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Molecular Cloning, Characterization, and Expression of TNF cDNA and Gene from Japanese Flounder Paralichthys olivaceus¹

Ikuo Hirono, Bo-Hye Nam, Tomofumi Kurobe, and Takashi Aoki²

We cloned a cDNA and the gene for Japanese flounder TNF. The TNF cDNA consisted of 1217 bp, which encoded 225 amino acid residues. The identities between Japanese flounder TNF and members of the mammalian TNF family were ~20–30%. The positions of cysteine residues that are important for disulfide bonds were conserved with respect to those in mammalian TNF-α. The Japanese flounder TNF gene has a length of ~2 kbp and consists of four exons and three introns. The positions of the exon-intron junction positions of Japanese flounder TNF gene are similar to those of human TNF-α. However, the length of the first intron of Japanese flounder is much shorter than that of the human TNF-α gene. There are simple CA or AT dinucleotide repeats in the 5'-upstream and 3'-downstream regions of the Japanese flounder TNF gene. Southern blot hybridization indicated that Japanese flounder TNF exists as a single copy. Expression of Japanese flounder TNF mRNA is greatly induced after stimulation of PBLs with LPS, Con A, or PMA. These results indicated that Japanese flounder TNF is more like mammalian TNF-α than mammalian lymphotoxin-α, with respect to its gene structure, length of amino acid sequence, number and position of cysteine residues, and regulation of gene expression. The Journal of Immunology, 2000, 165: 4423–4427.

Materials and Methods

Construction of cDNA library

Peripheral blood samples were taken from a single homocloned Japanese flounder P. olivaceus (16). Leukocytes were isolated by centrifugation, at 400 × g for 20 min, with Percoll solution (1.072 g/ml). Leukocytes were cultured in RPMI 1640 containing Con A (70 μg/ml) and PMA (0.35 μg/ml) and were sampled after 1, 2, and 3 h. mRNA was isolated using a micro mRNA purification kit (Amersham Pharmacia Biotech, Piscataway, NJ). The purified mRNAs of three different time periods were pooled and used to construct a cDNA library. cDNA was synthesized using a cDNA synthesis kit (Amersham Pharmacia Biotech) with an oligo(dT) primer. The cDNA library was constructed in AZAPIIs (Stratagene, La Jolla, CA) according to the instructions of the manufacturer.

Screening of cDNA

We conducted an expressed sequence tag analysis of the cDNA library prepared from Con A/PMA-treated leukocytes. Conversion of the recombinant AZAPIIs into the pBluescript plasmid was conducted by in vivo excision according to the protocol of the manufacturer (Stratagene). After conversion of phage clones into plasmids, we randomly selected clones from the library and sequenced them. cDNA clones were sequenced using Thermosequenase (Amersham Pharmacia Biotech) with M13 forward and/or M13 reverse primers and an automated DNA sequencer LC4200 (Li-Cor, Lincoln, NE). Each determined sequence was compared with all sequences available in DDBJ/EMBL/GenBank using the BLAST version 2.0 (17, 18) (http://www.ncbi.nlm.nih.gov).
FIGURE 1. Nucleotide and deduced amino acid sequence of Japanese flounder TNF cDNA. The TA-rich motifs (TTATTTAT) are underlined.

FIGURE 2. Alignment of Japanese flounder TNF with human TNF-α, lymphotoxin-α (TNF-β), and lymphotoxin-β. Sequences were obtained from DDBJ/EMBL/GenBank database. Amino acid identities with Japanese flounder are shown by dots. The position of residues identical in all sequences are shown with asterisks. Gaps (dashes) have been placed to maximize the identity.

Results

Isolation and analysis of Japanese flounder TNF-α cDNA and gene

From the expressed sequence tag analysis, we found a clone whose amino acid sequence had significant identity to that of human TNF-α (data not shown). This clone was used as a probe for screening the cDNA library and BAC genome library. The sequences of Japanese flounder TNF cDNA and gene have been deposited in the DDBJ/GenBank/EMBL database (accession numbers AB040448 and AB040449). The TNF cDNA consisted of 1217 bp, which coded 225 amino acid residues (Fig. 1). There is an N-glycosylation site on the 26th amino acid residue, but the phylogenetic analysis suggests that the TNF-α and lymphotoxin-α, respectively, although the length of the amino acid sequence of the Japanese flounder sequence is more similar to that of human TNF-α than to that of lymphotoxin-α, whereas these two human protein products are themselves 30% identical. The phylogenetic analysis suggests that the TNF-α and lymphotoxin-α diverged after the divergence of mammals from teleosts (Fig. 3).

Southern blot hybridization

Genomic DNA of two different lines of homocloned Japanese flounder, cloned lines 1 and 8, and two noncloned Japanese flounder were isolated as previously reported (19). Genomic DNA of two different lines of homocloned Japanese flounder, cloned lines 1 and 8, and two noncloned Japanese flounder were isolated as previously reported (19). The arrayed genomic BAC clones were screened for a TNF gene by using a TNF cDNA as a DNA probe. Hybridization was done as previously reported (19). BAC DNAs were isolated by the alkaline lysis method and then digested with EcoRI and subcloned into the pUC119 vector. The subclones were screened by the colony-hybridization method (20). The genomic clone was sequenced as described above.

Screening of gene

Previously, we constructed and arrayed a genomic bacterial artificial chromosome (BAC) library (19). The average insert size of clones in this library is ~165 kbp. Five micrograms of total RNA per lane was denatured at 65°C for 5 min in 50% formamide, electrophoresed through a 1.5% agarose gel containing 6.6% formaldehyde, and transferred to a nylon membrane (NEB, MA). Perfect RNA markers 0.2–10 kb (Novagen, Madison, WI) were used for size marker of agarose gel electrophoresis. The probe was the BAC genomic DNA of two different lines of homocloned Japanese flounder, cloned lines 1 and 8, and two noncloned Japanese flounder were isolated as previously reported (19). Total RNA was prepared from either untreated PBLs or PBLs that had been stimulated with LPS (500 μg/ml), or PMA (0.05 μg/ml), or Con A (50 μg/ml), or Trizol (Life Technologies, Rockville, MD). Five micrograms of total RNA per lane was denatured at 65°C for 5 min in 50% formamide, electrophoresed through a 1.5% agarose gel containing 6.6% formaldehyde, and transferred to a nylon membrane (NEB, Beverly, MA). Perfect RNA markers 0.2–10 kb (Novagen, Madison, WI) were used for size marker of agarose gel electrophoresis. The probe was the full length of a Japanese flounder TNF cDNA fragment and was labeled with [α-32P]dCTP using a random primer labeling kit (Takara Shuzo, Kyoto, Japan). Southern and Northern blot hybridizations were done as previously described (21).

RT-PCR analysis

Total RNA was extracted from healthy Japanese flounder brain, head kidney, trunk kidney, liver, spleen, erythrocytes, leukocytes, skin, muscle, gut, heart, intestine, gonad, and LPS-treated leukocytes using Trizol (Life Technologies). The purified total RNA (10 μg) was reverse transcribed into cDNA using the AMV Reverse Transcriptase First-strand cDNA Synthesis kit (Life Science, Arlington Heights, IL). The final volume of the cDNA synthesis reaction was 25 μl. The reverse-transcribed sample (1 μl) was used in 50 μl of PCR mixture. The PCR primers used in this study were 5'-agttaggtttaagcacagagg-3' and 5'-agttgactgtgagcatggtg-3'. The β-actin primer set was used for a positive control of RT-PCR (22). PCR was performed with an initial denaturation step of 2 min at 95°C, and then 20 cycles were run as follows: 30 s of denaturation at 95°C, 30 s of annealing at 55°C, and 1 min of extension at 72°C. The reacted products were electrophoresed on a 2.0% agarose gel.

Abbreviations used in this paper: BAC, bacterial artificial chromosome; UTR, untranslated region.
flounder TNF. We cloned the ~9-kb EcoRI DNA fragment from the isolated BAC clone and sequenced entirely. The Japanese flounder TNF gene has a length of ~2 kb and consists of four exons and three introns (Fig. 5). The positions of the exon-intron junctions of Japanese flounder TNF gene are similar to those of the human TNF-α gene. The lengths of the introns of the Japanese flounder TNF gene are shorter than those of the human TNF-α gene. This is especially true of the first intron of the Japanese flounder TNF gene, which is one-sixth the length of the first intron in the human TNF-α gene. The Japanese flounder TNF gene has six simple dinucleotide repeats, three in the 5’-upstream region and three in the 3’-downstream region (Fig. 5). Approximately 1 kb upstream from the ATG start codon, there are four repeats of TGGGGG (Fig. 5).

Expression of TNF mRNA

As shown in Fig. 6, mRNA from fresh PBLs and unstimulated PBLs in the medium did not express TNF-α mRNA. When the PBLs were stimulated with LPS, PMA, and a calcium ionophore, or Con A, for 1 and 3 h, TNF mRNA expression was greatly induced. However, when PBLs were stimulated with LPS, PMA, and the calcium ionophore, or Con A, for 6 h, the expression level of TNF mRNA was less than it was after 1- and 3-h stimulation. All RNA samples were intact because rehybridization of the same membrane with a Japanese flounder β-actin cDNA probe (23) revealed a 1.4-kb band in all lanes.

We tested several organs of healthy Japanese flounder for the presence of TNF mRNA by RT-PCR. None of the organs, tissues, or cells examined expressed TNF mRNA. Only LPS-treated leukocytes expressed TNF mRNA (data not shown).

Discussion

This is the first report of cloning and characterization of TNF from a teleost. The amino acid sequence alignment showed that the Japanese flounder TNF is equally homologous (~30%) to human TNF-α and lymphotoxin-α, although the identity between human TNF-α and lymphotoxin-α is ~30%. The human TNF-α has two cysteine residues in the mature protein region, which form a single disulfide bond, whereas the lymphotoxin-α does not have a disulfide bond (Fig. 2). These two cysteine residues are also conserved in the Japanese flounder TNF. The phylogenetic analysis suggests that the teleostei have one ancestral TNF gene and the mammalian TNF-α and lymphotoxin-α gene were duplicated and evolved after mammals diverged from teleosts (Fig. 3). The number of exons and introns and the positions of the exon-intron junctions of the Japanese flounder TNF gene are similar to those of the human TNF-α gene. The protein coding regions of the human lymphotoxin-α gene consist of three exons (15). We speculate that the Japanese flounder TNF has a role similar to that of the mammalian TNF-α based on similarities in the structures of the genes, the lengths of the amino acid sequences, and the existence of cysteine residues that form a disulfide bond.

There is no complete polyadenylation signal AATAAA in the 3’-untranslated region (UTR). However, TA-rich motifs (TATT...
The expression pathway of the Japanese flounder TNF gene might respond quickly to some inducers of TNF gene transcription. The present results also indicate that the expression of the Japanese flounder TNF gene might be controlled at the translational level. Interestingly, the expression level of the TNF gene in Japanese flounder was induced by LPS, Con A, and PMA, but the gene was not expressed in the cell culture medium (Fig. 6). The induction of the cytokine TNF in Japanese flounder PBLs was observed after a 1- and 3-h incubation with PMA (Fig. 6). Similarly, the expression level of the TNF gene in Japanese flounder was not expressed in the cell culture medium (Fig. 6).

In conclusion, all of the characteristics of the cloned gene in this study, i.e., its gene structure, amino acid sequence, and expression pattern, are similar to those of human TNF-α, and thus indicate that it is TNF-α. This is the first report of the TNF-α cDNA and gene from a nonmammalian vertebrate.

References


