Structure, Expression, and T Cell Costimulatory Activity of the Murine Homologue of the Human B Lymphocyte Activation Antigen B7

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Summary

Following occupancy of the T cell receptor by antigen, T cell proliferation and lymphokine production are determined by a second costimulatory signal delivered by a ligand expressed on antigen presenting cells. The human B cell activation antigen B7, which is expressed on antigen presenting cells including activated B cells and γ interferon treated monocytes, has been shown to deliver such a costimulatory signal upon attachment to its ligand on T cells, CD28. We have cloned and sequenced the murine homologue of the human B7 gene. The predicted murine protein has 44% amino acid identity with human B7. The greatest similarity is in the IgV and Ig-C like domains. Murine B7 mRNA was detected in murine hematopoietic cells of B cell but not T cell origin. Cells transfected with murine B7 provided a costimulatory signal to human CD28+ T lymphocytes. These results demonstrate the costimulatory activity of murine B7 and provide evidence that the ligand attachment site is conserved between the two species.

Although occupancy of the TCR complex by antigen in association with the MHC is necessary for the initiation of T cell activation, several lines of evidence suggest that a second costimulatory signal is essential for the induction of proliferation and lymphokine secretion (1–4). In murine and human systems, this costimulatory signal is delivered by APC and requires cell to cell contact (2, 4). Cells which can deliver this costimulatory signal include activated, but not resting B lymphocytes (5), INF-γ activated monocytes, and dendritic cells (2, 6).

Several recent studies in human systems have provided compelling evidence that the B cell activation antigen B7 can provide one such costimulatory signal (7–9). B7, a member of the Ig supergene family, has been shown to be a ligand for another member of this family, the T cell surface protein CD28, (10–13). CD28 is constitutively expressed on 95% of human CD4+ T cells, 50% of CD8+ T cells, and on thymocytes which coexpress CD4 and CD8 (14–16). Following suboptimal activation of T cells with anti-CD3 mAb (16), anti-CD2 mAb, or phorbol ester (17), crosslinking of CD28 by anti-CD28 mAb results in enhanced T cell proliferation and greatly augments synthesis of multiple lymphokines (18). B7 is likely to be an important regulator of T cell proliferation and lymphokine production as evidenced by its pattern of expression and functional activity. B7 is not expressed on resting B cells (19) but appears following crosslinking of surface Ig (10, 19) or class II MHC (9). Moreover, B7 is not expressed on unstimulated monocytes and is specifically induced by INF-γ but not other stimuli which activate monocytes (20). Human B7 (hB7)1 transfected cells or recombinant B7-Ig fusion protein augment proliferation and induce IL-2, but not IL-4, synthesis in T cells which have been treated with phorbol ester or anti-CD3 mAb (7–9).

In murine systems, a homologue for CD28 has recently been cloned (21); however, a conserved functional activity has not yet been demonstrated. In the present study, we have cloned, determined the nucleotide sequence, and structurally analyzed the murine homologue of B7. We demonstrate that murine B7 (mB7) is costimulatory for human CD28+ T cells, suggesting the existence of a highly conserved binding domain.

Materials and Methods

Isolation of Murine cDNA Clones. In preliminary experiments, low stringency hybridization of the human B7 cDNA insert (10) to blots of poly(A)+ RNA from the murine B cell lines 70Z, A20, 1

Abbreviations used in this paper: CHO, Chinese hamster ovary cells; hB7, human B7; mB7, murine B7.
beled by random oligonucleotide priming using a 32P-labeled
various organs isolated from a 4-wk-old Balb/c mouse. RNA was
the 3' untranslated region of the B7 mRNA. The protein coding region of the murine B7 cDNA was used as a probe
ping deletion clones on both strands were assembled to yield the
insert was sequenced using dye labeled primers and Taq polymerase
clone. 41110 was purified using a protein A Sepharose (Bio-Rad) as described (7). The anti-B7 mAb, 133, (IgM)
characterized in our laboratory (10, 19) and was used at a final
concentration of 10 μg/ml.

B7 Transfection. Transient expression of B7 cDNA clones in COS
cells was performed as previously described (11). COS cells trans-
fected with the pCDNAI vector alone were also prepared. Trans-
fected COS cells were used 72 h after the addition of DNA. A
stably transfected Chinese hamster ovary (CHO) cell line expressing
hB7 was constructed as previously described and is referred to as
CHO-hB7 (7).

Cell Fixation. COS and CHO cells were detached from tissue
culture plates and fixed with paraformaldehyde as described (7).
Proliferation Assay. The capacity of B7 to costimulate T cell prol-
eriferation was measured as described (7). Briefly, human CD28+
T lymphocytes were stimulated with phorbol myristate 13-ace-
tate (PMA) (Calbiochem, La Jolla, CA) at 1 ng/ml final concentration
(17). The fixed CHO-hB7 and COS cell transfectants were added at a concentration of 2 × 10^6 cells/well. The specificity of the
stimulation with COS-hB7 cells was assayed by the addition of anti-
B7 mAb to the cultures at a final concentration of 10 μg/ml. The
were pulsed with 1 μCi of ^H-thymidine (ICN Flow, Costa
Mesa, CA) during the last 8 h of a 72-h culture, harvested onto
filters, and counted.

Results

Isolation of Murine B7 cDNA, Sequencing, and Analysis. A murine cDNA clone was isolated by low stringency hybridization
with the hB7 cDNA employing a cDNA library prepared
from the murine pre-B cell line, 70Z. The murine cDNA
isolated from 70Z was composed of 1180 bases of intron fol-
lowed by a splice acceptor and sequences homologous to the
hB7 Ig-C, transmembrane, cytoplasmic, and 3' untranslated
domains. This cDNA was used to isolate additional clones
under stringent conditions from a cDNA library prepared
from the murine B cell line, A20. Comparison of the A20
cDNA clone with hB7 showed that the A20 cDNA con-
tained sequences homologous to the hB7 5' untranslated, signal
peptide, Ig-V, and Ig-C domains followed by a splice donor
and sequences homologous to the
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domains. This cDNA was used to isolate additional clones
under stringent conditions from a cDNA library prepared
from the murine B cell line, A20. Comparison of the A20
cDNA clone with hB7 showed that the A20 cDNA con-
tained sequences homologous to the hB7 5' untranslated, signal
peptide, Ig-V, and Ig-C domains followed by a splice donor
and 1900 bases of intron sequence. This intron DNA con-
tained murine B1 repetitive Alu-like elements. The two
murine cDNA clones share 320 bases of identical sequence cor-
responding to the Ig-C domain. The Ig-C domain contained
a unique BamHI site and a complete murine B7 cDNA clone
was constructed by ligating the two cDNA clones at this
BamHI site.

A search of the GenBank and EMBL databases with the
mB7 nucleotide sequence revealed that only the hB7 sequence
exhibited significant homology with the murine sequence
(sigma = 24 SDs above the mean). Comparison of the mB7
cDNA sequence with that of hB7 showed that the two were
60% identical. Homologous domains include the 5' (50%)
and 3' (40%) untranslated regions in addition to the protein
coding sequence (63%). A poly(A) tract following a con-
sensus polyadenylation signal (bases 1678-1863) was identified.

Analysis of the mB7 cDNA reveals a single, long open
reading frame of 918 bases initiated by one of three closely
spaced ATG codons beginning at nucleotides 225, 249, and

DNA Sequence Analysis. B7 cDNA inserts were subcloned into
the pKSII plasmid (Stratagene, La Jolla, CA). Nested deletions
were constructed using the Erase-A-Base kit according to the
manufacturer's directions (Promega, Madison, WI). The cDNA
insert was sequenced using dye labeled primers and Taq polymerase
(Applied Biosystems, Foster City, CA) and the sequencing reac-
tions were analyzed on an Applied Biosystems (model 373) auto-
mated fluorescent sequencer. Sequence data obtained from
overlapping deletion clones on both strands were assembled to yield the
final murine B7 sequence. These sequence data are available from
EMBL/GenBank/DDJB under accession number X60958.

B7 Hybridization Probe. A DNA fragment corresponding to the
protein coding region of the murine B7 cDNA was used as a probe
for RNA and DNA blot hybridizations because of a repetitive
element in the 3' untranslated region of the B7 mRNA. The complete murine B7 cDNA was used as a template for PCR ampli-
fication of the coding region using a sense primer (ATGGCTT-
GGTCT) corresponding to nucleotides 249-266 and 1169-1153 of
hB7 Ig-C, transmembrane, cytoplasmic, and 3' untranslated
domains. This cDNA was used to isolate additional clones
from the murine B cell line, A20. Comparison of the A20
cDNA clone with hB7 showed that the A20 cDNA con-
tained sequences homologous to the hB7 5' untranslated, signal
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sus polyadenylation signal (bases 1678-1863) was identified.

Analysis of the mB7 cDNA reveals a single, long open
reading frame of 918 bases initiated by one of three closely
spaced ATG codons beginning at nucleotides 225, 249, and
270. We have chosen the second of these ATG codons (nt 249) as the initiating methionine because the DNA sequence GCTATGG around this ATG is consistent with the consensus translation initiation sequence RCCATGG defined by Kozak (25). In addition, the region 5' of this ATG is highly similar (15 of 17 nucleotides identical) to the human start site. Initiation at this methionine predicts an open reading frame of 218 bases encoding a protein of 306 amino acids.

Fig. 1 shows the alignment of the murine and human B7 protein sequences and the structural features associated with these molecules. The structural domains shown for mB7 are based on a comparison with hB7 and with other members of the Ig supergene family. The initiatory methionine codon is followed by a 37 amino acid signal peptide. The length of the signal peptide was chosen to correspond to the signal cleavage site experimentally determined for hB7 expressed in CHO cells. Amino terminal sequencing of a soluble hB7 purified from the culture media of transfected CHO cells revealed that the mature hB7 began with the amino acid sequence valine, isoleucine, histidine, and valine (our unpublished results). Hydrophobicity analysis reveals that the putative translation initiation sequence agrees with the profile for a consensus signal sequence valine, isoleucine, histidine, and valine (our unpublished results). Hydrophobicity analysis reveals that the putative translation initiation sequence agrees with the profile for a consensus signal sequence valine, isoleucine, histidine, and valine (our unpublished results). Hydrophobicity analysis reveals that the putative translation initiation sequence agrees with the profile for a consensus signal sequence valine, isoleucine, histidine, and valine (our unpublished results). Hydrophobicity analysis reveals that the putative translation initiation sequence agrees with the profile for a consensus signal sequence valine, isoleucine, histidine, and valine (our unpublished results).

Comparison of murine and human B7 amino acid sequences. Amino acid identities are indicated with a vertical bar (1). Possible N-linked glycosylation sites are marked with an (*). The signal peptide, transmembrane, and cytoplasmic domains are indicated. Ig-like domains are defined by the cysteines at position 54 and 121 (Ig-V) and 166 and 220 (Ig-C). The cDNA sequence data are available from EMBL/GenBank/DDBJ under accession number X60958.
Figure 2. B7 mRNA expression is B cell restricted. RNA blot analysis of (A) lymphoid cell lines and (B) Balb/c mouse organs. 2 μg of poly(A)+ RNA were glyoxylated, electrophoresed on agarose gels, and transferred to nitrocellulose. The blot was hybridized with (a) 32P-labeled mB7 coding region cDNA and reprobed with (b) 32P-labeled rat actin cDNA. The lanes contain RNA from the murine pre-B cell lines, 38B9 and 300.19, the B cell lines, AJ9, CH1, and A20, the plasmacytoma lines, Ag8.653 and NS-1, and the T cell lines, EL-4, BW5147, RADA, and YAC. The mobility of rRNAs are indicated on the left.

kb transcript predominating. Thus, B7 expression is restricted in both murine and human lymphoid cells to mature B cells, some pre-B and plasmacytoma cell lines, but is not found in T cell lines.

DNA Blot Hybridization Analysis of B7. DNA blot analysis to determine the genomic organization of B7 was performed, using the B7 coding region probe described above (Fig. 3). When genomic DNA was digested with eleven different restriction endonucleases, the B7 coding region probe hybridized to between one and five restriction enzyme fragments. Digestion with ApaI or EcoRV produced a single DNA fragment, consistent with a single copy of the B7 gene per haploid genome. Digestion with SalI or BclI, which are not present in the B7 coding region, each produced 5 DNA fragments. These results suggest that the mB7 protein coding region encompasses approximately 20 kb and is divided into at least 5 exons. If these correspond to the hB7 genomic organization, these will encode the signal peptide, Ig-V, Ig-C,
transmembrane, and cytoplasmic domains (G. Freeman and G. Gray, manuscript in preparation).

**Murine and Human B7 Transfected Cells Stimulate the Proliferation of Phorbol Ester Activated Human CD28+ T Cells.** We examined whether mB7 might provide a costimulatory signal to human CD28+ T cells. Table 1 summarizes one of three representative experiments. Coincubation of paraformaldehyde fixed COS-mB7 or CHO-hB7 cells with PMA treated CD28+ human T cells resulted in 29-fold and 30-fold enhancement of proliferation, respectively, compared to T cells treated with PMA alone. Addition of anti-hB7 mAb could completely inhibit the costimulatory activity of COS-hB7 cells but not of COS-mB7 cells. Addition of paraformaldehyde fixed CHO-hB7 transfected cells resulted in a 51-fold increase in proliferation. In contrast, coinoculation of PMA treated T cells with paraformaldehyde fixed COS-Vector resulted in no increase in proliferation. Paraformaldehyde fixed CHO-hB7, COS-hB7, COS-mB7, and COS-Vector transfected cells did not induce untreated human CD28+ cells to proliferate above media controls.

**Discussion**

The mB7 homologue was cloned by probing murine B cell cDNA libraries with the human gene. DNA sequence analysis of the mB7 cDNA reveals that this gene is closely related to the hB7 gene and exhibits 63% identity in the protein coding region. Murine B7 exhibits structural features similar to the human gene and is composed of a signal peptide, Ig-V and Ig-C domains, a transmembrane region, and a cytoplasmic domain. The complete human and murine B7 protein sequences were 44% identical overall with 47% identity in the Ig-V domain and 57% in the Ig-C domain. Both murine and human B7 contain four essential cysteine residues and other conserved amino acids which define the Ig supergene family (13). In the transmembrane domain, mB7 has two and hB7 has three cysteines that maybe involved in lipid derivatization or crosslinking to other membrane proteins. Both murine and human B7 contain eight potential N-linked glycosylation sites of which four are located in conserved positions in each molecule. These conserved sites are found in the Ig-V and Ig-C domains and are presumably sites of functional glycosylation. The signal peptide, transmembrane, and cytoplasmic domains are less well conserved. An interesting difference between murine and human B7 is the presence of an Ig hinge-like sequence (26) adjacent to the transmembrane domain of the murine protein.

Expression of mB7 mRNA in tissues was restricted to splenocytes and was not detected in other organs. Transcription of mB7 was detected in mature murine B cell lines (AJ9, A20, and CH1) and a plasmacytoma line (Ag8.653) and yields two polyadenylated transcripts of 2.2 and 3.9 kb. A large 10 kb transcript was found in the pre-B cell line 38139. This pattern of transcription was reminiscent of the large number of hB7 transcripts which included 1.7, 2.9, 4.2, and 10 Kb mRNAs (10). The high degree of sequence homology and pattern of mRNA expression demonstrate that the hB7 gene and the murine gene reported here are closely related.

In humans, we and others have shown that a recombinant B7-Ig fusion protein or cells expressing B7 can augment proliferation of human T cells that have been stimulated with...
phorbol ester or anti-CD3 (7-9). This stimulation is delivered via attachment of B7 to the T cell CD28 glycoprotein and results in T cell proliferation and secretion of IL-2, but not IL-4. The specificity of this pathway has been demonstrated by inhibition of proliferation and IL-2 secretion by mAbs to either B7 or CD28. Murine T cells express a homologue of human CD28 which has 68% amino acid identity to the human gene (21). The expression of murine CD28 is T cell restricted; however, the existence of a pathway for T cell activation has not been demonstrated. COS cells transfected with mB7 can act as costimulators of human CD28+ T cells, enhancing proliferation of CD28+ T cells that have been treated with phorbol ester. The stimulation of human T cells by both mB7 and hB7 shows that the ligand binding site on B7 is conserved. The interspecies costimulatory activity of B7 was somewhat surprising since the protein sequence identity was only 44%.

In humans, engagement of the CD28 pathway greatly augments synthesis of multiple lymphokines including IL-2, INF-γ, tumor necrosis factor α (TNF-α), granulocyte macrophage colony stimulating factor (GM-CSF), but not IL-4 (7, 18, 27). This division of lymphokine synthesis corresponds to the phenotype of the T cell costimulatory signal present on murine APCs. Following occupancy of the T cell receptor by antigen in association with MHC, the outcome of T cell activation in the mouse is determined by a costimulatory signal (1). Schwartz and others have shown that if the costimulatory signal is not delivered, the T cell enters a prolonged period of anergy during which the T cell is unresponsive to signals of T cell activation (1, 3). Thus the failure to synthesize costimulatory factors such as B7 may be related to the generation of tolerance. Conversely, inappropriate expression of B7 might lead to unregulated costimulatory activity potentially resulting in autoimmune phenomena.

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