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Genomic Organization and Chromosomal Localization of the T-Cell Antigen 4-1BB[1]

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4-1BB is an inducible T cell surface receptor which belongs to the nerve growth factor receptor superfamily, a group of cysteine-rich cell-surface proteins. 4-1BB is a 30-kDa glycoprotein and exists as both a monomer and a 55-kDa dimer on the T cell surface. Cross-linking 4-1BB with monoclonal antibody resulted in the 2- to 10-fold enhancement of T cell proliferation. We have isolated and characterized 4-1BB genomic clones and have found that the 4-1BB gene contains two different 5′ untranslated regions, which are used alternately to form the 4-1BB mRNA. The two 5′ UTRs were encoded in the same chromosome and were separated from one another by an intron of ~2.5 kb. The entire gene spans approximately 13 kb of mouse chromosome 4. 4-1BB gene consists of 10 exons and 9 introns, in which there are two exons for 5′ untranslated regions and 8 exons for coding region. Most of the putative functional domains were encoded by separate exons. 4-1BB extracellular domain contains four potential C6 (CXn C XX C XX CXn C Xn C) motifs, of which the first motif is partial and the third is distinct from those of nerve growth factor receptor or TNF receptor 1. A comparison of exon-intron organization among the genes of the nerve growth factor receptor family indicated that most C6 motif is interrupted by an intron. *Journal of Immunology,* 1994, 152: 2256.

4-1BB cDNA was isolated from activated murine T cells by a modified differential screening procedure (1). Deduction of its amino acid sequence and analysis of mRNA expression indicated that 4-1BB is a T cell surface receptor the expression of which is inducible and sensitive to cyclosporin (2, 3). 4-1BB was categorized as an early activation gene because the protein synthesis inhibitor cycloheximide blocked the induction of 4-1BB mRNA (4).

4-1BB is a 30-kDa glycoprotein and exists as both a monomer and a 55-kDa dimer on T cell surfaces (4). Cross-linking of 4-1BB with monoclonal antibody 53A2 on anti-CD3-stimulated T cells results in a 2- to 10-fold enhancement of T cell proliferation (4). This result suggests that 4-1BB may function as a signaling molecule during T cell activation. Although the actual biochemical signals delivered through 4-1BB are not known, the cytoplasmic domain of 4-1BB contains a potential $p56^{lck}$ binding site, C-R-C-P-. We have recently shown that 4-1BB is physically associated with $p56^{lck}$, which suggests that this kinase may play a role in transmitting signals delivered through 4-1BB (5).

Recently, a number of cysteine-rich receptor proteins have been reported and have been designated as the nerve growth factor receptor (NGFR) superfamily (6). At present, other members of the NGFR superfamily include NGFR (7); the B-cell antigen CD40 (8); the rat T cell antigen OX-40 (9); two receptors for the TNF, TNFR-I and TNFR-II (10–12); an open reading frame in the Shope fibroma virus, SFV-T2 (13); a T cell surface antigen, CD27 (14, 15); a cell surface antigen, *Fas* (16); and a Hodgkin's lymphoma antigen, CD30 (17).

In the extracellular domain of these receptors, repeats of a cysteine-rich motif consisting of about 40 amino acids have been recognized. Cysteine residues are found in a conserved pattern with variable sequences interspersed (6). These molecules contain a hinge-like region immediately adjacent to the transmembrane domain, which is rich

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in serine, threonine, and proline residues (STP). This region is likely to be glycosylated with *O*-linked sugars. Although the receptors possess structurally similar extracellular domains, the cytoplasmic domains of these proteins are not conserved. This lack of conservation could allow for diversity in transmembrane signaling.

When we analyzed cDNA clones of 4-1BB, we noticed that there were two different species with different 5′ untranslated regions (UTR)[3]. If the two 5′ UTRs of 4-1BB are authentic, it is possible that 4-1BB uses two different promoters. If this possibility proves to be the case, it is important to understand the regulation of 4-1BB expression.

It is conceivable that the cysteine-rich repeats of these receptors are formed by gene duplication of a primordial unit, which may form a ligand-binding domain. Comparison of the genomic organization among the members of the receptor family may provide some clues to the functional unit; therefore, we determined the nucleotide sequence of the 4-1BB gene and compared its structural organization with the structural organization of NGFR (7), CD27 (18), CD40 (19), and TNFR1 (20).

Materials and Methods

Cells

CTLL-R8, a mouse cytolytic T cell line, was grown in DMEM (GIBCO, Grand Island, NY), containing 100 U/ml of penicillin, 100 μg/ml of streptomycin, 50 U/ml of mouse rIL-2 (Boehringer-Mannheim Corp., Indianapolis, IN), and 10% FBS. B16, a mouse melanoma cell line, was maintained in RPMI containing 10% FBS and antibiotics.

Primer extension analysis

Four micrograms of poly (A)$^+$ RNA from CTLL-R8 or B16 murine melanoma cells was annealed with 8 pmol of kinased oligonucleotide primer at 30°C overnight in a buffer containing 0.4 M NaCl, 40 mM PIPES (pH 7.0), 1.0 mM EDTA (pH 8.0), and 80% formamide. The mixture was ethanol-precipitated and resuspended in a buffer containing 50 mM Tris-HCl (pH 7.6); 60 mM KCl; 10 mM DTT; 1.0 mM each dATP, dGTP, dTTP, and dCTP; 1,000 U/ml RNAsin (Promega Corp., Madison, WI); and 20 U of AMV reverse transcriptase (Life Sciences, Inc., St. Petersburg, FL). The mixture was incubated at 37°C for 2 h, extracted with phenol-chloroform, and precipitated with ethanol. The precipitate was resuspended in 10 mM Tris-HCl (pH 7.4), 10 mM EDTA, and incubated with 50 μg/ml RNAse A at 37°C for 30 min. The reaction product was analyzed by sequencing gel.

Isolation and analysis of genomic 4-1BB clones

A BALB/c mouse genomic DNA library cloned in EMBL3 λ-phage vector was purchased from Clontech Laboratories, Inc. (Palo Alto, CA). The library was screened with 4-1BB cDNA and oligomers representing two different 5′ untranslated regions (UTR) under high stringency conditions. DNA was prepared from the positive λ-phage clones. To identify genomic fragments containing exons, 4 μg of phage DNA was digested with restriction enzymes, separated by electrophoresis on 1% agarose gels, and blotted on Gene Screen Plus (NEN Research Products, Boston, MA). The blot was hybridized with ^{32}P-labeled 4-1BB cDNA probe and ^{32}P-labeled oligomers representing the 5′ UTRs.

[3] Abbreviations used in this paper: *lck*, p56^{lck} protein tyrosine kinase locus; NGFR, nerve growth factor receptor; UTR, untranslated region; TPA, 12-0-tetradecanoylphorbol 13-acetate.

Sequence analysis of 4-1BB genomic clones

The genomic insert was digested with Sac I and the fragments were subcloned in the pGEM 7 vector. Each subclone was sequenced after generation of unidirectional deletions by means of the Erase-a-Base system (Promega Corp.). The nucleotide sequence was determined by the dideoxy chain termination method (21) using Sequenase Version 2.0 (United States Biochemicals, Cleveland, OH).

Chromosomal assignment of the 4-1BB gene

High molecular weight DNA was isolated from a previously characterized panel of 23 mouse-Chinese hamster hybrid cell lines (22). The DNA was digested to completion with a panel of restriction enzymes, subjected to electrophoresis through 0.8% agarose gels, transferred to Gene Screen Plus, and hybridized to ^{32}P-labeled 4-1BB cDNA probe. Stringent washes were carried out in 0.1X SSC/0.1% SDS at 65°C.

Results

Isolation and characterization of the 4-1BB gene

Two types of 4-1BB cDNA were isolated in screening mouse T lymphocyte cDNA libraries. The sequences of the two cDNAs were different only in the 5′ UTR beyond 27 base pairs immediately upstream of the initiation codon. We generated oligonucleotide probes corresponding to the two types of 5′ UTR, and cDNA probes corresponding to the coding region and 3′ UTR. These cDNA subfragments or oligonucleotide probes were used to screen a mouse EMBL-3 genomic library. Four overlapping genomic clones were isolated. The genomic inserts were digested with Sac I, blotted on Gene Screen Plus membrane, and hybridized with cDNA subfragments and oligonucleotide probes. The entire 4-1BB cDNA was included in five Sac I fragments. The lengths of the Sac I fragment from 5′ to 3′ were 2.7 kb, 5.5 kb, 2.8 kb, 1.0 kb, and 5.5 kb. These five Sac I genomic fragments were cloned in the PGem 7 vector. The first four Sac I fragments and a part of the fifth Sac I fragment, which span approximately 13 kb, were sequenced (Figures 1 and 2).

4-1BB gene consists of 10 exons and 9 introns, in which there are two exons for 5′ UTR and 8 exons for the coding region. The two types of UTR sequences were identified in the genomic sequence and found to be separated by an intron ~2.5 kb in length. The 5′ UTR sequence proximal to the 4-1BB gene body has been called the type I 5′ UTR, and the transcript using the proximal 5′ UTR has been called type I 4-1BB mRNA. The distal 5′ UTR has been named type II, and the transcript using the UTR has been named type II 4-1BB mRNA. The cysteine-rich extracellular domain was divided into six exons. The signal sequence, transmembrane region, and serine threonine and proline (STP)-rich region immediately outside the transmembrane domain were contained in separate exons. Finally, the cytoplasmic domain that contains the p56^{lck} – binding site was located in the last exon of the gene. The exon-intron boundaries were assigned by comparing the 4-1BB cDNA sequence with the genomic sequence (Fig. 1). Where the placement of splice donor and acceptor sites was ambiguous, assignment was made to

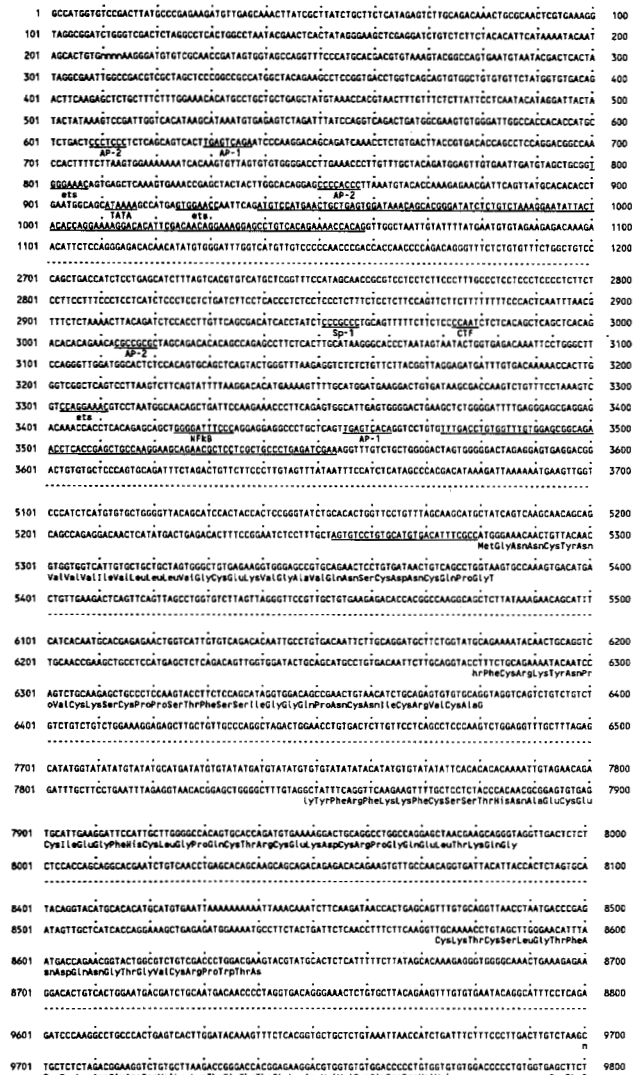

FIGURE 1. Nucleotide sequence of the 4-1BB gene. Nucleotide sequences of the 5′ UTR are underlined. Numerals at left and right indicate positions in the nucleotide sequence. The amino acid sequence of the coding region is shown. The dotted lines indicate intron sequences not shown. The stop codon (TGA) is indicated by – – –. The potential polyadenylation signal (AATAAA) is indicated by overhead stars. The potential transcription regulatory elements are underlined. The sequence has been deposited in the GenBank under accession no. UO2567.

optimize the consensus AG/GT sequence (23). The sizes of the exons and introns are indicated in Figure 2.

Transcription initiation sites

Primer extension analysis of poly (A)$^+$ RNA from Con A-stimulated CTLL-R8 was performed to determine the transcription start-site of the gene. Twenty-four mer oligonucleotides specific to each type I and type II mRNA were used to prime cDNA synthesis on poly (A)$^+$ RNA from Con A-stimulated CTLL-R8, or from B16 murine melanoma cells as a negative control.

Figure 3 shows the extension product of the I 5′ UTR. When the size of the extension product is calculated, the type I mRNA start predominantly at position −135 from the first A of the initiation codon and there is a minor species starting at position −140. Although we obtained cDNA of type II mRNA from a Con A-stimulated T cell cDNA library (1), we did not detect any extension product corresponding to the type II 5′ UTR. We believe that type II mRNA is very rare, because the type II 5′ UTR-specific probe did not hybridize to several Con A-stimulated T cell mRNAs, although the type I probe showed hybridization in the Northern blot analysis. The cDNA of the type II 5′ UTR contained 120 base pairs beyond the common 27 base pairs. Therefore, the type II mRNA start-site was assigned tentatively to position −147 (Fig. 2). This RNA start-site may be very close to the authentic start site because a ''TATA''-like sequence appears at position −28. Most TATA boxes appear at positions −23 to −27 from the mRNA start-site.

Potential regulatory elements

Potential regulatory elements within 600 base pairs of the promoter region of both mRNAs were located by comparison with known consensus sequences.

In the flanking sequence of the type I 5′ UTR, no TATA box-related elements were found. Instead, there are very good matches of the consensus TPA-responsive element (AP-1) (24) at positions −18 to −10, and of the NFκB-binding sequence (25) at positions −49 to −39. Upstream to these elements, this region contains a potential ets-binding site (26) at positions −169 to −162, an activator protein 2 (AP-2) binding site (27) at positions −460 to −453, a typical ''CAAT''-element (28) at positions −498 to −494, and a Sp-1 binding site (29) at positions −522 to −516.

The 5′ flanking region of the type II 5′ UTR contains a ''TATA''-related element at positions −28 to −23. Two potential ets-binding sites appear at positions −15 to −8 and −139 to −132, two potential AP-2 binding sites at positions −89 to −82 and −331 to −325, and a very good match of an AP-1 binding site at positions −311 to −302.

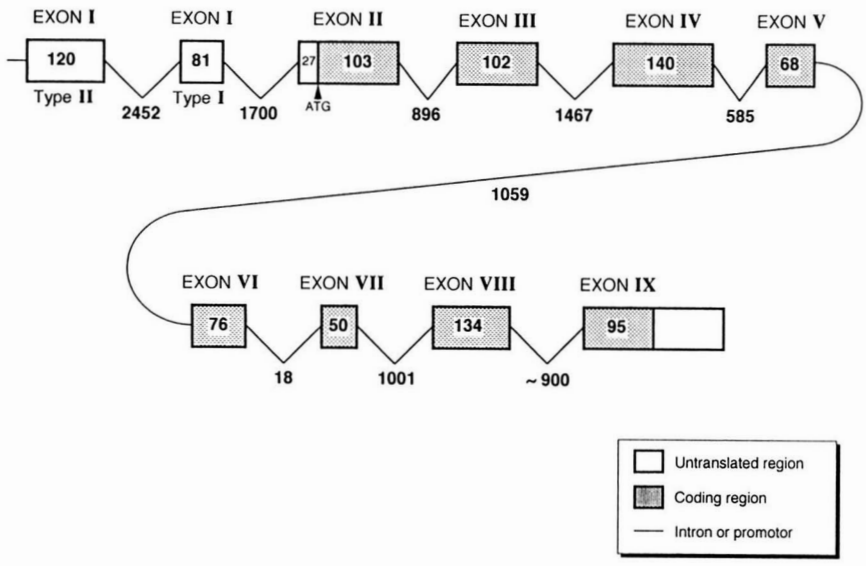

FIGURE 2. Organization of the 4-1BB gene. Exons are shown as boxes; UTRs are open and the protein coding region is dotted and indicated in Roman numerals. Lengths of exons and introns are shown in Arabic numerals (base pairs).

Chromosomal localization of the 4-1BB gene

To determine the chromosomal assignment of 4-1BB gene, a 0.6 kb 4-1BB cDNA probe representing part of the coding region was hybridized with DNA derived from a panel of 23 mouse-Chinese hamster hybrid cell lines. Digestion with Bam H1 produced a hybridizing band of 8.0 kb in mouse DNA. Mouse 4-1BB probe did not hybridize to hamster DNA. This informative band was found to correlate exactly with the presence or absence of chromosome 4 in each of the cell lines (Table I).

Discussion

Potential primordial unit of the NGFR gene superfamily

Recently, the gene structures of three other members of the NGFR superfamily were reported (18–20). The coding region of CD40 is separated into 8 exons, as is 4-1BB, whereas CD27 is separated into 6 exons and TNFR1 is separated into 10 exons. Although the predicted cysteine-rich motif was not separated by exons, other functional domains such as signal sequences and transmembrane, *O*-glycosylation, and cytoplasmic domains were embedded in separate exons (Fig. 4).

Mallett and Barclay (6) predicted a cysteine 6 (C6) motif of the extracellular domain in the members of NGFR superfamily. The crystal structure of the soluble human 55 kDa tumor necrosis factor receptor (TNFR)-human TNF beta complex by Banner et al. (30) provided strong evidence for this C6 structural unit. They showed that there were three disulfide bonds in each motif (30). Figure 5 shows the potential C6 motif and disulfide bonds in 4-1BB, CD40, CD27, NGFR, and TNFR1, the gene organization of which was published. We found that this functional C6 motif was not limited within an exon; rather, the cysteines that made up the motif usually span two different

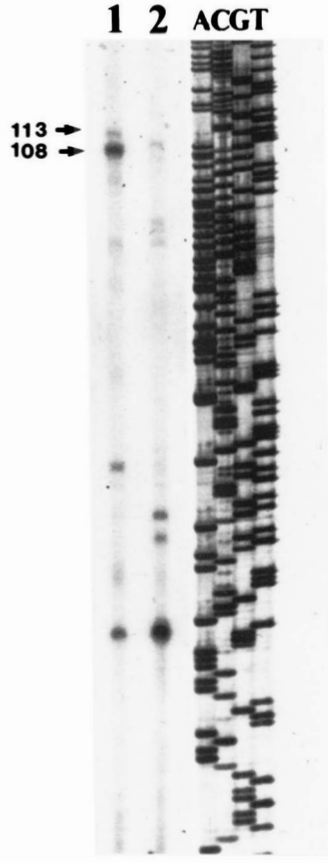

FIGURE 3. Primer extension analysis for determination of the start-site of type I 4-1BB mRNA. The amount of RNA for the extension was 4 μg poly $(A)^+$ for CTLL-R8 (*lane 1*) and B16 melanoma (*lane 2*). The nucleotide sequence ladder on the right is for calculation of the size of the extension products. The specific extension products for type I 4-1BB mRNA are indicated by arrows with numbers.

Table I. *Segregation of mouse chromosomes with 4-1BB cross-hybridizing bands in 23 somatic cell hybrids*

Mouse Chromosome	No. of hybrids (DNA hybridization/chromosome) +/+	−/−	+/−	−/+	% Discordance
1	8	8	0	4	20.0
2	7	6	2	6	38.1
3	2	7	1	4	35.7
4	8	11	0	0	0.0
5	3	9	6	2	40.0
6	7	7	2	5	33.3
7	8	6	1	6	33.3
8	6	11	2	1	15.0
9	5	11	4	1	23.8
10	0	12	8	0	40.0
11	0	11	9	1	47.6
12	4	3	1	8	56.2
13	7	8	2	4	28.6
14	2	9	7	3	47.6
15	6	2	0	9	52.9
16	6	8	3	3	30.0
17	6	4	2	7	47.4
18	6	7	2	5	35.0
19	7	9	2	2	20.0
20	6	6	6	6	40.0

This table is compiled from 23 cell hybrids involving mouse-Chinese hamster fusions. Symbols indicate the presence (+/) or absence (−/) of mouse *4-1BB* gene restriction fragments as related to the presence (/+) or absence (/−) of a particular mouse chromosome. The number of discordant observations is the sum of the +/− and −/+ observations. The lowest discordance is the basis for chromosomal assignment.

exons. The first motif of 4-1BB, which is partial, is made up of four cysteines. The third motif of 4-1BB appears to be classified with that of CD40 and CD27 and to differ from the motifs of NGFR and TNFR1 (Fig. 5). In the fourth motif of 4-1BB and CD40, C3 is absent and this coincides with the absence of C5. This observation corroborates with the finding that C3 and C5 are disulfide linked in the crystal structure of TNFR1 (30). The importance of each of the cysteines and other amino acids in ligand binding requires further study, such as of site-specific mutagenesis and its effect on ligand binding, analysis of gene organization of other members of this gene family, and physicochemical analysis of crystallized proteins.

4-1BB gene expression may be regulated by two different promoters

Although the differential expression of type I and type II mRNAs was not demonstrated in different tissues or different developmental stages of T cells in the present studies, 4-1BB appears to produce two types of mRNA, and their expression may be regulated differently. Two observations permit this conclusion: (1) the isolation of two types of cDNA; and (2) the genomic organization and the identification of potential regulatory elements in each of the two potential promoter regions.

The *lck* gene (31), the mouse α-amylase gene (32), and the *Drosophila melanogaster Adh* gene (33) are expressed from two promoters that produce mRNAs differing only in their 5′ UTRs. In these cases, transcription from the proximal promoter produces an mRNA that is present early in development. Transcription from the distal promoter begins later. In the case of *lck*, type I and type II *lck* mRNA were expressed at different levels at specific stages of T-cell differentiation in the adult, and during development from the fetus to the adult. It would be interesting to investigate whether 4-1BB expression is regulated developmentally, and whether the choice of promoter affects the level of 4-1BB expression.

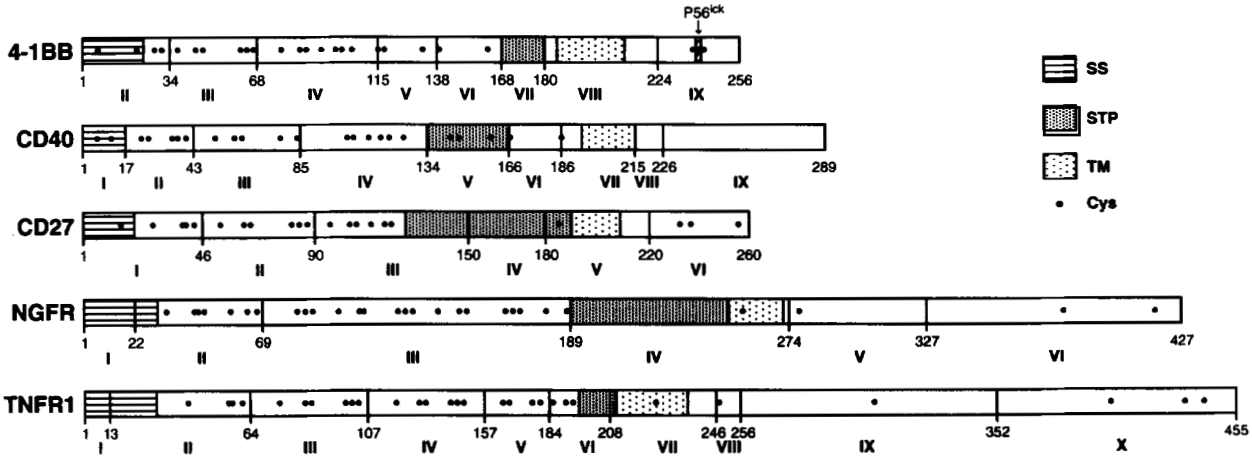

FIGURE 4. Comparison of the splice sites and arrangement of cysteines in 4-1BB, CD40, CD27 NGFR, and TNFR1 genes. The different domains of the proteins are indicated. The splice sites are indicated by vertical bars. Exons are indicated by Roman numerals. Positions of amino acids at each splice-site are indicated by Arabic numerals. $p56^{lck}$-binding site in the cystoplasmic domain of 4-1BB is indicated.

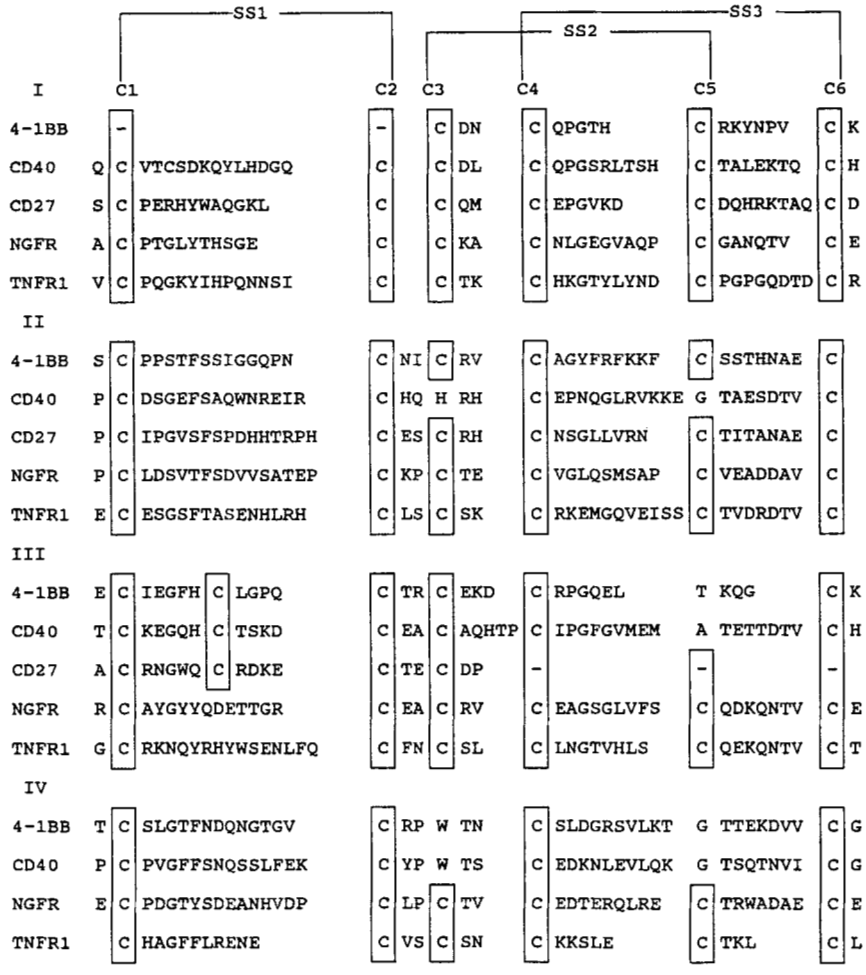

FIGURE 5. Aligned amino acid sequences of extracellular C6 motif of the NGFR superfamily. The C6 motif of TNFR1 was deduced from the crystal structure of soluble TNFR1. The amino acid sequence of 4-1BB, CD40, CD27, and NGFR was aligned with TNFR on the basis of sequence homology and conserved cysteines. The intramotif disulfide bonds, SS1, SS2, and SS3, are deduced from the data obtained from the TNFR1 crystal structure.

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