading frame for PGc9 is boxed. The transmembrane domains are indicated by black boxes and are numbered in white Arabic numerals. (B) Nucleotide and deduced amino acid sequences of the mouse PGD receptor gene. The nucleotides in PGc9 are numbered according to the first methionine of the open reading frame. The nucleotides in the intron but not in PGc9 are italicized. The deduced amino acids are shown under the nucleotide sequence. The position of the translation stop codon is marked with an asterisk and the positions of the putative transmembrane domains are underlined. N-glycosylation sites ( $\Delta$ ) and potential protein kinase C phosphorylation sites (*) are indicated below the amino acids. The nucleotide sequence corresponding to PG25 is boxed.

from several mouse tissues. Instead of expanding the screening process to isolate the full-length cDNA, we decided to isolate the gene encoding this prostanoid receptor-like sequence and to construct an open reading frame from a genomic clone for functional expression studies. Using a ³²P-labeled PG25 fragment as a probe, we screened a mouse genomic library and isolated four partially overlapping clones from  $2 \times 10^5$  recombinants. One of these clones had a 13-kb insert with 3.3-kb and 6-kb Xba I restriction fragments that hybridized to the probe. Analysis of these Xba I restriction fragments revealed that the 309-bp sequence corresponding to the 5' segment of PG25 was present in the 3.3-kb fragment. The rest of the PG25 sequence was located in the 6-kb fragment approximately 4 kb away from the upstream sequence (Fig. 1A). There is a potential translation initiation codon (28) 534 bp upstream of the PG25 sequence in the 3.3-kb fragment. This ATG initiates an open reading frame that codes for an amino acid sequence homologous to the first six transmembrane domains of the prostanoid receptors. The amino acid sequence of the open reading frame in the downstream 6-kb Xba I fragment contains sequence homologous to the seventh transmembrane domain of the prostanoid receptor followed by a short tail sequence and an in-frame stop codon (Fig. 1A). The intron has donor and acceptor splice sites compatible with the consensus sequence (29) (Fig. 1B), and its relative position in the coding sequence is identical to that of the human  $TXA_2$  receptor gene (30). Amplification by RT-PCR and Southern blotting analysis of the mouse genomic DNA revealed bands of the expected sizes on gels (data not shown).

A DNA fragment containing the open reading frame was constructed by connecting a genomic fragment to amplified cDNA. We performed RT-PCR with a pair of oligonucleotides: the 5' oligonucleotide corresponding to a sequence in the upstream exon, and the 3' oligonucleotide from a sequence 130 bp downstream of the putative in-frame stop codon in the 3' exon. We amplified an 824-bp fragment (PGc1) and constructed a 1.2-kb synthetic cDNA (PGc9) by ligating the Pst I-Nar I restriction fragments of the genomic fragment and PGc1 at their Nar I sites. Fig. 1A summarizes the relationship between the genomic clone, PG25, PGc1, and PGc9. PGc9 contains an open reading frame of 1071 bp that codes for a polypeptide of 357 amino acids (Fig. 1B). The translated product has a calculated molecular weight of 40,012, and its hydrophobicity profile (31) reveals the presence of seven hydrophobic regions which likely represent transmembrane domains. There are two asparagine residues for putative N-glycosylation: Asn-2 in the N-terminus and Asn-89 in the first extracellular loop. There are also two potential protein kinase C phosphorylation sites (32), Ser-47 and Thr-144, in the first and the second cytoplasmic loops, respectively. In addition, there are eight serines and threonines in the carboxyl-terminal tail, some of which may be sites for phosphorylation by a receptor kinase (21).

Fig. 2 shows the alignment of the deduced amino acid sequence from PGc9 compared with the sequences from other mouse prostanoid receptors. This analysis revealed that the PGc9 product has 39% and 32% sequence identity over the entire amino acid sequence with the PGI receptor (23) and the EP₂ subtype of the PGE receptor (18), respectively, but showed less than 25% amino acid sequence identity with other prostanoid receptors. Thus, the receptor encoded by PGc9, the PGI receptor, and the EP₂ subtype of the PGE receptor form a distinct subgroup within the prostanoid receptor family.

	I	П
DP	MNESYRCOTSTWVERGSSATMGAVLFGAGLLGNLLALVILARSGLGSC-RPGPLHPPPSV	EVVINCELT
IP	MKMMASDGHPGPPSVTPGSPLSAGGREWOGMAGSCWNITYVODSVGPATSTLMFVAGVVGNGLALGTLG	FAVIVICIA
EP2	-MAEVGGTIPRSNRELORCVLLTTTIMSIPGVNASFSSTPERLNSPVTIPAVMFTFGVVGNIVATVV	EVTIVECIA
EP3	MASMWAPEHSAEA-HSNLSST-TDDCGSVSVAFPTTMMVTGFVGNALAMLLVSRSYRR-RESKRKKS	ELLCTCWIA
EP1	MSPCGLNLSLADEAATCATPRLPNTSVVLPTGDNGTSPALPTFSMTLGAVSNVLALALLAGVAGRMPRPRSAAT	FILEVACLI
ΤP	MWPNGTSLGACFRPVNITLOFRATASPWFAASFCALGIGSNILALSVIA	ELALICCIV
FP	MSMNSSKOPVSPAAGLIANTICOTENRI-SVEESTTEMTVGTUSNSLATATUMKAYOREPO-KSKAS	
		LLLAJULV
DP	VTDLLGKCLTSPMVLAAYAONOSLKELLPASGNOLCETEAELWSEEGLASTIOLLAWAVECWLSLGWDEEYOPWYLDDCVLVADVVAACC	LAFCAL DEA
TP	VTDLIGTCFLSPAVEVAYARNSSIIGI-AHGGTNICDTFAFANTEFGIASTITLERMAVEDCIAISUDVIVAD DCDGAPEAD DCTVAFU	LAFCALPFA
FD2	VTDLLGTLLVGDVTTATYNKGOWDC_DOALGDVSTETLLEGTSGLSTTGANGTEDV ATNAAVECUVDVLAGTATTTATYNKGOW	CLECSLELL
FP3	TTDI VGOLT SOVVI AVI SODDWEOL, DOSC, DI CTESCI TUTVECI SCI VACAMATEMATEMATI SOTTU ARA TA DA DU WA CHA ATTA TA DA D	VLFCALPNM
FP1		LAFALLPVL
TD	TDELCH VICATVACQUALLEW DATDOC OLO CYENCKAWYEE CLEUC CAMACEKCYCY LOLINAAKYSYA AALAALAALAAMA	LAVALLPLV
ED	TTDEECHITMGTAVEUVACDUWTDEDO ONT LOSTECTOWECZO CHILIGAAMASERTVGITKPESKPIAIS-RKAMAIVGLWVAA	GALGLLPLL
I F	U V V	VEVAVLPIL
D D	CECKE-VOY/DETW/ETONT	-
TD	CI CELO AVEC DE MERCE AND COLORIS	5
ED2	CLOBEROV DOTWETDWTT NV TVVAESVAACTOETTATVI CUVACAL VISIFE RUVVCA	
ED2	CURSERVE DETWEETSTCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTETCHARVETTARVETTARVETTARVETTARVETTARVETTARVETTARVETTARVETTARVETTARVETTARVET	VACRGHAGA
ED1		
TD	ny oktel ut - Poince i slopko	GPRLASASS
E D		
٢٣	UT	
DP	RAYYGAFKLENKAEGDSEDLQALRFLSVISIVI	DPWIFIIFR
1P	CONTRACTOR CONTRA	DPWVFILFR
CPZ	SPALQRESUFRKRKSFRRIAGAEIQMVILLIAISLVVLICSIPLVVKVFINQLYQPNVVKDISRNPDLQAIRIASVNPIL	DPWIYILLR
EPJ	A STATATAT A SQS A A W GRI - I TE IA I QEM GI M CYLSY CW SPELIMMLKMI FNQMSVEQCKI QM GKEKECNSFLIAV RLASLNQILI	DPWVYLLLR
TD	ASSITSATATLKSSKUUGSARKVTATDVEMVUOLVUTAVUSLILWSPLLVLVVLAIGGWNSNSLQRP-LFLAVRLASWNQIL	DPWVYILLR
I P E D	VTHIREATQRPRDCEVEMMVQLVGIMVVAIVUMPLLVFIMQILLQIPPVMSFSGQLLRATE-HQLLIYLRVATWNQIL	DPWVYILFR
ГР	IMANIAINGNNSPVICEIT-LFALRMATWNQILI	<b>PWVYILLR</b>
DD	TENERMI EN AV ETROITVONWEEUEDOCNVEETI	
TD		
ED2	KTYL CVATEVICIC AS TACCO DE CANCELCARE CLARE CURRECS MYPLSS MGT GUYAPLIAVPLIGUDG SVGMPSKSEAIAACSL	C
ED3	KILLSKALENINGLE GALANSANDASANGHUSESKALSANSANSANSANSANSANSANSANSANSANSANSANSANS	ITSLR(56)
EPJ		
TD		
ED		
r P	NAWERNW # IN LA SUCCANNIT # STUTIEL # STUE # STUE A VAUT SE SLAVE K F SÅÅY STUE A VAUT SE SLAVE F F SÅY STUE A VAUT SE SLAVE F F SÅÅY STUE A VAUT SE SLAVE F F SÅY STUE A VAUT SE SLAVE F SÅY STUE A VAUT SE SLAVE F SÅY STUE A VAUT SE STUE A VAUT SE SLAVE F SÅY STUE A VAUT SE STUE A VAUT SE STUE A VAUT SE STUE A VAUT STUE A VAUT SE S	

FIG. 2. Comparison of the amino acid sequence of the receptor encoded by PGc9 with other prostanoid receptors. The amino acid sequences from PGc9 (DP), the mouse PGI receptor (IP), the EP₂, EP₃, and EP₁ subtypes of the PGE receptor, the TXA₂ receptor (TP), and the PGF receptor (FP) were aligned to achieve maximal sequence similarity. The shaded areas indicate amino acids that are conserved between the receptor encoded by PGc9 and other prostanoid receptors. Putative transmembrane domains are indicated by horizontal lines above the sequence.

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FIG. 3. Pharmacologic characteristics of the PGD receptor stably expressed in CHO cells. (A) Binding of [³H]PGD₂ to CHO-J cells and its displacement by unlabeled ligands. Cell homogenate and [³H]PDG₂ were incubated with and without unlabeled compounds at the indicated concentrations. The compounds used were: PGD₂ ( $\Box$ ), BW 245C ( $\triangle$ ), BW A868C ( $\bigcirc$ ), STA₂ ( $\bullet$ ), PGE₂ ( $\triangle$ ), iloprost ( $\diamondsuit$ ), and PGF_{2a} ( $\blacktriangledown$ ). (*Inset*) Scatchard plot of the equilibrium binding of [³H]PGD₂. B, bound [³H]PGD₂; F, free [³H]PGD₂. (B) cAMP levels in CHO-J cells treated with PGD₂ and analogues. CHO-J cells were incubated with 3-isobutyl-1-methylxanthine and the indicated concentrations of PGD₂ ( $\Box$ ), BW 245C ( $\triangle$ ), BW A868C ( $\bigcirc$ ), or solvent alone ( $\bullet$ ) and the cAMP content was determined as described in *Materials and Methods*.

The PGI receptor, the  $EP_2$  subtype of the PGE receptor, and the PGD receptor are classified pharmacologically as relaxant prostanoid receptors (4). Since PGc9 codes for a receptor that is closely related to two of these receptors, it is possible that PGc9 encodes a PGD receptor. This was tested by expressing PGc9 in CHO cells. CHO-J cells expressing PGc9 displayed a saturable, high-affinity binding of [³H]PGD₂, and Scatchard analysis revealed a single class of binding sites with a  $K_d$  of 40 nM and a  $B_{max}$  of 93 fmol/mg of homogenate protein (Fig. 3A Inset). Displacement studies showed that the PGD agonist BW 245C (5, 33) and the antagonist BW A868C (34-36) competitively displaced  $[^{3}H]PGD_{2}$  binding at a concentration range of 10 nM to 5  $\mu$ M (Fig. 3A). PGE₂, PGF_{2 $\alpha$}, and iloprost showed little displacement at 10  $\mu$ M, whereas the TXA₂ agonist STA₂ displaced the binding in a concentration-dependent manner from 100 nM. Parental CHO-dhfr⁻ cells showed no specific [³H]PGD₂

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FIG. 4. Northern blotting analysis of RNA isolated from various mouse tissues. Twenty micrograms of  $poly(A)^+$  RNA isolated from the tissues was applied to each lane. Hybridization was performed with ³²P-labeled PG25 fragment as a probe. Lane 1, brain; lane 2, thymus; lane 3, lung; lane 4, heart; lane 5, spleen; lane 6, stomach; lane 7, liver; lane 8, ileum; lane 9, kidney; and lane 10, uterus. The positions of 28S and 18S rRNA on the gel are given by arrows at the left side of the figure.

binding (data not shown). As shown in Fig. 3B, PGD₂ raised cAMP levels in CHO-J cells in a concentration-dependent manner with an EC₅₀ of 6.8 nM. BW 245C was about 10 times more potent than  $PGD_2$ , with an  $EC_{50}$  of 0.54 nM. BW A868C also increased cAMP levels at concentrations greater than 100 nM; however, the increase reached a plateau at 10  $\mu$ M, with the efficacy being about 35% of the maximal stimulation obtained with PGD₂. These results suggest that BW A868C is a partial agonist on a mouse PGD receptor. PGD₂, BW 245C, and BW A868C had no effect on cAMP levels in the parental CHO-dhfr⁻ cells (data not shown). PGD₂ did not induce significant inositolphospholipid breakdown in CHO-J cells (data not shown). These properties are compatible with those of the pharmacologically defined PGD receptor (4, 5), and we therefore conclude that PGc9 codes for a functional PGD receptor.

Northern blotting analysis of various mouse tissues with the PG25 fragment revealed a major hybridization signal at 3.5 kb (Fig. 4). This species of mRNA was most abundant in the ileum, followed by the lung, the stomach and the uterus. In the lung and ileum lanes, a band of about 7 kb was also apparent. In addition, a band at around 2 kb in ileal RNA was present.

### DISCUSSION

With the PGD receptor described herein, we have now reported the primary structures of all seven pharmacologically defined prostanoid receptors. There are two motifs that are highly conserved in these receptors. The first conserved motif is the QYCPG(T/S)WCF sequence (amino acids 175-183 of the PGD receptor) in the second extracellular loop. Although the cysteine residue in this motif (Cys-182) is present in the majority of the G protein-coupled receptors, and is believed to form a disulfide bond with another conserved cysteine residue in the first extracellular loop (Cys-104) (37), the above motif can be found only in the prostanoid receptors. The second conserved motif, RXX(S/T)X(N/I)-XI(V/L)DPW(I/V)(F/Y)(I/L) (amino acids 306-320 of the PGD receptor) is found in the seventh transmembrane domain and contains two charged amino acids, Arg-306 and Asp-315. These conserved residues may interact with the common structure of the ligands for these receptors. Recently, it has been shown that a mutation in this arginine residue in the human TXA₂ receptor results in the loss of ligand binding activity (38).

There are two potential phosphorylation sites for protein kinase C: one in the first cytoplasmic loop and one in the

second cytoplasmic loop. The former site is conserved in the  $EP_2$  and  $EP_3$  subtypes of the PGE receptor, whereas the latter is found in the  $TXA_2$  and the PGF receptors. In human platelets, phorbol ester treatment abolishes adenylate cyclase activation mediated by the PGD receptor, but spares the response mediated by the PGI receptor (39). Phorbol ester treatment also attenuates TX agonist-induced GTP hydrolysis in platelet membranes (F.U. and S.N., unpublished observation). These observations indicate that phosphorylation at these sites would affect the coupling of receptor and G protein to produce receptor desensitization.

The PGI receptor, the EP₂ subtype of the PGE receptor, and the PGD receptor have all been classified as relaxant prostanoid receptors (4). In agreement with this functional classification, their primary structures are more closely related to each other than to the other four prostanoid receptors. In contrast, the  $TXA_2$  receptor, the EP₁ subtype of the PGE receptor, and the PGF receptor all preferentially raise intracellular calcium and can be functionally classified as contractile prostanoid receptors. They are also more closely related to each other in their primary structure than they are to any of the others (22). Thus, within the prostanoid receptor family, there are two groups of functionally and structurally related receptors. The genomic organization for these receptors may have been conserved during their evolution, because both the human  $TXA_2$  receptor gene (30) and the mouse PGD receptor gene contain an open reading frame consisting of two exons separated by an intron at a homologous position.

Northern blotting for the PGD receptor identified a 3.5-kb mRNA in the lung, the gastrointestinal tract, and the uterus. The intestine is one tissue where high PGD synthase activity has been detected (40). It has also been reported that treatment with PGD₂ modifies the chloride transport across the intestinal mucosa (10, 41). In addition to the tissues identified by RNA blotting, RT-PCR detected the PGD receptor expression in the thymus and brain (M.H. and H. Oida, unpublished observations), suggesting that the PGD receptor expression in these organs may be confined to small region(s) or specific cell(s). PGD₂ is one of the major PGs formed in the brain and has been suggested to be a physiological regulator of sleep (14). The brain-type PGD synthase mRNA has been found to be abundantly expressed in the leptomeninges (42). PGD₂ is formed in the lung under allergic conditions and produces bronchoconstriction (5, 43). Our results have demonstrated that the PGD receptor preferentially mediates adenylate cyclase activation but not inositolphospholipid breakdown. The contractile responses to PGD₂ may therefore be a result from its action on contractile prostanoid receptors, such as the TXA₂ receptor (5, 6, 44).

In summary, we have identified the gene and a cDNA for a mouse PGD receptor. Availability of the nucleotide and amino acid sequences of the PGD receptor will permit the identification of cells expressing this receptor and allow elucidation of the role for PGD₂ in physiological and pathological conditions.

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