Volume 302, number 2, 161–165 FEBS 11010 May 1992

Determination of the sequence coding for the β subunit of the human high-affinity IgE receptor

Kazuhiko Maekawa[a], Noriko Imagawa[a], Yoshiaki Tanaka[b] and Shigenori Harada[a]

[a]*Shionogi Institute for Medical Science, 2-5-1, Mishima, Settsu-shi, Osaka 566, Japan* and [b]*Department of Obstetrics and Gynecology, Suita Municipal Hospital, Suita, Osaka 564, Japan*

Received 26 February 1992; revised version received 26 March 1992

The cDNA encoding the β subunit of the human high-affinity IgE receptor was cloned by a combination of various polymerase chain reactions (PCR). A major portion of the β cDNA was amplified using primers homologous within the sequences of rat and mouse. The 3′ unknown sequence was preferentially amplified using the RNA template-specific PCR and the improved two-step PCR. The 5′ unknown sequence was specifically amplified by our newly developed PCR walking. Random heptanucleotides tagged with a unique sequence at the 5′ end were used as the walking primer. Finally, the entire coding region was amplified and sequenced. The two extracellular loops of the human β subunit were the least homologous to those of rat and mouse.

High-affinity IgE receptor; Human β subunit; Cloning; Sequencing; Polymerase chain reaction; Polymerase chain reaction walking

1. INTRODUCTION

The high affinity receptor for IgE (FcεRI) is found exclusively on mast cells and basophils. When this receptor is aggregated by binding multivalent allergens via receptor-bound IgE, these cells are stimulated and release allergic and inflammatory mediators. FcεRI is composed of four polypeptide chains: a single α chain, a single β chain, and two disulfide-linked γ chains [1,2]. cDNAs coding for the rat α [3–5], β [6] and γ [7] subunits were cloned and sequenced from basophilic leukemia (RBL) cells. Mouse cDNAs for all three subunits were cloned from a mast cell line PT18 [8]. cDNAs corresponding to the human α and γ subunits were cloned from a mast cell line KU812 [4,9] and a leukocyte genomic library [10], respectively. The human β subunit, however, remains to be cloned. Despite findings that the extracellular domain of the human α subunit was sufficient for high affinity IgE binding [11,12] and that the rat and mouse β subunits were indispensable for expression of the corresponding FcεRIs on transfected cells [8,13] and for the receptor-mediated signal transduction [14], the role of the human β subunit is still obscure: the human α subunit that co-transfected with the γ but not with the β subunit was expressed on the cell surface [8,13] and transduced weak but substantial signals [14].

In this study, the cDNA encoding the human β subunit was cloned by a combination of various PCR amplifications and the sequence was compared with those of the rodent. Since KU812 cells failed to release histamine after FcεRI aggregation and mutations on the receptor cannot be denied entirely [15], basophils obtained by culture of mononuclear cells from human umbilical cord blood [16] were used for isolation of the β cDNA.

Correspondence address: S. Harada, Shionogi Institute for Medical Science, 2-5-1, Mishima, Settsu-shi, Osaka 566, Japan. Fax: (81) (6) 3822598.

2. MATERIALS AND METHODS

2.1. *Cell cultures*

Mononuclear cells from human umbilical cord blood were cultured in media facilitating the growth and differentiation of basophils as described [16]. Briefly, mononuclear cells were obtained from heparinized human umbilical cord blood using Ficoll-Paque (1.077 mg/ml; Pharmacia LKB), suspended at 2×10^6 cells/ml in RPMI 1640 medium supplemented with 10% fetal calf serum, 0.05 mM 2-mercaptoethanol, 2 mM L-glutamine, antibiotics (100 U of penicillin, 0.05 mg of streptomycin, and 0.025 mg of gentamycin per ml) and 200 U/ml of recombinant human IL-3 (Genzyme, Boston, MA) and cultured for 2–4 weeks. The nonadherent cells, 15–30% of which were FcεRI positive (data not shown), were used for isolation of RNA.

2.2. *Isolation of RNA*

Total RNA was isolated from approximately 10^7 cultured cells by the acid guanidium–phenol–chloroform method [17]. mRNA was isolated from approximately 10^7 cultured cells using a QuickPrep mRNA Purification Kit as recommended by the supplier (Pharmacia LKB).

2.3. *Oligonucleotide synthesis*

Oligonucleotides were synthesized using a DNA synthesizer (CYCLONE, MilliGen, Bedford, MA), purified through Oligo-Pak column (MilliGen) and used as the primer for PCR or sequencing. The positions of the representative PCR primers are indicated in Fig. 3. Primers designated as EB are eicosanucleotides that are homologous in the rat and mouse β cDNA sequences: EB1 primer is identical to the sequence in the NH_2-terminal cytoplasmic (IC) tail of the rat (nucleotides 75–94, ref. [6] and mouse (nucleotides 43–62, ref. [8] β

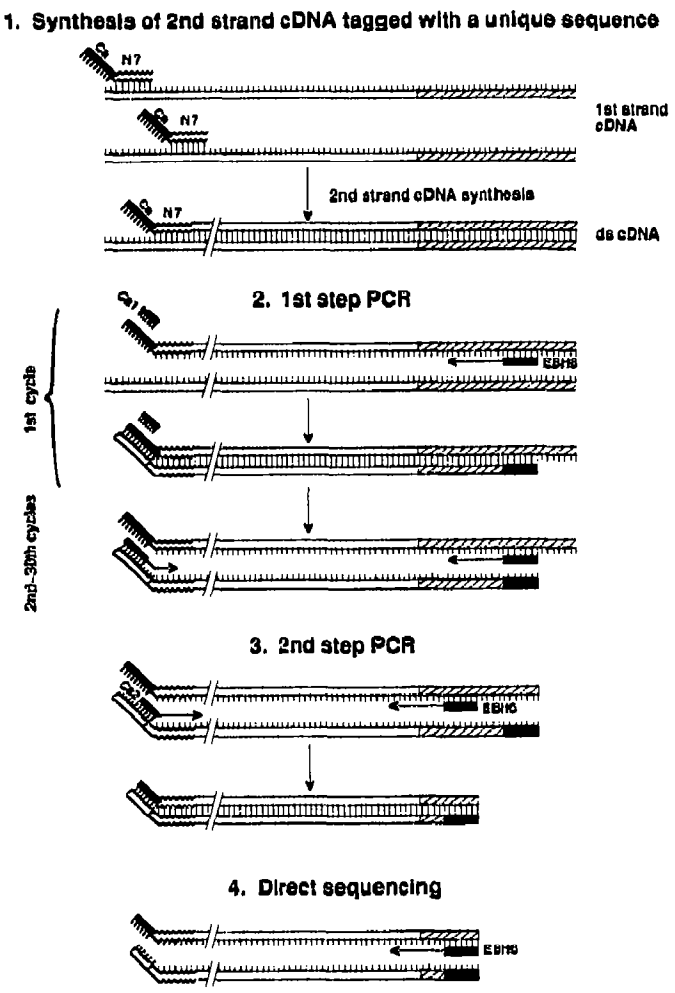

Fig. 1. Amplification and sequencing of the 5' unknown region of the human FcεRI β cDNA by a novel PCR walking. Some of walking primers, random heptanucleotides (N7) tagged with a unique sequence (Ca) at the 5' end, hybridized to various sites in the 5' unknown sequence (open bar) upstream from the known sequence (hatched bar) of the first-strand human β cDNA and primed to synthesize second-strand cDNAs. The resulting double-stranded cDNAs of various lengths were subjected to the subsequent PCR amplification. During the first cycle of the first-step PCR, only the known sequence-specific primer EBH8 hybridized to the second-strand β cDNAs and primed to synthesize the double-stranded human β cDNA fragments containing a unique sequence at 5' end. The resulting cDNA fragments of β subunit were specifically amplified during the 2nd–30th cycles of the first-step PCR using a pair of primers (EBH8 and the unique sequence-specific Ca1). By use of a pair of inner primers (EBH6 and Ca2), the 5' unknown sequences of the human β cDNA were further specifically amplified by the 2nd-step PCR. The amplified DNA fragments, even though different in length, were sequenced directly by using the known sequence-specific primer EBH6.

cDNAs. EB6 primer is complementary to the sequence in the COOH-terminal transmembrane (TM) 4 segment of the rat (606–625) and mouse (550–569). EB8 primer is complementary to the sequence in the COOH-terminal IC tail (726–745 for rat; 665–684 for mouse). Primers designated as EBH (odd numbers) and EBH (even numbers) are respectively identical and complementary to the human β cDNA sequenced in this study (Fig. 4): EBH5 (nucleotides 532–551), EBH6 (90–109), EBH7 (505–524), EBH8 (134–153), EBH15 (−55 to −32), EBH17 (−28 to −9), EBH18 (767–786), EBH22 (829–844). NPA5 primer (5'GACTAGTTCTAGATCGCG3') and NPA3 primer (5'AGCGGCCGCCCTTTTT3') are identical to the sequences in the 5' end and middle of *Not*I Primer-Adapter (Cat No. 8248SA, A1; BRL, Gaithersburg, MD), respectively. CaN_7 primer is random heptanucleotides (N_7) tagged with a unique sequence (Ca, 5'GTACATATTGTCGTTAGAACGCGTAATACGACTCACTATAGGGAGA3') at 5' end. Ca1 and Ca2 primers are the 5'- and 3'-halves of the unique sequence (Ca), respectively.

2.4. *PCR*

2.4.1. Amplification of a major portion of the β cDNA

Using the isolated total RNA as the template and oligo $(dT)_{15}$ (Boehringer, Mannheim, Germany) as the primer, first strand cDNA synthesis was catalyzed by M-MLV reverse transcriptase (BRL) according to the recommended protocols. By use of the resulting first-strand cDNA as the template and a pair of EB1 and EB6 primers, the cDNA encoding the sequence between the NH_2-terminal IC tail and the COOH-terminal TM4 segment was amplified by PCR. First-strand cDNA equivalent to approximately 2.5 μg of total RNA and 100 pmol of each primer were used in a 100 μl reaction mixture containing 20 pmol of each deoxynucleotide and 2.5 U of AmpliTaq DNA polymerase from a GeneAmp DNA Amplification Reagent Kit (Cetus, Norwalk, CT). After 30 cycles of denaturation (94°C for 1 min), annealing (60°C for 1 min) and extension (72°C for 90 s) in a DNA Thermal Cycler (Cetus), the amplified cDNA fragment was purified by electrophoresis in a 2.5% agarose gel.

2.4.2. Amplification of the 3'-end sequences of the β cDNA

Using the isolated mRNA as the template, the first-strand cDNA was synthesized using SuperScript Plasmid System (BRL): SuperScript RT as the reverse transcriptase and *Not*I Primer-Adapter as the primer. By use of the resulting first-strand cDNA containing a unique sequence of *Not*I Primer-Adapter at 5' end as the template, the cDNA coding for the COOH-terminal TM4 to the poly(A) segment was amplified using the improved two-step PCR [18]. Briefly, first-strand cDNA corresponding to about 0.14 μg of mRNA, was first amplified as described in 2.4.1, with the following modifications: (1) as little as 10 pmol each of EBH7 and NPA5 primers were used; (2) the mixture was incubated for 25 cycles (94°C for 1 min, 50°C for 1 min, and 72°C for 3 min). A 1-μl aliquot of the reaction mixture was again amplified for 30 cycles as in the first process except that 100 pmol each of EBH5 and NPA3 primers were used. The amplified DNA fragment was purified by 2.5% agarose gel electrophoresis.

2.4.3. Amplification of the 5'-end sequences of the β cDNA

Our newly developed PCR walking in the 5' unknown sequence is outlined in Fig. 1. The second-strand reaction was catalyzed by AmpliTaq DNA polymerase (Cetus) using the first-strand cDNA synthesized as described in 2.4.1, as the template and CaN_7 oligonucleotides as the walking primers. The mixture was incubated in a DNA thermal Cycler (Cetus) for one cycle (94°C for 10 min, 37°C for 30 min, 37 → 72°C for 30 min, and 72°C for 30 min). By use of the resulting double- stranded cDNAs as the template, the cDNAs encoding the NH_2- terminal IC tail and the preceding 5' upstream non-coding region were amplified using the improved two-step PCR [18]. After the first-step PCR using 10 pmol each of EBH8 and Ca1 primers for 30 cycles (94°C for 1 min, 55°C for 1 min, and 72°C for 2 min), a 1-μl aliquot of the reaction mixture was subjected to the second-step PCR using 100 pmol each of EBH6 and Ca2 primers, using the same cycle incubation as in the first-step PCR, and purified by electrophoresis.

2.4.4. Amplification of the entire coding sequence of the β cDNA

Using the first-strand cDNA synthesized as described in 2.4.2, as the template, the cDNA containing the full coding region was amplified by the improved two-step PCR [18]. Briefly, first-strand cDNA corresponding to approximately 0.19 μg of mRNA was first amplified using 10 pmol each of EBH15 and EBH22 primers for 30 cycles of incubation (94°C for 1 min, 45°C for 80 s, and 72°C for 3 min). A 3-μl

aliquot of the reaction mixture was re-amplified using 100 pmol each of EBH17 and EBH18 primers for 25 cycles of incubation (94°C for 1 min, 55°C for 1 min, and 72°C for 2 min), and purified by electrophoresis.

2.5. *Sequencing*

PCR-amplified cDNA fragments were directly sequenced with Sequenase Version 2.0 (Code No. 70772; United States Biochemical, Cleveland) using [α-^{32}P]dCTP or with the dsDNA Cycle Sequencing System (BRL) according to the manufacturer's instructions. The cDNA fragment subcloned into an *Eco*RV-digested pBluescript SK(−) vector (Stratagene, La Jolla, CA) was also sequenced using SK and KS primers following the supplier's protocol.

3. RESULTS AND DISCUSSION

3.1. *Amplification and sequencing of a major portion of the β cDNA*

Based on the cDNA sequences coding for the β subunit of rat [6] and mouse [8], eight 20-mer oligonucleotides (designated as EB1–8) homologous with the rat and mouse sequences were synthesized. A total of 10 PCR amplifications were performed using a pair of EB (odd numbers) and EB (even numbers) oligonucleotides as primers. All four amplifications using the EB8 primer were unsuccessful, and the pair of EB1 and EB6 primers gave the largest and reasonable sized product 550 bp (arrowed in Fig. 2A). Only the EB8 primer had a 3′-end nucleotide mismatched with the human β cDNA sequenced in this study (nucleotide 670 in Fig. 4), which may have resulted in poor amplification.

The PCR-amplified cDNA fragment (EB1–EB6) encoding a major portion of the human β subunit between the NH_2-terminal IC tail and the COOH-terminal TM4 segment (Fig. 3) was sequenced directly or after subcloning into pBluescript (data not shown).

3.2. *Extension to the 3′-end sequences*

The RNA template-specific PCR (RS-PCR) [19] and the improved two-step PCR [18] were used to amplify

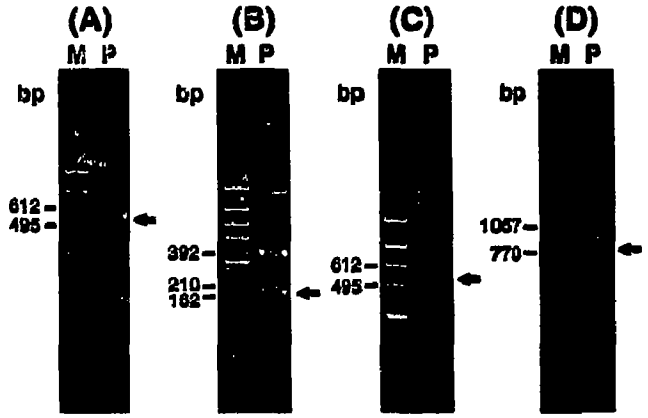

Fig. 2. PCR amplification of a major portion (A), the 5′ end (B), the 3′ end (C) and the entire coding sequence (D) of the human FcεRI β cDNA. Amplified products (lane P) were separated by 2.5% agarose gel electrophoresis and stained with ethidium bromide. The arrowed bands were excised from the gel and purified. Lane M is a DNA size marker, ϕX174/*Hin*clI digest.

specifically the 3′-end sequence of the human β subunit. In the RS-PCR, RNA-derived sequences are preferentially amplified by using a first-strand cDNA containing a unique sequence at the 5′ end as the template and a pair of known sequence-specific and unique sequence-specific primers. In the improved two-step PCR, high specificity and sensitivity by the conventional two-step PCR [20] using first outer, then inner primers nested within the first primers, are further improved by reducing the amount of both the first primers and the second template (i.e. the first reaction mixture).

*Not*I Primer-Adapter, containing a unique 29-base sequence to the 5′ side of oligo $(dT)_{15}$, hybridized to the poly(A) segment of mRNA and primed to synthesize a first-strand cDNA, which was used as the template in PCR-amplification of the 3′-end sequence. NPA5 and NPA3 oligonucleotides corresponding to the 5′ end and middle of *Not*I Primer-Adapter, respectively, were used

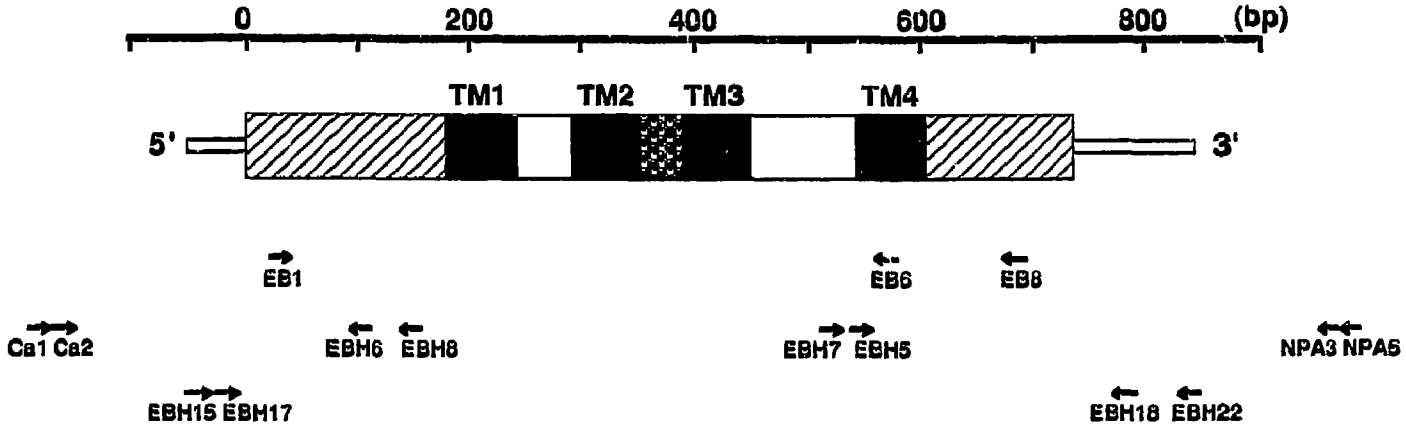

Fig. 3. The positions of the representative PCR primers. The scale indicates nucleotide positions (in bp) beginning from the first base of the initiation codon for the human FcεRI β cDNA. Beneath the scale the coding region of the human β subunit sequenced in this study is indicated by boxes: solid, TM segment; hatched, IC tail; cross-hatched, IC loop and open, EC loop. Bars represent non-coding sequences. The arrows illustrate the direction and extent of DNA sequences of the representative PCR primers.

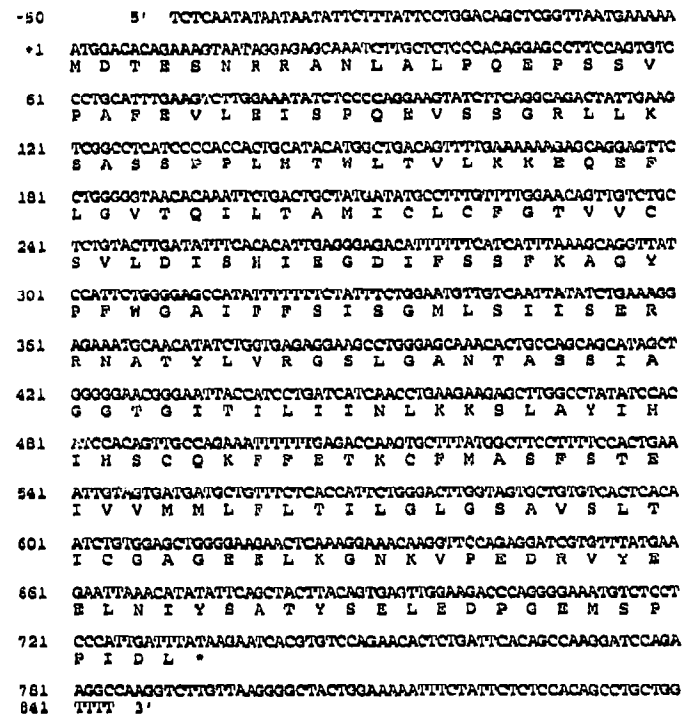

Fig. 4. Nucleotide sequence and predicted amino acid sequence of the human FcεRI β subunit. The numbers on the left refer to the nucleotide number beginning at the start codon at position +1; nucleotides preceding it are represented by negative numbers.

as unique sequence-specific primers. EBH5 and EBH7 oligonucleotides corresponding to the 3′ end sequences of the PCR-product (EB1~EB6) were used as known sequence-specific primers (Fig. 3).

The PCR-amplified cDNA fragment (EBH5–NPA3) encoding the TM4 to the poly(A) segment (approximately 550 bp as arrowed in Fig. 2C) was sequenced directly (data not shown).

3.3. *Extension to the 5′ end sequences*

The 5′ unknown region of the human β subunit was successfully amplified and sequenced by our newly developed PCR walking (Fig. 1). Random heptanucleotides (N_7) tagged with a unique 46-base sequence to their 5′ side (CaN_7 oligonucleotides) were used as the walking primer. Some of them hybridized to the 5′ upstream from the known sequence (EB1–EB6) of the first-strand β cDNA and primed to synthesize second-strand cDNAs containing a unique sequence at the 5′ end. The second-strand cDNAs derived from the human β subunit were specifically amplified by a combination of the RS-PCR and the improved two-step PCR as described in 3.2. Ca1 and Ca2 primers equivalent to the 5′ and 3′ sides of the unique 46-base sequence (Ca), respectively, were used as unique sequence-specific primers. EBH6 and EBH8 oligonucleotides corresponding to the 5′-end sequences of the PCR-products (EB1–EB6) were used as known sequence-specific primers (Fig. 3). A total of 12 PCR-amplifications produced major DNA fragments of around 200 bp. In some amplifications, larger- and some smaller-sized DNA fragments were produced. A representative amplification is shown in Fig. 2B. The major fragments of around 200 bp (arrowed in Fig. 2B) from the six amplifications were sequenced and found all identical to the sequence coding for the region upstream from the NH_2-terminal IC tail (data not shown). Other larger fragments were revealed as being unrelated. These 200 bp fragments are thought to cover the most 5′ end of the human β cDNA amplified.

3.4. *Amplification and sequencing of the entire coding region of the β cDNA*

As shown in Fig. 3, the three PCR products (EB1–EB6, EBH5–NPA3 and Ca2–EBH6) could be connected in sequence, and the human β cDNA sequence containing the entire coding region was presumed.

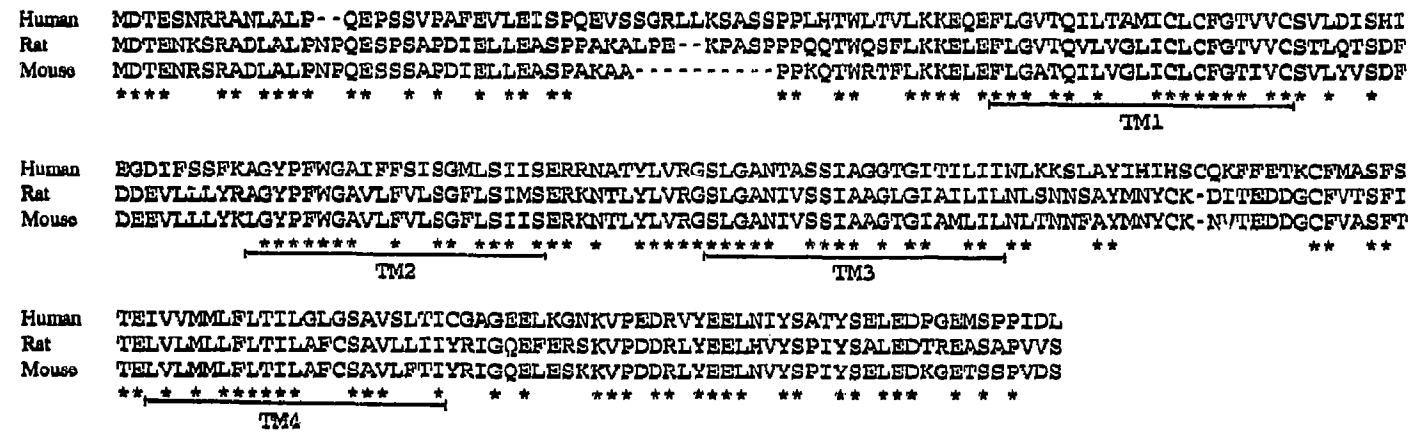

Fig. 5. Alignment of the predicted amino acid sequences of the human, rat, and mouse FcεRI β subunits. The three proteins are aligned to obtain maximum identity using the ALIGN program developed by Dr. Yasuo Ina (National Institute of Genetics, Japan). Dashes indicate gaps introduced to improve the alignment. Residues identical in all three species are indicated with asterisks. TM segments 1–4 underlined are presumed with reference to those of the rat [6] and mouse [8] β subunits.

Based on the putative sequence thus obtained, a total of 27 eicosanucleotides (designated as EBH) were synthesized across the sequence in both directions, and used as primers to amplify and confirm the whole coding sequence.

Using nested primers corresponding to 5′ upstream and 3′ downstream non-coding sequences, a cDNA containing the full coding region was amplified using the improved two-step PCR as shown by an arrow in Fig. 2D. The PCR-amplified cDNA was sequenced directly using various EBH primers (data not shown), and the conclusive nucleotide sequence and predicted amino acid sequence of the human FcεRI β subunit was obtained as described in Fig. 4.

3.5. *Characteristics of the predicted amino acid sequence*

The hydrophobicity profile analyzed for the predicted amino acid sequence of the human β subunit is similar to those for the rat [6] and mouse [8] β subunits and has four characteristic hydrophobic regions corresponding to the TM segments 1–4 (data not shown). The amino acid sequences of the β subunits in three species were compared (Fig. 5). All four TM segments and an IC loop between TM2 and TM3 are well conserved, whereas the two extracellular (EC) loops are the least conserved regions. The percentages of identical residues of the EC loops are 18% between TM1 and TM2 and 33% between TM3 and TM4. Since the rat and mouse β subunits are highly homologous (82% in the entire sequence and more than 70% in any corresponding regions; refs. [6] and [8]), the two EC loops of the human β subunit are especially less homologous to those of the rodents. Furthermore, the EC loop between TM3 and TM4 of the human β subunit is characteristic in the number of acidic and basic residues resulting in being positively charged (+2) for human, but negative (−4 and −3) for rat and mouse, respectively. According to the positive- inside rule for integral membrane proteins [21,22], segments with positively charged residues flanking the apolar regions are found on the intracellular, non-translocated side. We are now investigating whether the human β subunit is expressed in the same topology as the rat [6] and mouse [8] β subunits, or following the positive- inside rule, i.e. the loop between TM3 and TM4 remains on the inside of the membrane.

Acknowledgements: We are grateful to Dr. Yorio Hinuma, director of the Shionogi Institute for Medical Science, for his encouragement. We would like to thank Miss Yumi Kosada for technical assistance.

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