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Characterization of Mouse and Human B7-H3 Genes¹

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Alexander Rudensky,*[†] and Chen Dong^{3*}

T cell activation and immune function are regulated by costimulatory molecules of the B7 superfamily. Human B7-H3 is a recent addition to this family and has been shown to mediate T cell proliferation and IFN- γ production. In this work we describe the identification of the mouse B7-H3 homolog, which is ubiquitously expressed in a variety of tissues. Activated CD4 and CD8 T cells express a putative receptor that can be recognized by soluble mouse B7-H3-Ig molecules. While the mouse B7-H3 gene was found to contain a single copy, we discovered a novel isoform of human B7-H3 (named as B7-H3b hereafter) with four Ig-like domains that results from gene duplication and differential splicing. B7-H3b is the major isoform expressed in several tissues. This structural information suggests a genetic variation of the B7-H3 gene in mammalian species. *The Journal of Immunology*, 2002, 168: 6294–6297.

T helper cell activation, differentiation, and function are regulated by costimulatory molecules (1). CD28, a receptor for the B7 gene products CD80 and CD86, plays a major role in initiating T cell immune responses (2). CTLA4, which also binds both B7 molecules with a higher affinity, is induced after T cell activation and plays a role in down-regulating T cell responses (3). In addition to this well-studied pair of receptors with opposing regulatory functions, other costimulatory molecules were discovered more recently. PD-1 is a receptor for the B7 homolog B7-H1 and PD-L2/B7DC (4–6). Mice deficient in PD-1 develop lupus-like proliferative arthritis, glomerulonephritis, and autoimmune dilated cardiomyopathy (7, 8). Inducible costimulator (ICOS),⁴ a third member of the CD28/CTLA4 family, is expressed on activated T cells (9, 10). Its ligand, B7 homolog (also named B7RP-1) is expressed constitutively on B cells and is induced in nonlymphoid tissues by TNF (9, 11). Recently, we and others generated and analyzed ICOS-deficient mice (12–14). We showed that ICOS is required for humoral immune responses after immunization with several Ags (12, 15). In addition, ICOS^{-/-} mice exhibited greatly enhanced susceptibility to experimental autoimmune encephalomyelitis, suggesting that ICOS plays a protective role in inflammatory autoimmune diseases. Thus, members of the B7 costimulator family are essential regulators in the immune response.

Human B7-H3 is a new member of the B7 family (16). It is broadly expressed and its expression in dendritic cells can be in-

duced by inflammatory cytokines. Soluble human B7-H3 binds to activated T cells and costimulates their proliferation and, most potently, IFN- γ production. Therefore, B7-H3 represents a novel regulatory means for cell-mediated immunity.

In this paper, we describe for the first time the mouse B7-H3 homolog, which is ubiquitously expressed in almost all tissues. Soluble B7-H3-Ig fusion protein binds to activated T cells. We also identify a new isoform of human B7-H3 gene with four Ig-like domains, which we name B7-H3b. Structural analysis B7-H3 genes in two species reveals that a gene duplication event happened after separation of mouse and human ancestors.

Materials and Methods

EST clones

Mouse B7-H3 and human B7-H3b expressed sequence tag (EST) clones (GenBank accession nos. BF450618 and BF984597, respectively) were purchased from Incyte Genomics (Palo Alto, CA). The plasmid DNA was prepared and sequenced by use of Big-Dye reagents from Qiagen (Valencia, CA).

Northern blot analysis

Mouse tissue Northern blot was purchased from Seegene (Seoul, Korea). A PCR fragment consisting of two Ig-like domains of the mouse B7-H3 gene was used for the analysis. The final wash of the blot was performed with 0.1 \times SSC/0.1% SDS at 65°C.

Expression and purification of B7-H3-Ig fusion protein

cDNA sequence encoding the extracellular portion of mouse B7-H3 protein was amplified by PCR and sequenced before subcloning into DES-Ig vector consisting of an insect expression plasmid pMT/BiP/V5-His A (Invitrogen, San Diego, CA) backbone and a human IgG1 Fc tag (a kind gift of Dr. E. Clark, University of Washington, Seattle, WA) (see Fig. 3A). Transfection of the new vector, which we named as DES-H3Ig, into the *Drosophila* cell line S2 results in CuSO₄-inducible expression of B7-H3-Ig fusion protein in the culture supernatant. To yield a large quantity of the protein, we constructed a stable cell line of DES-H3Ig by cotransfection of a hygromycin resistance plasmid. B7-H3-Ig fusion protein produced by this stable line was further purified on a protein A-Sepharose column (Sigma-Aldrich, St. Louis, MO) and labeled by sulfo-NHS-LC-biotin (Pierce, Rockford, IL). To examine expression of a putative B7-H3 receptor, C57BL/6 lymph node cells, before and after 2 days of activation with Con A (2.5 μ g/ml), were first incubated with human IgG1 (10 μ g/ml; Sigma-Aldrich) to block nonspecific binding and then stained with biotinylated B7-H3-Ig fusion protein at 2 μ g/ml. For flow cytometry analysis, B7-H3-Ig staining was revealed by streptavidin-R-PE, and T cell population was determined by anti-CD8-FITC and anti-CD4-Spectral Red-labeled (Southern Biotechnology Associates, Birmingham, AL).

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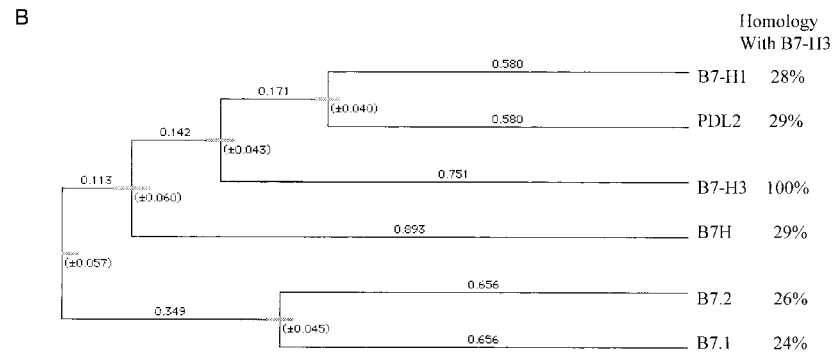
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⁴ Abbreviations used in this paper: ICOS, inducible costimulator; EST, expressed sequence tag; BLAST, basic local alignment search tool.

A Leader
 MLRGWGGPSVGVCVRTALGVLCLCLTGAVEVQVSEDPVVALVDTDATLRCSFS
 IgV
 PEPGFLAQLNLIWQLTDTKQLVHSFTEGRDQGSAYSNRTALFPDLLVQGNASLR
 IgC
 LQRVRVTDEGSYTCFVSIQDFDSAAVSLQVAAPYSKPSMTLEPNKDLRPGNMVTI
 TCS^uSYQGYPEAEVFWKDGQGVPLTGNVTTSSQMANERGLFDVHSLRVVLGANG
 Transmembrane
 TYSCLV^uRNPVLQQDAHGSVTITGQPLTFPPEALWVTVGLSVCLVLLVALAFVC
 WRKIKQSCEEENSGAEDQDGDGEGSKTALRPLKPSENKEDDGQEIA

FIGURE 1. Identification of mouse B7-H3 gene. *A*, Deduced amino acid sequence of mouse B7-H3 cDNA. The leader peptide and transmembrane region are indicated by solid lines; Ig-like domains are indicated by dotted lines. Conserved cysteine residues of the Ig domains are underlined and displayed in bold. *B*, Phylogenetic analysis of the B7 family members. The scores at each branch represent the degree of sequence variability between proteins (0 for identical sequences). Percentages of sequence identity of other known B7 proteins to B7-H3 are indicated.



Analysis of human B7-H3 gene splicing

Human B7-H3b cDNA, which consists of two pairs of IgV-IgC domains, was PCR-amplified from a human colon cDNA and sequenced. The primers used are the forward, agcactgtggttcgcctca, and the reverse, tgcattcctcctcacagc. To analyze B7-H3 splicing in different tissues, a pair of primers designed from the common sequences of B7-H3a and B7-H3b, which lie in exons 1 and 8 of the B7-H3 gene (see Fig. 4A), was used to amplify cDNA samples of multiple human tissue cDNA panel (Clontech Laboratories, Palo Alto, CA).

Sequence analysis

The Ig domains of mouse B7-H3 protein were predicted by CD-Search program of the National Center for Biotechnology Information. Genomic structure of mouse and human B7-H3 genes was analyzed by use of standard nucleotide basic local alignment research tool (BLASTN) and translate BLAST (TBLASTN) methods in the public human genome database and in the mouse and human genome database of Celera (Rockville, MD). The alignment of mouse and human B7-H3 protein sequences was performed using Jellyfish software (Biowire, San Francisco, CA). Comparison

of mouse B7-H3 and other B7 family members was performed using basic local alignment search tool 2 of the National Center for Biotechnology Information, and the phylogeny tree was constructed with GeneWorks software (Gene Works, Australia).

Results and Discussion

T cell activation and function are regulated by costimulatory molecules, most notably those of the B7 family (1). It has been recently estimated that ~20 B7 family members exist in humans

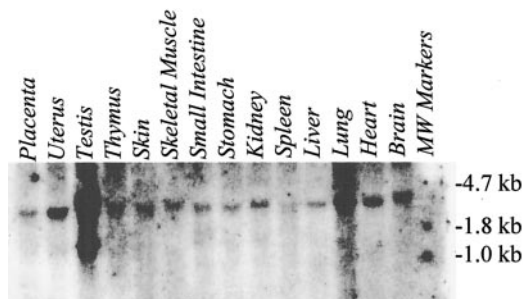


FIGURE 2. Northern blot analysis of mouse B7-H3 gene expression. A PCR fragment consisting of two Ig-like domains of the mouse B7-H3 gene was used to hybridize mouse tissue Northern blot (Seegene).

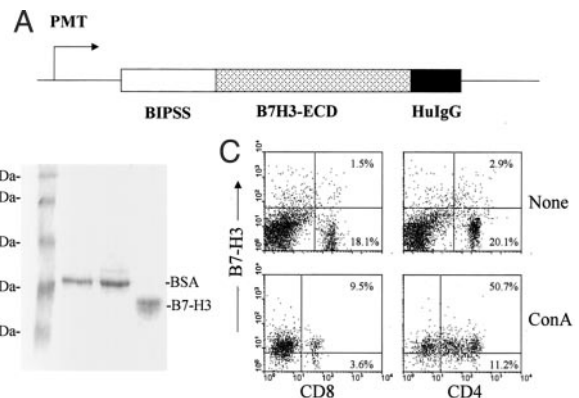


FIGURE 3. B7-H3 soluble protein binds to activated T cells. *A*, Construction of B7-H3-Ig vector for insect cell expression. *B*, B7-H3-Ig fusion protein was purified from insect cell supernatant by protein A-Sepharose and analyzed by SDS-PAGE and Coomassie blue staining. *C*, Biotinylated B7-H3-Ig protein binds to activated CD4 and CD8 cells. Lymph node cells from a C57BL/6 mouse were activated with Con A for 48 h, and cells before and after activation were analyzed for B7-H3 binding together with Abs for CD4 and CD8.

(17). Therefore, structural and functional analysis of B7 family genes will benefit our understanding of the regulation of the adaptive immune system.

Human B7-H3 is among the most recently discovered B7-like molecules (16). Soluble human B7-H3 molecule was previously reported to bind to activated T cells and costimulate their proliferation and IFN- γ production (16). To characterize the mouse counterpart, we did a search of the mouse EST database and discovered a clone with significant homology with the described human sequence. We obtained the clone and completely sequenced the coding region. The predicted mouse B7-H3 protein contains 315 amino acids (Fig. 1A) with 88% identity and 93% similarity with the human molecule, while sharing ~20–30% identity with the other B7 family members known to date (Fig. 1B). Murine B7-H3 has a leader peptide and a transmembrane domain, indicating that, as the human molecule, it is a type 1 transmembrane glycoprotein. The extracellular portion of mouse B7-H3, like those in many other B7 family members, consists of one IgV- and one IgC-like domain (Fig. 1A).

We then examined the expression of mouse B7-H3 mRNA in multiple tissues by Northern blot analysis. Quite similar to its human homolog (16), mouse B7-H3 is widely expressed in all tissues we analyzed (Fig. 2). The size of its mRNA is ~1 kb bigger than that of the EST clone, suggesting that the transcript has a long 5' untranslated region. Ubiquitous expression of B7-H3 in nonlymphoid tissues suggests its role in modulating immune responses in these tissues. However, it is unclear at this stage what specific cell types express B7-H3.

To assess whether mouse B7-H3, like its human counterpart, binds to a receptor on activated T cells, we produced soluble mouse B7-H3 protein with a human IgG1 Fc tag (Fig. 3B). When we used biotinylated fusion protein to examine expression of a putative mouse B7-H3 receptor, we found that the soluble protein does not bind significantly to CD4 or CD8 cells from C57BL/6 lymph node cells (Fig. 3C). After these cells were activated with 2.5 μ g/ml Con A for 48 h, they could all be recognized by the fusion protein (Fig. 3C). This result suggests a mouse B7-H3 receptor is induced upon T cell activation. Therefore, the B7-H3

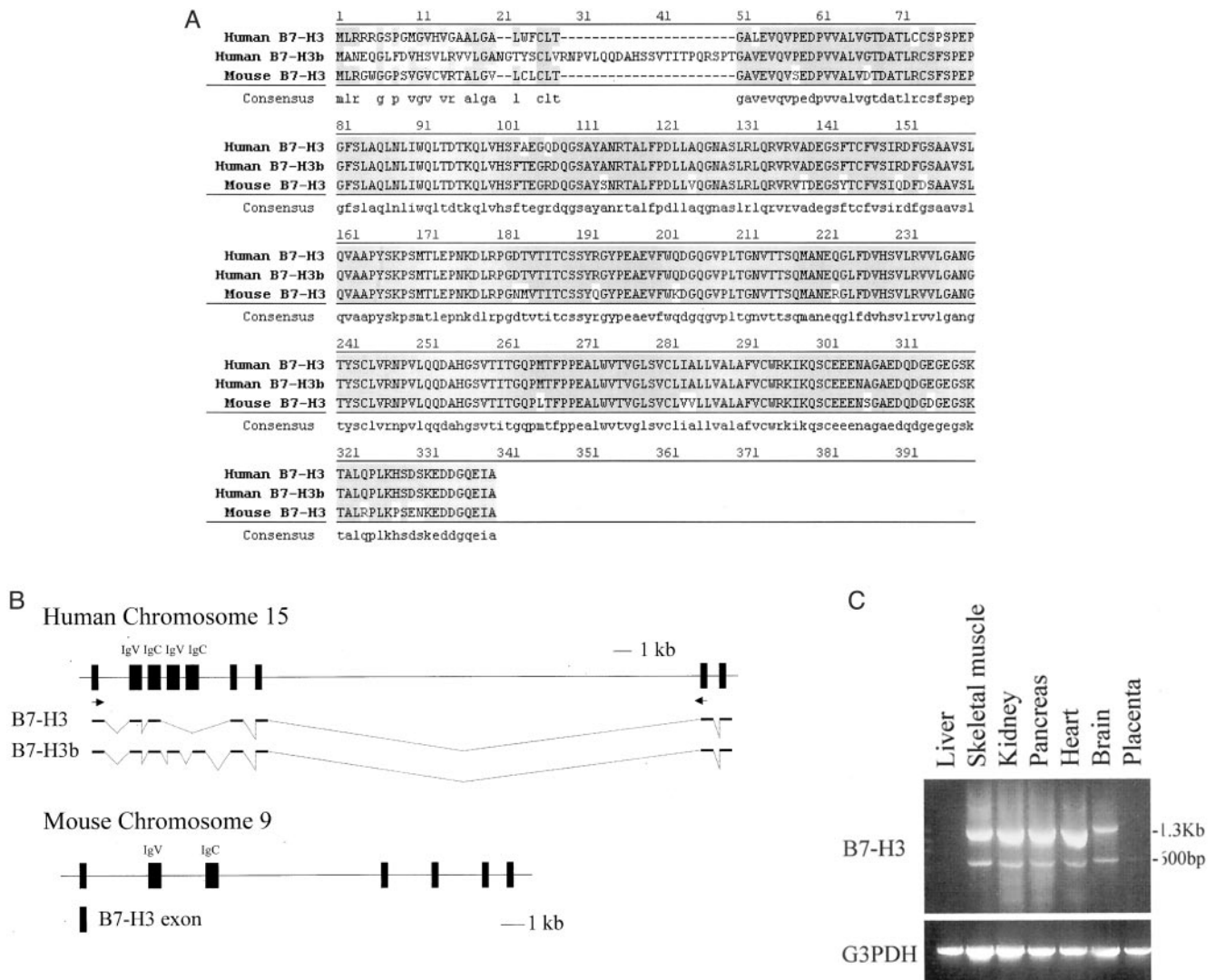


FIGURE 4. Identification of human B7-H3b isoform. *A*, The deduced amino acid sequence from a human B7-H3b EST clone is aligned with those of human B7-H3a and mouse B7-H3. Conserved residues are shaded in gray and consensus amino acids are indicated. *B*, Genomic structure of human and mouse B7-H3 genes. The amino acid-coding exons are indicated. The scale of 1 kb is indicated. Small arrows below exons 1 and 8 indicate the primers used for PCR analysis presented in *C*. *C*, Expression of B7-H3b in multiple human tissues analyzed by RT-PCR analysis. B7-H3b gives a 1.3-kb PCR product, while B7-H3 gives a 0.6-kb PCR product.

ligand-receptor interaction may possess a potential function in modulating T cell effector activity.

When we searched the human EST database with mouse B7-H3 amino acid sequences, we found several of them with minor sequence differences with the reported B7-H3 sequence. We ordered one of the clones and completely sequenced it. This clone appears to encode a novel isoform of human B7-H3, because it has a different N-terminal sequence and a few amino acid substitutions in the two extracellular Ig domains (Fig. 4A). However, the rest of the coding sequence is identical to that of B7-H3. We name this new isoform B7-H3b. The sequence we obtained from our own analysis was confirmed by those of multiple clones in the EST database, indicating that the difference we observed between B7-H3 and B7-H3b was not an artifact. The alignment of human B7-H3, B7-H3b, and mouse B7-H3 is shown in Fig. 4A. Although the human B7-H3 and mouse B7-H3 have similar N-terminal amino acid sequences, the extracellular portion of B7-H3b and mouse B7-H3 share closer homology.

To identify the molecular origin of B7-H3 and B7-H3b isoforms, we searched the public human genome database using B7-H3 and B7-H3b nucleotide and amino acid sequences. A fragment of human chromosome 15 contains sequences homologous to both isoforms (Fig. 4B). From this analysis, we deduced the genomic structure of the human *B7-H3* locus (Fig. 4B), which was confirmed by analysis using the Celera database (data not shown). It appears the B7-H3 and B7-H3b isoforms are the result of a gene duplication of two exons encoding the IgV-IgC domains of the B7-H3 molecule. These two isoforms share the same exons 4–7, which result in the identical transmembrane and intracellular sequences as we described above (Fig. 4A).

Interestingly, the most N-terminal amino acids present in the B7-H3b EST clone we sequenced lie in the middle of exon 3 of the B7-H3 gene (Fig. 4B). Because there is no stop codon in front of the first ATG sequence, this raised a possibility that the EST may not reflect the full-length B7-H3b-encoding cDNA, which may be even longer at the 5' and may contain two pairs of IgV-IgC domains. To assess this, we designed a pair of primers corresponding to the sequence from exons 1 and 8 (Fig. 4B), respectively, so that they would amplify a 1300-bp product if there were an isoform containing four Ig domains and 600 bp for the two-Ig isoform. When we used as a template human colon cDNA, which gave high B7-H3 expression in the Northern blot analysis (16), we observed a 1300-bp PCR product (data not shown). Subsequent DNA sequencing analysis confirmed that this product does correspond to the four-Ig isoform (data not shown). In this PCR analysis, a 600-bp product was also found, although present at much lower levels (data not shown).

To assess whether there is any tissue-specific splicing of the B7-H3 gene, we performed analogous PCR analysis of a cDNA panel of multiple human tissues (Clontech Laboratories) (Fig. 4C). In most tissues examined, except for brain and placenta, B7-H3b represented the major isoform. Interestingly, placenta does not express B7-H3b at all. When we sequenced the 600-bp PCR products from several tissues, we found that they contain the first IgV-IgC domains (data not shown). Therefore, they are the previously described B7-H3 isoform.

To assess whether the mouse B7-H3 gene has multiple isoforms, we searched the Celera mouse genome database and found seven exons encoding the B7-H3 protein on chromosome 9. There is no evidence of gene duplication in this case. This indicates that the two B7-H3 isoforms in humans must be derived by a duplication

of two exons after separation of human and mouse ancestors. The functional significance of this gene duplication event is unclear right now, although it is possible that B7-H3b molecules with four Ig-like domains may ligate two receptors at once and therefore be a stronger cross-linking ligand. Nonetheless, in light of the potentially important immunomodulatory function of B7 family member, it is interesting that the mammalian species are still evolving new molecules to regulate the adaptive immune function.

In summary, we report in this work, for the first time, the identification and expression pattern of mouse homolog of B7-H3, and a new human B7-H3b isoform generated by gene duplication and differential splicing. Our results revealed a variation of this gene in mammalian species. B7-H3 molecules are widely expressed in mouse and humans and therefore may possess fundamental immunoregulatory roles, especially in nonlymphoid tissues, to engage receptors on activated T cells. With the structural information described in this paper and the reagents prepared from it, exciting data may emerge in the near future on the function and regulation of B7-H3 as a novel costimulatory molecule.

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