A murine platelet-activating factor receptor gene: cloning, chromosomal localization and up-regulation of expression by lipopolysaccharide in peritoneal resident macrophages

Satoshi ISHII*†, Yoichi MATSUDA‡, Motonao NAKAMURA*†, Iwao WAGA*, Kazuhiko KUME*, Takashi IZUMI* and Takao SHIMIZU*§

*Department of Biochemistry, Faculty of Medicine, The University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113, †Life Science Research Laboratory, Japan Tobacco Inc., Umegaoka 6-2, Aoba-ku, Yokohama-shi, Kanagawa 227, and ‡Genome Research Group, National Institute of Radiological Sciences, Anagawa 4-9-1, Inage-ku, Chiba-shi Chiba 263, Japan

A murine gene encoding a platelet-activating factor receptor (PAFR) was cloned. The gene was mapped to a region of the D2.2 band of chromosome 4 both by fluorescence *in situ* hybridization and by molecular linkage analysis. Northern blot analysis showed a high expression of the PAFR message in peritoneal macrophages. When C3H/HeN macrophages were treated with bacterial lipopolysaccharide (LPS) or synthetic lipid A, the PAFR gene expression was induced. Bacterial LPS, but not lipid A, induced the level of PAFR mRNA in LPS-unresponsive C3H/HeJ macrophages. These induction patterns were parallel to those of tumour necrosis factor-α mRNA. Thus the PAFR in macrophages is important in LPS-induced pathologies.

INTRODUCTION

Platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a lipid mediator that has pleiotropic biological activities besides platelet activation (reviewed in [1–4]). A pathophysiological role of PAF in anaphylaxis or endotoxin shock has been revealed [5–9]. [^{3}H]PAF binding studies revealed that macrophages from murine peritoneal exudate and murine P388D1 macrophage-like cells possessed specific PAF receptor (PAFR) [10,11]. Honda et al. [12] cloned a cDNA for guinea-pig PAFR, and subsequently human and rat homologues were isolated [13–17]. Lipopolysaccharide (LPS), derived from the cell wall of Gram-negative micro-organisms, evokes endotoxic shock under certain pathophysiological conditions. It has been shown that many actions of LPS are mediated by macrophages [18,19] that produce potent mediators of the shock including eicosanoids, PAF and tumour necrosis factor-α (TNF-α) [20–23]. LPS priming of P388D1 cells amplified the effect of PAF on releasing arachidonic acid *in vitro* [20]. Regarding the priming effect of LPS *in vivo*, Heuer et al. [24] demonstrated that pretreatment of mice with LPS enhanced the activity of PAF, resulting in increased mortality. In IC-21 murine macrophage-like cells, LPS was found to increase the binding activity of PAF and to up-regulate PAFR-mediated calcium mobilization [25]. However, little is known about the structure and molecular mechanisms up-regulating the PAFR protein by LPS. For this purpose we attempted to isolate a PAFR gene of mouse, the most useful animal for studying the effects of LPS. Here we characterize the primary structure and chromosomal localization of the murine PAFR gene, and demonstrate up-regulation of the mRNA expression by LPS in C3H/HeN murine peritoneal resident macrophages.

MATERIALS AND METHODS

Materials

Materials and chemicals were obtained from the following sources. [α-^{32}P]dCTP (111 TBq/mmol) was from Du Pont/NEN (Tokyo, Japan) or Amersham (Tokyo, Japan); ^{3}H-labelled WEB 2086 (521.7 GBq/mmol) from Du Pont/NEN; restriction enzymes and DNA-modifying enzymes from Takara (Kyoto, Japan) or Toyobo (Tokyo, Japan); agarose (Sea Kem and Sea Plaque) from FMC (Rockland, ME, U.S.A.); PAF from Cayman Chemical (Ann Arbor, MI, U.S.A.); screening filters (Biodyne A, 1.2 μm) from Pall (Tokyo, Japan); transfer membranes (Hybond-N, Hybond-N+) from Amersham; salmon sperm DNA from Stratagene (La Jolla, CA, U.S.A.); BSA and bacterial LPS (L-3129, *Escherichia coli* Serotype O127:B8) from Sigma (St. Louis, MO, U.S.A.); synthetic *Escherichia coli* lipid A [LA-15-PP (506)] from Daiichi Pure Chemicals (Tokyo, Japan). Bacterial LPS was prepared as a 20 μg/ml solution in PBS before use. Synthetic lipid A was prepared as a 20 μg/ml solution in PBS containing 0.001% triethylamine. Unlabelled WEB 2086 and Y-24180 were gifts from Boehringer Ingelheim (Ingelheim, Germany) and Yoshitomi Pharmaceutical Industries (Osaka, Japan) respectively. Other materials and reagents were of analytical grade.

Abbreviations used: FISH, fluorescence *in situ* hybridization; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LPS, lipopolysaccharide; *Lps*, murine LPS response locus designation; PAF, platelet-activating factor; PAFR, PAF receptor; Pafr, murine PAFR gene designation; *Pafr*, murine PAFR chromosomal locus designation; RFLVs, restriction fragment length variants; TNF-α, tumour necrosis factor-α.

§ To whom correspondence should be addressed.

The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases with accession number D50872.

Screening of a murine genomic library

A murine genomic DNA library in λFixII vector (Stratagene) was used in the study. A 933 bp *Sma*I–*Sma*I fragment of the guinea-pig lung PAFR cDNA [12] was labelled with [α-^{32}P]dCTP by a Multiprime DNA labelling system (Amersham). Using *E. coli* P2392 as host cells, and an approximate density of 5×10^4 plaque-forming units/150 mm plate, we screened about 5×10^5 plaque-forming units. Hybridization was carried out at 55 °C in 6 × SSC, 5 × Denhardt's solution, 0.1 % SDS, 100 μg/ml sonicated salmon sperm DNA overnight. The filters were washed with 4 × SSC, 0.1 % SDS at 58 °C and exposed to X-ray films (Fuji, Tokyo, Japan) with intensifying screens at −80 °C for 3 days (primary screening) or less. Positive phage clones were purified by three rounds of screening.

DNA of a positive phage clone, MPRG1211, was prepared by the plate lysate method [26] and was digested with several restriction enzymes. Enzyme-digested phage DNA (2 μg) was separated on 1 % (w/v) agarose gel. The gel was denatured and blotted onto a Hybond-N membrane with 0.4 M NaOH. The membrane was hybridized and washed in the same condition as described for screening of a library. Fragments that hybridized to the guinea-pig PAFR probe were subcloned into the plasmid vector pBluescript SK(−) (Stratagene).

DNA sequencing

With alkali-denatured double-stranded plasmid DNA as template, the nucleotide sequence of murine PAFR gene was obtained by the dideoxy chain termination method. The coding region and its flanking regions, comprising 1477 nucleotides, were sequenced on both strands. Oligonucleotides were synthesized by a MilliGen/Biosearch Cyclone Plus DNA synthesizer (Burlington, MA, U.S.A.). [α-^{32}P]dCTP and Sequenase version 2.0 (U.S.B., Cleveland, OH, U.S.A.) were used in the sequence reactions.

Expression of the murine PAFR gene in *Xenopus* oocytes

A 1219 bp *Eco*47III–*Spe*I fragment of MPRG1211 which contained the complete open reading frame was subcloned into the plasmid vector pBluescript SK(−). With the resultant plasmid (pmPAFR) as a template, complementary RNA was transcribed *in vitro* by T7 RNA polymerase (Stratagene). The transcripts were injected into *Xenopus* oocytes and the PAF-evoked chloride currents were detected as described previously [12].

Expression of the murine PAFR gene in COS-7 cells

An *Xho*I–*Xba*I fragment containing the complete open reading frame was excised from the pmPAFR and subcloned into the expression vector pEUK-C1 (Clontech, Palo Alto, CA, U.S.A.). It was transfected into COS-7 cells by the DEAE-dextran method, and the ligand binding assay was performed with ^{3}H-labelled WEB 2086 as described previously [13].

Isolation of DNA and genomic Southern blot analysis

Genomic DNA was isolated from C3H/HeN mouse liver by using a DNA extraction kit (Stratagene) as recommended by the manufacturer. Enzyme-digested murine liver genomic DNA (5 μg) was electrophoresed on a 0.7 % agarose gel [26]. The gel was blotted onto a Hybond-N+ membrane. The membrane was hybridized with the ^{32}P-labelled *Eco*47III–*Spe*I fragment (−35 to 1182 with reference to the nucleotide sequence in Figure 1) in Rapid-hyb buffer (Amersham). The final washing condition was 2 × SSC, 0.1 % SDS for 30 min at 65 °C. The membrane was exposed to an X-ray film (Fuji) with an intensifying screen for 48 h at −80 °C.

Chromosome preparation and *in situ* hybridization

The direct R-banding FISH method was used for chromosomal assignment of Pafr gene. Preparation of R-banded chromosomes and FISH were performed as described by Matsuda et al. [27] and Matsuda and Chapman [28]. The chromosome slides were hardened at 65 °C for 2 h, and then denatured at 70 °C for 2 min in 70 % formamide in 2 × SSC, and dehydrated in a 70–85–100 % ethanol series at 4 °C. The genomic 8.9 kbp *Eco*RI–*Eco*RI fragment inserted in pBluescript SK(−) was labelled by nick translation with biotin 16-dUTP (Boehringer Mannheim Yamanouchi, Tokyo, Japan) following the manufacturer's protocol. The labelled DNA fragment was ethanol-precipitated with a 10-fold excess of mouse COT-1 DNA (Life Technologies, Tokyo, Japan) and sonicated salmon sperm DNA and tRNA, and then denatured at 75 °C in 100 % formamide. The denatured probe was mixed with an equal volume of hybridization solution to make a final concentration of 50 % (v/v) formamide, 2 × SSC, 10 % (w/v) dextran sulphate, and 1 mg/ml BSA; 20 μl of mixture containing 250 ng labelled DNA was put on the denatured slide, covered with Parafilm and incubated overnight at 37 °C. The slides were washed for 20 min in 50 % formamide in 2 × SSC at 37 °C, and in 2 × SSC and 1 × SSC for 20 min each at room temperature. After rinsing in 4 × SSC, they were incubated under a coverslip with fluoresceinated avidin (Vector Laboratories, Burlingame, CA, U.S.A.) at a 1 : 500 dilution in 1 % BSA/4 × SSC for 1 h at 37 °C. They were washed sequentially with 4 × SSC, 0.1 % Nonidet P-40 in 4 × SSC and 4 × SSC for 10 min each on a shaker, rinsed with 2 × SSC and then stained with 0.75 μg/ml propidium iodide (Sigma). Excitation at wavelength 450–490 nm (Nikon filter set B-2A) or at approx. 365 nm (UV-2A) was used for observation. Kodak Ektachrome ASA100 films were used for microphotography.

Linkage mapping of Pafr gene

Recombinant animals for this study were generated by mating males of the feral-derived mouse stocks, *Mus spretus*, with C57BL/6J females and backcrossing the F_1 females with *Mus spretus* in the National Institute of Radiological Sciences, Chiba, Japan. Whole genomic DNAs prepared from kidneys of the backcrossed mice were digested with restriction endonucleases. The resulting fragments were separated by electrophoresis on 0.8 % agarose gels and transferred to nylon membranes. A radiolabelled 0.5 kbp *Hin*dIII–*Eco*RI fragment of MPRG1211 was used to determine the genotype of individuals. Microsatellite DNA marker loci for linkage analysis were chosen following the result of cytogenetic mapping by FISH, and purchased from Research Genetics (Huntsville, AL, U.S.A.). All PCRs were performed in 15 μl of solution containing 75 ng of genomic DNA and 15 pmol of each oligonucleotide primer. Amplification conditions were 94 °C for 3 min; 30 cycles of 94 °C for 0.5 min, 55 °C for 0.5 min and 72 °C for 0.5 min; and 72 °C for 10 min. The PCR products were analysed by agarose gel electrophoresis.

Macrophage isolation and culture

Specific pathogen-free C3H/HeN mice and C3H/HeJ mice (female, 6 weeks old) were purchased from Clea Japan (Tokyo, Japan). Resident peritoneal macrophages were obtained by washing the peritoneal cavity three times with 2 ml of ice-cold PBS. The cells were resuspended in RPMI 1640 (Nissui, Tokyo, Japan) supplemented with 2 mM glutamine, 0.24 % sodium

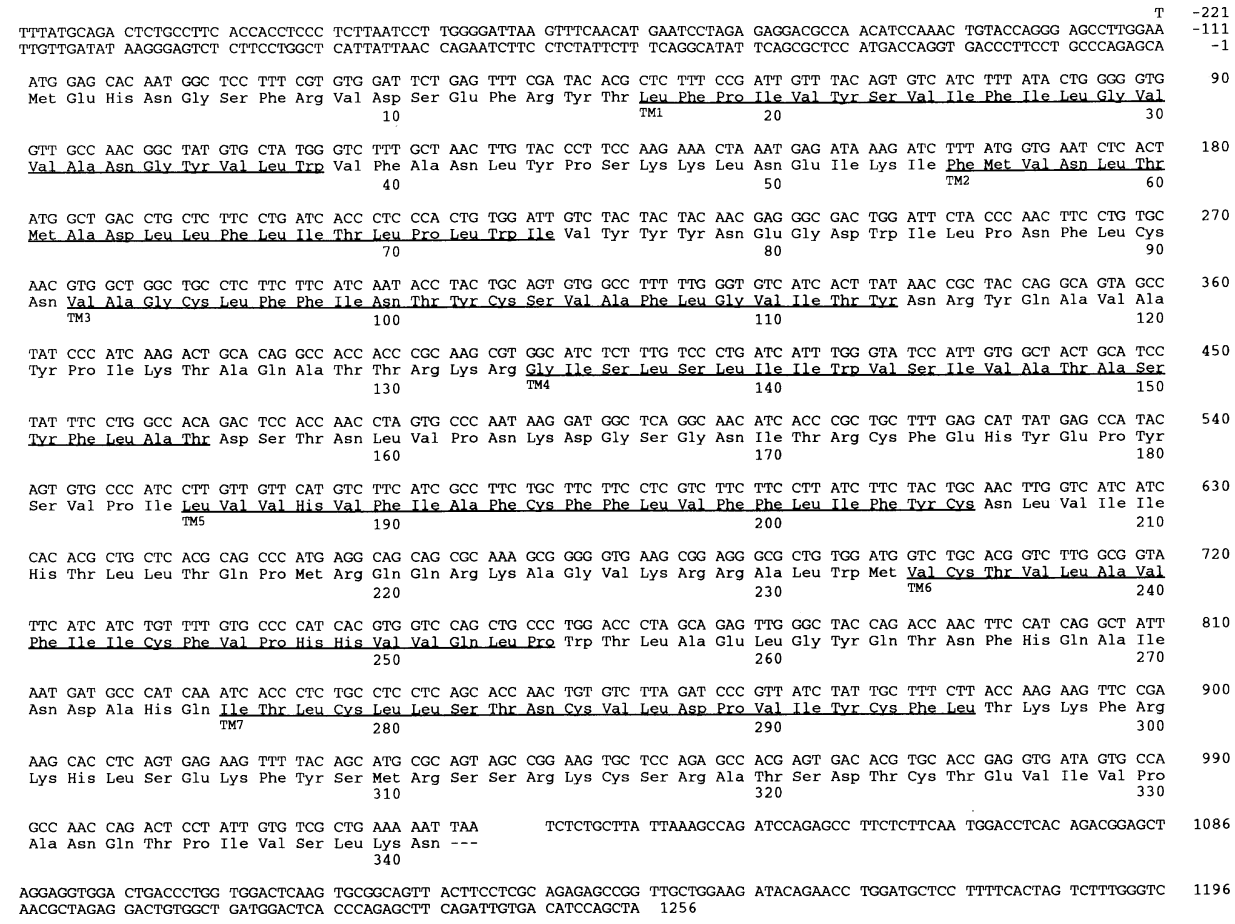

Figure 1 Nucleotide and deduced amino acid sequences of murine genomic DNA containing the open reading frame encoding the PAF receptor

Nucleotides are numbered from the first base of the putative ATG initiator codon. Numbering of amino acid residues begins from the first methionine. The seven putative transmembrane (TM) domains are underlined (TM1–TM7).

bicarbonate, 1 × Antibiotic–Antimycotic (100 i.u./ml penicillin G, 100 μg/ml streptomycin sulphate, 0.25 μg/ml amphotericin B; Life Technologies) and 10% heat-inactivated fetal bovine serum (Moregate, Melbourne, Australia). They were cultured at 37 °C in 5% CO_2 on polystyrene dishes 100 mm in diameter (Corning Glass Works, Corning, NY, U.S.A.) at 6×10^6 cells per dish in a total volume of 14 ml. After a 1.5 h adherence period, non-adherent cells were removed by washing three times with 5 ml of PBS prewarmed at 37 °C. Adherent macrophages were treated either with bacterial LPS after a 0.5 h incubation or synthetic lipid A after a 1 h incubation, and cultured for an additional period as described. WEB 2086 was added to the medium 0.5 h before stimulation with synthetic lipid A. Peritoneal exudate macrophages were obtained 3 days after intraperitoneal injection of 2 ml of sterile 4% brewer thioglycollate (Difco, Detroit, MI, U.S.A.). The cells were extracted as described above.

Isolation of RNA from tissues, and Northern blot analysis

Total RNAs from C3H/HeN mouse organs were obtained by using 6 ml of Isogen (Wako, Osaka, Japan) according to the manufacturer's instructions. Poly(A)$^+$ RNA was obtained from 500 μg of the total RNA by using Oligotex-dT30 Super (Takara) according to the manufacturer's instructions. To obtain total RNA from thioglycollate-elicited C3H/HeN macrophages, about 2×10^7 cells were passed through a nylon screen, pelleted and lysed in 5 ml of Isogen. Total RNA from adhered resident macrophages from C3H/HeN mice or C3H/HeJ mice was obtained by using 1 ml of Isogen per dish. The RNAs were separated by electrophoresis on 0.8% agarose/0.66 M formaldehyde gel [26] and transferred to a Hybond-N+ membrane by using 8 mM NaOH with the method of Chomczynski [29]. When the transfer had completed, residual RNA was not observed in the gel stained with ethidium bromide after neutralization. The membrane was hybridized under the same conditions as the genomic Southern blot analysis. The final washing condition was 1 × SSC, 0.1% SDS for 30 min at 65 °C. The membrane was exposed to an X-ray film (Fuji) with an intensifying screen for 48 h at −80 °C. Alternatively, the same membrane was sequentially dehybridized and hybridized with the 1105 bp *Eco*RI–*Eco*RI fragment of murine TNF-α cDNA probe (a gift from Dr. S. Narumi, Pharmaceutical Basic Research Laboratories, Japan Tobacco Inc.) [30], and with a human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA probe (Human G3PDH cDNA Probe, Clontech). The hybridized membrane was exposed to the FUJIX Imaging Plate (Fuji) and analysed with a FUJIX BAS-2000 bioimage analyser. Normalization with respect to the GAPDH signal was obtained by calculating the ratio between intensity of PAFR or TNF-α and GAPDH.

RESULTS

Isolation of a murine PAFR gene

A mouse genomic library was screened with a guinea-pig PAFR cDNA. We isolated three independent clones under conditions of low stringency. Each clone had a 17 kbp insert and the same restriction map. One of them, designated MPRG1211, was analysed further.

A 1026 bp open reading frame was identified (Figure 1) with considerable similarity to the PAFR cDNAs of guinea-pig, human and rat [12–17]. An in-frame termination codon at 21 bp upstream of the ATG codon was conserved [12,17]. In addition, the sequence around the codon closely agreed with the Kozak's consensus sequence (results not shown) [31]. Thus we assigned the first ATG codon as a putative initiation site. The deduced amino acid sequence of MPRG1211 revealed a protein of 341 residues with an estimated molecular mass of 39148 (Figure 1). The MPRG1211 polypeptide sequence was 78.9 % identical to the sequence of guinea-pig PAFR, 81.6 % to human PAFR and 91.2 % to rat PAFR.

The coding region of the clone MPRG1211 was expressed in different eukaryotic systems to confirm that it encoded a PAFR and to characterize the functional and pharmacological properties of the receptor. Defolliculated oocytes of *Xenopus laevis* were injected with the complementary RNA transcribed *in vitro* from the cloned gene. The injected oocytes displayed chloride currents (more than 500 nA) in response to 100 nM PAF. Y-24180, a PAFR-specific antagonist [9], inhibited the PAF-evoked response at 1 μM. Next the cloned gene was transiently expressed in COS-7 cells. We observed specific binding of ^{3}H-labelled WEB 2086, a PAF antagonist [6,8], to membranes of the transfected cells (B_{max} 230–950 fmol/mg of protein; K_d 32.8 $\pm$ 3.0 nM, mean $\pm$ S.D., $n = 3$). These results demonstrate that the genomic clone contains the functional murine PAFR open reading frame without intron, as does the human PAFR gene [32,33].

Genomic Southern blot analysis

For Southern blot analysis, murine genomic DNAs were digested with several restriction enzymes. A radiolabelled DNA fragment of MPRG1211 contained the entire coding region. The digestion with *Eco*RI, *Hin*dIII or *Bam*HI gave a single hybridizing fragment of 8.8, 6.8 or 10.2 kbp respectively, whereas two *Pst*I fragments, 8.0 and 5.1 kbp, hybridized with the probe.

Chromosomal localization of Pafr gene

The chromosomal localization of the murine PAFR gene (Pafr) was assigned by direct R-banding fluorescence *in situ* hybridization (FISH) with a genomic DNA fragment of MPRG1211 as a probe. As shown in Figure 2, the signals were localized on the R-positive D2.2 band of mouse chromosome 4.

Genomic DNAs of C57BL/6J and *Mus spretus* were digested separately with *Eco*RI, *Hin*dIII, *Bam*HI and *Pst*I, and analysed by Southern blot hybridization for informative restriction fragment length variants (RFLVs) by using DNA fragments of MPRG1211. Among the restriction enzyme digestions, RFLVs between the two *Mus* species were identified by *Bam*HI digestion as follows: a 2.3 kbp fragment in C57BL/6J and a 2.0 kbp fragment in *Mus spretus* (results not shown). Linkage mapping of the Pafr gene was performed with *Bam*HI-digested DNA samples from a total of 146 interspecific backcross mice between (C57BL/6J $\times$ *Mus spretus*) F_1 females and *Mus spretus* males. We examined the concordance of the segregation of the RFLVs

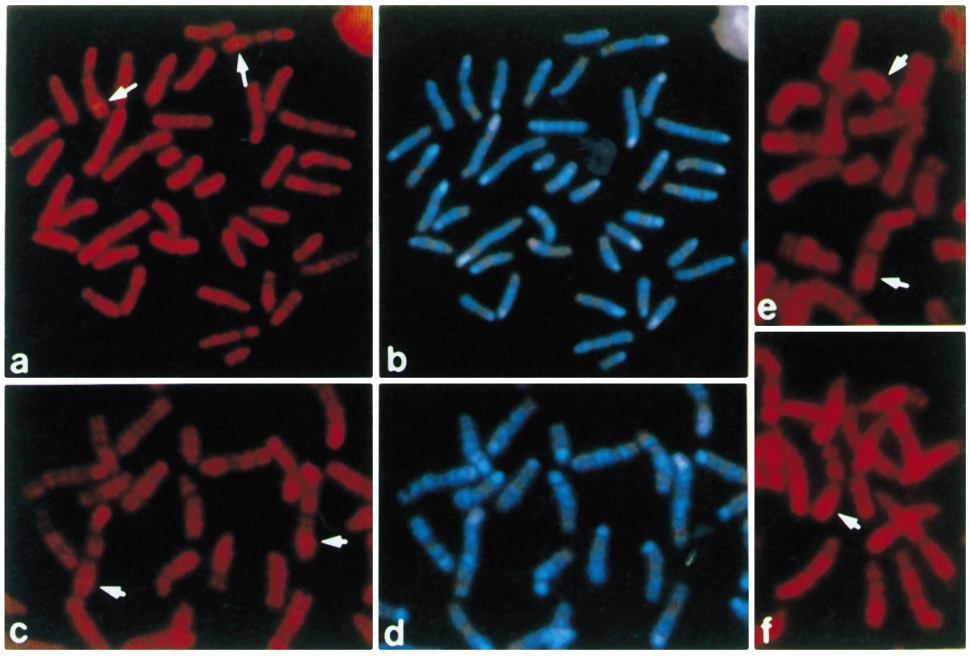

Figure 2 Chromosomal localization of the Pafr gene on mouse R-banded chromosomes by FISH

The hybridization signals are indicated by arrows. The signals are localized in the R-positive D2.2 of mouse chromosome 4. The metaphase spreads were photographed with Nikon B-2A (**a**, **c**, **e**, **f**) and UV-2A (**b**, **d**) filters. R-band and G-band patterns are demonstrated in (**a**, **c**, **e**, **f**) and (**b**, **d**) respectively.

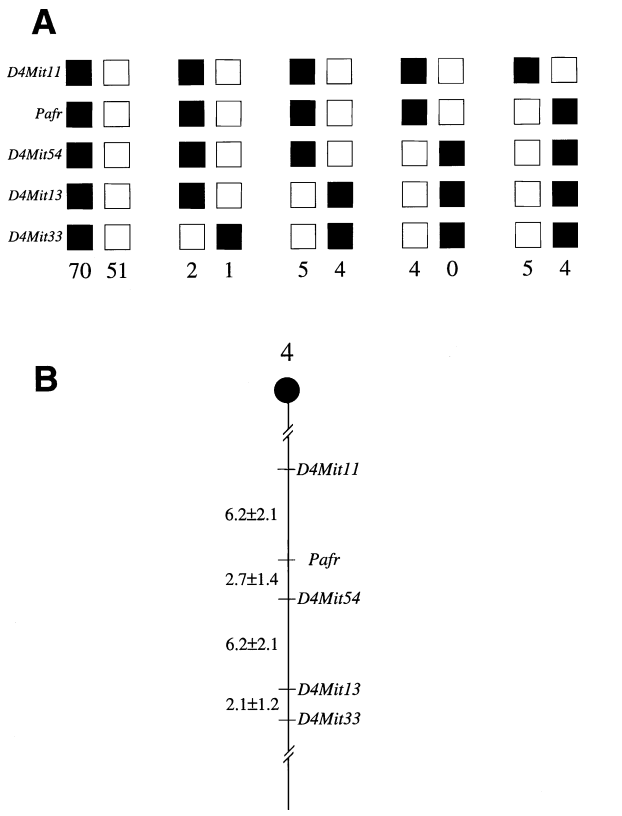

Figure 3 Chromosomal localization of the Pafr gene by interspecific backcross analysis

The Pafr gene was mapped in the distal region of chromosome 4 by interspecific backcross analysis. The segregation patterns of *Pafr* locus and flanking marker loci from 146 interspecific backcross mice are shown in **A**. Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J × *Mus spretus*) F_1 female parent. The black boxes represent the presence of a C57BL/6J allele, and the white boxes represent the presence of a *Mus spretus* allele. The numbers of offspring inheriting each type of chromosome are listed at the bottom of each column. A partial chromosome 4 linkage map showing the location of the *Pafr* locus in relation to linked marker loci is shown in **B**. Recombination distances between loci in centimorgans ± standard error are shown to the left of the chromosome.

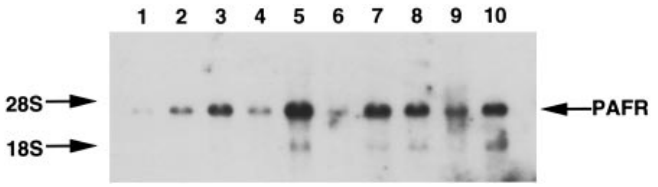

Figure 4 Northern blot analysis of murine PAFR mRNA

Poly(A)$^+$ RNA (5 μg) (lanes 1–8) or total RNA (5 μg) (lanes 9 and 10) was loaded into each lane. Northern blot analysis was performed with a murine PAFR genomic DNA fragment. Lane 1, brain; lane 2, heart; lane 3, lung; lane 4, liver; lane 5, spleen; lane 6, kidney; lane 7, skeletal muscle; lane 8, small intestine; lane 9, resident peritoneal macrophages; lane 10, thioglycollate-elicited macrophages. The locations of 28 S and 18 S ribosomal RNAs are indicated.

with that of four microsatellite DNA marker loci, *D4Mit11*, *D4Mit54*, *D4Mit13* and *D4Mit33*. The segregations of the murine PAFR locus, which is designated *Pafr*, in the backcross mice are shown with the microsatellite DNA marker loci as anchors in Figure 3(A). Comparative pairwise analysis of loci shows that the most likely gene order and recombination frequencies for each pair of loci on chromosome 4 are: centromere (*Hc4*)–*D4Mit11*–(9/146)–*Pafr*–(4/146)–*D4Mit54*–(9/146)–*D4Mit13*–(3/146)–*D4Mit33*–telomere (Figure 3B). The corresponding map distances (±standard errors) in centimorgans between loci are: *Hc*4–*D4Mit11*–(6.2±2.1)–*Pafr*–(2.7±1.4)–*D4Mit54*–(6.2±2.1)–*D4Mit13*–(2.1±1.2)–*D4Mit33* [34,35].

Tissue distribution of murine PAFR gene expression

The PAFR messages were detected in all tissues examined as a major band of approximately 4 kb (Figure 4). It should be noted that 5 μg of total RNA from peritoneal resident macrophages was separated by gel electrophoresis, whereas 5 μg aliquots of poly(A)$^+$ RNA from various organs were electrophoresed. Thus murine peritoneal resident macrophages expressed an enormous amount of the PAFR mRNA. Thioglycollate stimulation further augmented the accumulation of the messages. Spleen was the organ richest in PAFR mRNA content, followed by skeletal muscle, lung and small intestine. A moderate amount of expression was also observed in heart tissue. A relatively low but appreciable expression was detected in brain, liver and kidney.

Modulation of murine PAFR gene expression by bacterial LPS in peritoneal resident macrophages

Although the signal transduction system of LPS is equivocal, LPS is likely to trigger the stimulation signal(s) by interacting with one or more specific receptors on the cell membrane [18]. A mouse strain C3H/HeJ is unresponsive to LPS because of mutation at a genetic locus, *Lps*, on chromosome 4 [36,37]. Despite extensive investigation, the molecular basis for this defect is still unknown. Because the unresponsiveness of C3H/HeJ mice is specific to LPS, the defect has been suspected of impairing an effector molecule functioning very early in LPS-signalling, e.g. a receptor (reviewed in [38]). A closely related strain of mice, C3H/HeN, is fully responsive to LPS [39]. Comparisons of the differences in immune responses between these two strains have provided significant information about the cellular mechanisms underlying LPS-signalling.

We observed that macrophages accumulated an enormous amount of the PAFR mRNA (Figure 4), further indicating the pathophysiological importance of the cells as targets of PAF bioactions. We therefore investigated the modulation of PAFR gene expression by LPS in peritoneal resident macrophages from LPS-sensitive C3H/HeN mice. Total RNAs from untreated or 100 ng/ml bacterial LPS-treated macrophages were prepared and subjected to Northern blot analysis. The untreated cells constitutively expressed PAFR mRNA (Figure 5). Treatment of the macrophages with bacterial LPS increased the receptor gene expression. The maximal accumulation was achieved 4 h after the stimulation. To assess macrophage activation [40], the blot was rehybridized with a TNF-α cDNA probe. In the untreated C3H/HeN macrophages, TNF-α gene expression was barely observed (Figure 5). The amount of messages peaked 1 h after the stimulation.

Equivalent results were obtained with LPS-unresponsive C3H/HeJ macrophages. The untreated macrophages also constitutively expressed PAFR mRNA, and the mRNA level peaked 4 h after treatment with bacterial LPS (Figure 5). Both the level and the kinetics of PAFR transcripts were comparable between these two strains. Bacterial LPS-induced TNF-α expression in C3H/HeJ macrophages was also observed (Figure 5), although at a significantly lower level than in C3H/HeN macrophages, as has been reported previously [40,41].

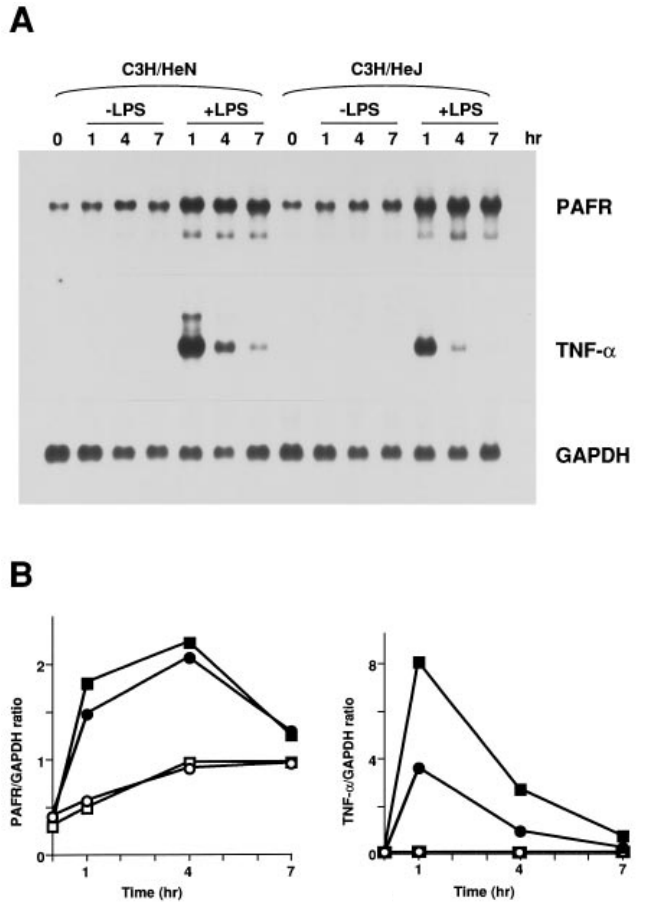

Figure 5 Kinetic studies of murine PAFR mRNA accumulation in macrophages induced by bacterial LPS

Resident peritoneal macrophages from C3H/HeN or C3H/HeJ mice were cultured in the absence or presence of bacterial LPS (100 ng/ml) for different periods. Cells were then lysed and total RNA was prepared. Equal amounts of total RNA (4 μg per lane) were electrophoresed, blotted onto a nylon membrane and hybridized with either a PAFR genomic DNA, a murine TNF-α cDNA or a human GAPDH cDNA probe. Two independent experiments were performed with equivalent results. The results of one experiment are shown. (**A**) Northern blots; (**B**) corresponding densitometric values expressed as PAFR/GAPDH or TNF-α/GAPDH ratios. Symbols: □, C3H/HeN, without LPS; ■, C3H/HeN, with LPS; ○, C3H/HeJ, without LPS; ●, C3H/HeJ, with LPS.

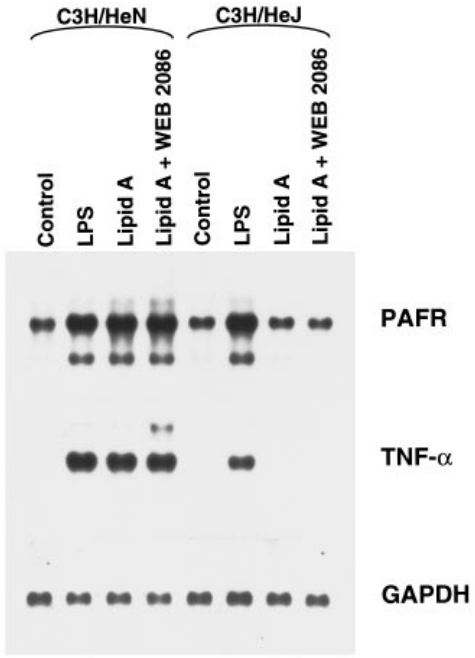

Figure 6 Effects of bacterial LPS, synthetic lipid A and WEB 2086 on murine PAFR mRNA accumulation in macrophages

Resident peritoneal macrophages from C3H/HeN or C3H/HeJ mice were cultured in the absence or presence of bacterial LPS (100 ng/ml), synthetic lipid A (100 ng/ml) or synthetic lipid A (100 ng/ml) + WEB 2086 (1 μM). After 4 h, cells were lysed and total RNA was prepared. Equal amounts of total RNA (3 μg per lane) were analysed by using either a murine PAFR genomic DNA, a murine TNF-α cDNA or a human GAPDH cDNA probe. Two independent experiments were performed with equivalent results; the results of one experiment are shown.

Modulation of murine PAFR gene expression by synthetic lipid A in peritoneal resident macrophages

The lipid A moiety of the LPS molecule is the active centre of LPS, because this structure was found to reproduce most of the toxic and macrophage-stimulatory effects of LPS [19]. A recent report has shown that C3H/HeJ macrophages were induced by crude preparation of LPS to express various genes, but did not respond to purified LPS or synthetic lipid A [42]. It was claimed that the crude preparation of LPS contained endotoxin protein(s), to which C3H/HeJ mice responded [42].

We observed the responses of C3H/HeJ mice to bacterial LPS with regard to both the PAFR mRNA and TNF-α mRNA (Figure 5). To exclude the effect of endotoxin protein(s) possibly present in bacterial LPS, both C3H/HeN and C3H/HeJ macrophages were cultured with 100 ng/ml synthetic *E. coli* lipid A. The culture period was 4 h, at which the optimal PAFR gene expression was observed. As shown in Figure 6, the synthetic lipid A induced the expression of PAFR gene in C3H/HeN macrophages; a comparable level of the gene expression was induced by bacterial LPS and by synthetic lipid A. In contrast, the PAFR gene expression in C3H/HeJ macrophages was not induced detectably after stimulation with synthetic lipid A. Equivalent results were obtained with TNF-α gene expression (Figure 6). All these results show that, in terms of the mRNA levels of PAFR and TNF-α, C3H/HeN macrophages responded to both synthetic lipid A and bacterial LPS; C3H/HeJ cells did not respond to synthetic lipid A, but responded to bacterial LPS.

Effect of WEB 2086 on PAFR gene expression

We have previously reported that both LPS and synthetic lipid A transduced Ca^{2+} signalling through PAFR [43,44]. To evaluate the effect of LPS signalling through PAFR on LPS-inducible gene expression, macrophages were treated with 1 μM WEB 2086 before stimulation with synthetic lipid A. The expression of neither the PAFR gene nor the TNF-α gene was affected by WEB 2086, either in C3H/HeN mice or in C3H/HeJ mice (Figure 6).

DISCUSSION

In this study we have isolated a gene that contained an open reading frame encoding 341 amino acids (Figure 1). The gene was expressed both in *Xenopus* oocytes and in COS-7 cells. Electrophysiological and pharmacological profiles indicate that

the intronless open reading frame encoded a murine PAFR. The Southern blot analysis suggests the existence of a single-copy gene for murine PAFR in a haploid genome.

The Pafr gene was localized to a region of the R-positive D2.2 band of chromosome 4 by direct R-banding FISH (Figures 2 and 3). Consistent with previous data for the human PAFR gene [32,33], this region of mouse chromosome 4 is syntenic with human chromosome 1p [34]. LPS transduced Ca^{2+} signalling through PAFR [43,44], raising a possibility that PAFR protein is encoded by a gene in the *Lps* locus [36]. Nevertheless, *Pafr* is localized to a specific region distinct from that previously reported for *Lps* [36,37]. We have compared our interspecific map of chromosome 4 (Figure 3) with a composite mouse linkage map that reports the locations of many uncloned mouse mutations [results were obtained from the Mouse Genome Database (MGD), Mouse Genome Informatics Project, The Jackson Laboratory, Bar Harbor, ME, U.S.A.]. *Pafr* was localized in a region of the composite map that lacks mouse mutations with a phenotype (results not shown).

Northern blot analysis shows a ubiquitous distribution of the PAFR mRNA, with the highest expression in peritoneal macrophages (Figure 4). Previous reports have shown that the PAFR transcripts were abundant in guinea-pig and human leucocytes [12,13]. Spleen was the organ richest in the PAFR mRNA, followed by skeletal muscle, lung, small intestine, heart, brain, liver and kidney in that order (Figure 4). Although the tissue distribution of the murine PAFR mRNA was different from those of other species [12,14,17], both spleen and lung appear to express PAFR mRNA highly, irrespective of the species. Because both organs contain macrophages, a part of the detected mRNA in these organs could derive from the cells. *In situ* hybridization analysis is necessary to identify authentic cells expressing the PAFR mRNA.

LPS primes P388D1 murine macrophage-like cells for enhanced arachidonic acid metabolism in response to PAF [20]. Furthermore LPS increases the surface expression of PAFR protein in IC-21 cells, as judged by binding parameters and Ca^{2+} responses [25]. The effect of LPS was almost abolished by actinomycin D, suggesting the activation of gene transcription in this event [25]. We show here the up-regulation of PAFR mRNA accumulation in peritoneal resident macrophages by bacterial LPS (Figure 5). LPS therefore increases the surface expression of PAFR by inducing the gene expression in macrophages. Treatment with WEB 2086 had no effects on the gene induction of PAFR and TNF-α by lipid A (Figure 6), indicating that the effect of LPS is not through PAFR, which is different from LPS-induced Ca^{2+} signalling in various cells [43,44]. The increased PAFR mRNA accumulation in the activated macrophages may enhance the sensitivity to PAF *in vivo* and contribute to the marked facilitation of PAF-elicited shock in the LPS-treated mice [24].

We further demonstrated that, in terms of the mRNA levels of PAFR, C3H/HeN macrophages responded to both synthetic lipid A and bacterial LPS; LPS-unresponsive C3H/HeJ macrophages failed to respond to synthetic lipid A, but significantly responded to bacterial LPS (Figure 6). Manthey et al. [42] have shown that C3H/HeJ macrophages were unresponsive to concentrations of purified LPS and synthetic lipid A in the μg/ml range. They also reported that unidentified phenol-extractable contaminant(s), called endotoxin protein(s), induced C3H/HeJ macrophages to express a subset of LPS-inducible early genes. According to the manufacturer of the bacterial LPS, our LPS preparation contained less than 3% protein; the crude LPS possibly contained endotoxin protein(s). Hence the murine PAFR gene appears to be induced by not only purified LPS but also endotoxin protein(s). There are two subsets of LPS-inducible early genes depending on responsiveness to endotoxin protein(s) [42]; the one that is responsive to the contaminant(s) is termed the TNF-α subset. Because our present data show that the induction pattern of the PAFR gene is parallel to that of TNF-α gene, the PAFR gene seems to belong to the TNF-α subset.

Since the guinea-pig PAFR cDNA was cloned [12], modulations of PAFR gene expression by various exogenous stimuli have been demonstrated. Both differentiations of HL-60 cells and of human eosinophilic EoL-1 cells were associated with the induction of PAFR gene transcripts [13,45,46]. In human monocytes, PAFR gene expression was up-regulated and down-regulated by interferon-γ and dibutyryl cyclic AMP respectively [47,48]. These exogenous stimuli, as well as LPS, could alter the responsiveness to PAF by modulating PAFR gene expression. LPS-inducible gene expression in macrophages has been shown to be regulated by both transcriptional and post-transcriptional mechanisms [49,50]. The promoter for murine TNF-α gene, a representative of the LPS inducible genes, possesses binding motifs for NF-κB [51]. NF-κB is a predominant factor for transcriptional induction of the TNF-α gene by LPS [52]. We have clarified the positive transcriptional regulation of the human PAFR gene expression by PAF or phorbol 12-myristate 13-acetate through NF-κB in JR-St human stomach cells [53]. The identification of a functional promoter for the murine PAFR will serve as a valuable tool for studying the transcriptional modulation of PAFR gene expression. The acquisition of the murine PAFR gene enables the establishment of PAFR-deficient mice by gene targeting strategy.

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