Murine B7-2, an Alternative CTLA4 Counter-receptor that Costimulates T Cell Proliferation and Interleukin 2 Production

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Summary

The B7-1 molecule, expressed on antigen-presenting cells (APC), provides a crucial costimulatory signal for T cell activation. Recent studies demonstrate the existence of alternative, non-B7-1 CTLA4 counter-receptors in mice and humans. Here, we describe the molecular cloning and demonstrate costimulatory function of the murine B7-2 (mB7-2) gene. Murine B7-2 cDNA encodes a member of the Ig supergene family that binds CTLA4-Ig and stains with the GL1 but not anti-mB7-1 mAb. Murine B7-2 costimulates the proliferation and interleukin 2 production of CD4+ T cells and this costimulation can be inhibited by either CTLA4-Ig or GL1 mAb. Identification of the B7-2 molecule will permit further manipulation of the B7:CD28/CTLA4 costimulatory pathway which has been shown to be involved in the prevention of tolerance, induction of tumor immunity, and most recently, in the pathogenesis of autoimmunity.

To induce antigen-specific T cell clonal expansion, two signals provided by APC must be delivered to resting T lymphocytes (1, 2). The first signal, which confers specificity, is mediated via the TCR after recognition of an antigenic peptide presented by the MHC. The second signal, termed costimulation, induces T cells to proliferate. Costimulation is neither antigen specific, nor MHC restricted, and is delivered by cell surface molecules expressed by APCs (3, 4). Originally termed B7 in mice and humans, B7-1 is one such critical costimulatory molecule (5–7). B7-1 is the counter-receptor for two ligands expressed on T lymphocytes (8, 9). The first ligand, termed CD28, is constitutively expressed on T cells, and after ligation, induces IL-2 secretion and proliferation (10). The second ligand, termed CTLA4, is homologous to CD28 and appears on T cells after activation (11). Although CTLA4 has a significantly higher affinity for B7 than does CD28, its role in T cell activation remains to be determined (9).

We and others have considerable evidence for the existence of multiple non-B7-1, CTLA4 counter-receptors which costimulate T cell proliferation and IL-2 production. APCs from B7-1-deficient mice bind CTLA4-Ig (12) and costimulate T cell proliferation which is inhibited by CTLA4-Ig. Activated murine B lymphocytes express functional non-mB7-1, CTLA4 counter-receptor(s) which appears significantly earlier after activation than murine B7-1 (mB7-1)1 (13). The GL1 mAb identifies one such non-B7-1, CTLA4 counter-receptor (14). Similarly, activated human B cells express multiple CTLA4 counter-receptors distinguished by their selective reactivity with mAbs and their temporal expression (15).

Here, we report the cloning and functional analysis of the murine B7-2 cDNA (mB7-2). Murine B7-2 binds CTLA4-Ig, costimulates T cell proliferation and IL-2 production, and is defined by the GL1 mAb.

1 Abbreviation used in this paper: mB7-2, murine B7-2.
Materials and Methods

Isolation of mB7-2 cDNA. A cDNA library was prepared in the pCDM8 expression vector using poly(A) RNA from cyclic AMP (300 μg/ml)-stimulated M12 murine B cell line (RNA harvested at 0, 8, 19, 27, 32, and 48 h) (16). COS cells were transfected with the activated M12 cDNA library DNA by DEAE-dextran transfection (17). Cells were harvested after 47 h, incubated with 10 μg/ml hCTLA4-Ig and mCD28-Ig, and binding cells were isolated by panning on goat anti-human IgG coated plates (18). Episomal DNA was isolated, transformed into Escherichia coli DH10B/P3, and the plasmid DNA reintroduced into COS cells via spheroplast fusion. Transfected cells were harvested after 47 h. Cells expressing B7-1 were removed by incubation with anti-mB7-1 mAb (16-10A1) and immunomagnetic bead depletion using anti-mouse IgM and IgG coated beads. Transfectants were selected by panning with hCTLA4-Ig and mCD28-Ig as described above, and plasmid DNA was transformed into E. coli. A third round of selection, identical to the second round was performed, after which plasmid DNA was isolated from individual transformants. Six of seven isolated plasmids contained a 1.2-kb cDNA insert. COS cells transfected with these plasmids bound CTLA4-Ig but not control Ig fusion protein.

DNA Sequence Analysis. Murine B7-2 cDNA insert (clone 4) was subcloned into the pKSII- plasmid. The cDNA insert was sequenced on both strands using synthetic oligonucleotide primers and dye-labeled terminator/Taq polymerase chemistry, and analyzed by panning on goat anti-human IgG coated plates (18). A very slight shift in fluorescence intensity was observed when incubated with vector alone did not bind any of the aforementioned reagents (Fig. 1). Furthermore, the mB7-2 COS transfectants stained with the GL1 mAb which identifies a non-mB7-1, CTLA-4 counter-receptor expressed on activated murine B cells. This mAb is a potent inhibitor of costimulation delivered by murine APCs (14). A very slight shift in fluorescence intensity was observed when anti-mB7-1 mAb was used to stain mB7-2 transfected COS cells. In contrast, mB7-1 COS transfectants bound CTLA4-Ig and stained with anti-mB7 but not GL1 mAb. None of these transfected COS cells bound control Ig fusion protein or isotype-matched control mAbs. Similarly, COS cells transfected with vector alone did not bind any of the aforementioned reagents (Fig. 1 and data not shown).

The mB7-2 cDNA is comprised of 1,183 nucleotides, and predicts a protein of molecular weight of 32,195 before predicted to have a molecular weight of 32,195 before membrane domain of about 20 amino acids, and a cytoplasmic tail of 222 amino acid extracellular region containing a 22-amino acid cytoplasmic tail of 44 amino acids. The mB7-2 protein is predicted to have a molecular weight of 32,195 before modification. The B7-2 extracellular domain contains nine
potential N-linked glycosylation sites. The glycoprotein immunoprecipitated by the GL1 mAb has a molecular weight of 60–100 kD, which reduces to 34 kD after N-glycanase treatment (14), suggesting that the mB7-2 protein is heavily glycosylated. Comparison of both nucleotide and amino acid sequences of mB7-2 with the GenBank and EMBL databases and with our recently identified human B7-2 has shown that only the human B7-2 protein is closely related, with 50% amino acid identity, and that murine and human B7-1 are distantly related. The alignment of murine and human B7-1 and B7-2 protein sequences shows that the homology is concentrated in the Ig-like domains with the transmembrane, and cytoplasmic domains being much less conserved (Fig. 2 B). Comparison of the four characterized B7 proteins (Fig. 2 B) reveals that the IgV- and Ig-C-like domains demonstrate 14% and 18% amino acid identity, respectively. In general, conserved amino acids other than those involved in the canonical Ig structure tend to be concentrated in the sequences at the bottom of the IgV and the top of the IgC domains. In particular, the sequence SQD(P/N)(E/V)(S/T)EL(Y/F), predicted to be loop between the D and E strands of the IgC domain, is conserved (22). Whether this sequence is required for the structural integrity of B7-like molecules or represents a required motif for CTLA4 binding is not yet clear. But since human B7-1, human B7-2, murine B7-1, and murine B7-2 all bind to human CTLA4, the homologous amino acids probably represent those comprised to c a CTLA4 binding sequence.

Expression of mB7-2 mRNA. Two mRNA transcripts of 1.8 and 3.1 kb were identified by hybridization to the mB7-2 cDNA (Fig. 3). RNA blot analysis of c-AMP-treated M12 cells demonstrates that B7-2 is not expressed in unstimulated M12 cells, but is induced by c-AMP treatment by 8 h and

Figure 1. mB7-2 binds CTLA4-Ig and GL1 mAb but not anti-mB7-1 mAb. COS cells were transfected with pCDNA1 alone (vector) or expression plasmid encoding cDNAs for mB7-2 or anti-mB7-1 Abs or control Ig fusion protein; vector alone transfects COS cells by indirect immunofluorescence and flow cytometric analysis. Staining of mB7-2 transfected cells (top), mB7-1 transfected cells (middle), and vector alone (bottom). (Thin lines) Staining with isotype-matched control Ab binding was detected (bottom). (Thin lines) Staining with control Ig, anti-mB7-1 mAb (16-10A1), GL1 mAb or appropriate isotype-matched controls, Ab binding was detected.

Figure 2. Comparison of murine and human B7-1 and B7-2 amino acid sequences. (A) All amino acid identities between mB7-2 and hB7-2 are indicated with a vertical bar. Only those amino acids present in hB7-1 and mB7-1 which are common to murine and human B7-1 are marked. Amino acids at B7-1 exon boundaries are boxed and underlined.
Figure 3. mB7-2 mRNA expression in unstimulated and cAMP-stimulated M12 cells and in B cell lines. 20 μg of total RNA (A) or 5 μg of poly(A)+ RNA (B) were electrophoresed on agarose gels and transferred to nitrocellulose membranes. The blots were hybridized with 32P-labeled mB7-2 cDNA. The lanes contain RNA prepared from (A) unstimulated M12 B cells or activated with cAMP as described in Materials and Methods for the indicated times or (B) the 70Z pre-B cell line, the NS-1 plasmacytoma line, and the A20 and TA3 B cell lymphoma cell lines. The mobility of rRNAs is indicated.

declines only slightly by 27–48 h. In contrast, mB7-1 expression induced by c-AMP is highest after 27 h (23). mB7-2 mRNA expression was detected in the TA3 B cell line, a frequently used murine APC line. Very low levels of mB7-2 mRNA were seen in the A20 B cell line but are sufficient for moderate levels of mB7-2 cell surface expression as shown by GL1 staining of A20 cells (data not shown). However, mB7-2 mRNA was not detected in the NS-1 plasmacytoma or the pre-B cell line 70Z. In contrast, B7-1 mRNA is expressed in NS-1 and A20 cells but not 70Z or TA3 (5).

Induction of mB7-2 on LPS/Dextran Stimulated Murine B Cells. Murine B cells from wild-type and B7-1-deficient mice were isolated and activated with LPS/dextran for 72 h. As seen in Fig. 4, upon stimulation both B7-1 wild-type and deficient B cells bind CTLA4-Ig. When GL1 mAb was used to stain B7-1 wild-type and B7-1-deficient B cells, a similar pattern of staining was observed. However, low levels of GL1 staining were seen on unstimulated B cells. In contrast, anti-mB7-1 mAb stained LPS/dextran-stimulated wild-type B cells

Figure 4. Expression of mB7-1 and mB7-2 in unstimulated and stimulated murine B cells. Highly purified B cells were prepared from wild-type and B7-1-deficient mice (12). Both populations were used for expression studies either without stimulation or at 72 h after stimulation with LPS/dextran. (Thin line) Isotype-matched control Ab; (thick line) anti-mB7-1 mAb, GL1 mAb, or CTLA4-Ig staining. Ab binding was detected by indirect immunofluorescence and flow cytometric analysis. This data is representative of three experiments.
but not B cells isolated from mB7-1-deficient mice. These results are consistent with those recently observed by Freeman et al. (12).

Costimulatory Activity of mB7-2. To determine whether mB7-2 can costimulate submitogenically activated murine T cells, CD4+ murine T cells were isolated and activated with either PMA or soluble anti-CD3 mAb (145-2C11). As shown in Fig. 5 A, PMA-activated T cells were costimulated by both mB7-1 and mB7-2 but not vector-transfected COS cells. Similarly, anti-CD3-activated T cells demonstrated significant proliferation when cocultured with either mB7-1 or mB7-2 COS transfectants but not vector transfectants. (Fig. 5 B). Both proliferation and IL-2 accumulation induced by mB7-2 costimulation were inhibited by CTLA4-Ig and by the GL1 mAb (Fig. 6). This result is consistent with the recently demonstrated inhibitory effect of GL1 on costimulatory responses induced by heterologous APCs (14). While mB7-1 mAb stained mB7-2 cDNA transfected COS cells only very weakly, mB7-1 mAb consistently inhibited proliferation and IL-2 accumulation induced by mB7-2 by ~ 30-50% (five experiments), suggesting the existence of a weakly cross-reactive epitope. In contrast, mB7-1 costimulatory activity was blocked by CTLA4-Ig and by the anti-mB7-1 mAb but not by GL1 mAb.

Both human B7-1 and murine B7-1 transfected COS cells have been previously shown to provide costimulation to both human and murine T cells (5). We, therefore, also examined the capacity of human and murine B7-2 transfected COS to

![Figure 5](image1.png)

**Figure 5.** mB7-1 and mB7-2 COS cell transfectants costimulate T cell proliferation. The costimulatory capacity of COS cells expressing mB7-1 or mB7-2 was determined by mixing transfected COS cells with 10^6 BALB/c T lymphocytes stimulated with submitogenic levels of PMA (A) or anti-CD3 (B). All T cell populations were depleted of detectable accessory cells, as demonstrated by a lack of an anti-CD3 or PMA response in the absence of a source of costimulatory activity. Wells contained 2 x 10^4 mitomycin C-treated COS cells transfected with vector alone, mB7-1, or mB7-2. Cultures were pulsed after 48 h with 1 μCi of [3H]thymidine per well for the last 16 h of the incubation period to assay T cell proliferation. This data is representative of two experiments.

![Figure 6](image2.png)

**Figure 6.** Inhibition of mB7-1 and mB7-2 costimulation. Microcultures were set up as described with 10^6 BALB/c T lymphocytes. All T cell populations were depleted of detectable accessory cells, as demonstrated by a lack of an anti-CD3 (1 μg/ml) or PMA (5 ng/ml) response in the absence of a source of costimulatory activity. Wells contained 2 x 10^4 mitomycin C-treated COS cells transfected with vector alone, mB7-1, or mB7-2. Ab reagents were added at a final concentration of: CTLA4-Ig and control Ig fusion protein, 2 μg/ml; GL1 mAb and rat control mAb, 0.1 μg/ml; and anti-B7-1 mAb and hamster control mAb, 1 μg/ml. Supernatants were harvested at 24 h for IL-2 ELISA assay. Cultures were pulsed after 48 h with 1 μCi of [3H]thymidine per well for the last 16 h of the incubation period to assay T cell proliferation. This data is representative of three experiments.

![Figure 7](image3.png)

**Figure 7.** Human and murine B7-2 costimulate T cell proliferation across species. Human CD28+ T lymphocytes (A) and murine CD4+ T cells (B) were treated with PMA (5 ng/ml) and mixed with 2 x 10^4 mitomycin C-treated COS cells expressing hB7-2, mB7-1, mB7-2, or transfected with vector alone. All T cell populations were depleted of detectable accessory cells, as demonstrated by a lack of an anti-CD3 or PMA response in the absence of a source of costimulatory activity. Wells contained 2 x 10^4 mitomycin C-treated COS cells transfected with vector alone, mB7-1, or mB7-2. Cultures were pulsed after 48 h with 1 μCi of [3H]thymidine per well for the last 16 h of the incubation period to assay T cell proliferation. This data is representative of two experiments.
costimulate across species. Fig. 7 B demonstrates that sub-
mitogenically activated murine T cells proliferate in response
to hB7-2 COS transfectants, as well as to mB7-1 and mB7-2
COS transfects. Similarly, human PMA-activated T cells
proliferate in response to mB7-2, as well as to hB7-2. (Fig.
7 A).

Discussion

In this report, we describe the molecular cloning, expres-
sion, and costimulatory function of the mB7-2 gene. Although
demonstrating only 25% amino acid identity with mB7-1,
mB7-2 cDNA encodes a member of the Ig supergene family
that binds CTLA4-Ig and costimulates T cells to produce IL-2
and proliferate. In contrast to mB7-1, mB7-2 transfectants
bind with the GL1 mAb but do not stain with mB7-1
mAb. Costimulation induced by mB7-2 transfectants is in-
hibited by CTLA4-Ig and GL1 mAb. These results demon-
strate that the mB7-2 cDNA encodes a second CTLA4
counter-receptor that is identified by the GL1 mAb and which
costimulates T cell proliferation and IL-2 production.

The cloning of mB7-2 and its reactivity with the GL1 mAb
confirms the existence of a novel murine CTLA4 counter-
receptor expressed within hours after B cell activation. These
data are consistent with recent experiments suggesting the
existence of additional ligands for CTLA4, distinct from
mB7-1, on activated splenic B cells (12, 13, 24). In this manu-
script, we show that LPS/dextran-activated murine B cells
derived from the mB7-1-deficient mouse strain bind CTLA4-
Ig and stain with the GL1 but not with anti-mB7-1 mAb.
Recently, Lenschow et al. (13) demonstrated that by 6 h af-
after activation, murine B cells express a CTLA4 counter-receptor
distinct from mB7-1. This molecule appears on the cell sur-
facer ~24 h earlier than mB7-1 and unlike mB7-1, its expres-
sion is induced by Con A. GL1 is expressed at low levels on
unstimulated murine B cells and rapidly increases with activa-
tion. The GL1 mAb abrogates costimulation induced by
populations containing activated murine B cells (14). Cyclic
AMP induces mB7-2 mRNA more rapidly than mB7-1
mRNA, suggesting that mB7-2, like mB7-1, is upregulated
by cross-linking of MHC class II (23) but is expressed sooner
than mB7-1. Taken together, these results suggest that the
mB7-2 molecule appears earlier than mB7-1 and is respon-
sible, at least in part, for the costimulation delivered by APCs
within the first 24 h.

Many experiments examining the costimulatory capacity of
natural APCs use chemically fixed APCs (1, 30). We have
found that stimulators have very different sensitivities to
fixation. The costimulatory capacity of B7-2 is partially sen-
titive to paraformaldehyde fixation, whereas B7-1 costimula-
tion is insensitive to paraformaldehyde fixation (5, Freeman,
G., unpublished results). ICAM-1 costimulation (27) is elim-
ninated by paraformaldehyde fixation. Thus the costimulatory
capacity of native APCs may be different than those of chem-
ically fixed APCs and should be considered when interpreting
experiments using fixed APCs.

Virtually identical evidence exists for an early, non-B7-1,
CTLA4 counter-receptor in humans (hB7-2) (15, 25). Be-
tween 12 and 24 h after activation, human B cells express
a non-hB7-1, CTLA4 counter-receptor which costimulates
CD28+ T cells to proliferate and secrete IL-2. This costimu-
lation is abrogated by blocking with CTLA4-Ig and anti-
CD28 Fab but not by anti-hB7-1 mAb (133) (15). Recently,
we have molecularly cloned the human B7-2 cDNA (25).
The hB7-2 cDNA demonstrates 26% amino acid identity
with hB7-1 and 50% identity with mB7-2. Like hB7-1, hB7-2
costimulates T cell proliferation and IL-2 secretion and this
costimulation is inhibited by both CTLA4-Ig and anti-CD28
Fab (25). Whereas hB7-1 mRNA is not detected in unstimu-
lated B cells, hB7-2 mRNA is constitutively expressed in un-
stimulated B cells and increases after activation. Since no
CTLA4 counter-receptors are expressed on the surface of un-
stimulated B cells (15), the constitutive expression of hB7-2
mRNA suggests a posttranscriptional regulation of hB7-2
expression.

Although mAbs directed against hB7-2 are currently un-
available, the constitutive expression of hB7-2 mRNA and
the observed costimulatory capacity of 24-h activated human
B cells suggests that hB7-2 also appears early after B cell
activation. Whereas the signaling function of CTLA4 in mu-
rine and human systems has yet to be resolved, hB7-1 and
hB7-2 appear to signal via CD28 (15, 25). Although we do
not yet have an antimurine CD28 Fab to address this issue
directly, we postulate that mB7-2 also signals via CD28.
Indirect evidence for this hypothesis is derived from the obser-
vation that mB7-2 and hB7-2, like mB7-1 and hB7-1, costimu-
late T cell proliferation and IL-2 production across species
barriers. These results demonstrate the striking evolutionary
conservation displayed by murine and human B7-1 and B7-2
for their receptors CD28 and CTLA4 expressed on T lympho-
cytes.

These results indicate that the B7-CD28/CTLA4 pathway
has additional levels of complexity. The biologic function(s)
for multiple, sequentially induced CTLA4 counter-receptors
remains to be elucidated. However, the early appearance
of B7-2 provides one mechanism for B cell–induced costimula-
tion at a time before the induction of B7-1. Therefore, the
eye expression of B7-2 suggests that it may provide a criti-
cal costimulatory signal involved in the decision between
immunity and anergy that is made by T cells within 24 h
after activation (2, 26, 27). Furthermore, it may be that B7-1
serves to amplify rather than initiate an immune response.

Recent data in the human system demonstrating a third
CTLA4 counter-receptor suggest that the B7-CD28/CTLA4
pathway is even more complex. Specifically, B7-1–negative,
activated human B cells express a BB1 molecule which is dis-
tinct from hB7-1 and hB7-2 (15). It may be that subpopu-
lations of APCs differentially express these CTLA4 counter-
receptors. It is presently unknown whether additional T cell
receptors for the B7 family of costimulatory molecules exist.
The capacity of the CD28-deficient mouse to generate an
immune response and produce low levels of IL-2 is consis-
tent with this hypothesis (28). Since this pathway appears
to be central to the initiation and amplification of a T cell-
mediated immune response, it is not surprising that it is highly regulated. Elucidating these levels of regulation should enable us to manipulate this critical costimulatory pathway which already has been shown to be involved in the regulation of tolerance (2, 29, 30), induction of tumor immunity (31, 32, 33), and most recently in the pathogenesis of autoimmunity (Harlan, D. M., H. Hengartner, M. L. Huang, Y.-H. Kang, R. Abe, R. W. Moreadith, H. Pircher, G. S. Gray, P. S. Ohashi, G. Freeman, L. M. Nadler, C. H. June, and P. Aichele, manuscript submitted for publication).

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