The IgV domain of human B7-2 (CD86) is sufficient to co-stimulate T lymphocytes and induce cytokine secretion

Paul Rennert1,4, Kimberly Furlong1, Cindy Jellis1, Edward Greenfield1, Gordon J. Freeman3, Yuji Ueda2, Bruce Levine2, Carl H. June2 and Gary S. Gray1,5

1Departments of Molecular Biology and Immunology, Repligen Corp., Cambridge, MA 02139, USA
2Immune Cell Biology Program, Naval Medical Research Institute, Bethesda, MD 20814, USA
3Division of Hematologic Malignancies, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02115, USA
4Present address: Biogen, Inc., 14 Cambridge Center, Cambridge, MA 02142, USA
5Present address: Department of Molecular Immunology, Genetics Institute, Cambridge, MA 02140, USA

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Abstract

B7-1 (CD80) and B7-2 (CD86) are genetically and structurally related molecules expressed on antigen-presenting cells. Both bind CD28 to co-stimulate T lymphocytes, resulting in proliferation and cytokine production. The extracellular portions of B7-1 and B7-2 which bind to CD28 and CTLA-4 are related to Ig variable (V) and Ig constant (C) domain sequences. Recent reports have described splice variant forms of B7 proteins which occur in vivo and are of unknown function. Here we describe soluble recombinant forms of B7-1 and B7-2 containing either both of the Ig-like extracellular domains or the individual IgV or IgC domains coupled to an Ig Fc tail. Soluble B7-1 and B7-2 bind to CD28 and CTLA-4, and effectively co-stimulate T lymphocytes resulting in their proliferation and the secretion of cytokines. Furthermore, the IgV domain of B7-2 binds CD28 and CTLA-4, competes with B7-1 and B7-2 for binding to these receptors, and co-stimulates T lymphocytes. Cross-linked soluble B7-2v was the most potent co-stimulatory molecule tested and was active at a concentration ~100-fold lower than cross-linked soluble B7-1 or B7-2 proteins. When bound to tosyl-activated beads, B7-2v was capable of sustaining multiple rounds of T cell expansion. These data complement the description of naturally occurring variants to suggest that T cell co-stimulation in vivo may be regulated by soluble or truncated forms of B7 proteins.

Introduction

The requirement for a co-stimulatory signal in T cell activation is well known (1,2) and the function of CD28 in transducing this signal has been demonstrated (3). Engagement of CD28 on the T lymphocyte surface is required for IL-2 up-regulation leading to a sustained immune response (4). Blockage of the CD28 signal concurrent with the delivery of the primary activation signal leads to a T lymphocyte unresponsive state termed anergy (5) or to cell death (6). The B lymphocyte activation antigens B7-1 (CD80 (7)) and B7-2 (CD86 (8,9)) are related membrane bound proteins expressed by antigen-presenting cells. Both are ligands for CD28 and can deliver a co-stimulatory signal to T lymphocytes via the CD28 pathway (9–11). Most reports have shown that B7-1 and B7-2 are genetically, structurally and functionally similar, nonetheless it is possible that they have distinct roles in the regulation of an immune response (12–16). B7-1 and B7-2 are also ligands for the T cell activation antigen CTLA-4 (17,18) whose function can be down-regulatory (19,20) or co-stimulatory (21). Analyses of B7-1 and B7-2 function have used antigen-presenting cells or transfectants to present the co-stimulatory signal to T cells. Although blocking mAb used in these studies demonstrate specificity, they do not formally rule out effects of other cell surface molecules or secreted factors. This may be particularly relevant in analyses of T cell cytokine profiles resulting from co-stimulation. The possible expression of variant forms of B7-1 and B7-2 in vivo could further complicate the study of T cell activation.
In this study, we have used domain-specific constructs of human B7-1 and B7-2 and tested the ability of these constructs to bind to CD28 and CTLA-4 and to co-stimulate T cells. We demonstrate that soluble B7-1, B7-2 and the IgV-like domain of B7-2 bind CTLA-4 and CD28. In contrast, the B7-1 IgV and IgC domains and the B7-2 IgC domain were unable to bind CD28 or CTLA-4. Importantly we provide the first demonstration that soluble B7-2 V domain proteins can co-stimulate T lymphocytes leading to proliferation and the production of cytokines. Our results indicate that B7-1 and B7-2 have different structural requirements for binding to CD28 and CTLA-4, in agreement with recent reports (22,23). Differential expression of membrane bound or soluble full length and domain-specific forms of B7-2 could have independent and distinct effects on the generation of immune responses.

**Methods**

**Cloning, expression and purification of reagents**

PCR was used to create extracellular and domain specific gene expression constructs using B7-1 and B7-2 cDNAs as templates for the coding regions. All expression constructs were composed of a signal sequence coupled to the respective B7 domain(s) coupled to the human IgG1 modified hinge—

C_{1i}-C_{i2}-C_{i3} sequences as previously described (14). The boundaries of the IgV and IgC domains were selected to correspond to the genomic structure of the human B7-1 and B7-2 genes (24,25), and to the predicted N-termini (7,9). Briefly, the full length B7-1 extracellular domain extended from predicted amino acid 1 (glycine) to amino acid 215 (aspartic acid). The IgV domain of B7-1 extended from amino acid 1 (glycine) to 115 (aspartic acid). The IgC domain of B7-1 extended from amino acid 114 (alanine) to 215 (aspartic acid). The B7-2 extracellular domain extended from amino acid 23 (alanine) to 237 (glutamic acid). The B7-2 IgV domain extended from amino acid 23 (alanine) to 133 (leucine). The B7-2 IgC domain extended from amino acid 134 (alanine) to 237 (glutamic acid). All PCR primers were designed such that the final reaction products were bounded on the 5′ end by a unique HindIII restriction site and on the 3′ end by a unique BclI restriction site to facilitate insertion into the expression vector.

The final gene expression constructs were prepared by ligating the HindIII–BclI digested PCR fragments into the expression vector pNRDSH-Ig. This vector featured a CMV promoter, HindIII and BclI restriction sites for insertion of the B7 segment, a human hinge, C_{i2} and C_{i3} domains from IgG1, and a polyadenylation signal. All cistrons were replaced by serines in the IgG1 hinge region (14).

All recombinant proteins were prepared by transient expression in COS-7 cells using standard techniques (26). Culture medium containing the various B7Ig proteins was harvested at 72 h post-transfection. The various B7Ig proteins were purified by affinity chromatography on Protein A–Sepharose (Repligen, Cambridge, MA). The amount of protein expressed per milliliter of transfection media varied, but in all cases high-purity protein (>90%) of the expected mol. wt was prepared as measured by SDS–PAGE gels. B7Ig recombinant protein preparations were predominantly non-covalently linked dimers. The remainder of the proteins existed primarily as higher order multimers with a small amount of monomer also present. CTLA-4-Ig existed predominately as a covalently linked dimer with higher order multimers also present. Protein concentration was determined by spectroscopy. Endotoxin levels were determined by ELISA (BioWhittaker, Walkersville, MD) for all preparations and were <5 EU/mg purified protein.

**mAb**

mAb to human B7-1 and B7-2 were prepared using SP2/0 cells and standard protocols (27). Briefly, BALB/c female mice (Taconic, Germantown, NY) were immunized i.p., with either 50 µg B7-2Ig emulsified in complete Freund's adjuvant (Sigma, St Louis, MO), 10^{6} CHO/B7-2 cells (14) or 10^{6} CHO/B7-1 cells (14) and boosted twice at 14 day intervals. Hybridoma colonies were established in 96-well tissue culture plates and the culture supernatants were assayed by ELISA for direct binding to either B7-1Ig or B7-2Ig. All mAb were purified from ascites fluid on Protein A–Sepharose as described above. mAb B70 was purchased from Pharmingen (San Diego, CA). Anti-CD3 mAb OKT3 was prepared from ascites using hybridoma CRL8001 (ATCC, Rockville, MD).

Purified mAb were tested for their ability to bind to the various B7Ig forms as follows. Maxisorp plates were coated overnight at room temperature with 20 µg/ml of purified B7Ig protein in PBS. The plates were then blocked with PBS/1% BSA for 1 h. Purified antibody (5 µg/ml in PBS) was added to the test wells, the plates incubated for 1 h and then washed five times with PBS/0.05% Tween 20. Goat anti-mouse IgG–horse radish peroxidase (HRP) (1:1000 dilution in PBS; Zymed, San Francisco, CA) was added for 1 h. The plates were washed five times and developed using ABTS (Zymed). Absorbance was read at 405 nm. Anti-B7 mAb was scored as having positive (+) reactivity for the various B7Ig proteins when absorbance values were >1.5 and negative (−) reactivity for values <0.2. None of the mAb reported here exhibited an intermediate level of reactivity.

**CTLA-4 binding assays**

Maxisorp plates were coated overnight at room temperature with 50 µl/well of a 20 µg/ml stock of the various B7Ig proteins or purified human IgG (Zymed) in PBS. The plates were blocked with PBS/1% BSA for 1 h then incubated with varying amounts of biotinylated CTLA-4–Ig for 2 h at room temperature. After five washes with PBS, streptavidin–HRP (Zymed, 1:1000 dilution) was added for 20 min. The plates were washed with PBS and the HRP reactivity was measured as described above.

The ability of the various B7Ig forms to bind to CTLA-4–Ig was also analyzed in a competition format. Maxisorp plates were coated and blocked as described for the direct ELISA assay, using B7-1Ig or B7-2Ig. Biotinylated CTLA-4–Ig was added at 35 ng/ml, a concentration determined to be one-half saturation in the direct binding curve for B7-1. Purified protein of the various forms of B7Ig was added in varying amounts and the plate was incubated for 2 h at room temperature. The plates were incubated with streptavidin–HRP, washed and developed as described above.

The ability of the anti-B7-2 mAb to inhibit the binding of CTLA-4–Ig to B7-2 was tested using a competition ELISA.
format. The mAb (5 μg/ml) were mixed with 35 ng/ml biotinylated CTLA-4-Ig (NHS- LC-biotin; Pierce, Rockford, IL) and then added to microtiter plates coated with B7-2-Ig. After 1 h the plates were washed five times with PBS/0.05% Tween 20. Streptavidin-HRP (Zymed; at a 1:1000 dilution in PBS) was added for 1 h then washed, developed and read as described above. The ability of mAb to inhibit the binding of CTLA-4-Ig to B7-2 was scored as positive (+) when the absorbance value was reduced to <0.2 and as negative (−) when the absorbance value was within 10% of the uninhibited control (absorbance ~1.5). None of the mAb reported here exhibited an intermediate level of reactivity.

FACS analysis
The ability of soluble forms of B7-Ig to bind to CD28 was tested by FACS (FACScan; Becton Dickinson, San Jose, CA) using Jurkat cell lines E6.1 (CD28+/CTLA-4+) and RT3 (CD28+/CTLA-4+), CHO and CHO/CD28. The flow cytometer was calibrated using CaliBRITE beads and AutoComp software (Becton Dickinson). All data acquisition and analysis was performed using the Lysys II program (Becton Dickinson). Three micrograms of various B7-Ig proteins were cross-linked by incubation with 10 μg of affinity-purified FITC-labeled goat anti-human IgG Fc (Cappel, Durham, NC) for 30 min on ice. The cross-linked B7-Ig proteins were added to 10^6 Jurkat or CHO cells previously washed and suspended in 100 μl cold HBSS (BioWhitaker). The cells were washed twice with cold HBSS, suspended with 250 μl cold HBSS and fixed using 250 μl of 2% paraformaldehyde in HBSS. In mAb blocking experiments, anti-B7-2 mAb were added at 30 μg/ml prior to re-stimulation. Exogenous cytokines were added for 1 h then washed, developed and read as described above. The ability of mAb to inhibit the binding of CTLA-4-Ig to B7-2 was scored as positive (+) when the absorbance value was reduced to <0.2 and as negative (−) when the absorbance value was within 10% of the uninhibited control (absorbance ~1.5). None of the mAb reported here exhibited an intermediate level of reactivity.

T cell proliferation assays
CD28+ T cells were isolated from normal human volunteers as described (28). T lymphocytes were used at 1.2×10^5/well in 96-well format. T cell proliferation was induced using anti-CD3 (OKT3; coated on plates at 10 μg/ml overnight) or phorbol myristate acetate (PMA; 1 ng/ml) to deliver a primary signal and anti-CD28 (10 μg/ml; mAb 3D10 (14)), CHO/CD28-IgV cells (5×10^6 cells/well), CHO/CD28-IgV cells (5×10^6 cells/well) or purified IgG proteins to deliver the co-stimulatory signal. CHO cells were pretreated with mitomycin C (Sigma) overnight and extensively washed prior to use. Purified Ig-tailed proteins were pre-incubated with a 3-fold excess (w/w) of affinity-purified goat anti-human IgG Fc (Cappel) for 30 min on ice prior to use. After 60 h incubation, the cells were pulsed overnight with [3H]thymidine (Dupont/NEN, Boston, MA) and then harvested for counting. [3H]Thymidine incorporation was calculated from triplicate points and the results shown are representative of at least three separate experiments. Cytokine levels were determined in duplicate by ELISA (Endogen, Cambridge, MA) using cell culture supernatants harvested after 18 and 120 h of co-stimulation. When tested as competitors of co-stimulation, anti-B7-2 mAb were added at 20 μg/ml final concentration. Co-stimulation inhibition was scored as positive (+) when the amount of [3H]thymidine incorporation was reduced to <10% of the uninhibited control value or negative (−) when the amount of [3H]thymidine incorporation remained the same as the uninhibited control value.

Long-term cell culture
Peripheral blood lymphocytes were isolated by Percoll gradient centrifugation from leukopacks obtained by apheresis of healthy donors. CD28+CD4+ T cells were purified by a previously described negative selection method (28). In each experiment the purity of the cell preparations was monitored by flow cytometry: the cells from such preparations were >99% CD2+, >98% CD3+, >98% CD28+ and contained <3% CD8+ cells.

Dynal M450 tosyl activated beads (Dynal, Great Neck, NY) were coated with mAb or recombinant proteins according to the manufacturer’s instructions. Beads were coated with anti-CD3 (OKT3) alone or approximately equimolar ratios of anti-CD3 and anti-CD28 mAb 9.3 or the B7-Ig proteins. Proteins were prepared in borate buffer (pH 9.5) at 75 μg/ml prior to coupling to the beads.

Purified CD4+ T cells were cultured at 1×10^6 cells/ml in R10 complete medium (Gibco/BRL, Gaithersburg MD). T cells were stimulated with anti-CD3-coated beads, anti-CD3 plus anti-CD28-coated beads or anti-CD3 plus B7-Ig protein-coated beads. The coated beads were initially added at a ratio of 3 beads/cell. The cell cultures were fed at 2–3 day intervals with fresh medium to maintain cell concentrations between 0.5 and 1.5×10^6 T cells/ml; antibody-coated beads were diluted progressively until re-stimulation. Exogenous cytokines or feeder cells were not added to the cultures.

Results
Antibodies to the B7-2 V domain block B7-2 function
In order to study the functional capacity of the B7-1 and B7-2 extracellular domains, fusion proteins consisting of the individual IgV and IgC domains coupled to the IgG1 Fc region were prepared (Fig. 1). In ELISA analyses anti-B7-2 mAb recognized the entire extracellular regions and either the IgV or IgC domains of B7-2 (Table 1). All of the anti-B7-2 mAb tested specifically bound to CHO/CD28 cells and to activated human B cells (data not shown) in FACS analyses. The mAb did not recognize B7-2 proteins in Western blot assays following SDS-PAGE demonstrating that denaturation of the protein destroyed the epitopes recognized by the antibodies. None of the anti-B7-2 mAb showed any cross-reactivity with B7-1 nor did anti-B7-1 mAb recognize B7-2 (data not shown).

Competition assays showed that mAb to B7-2 could be divided into two groups on the basis of their ability to interrupt B7-2 binding to CD28 and CTLA-4 and inhibit T cell co-stimulation (Table 1). Anti-B7-2v, but not anti-B7-2c mAb blocked binding of the B7-2v Ig fusion protein to CD28 and CTLA-4, in FACS and ELISA assays respectively. Furthermore, anti-B7-2v but not anti-B7-2c domain-specific mAb blocked B7-2-mediated co-stimulation in T cell proliferation assays (Table 1).

The variable domain of B7-2 binds to CD28 and CTLA-4 Since the mAb blocking experiments suggested that only the IgV domain of B7-2 was required for binding, soluble
B7-2 IgV domain is co-stimulatory for T lymphocytes

Table 1. Characterization of mAb to B7-2a

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Isotype</th>
<th>Recognition Inhibition</th>
<th>CTLA-4 binding</th>
<th>CD28 binding</th>
<th>T cell proliferation</th>
</tr>
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<tbody>
<tr>
<td>Anti-B7-2 mAb</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>HA5.1F9</td>
<td>IgG1</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HA5.2B5</td>
<td>IgG2b</td>
<td>+</td>
<td>–</td>
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<td>+</td>
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<td>HF2.3D11</td>
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<td>+</td>
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<tr>
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<td>+</td>
<td>–</td>
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<tr>
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<td>IgM</td>
<td>+</td>
<td>–</td>
<td>–</td>
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</tr>
<tr>
<td>HF4.6B1</td>
<td>IgG2a</td>
<td>+</td>
<td>+</td>
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<tr>
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<tr>
<td>B70</td>
<td>IgG2b</td>
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mAAb binding to B7-2Ig, B7-2vIg or B7-2cIg was determined by reacting the various mAAb with the recombinant B7Ig proteins previously coated on microtiter plates. Bound mAAb were detected using HRP-labeled goat anti-mouse IgG and ABTS. Antibody recognition of recombinant proteins was scored (+) for absorbance values >1.5 and (–) for absorbance values <0.1. mAAb inhibition of B7Ig protein binding to CTLA-4-Ig was tested by mixing the anti-B7 mAAb and biotinylated CTLA-4-Ig and reacting this mixture with B7Ig coated plates. mAAb inhibition of CTLA-4/B7 binding was scored (+) for absorbance values <0.2 and (–) for absorbance values >1.5. Inhibition of B7Ig protein binding to CD28 was tested by adding the anti-B7 mAAb to the B7Ig proteins and CD28 Jurkat cells. Binding was monitored by flow cytometry. mAAb inhibition was scored as (+) inhibition of B7Ig binding when the profile overlay that determined for the FITC-labeled secondary mAAb alone and (–) inhibition when the profile overlay the uninhibited state. Inhibition of T lymphocyte proliferation was tested by adding the anti-B7 mAAb to T cells stimulated with PMA and the cross-linked B7Ig proteins. mAAb inhibition was scored as positive (+) inhibition when the [3H]thymidine incorporation was reduced to <10% of the uninhibited control value and negative (–) inhibition when no change in [3H]thymidine incorporation was observed.

ND, not determined.

Fig. 1. Structure of B7Ig proteins. The Ig V and C domains of B7-1 and B7-2 are shown for the various B7Ig recombinant proteins. All recombinant proteins are composed of B7-derived segments (black) and the IgG1 Fc Ig tail composed of a hinge–C(H2)–CH3 segment (striped).

Fig. 2. Direct binding of B7Ig forms to CTLA-4-Ig. Purified recombinant B7Ig proteins were coated overnight in 96-well microtiter trays and detected using serial dilutions of biotinylated CTLA-4-Ig and streptavidin–HRP. All determinations were made in triplicate. This figure represents the results of one of six experiments.

recombinant forms of B7-1Ig, B7-2Ig, and the individual IgV and IgC domains were tested for binding to CD28 and CTLA-4. In direct binding experiments with CTLA-4, the recombinant proteins containing the entire extracellular domains of B7-1 and B7-2 or the V domain of B7-2 (B7-2vIg) bound to CTLA-4. In this assay format, half-saturation occurred at 1 nM for B7-1Ig, 5 nM for B7-2Ig and 8 nM for B7-2vIg. Neither subfragment of B7-1 (B7-1vlg or B7-1clg) nor B7-2clg showed any reactivity with CTLA-4 (Fig. 2). The B7Ig forms were also tested for their ability to compete with either B7-1Ig or B7-2Ig for binding to CTLA-4. B7-1Ig, B7-2Ig and B7-2vIg competed with B7-2Ig for binding to CTLA-4 and exhibited IC50 values of 1, 16 and 35 nM respectively. B7-2vIg, B7-1vlg and B7-1clg were not able to compete with the binding of B7-2Ig for CTLA-4. Similarly, B7-1Ig, but not B7-1vlg or B7-1clg, competed with B7-1Ig for binding to CTLA-4. Thus B7-2vIg was the only one of the IgV or IgC domain-specific proteins...
B7-2 IgV domain is co-stimulatory for T lymphocytes

Fig. 3. Flow cytometry of CD28+ Jurkat cells using purified recombinant B7lg proteins cross-linked with FITC–goat anti-human IgG Fc. B7lg proteins or control Ig were mixed with FITC–goat anti-human IgG Fc (3:10; w:w) for 30 min before addition to the Jurkat cells. Anti-CD28 mAb E5X.3D10 was added to the Jurkat cells for 30 min followed by the addition of FITC–goat anti-mouse Ig. The relative fluorescent intensity of the CD28+ Jurkat cells after staining is shown for the control Ig (light stripe), B7-2lg (white), B7-1lg (medium gray), B7-2vlg (light gray) and anti-CD28 (dark stripe). Flow cytometry using B7-2clg, B7-1vlg or B7-1clg and the CD28+ Jurkat cells was negative and resembled that for the control Ig. No staining of the CD28+ Jurkat cells was observed with any of the reagents tested. This figure represents the results of one of six experiments.

Fig. 4. Proliferation of CD28+ T lymphocytes. Purified CD28+ T lymphocytes were incubated with PMA and either CHO cells expressing B7-1 or B7-2 on their surface or with cross-linked B7lg recombinant proteins. Proliferation was measured after 3 days by the overnight addition of [3H]thymidine. Untransfected CHO cells and cross-linked B7-2clg, B7-1vlg or B7-1clg recombinant proteins all failed to induce proliferation of the CD28+ T cells in conjunction with PMA. The T cell proliferation induced by B7-1lg, B7-2lg or B7-2vlg could be blocked by the addition of CTLA-4-Ig or mAb to B7-1 or B7-2. All determinations were made in triplicate. This figure represents the results of one of six experiments. Similar results were obtained when immobilized anti-CD3 was used in place of the PMA.

The V domain of B7-2 co-stimulates T lymphocytes

B7lg proteins were cross-linked using affinity-purified goat anti-human IgG Fc and added to CD28+ T lymphocytes in the presence of PMA. T cell proliferation was measured after 3 days. Cross-linked B7-1lg protein delivered a weak proliferative co-stimulus similar to previous results from Damle and coworkers (29). Cross-linked B7-2lg protein also delivered a weak co-stimulatory signal to PMA-activated T lymphocytes, whereas an equivalent amount of cross-linked B7-2vlg provided a much stronger signal that was similar in strength to that provided by CHO cells expressing B7-1 or B7-2 on their surface (Fig. 4). Cross-linked isotype-matched control Ig protein failed to induce proliferation in this assay, as did cross-linked B7-2clg, B7-1vlg or B7-1clg (Fig. 4 and data not shown). Blocking experiments with anti-B7-2 mAb again demonstrated that mAb specific for the V domain of B7-2 could block co-stimulation, while those specific for the C domain could not (Table 1).

Titrated amounts of B7-1lg, B7-2lg or B7-2vlg were added to T lymphocytes in the presence of immobilized anti-CD3, and the accumulation of IL-2 (18 h) and cell proliferation were measured (72 h; Fig. 5). Cross-linked B7-1 or B7-2 effectively co-stimulated CD28+ T lymphocytes resulting in their proliferation and the production of IL-2 in a dose-dependent manner. The proliferative response rapidly decreased with decreasing B7-1lg or B7-2lg and was only slightly above control levels when present at 1 µg/ml. In contrast, B7-2vlg was much more effective as a co-stimulatory molecule, driving appreciable proliferation and IL-2 production at levels of only 0.01 µg/ml.
**Fig. 5.** Proliferation of CD28⁺ T lymphocytes and the secretion of IL-2 after co-stimulation by B7-1Ig, B7-2Ig or B7-2vIg. Purified CD28⁺ T lymphocytes were incubated in the presence of immobilized anti-CD3 and varying amounts of B7Ig proteins and the production of IL-2 (18 h) and the proliferative response (72 h) measured. IL-2 levels were determined by quantitative ELISA and proliferation was measured by the addition of [³H]thymidine for overnight after 72 h. The T cell proliferation induced by B7-1Ig, B7-2Ig or B7-2vIg could be blocked by the addition of CTLA-4–Ig or mAb to B7-1 or B7-2. All determinations were made in triplicate. This figure represents the results of one of six experiments.

**Table 2.** Production of cytokines after co-stimulation by B7-2vIg

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<tr>
<th>Cytokine (pg/ml)</th>
<th>18 h</th>
<th>120 h</th>
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<tr>
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<td></td>
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<tr>
<td>None</td>
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<td>7</td>
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</tr>
</tbody>
</table>

| **IL-4**         |      |       |      |       |      |      |
| **IFN-γ**        |      |       |      |       |      |      |
| **GM-CSF**       |      |       |      |       |      |      |

*Cytokine (pg/ml) for B7-2vIg co-stimulation led to increased IL-2 accumulation over a 5 day period as compared to B7-1Ig or B7-2Ig or CHO/B7-1 or CHO/B7-2 (Table 2 and data not shown). B7-2vIg drives multiple cycles of T cell proliferation*

Half maximal concentration was ~0.03 µg/ml for B7-2vIg and 3 µg/ml for B7-2Ig, an ~100-fold difference.

CD28⁺ T lymphocytes were cultured in the presence of immobilized anti-CD3 and either B7-1Ig (10 µg/ml), B7-2Ig (10 µg/ml) or B7-2vIg (3 µg/ml) and the accumulation of individual cytokines measured by quantitative ELISA in the culture medium after 18 and 120 h.

B7-2vIg co-stimulation led to increased IL-2 accumulation over a 5 day period as compared to B7-1Ig or B7-2Ig or CHO/B7-1 or CHO/B7-2 (Table 2 and data not shown).

B7-2vIg drives multiple cycles of T cell proliferation

We have recently shown that CHO cells expressing either B7-1 or B7-2 can mediate long-term autocrine proliferation of CD4⁺ T lymphocytes in conjunction with anti-CD3 stimulation (28). To further test the function of the various B7 fusion proteins, CD4⁺ T cells were cultured with beads coated with anti-CD3 mAb plus anti-CD28 mAb, B7-1Ig, B7-2Ig or B7-2vIg and the total number of cells monitored (Fig. 6). T cells stimulated with anti-CD3 alone failed to proliferate while those stimulated with anti-CD3 plus B7-1Ig went through two cycles of replication and then underwent apoptosis as evidenced by the fragmentation of their DNA on day 5 (data not shown). It seems likely that the signal induced by cross-linked B7-1Ig did not generate sufficient IL-2 to allow T cell survival. In contrast, co-stimulation by anti-CD3 plus anti-CD28, B7-2Ig or B7-2vIg drove T lymphocytes through multiple rounds of replication with the total number of cells increasing 20-fold over a 10 day period (Fig. 6). Continued stimulation by anti-CD3 plus anti-CD28, B7-2Ig or B7-2vIg resulted in an identical, continued logarithmic increases in cell numbers over a 30 day period (data not shown) indicating that the IgV domain of B7-2 alone can provide a co-stimulatory signal as effective as anti-CD28 mAb or full length B7-2Ig.
Discussion

Although they share only a moderate level of homology, the B7-1 and B7-2 molecules recognize the same ligands and co-stimulate T cell proliferation through the CD28 receptor pathway (8–11). Although differences in the pattern of expression of B7-1 and B7-2 on antigen-presenting cells have been described, analyses of the functional consequences of CD28 receptor engagement by B7-1 and B7-2 have yielded disparate results (12–16,28,30). In addition, splice variants of B7 proteins which yield domain-specific forms have been described and a possible soluble form has been identified (31–34). The function of these proteins is largely unknown. In an effort to determine the functionality of soluble B7 proteins in general, and V or C domains in particular, we constructed individual IgV and IgC domains of B7-1 and B7-2 as IgG Fc fusion proteins and determined whether they retained activity.

Our results show that soluble B7-1Ig and B7-2Ig proteins retain all of the binding and co-stimulatory activities of the membrane bound proteins. Furthermore the V domain of B7-2 retains all of the binding and co-stimulatory functions of the full length protein. Soluble B7-1Ig, B7-2Ig and B7-2vlg bound soluble CTLA-4-Ig in ELISA format at 1, 5 and 8 nM respectively in fair agreement with affinity measurements using soluble B7 proteins or CHO/B7 transfectants (23,35). Competition studies showed that B7-2Ilg competed for binding to CTLA-4 as well as B7-2lg. Thus we conclude that all of the sequences required for the binding of B7-2 to CTLA-4 are contained within the V domain. We have refrained from labeling the interactions of the soluble proteins with CTLA-4 as affinities since the purified proteins contained a mixture of multimeric forms with potentially different binding capacities. Soluble B7-1Ig, B7-2Ig and B7-2vlg also bound to CD28-expressing cells in FACS analyses. Surprisingly the FACS signal obtained using B7-2vlg was significantly brighter than that of the full length proteins and could not be accounted for by the small increase in molar concentration of the B7-2vlg form. It is possible that the CD28 binding site is more accessible on the B7-2vlg protein than in the full length forms or that the B7-2 C domain contains a down-regulatory function. Alternatively the B7-2vlg protein may cross-link more readily than either B7-1lg or B7-2lg. We have not characterized the binding of the various B7 proteins to CD28 as affinity, since by cross-linking these proteins with anti-human IgG antiserum to presenting them to CD28+ cells we have presumably added a significant avidity effect. The difference in CD28 binding seen in the FACS experiments could be readily explained by different avidities produced by greater or lesser cross-linking of the Ig fusion proteins. An explanation for the B7-2vlg molecule’s increased binding activity will require thorough biochemical characterization. Nonetheless, the ability of B7-2vlg to compete with B7-2lg for binding to CD28+ cells indicates that all of the sequences required for binding to CD28 are contained within the V domain of B7-2.

The greatly increased co-stimulatory activity of B7-2vlg shows that the increased binding to CD28 has a functional consequence. Cross-linked B7-2vlg added with either PMA or anti-CD3 mAb induced significant T cell proliferation and accumulation of IL-2 that was several fold greater than that seen with either B7-1 or B7-2. Also, the proliferative activity of B7-2vlg persisted down to 10 ng/ml (250 pM B7-2vlg) when using anti-CD3 to provide the first signal; neither B7-1 nor B7-2 were co-stimulatory at 1 μg/ml. Co-stimulation with cross-linked B7-1Ig, B7-2Ig or B7-2vlg induced IL-2, IFN-γ and IL-4 at levels that reflected the amount of proliferation, rather than a fundamental difference in the array of cytokines produced. Multiple rounds of co-stimulation with B7-2 can drive T cells toward a Th2 cytokine profile as has been shown using antigen specific antigen-presenting cells and with transfectants (14,30). We have not yet determined if multiple rounds of co-stimulation using soluble B7-2Ig or B7-2vlg will have a similar effect. Multiple rounds of co-stimulation do result in the significant expansion of T cell populations, as shown in the studies using protein-coated tosyl-activated beads. Curiously, B7-1-coated beads were unable to sustain T cell expansion beyond two rounds of co-stimulation. Since the cells died by apoptosis in these experiments we hypothesize that insufficient IL-2 was being produced to maintain the viability of the activated cells.

Several attempts have been made to identify the ligand binding sites on B7-1 and B7-2, and on CD28 and CTLA-4, using sequence homology data, hybrid CD28/CTLA-4 proteins and mutational analysis (22,23,35–37). These studies defined regions of CD28 and CTLA-4 required for binding to B7-1 and B7-2 within the highly conserved CDR3-like domain and in the CDR-1 like region (22,23). Replacement of one amino acid in the CDR3 region of CTLA-4 eliminated binding to B7-2 but only partially diminished binding to B7-1 (35), indicating differential binding sites. Mutational analysis of B7-1 and B7-2 suggested that conserved residues within the V domains of these proteins were critical for binding to CTLA-4 and CD28 (36,37). Moreover the latter study demonstrated that neither IgV nor IgC domain-specific B7-1 fusion proteins were capable of interacting with CTLA-4 and CD28, in agreement with results presented here.

Membrane-bound splice variants of murine B7-1 have been described (31,32). While the C domain form is inactive (31), the V domain form appears to have binding and functional activity. When expressed on the surface of transfectants this variant induced the proliferation of activated, but not resting, T cells (32). Other transmembrane-containing splice variants of murine B7-2 have been described (33) but their functional capacity is not yet known. Soluble forms of B7-1 or B7-2 have not been described in either mouse or human, and all known splice variants retain the transmembrane domain at the mRNA level. Recently, a soluble CD28-dependent co-stimulatory activity produced by B7-2-positive porcine endothelial cells was described (34). The authors speculated that this co-stimulatory activity was derived from a soluble form of B7-2. Thus there may be precedence for soluble forms in other species.

We show here that the soluble forms of B7-1Ig, B7-2Ig and B7-2vlg become effective co-stimulators of T lymphocytes when cross-linked using an anti-IgG fusion protein or attached to beads, and that B7-2vlg stimulat can be as strong as that provided by cell surface expressed B7-1 or B7-2. These studies demonstrate that human B7-1, B7-2 and B7-2v-pro-
teins themselves are sufficient to co-stimulate T lymphocytes and that additional signals derived from other antigen-presenting cell surface or secreted proteins are not required for co-stimulation. Such reagents should prove useful in further unravelling the role of B7-1 and B7-2 in T cell co-stimulation. Finally, these results suggest that secreted forms of intact or truncated B7 proteins could have a physiological role in regulating the co-stimulation of T cells.

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Abbreviations

GM-CSF  granulocyte macrophage colony stimulating factor
HRP  horseradish peroxidase
PMA  phorbol myristate acetate

References

28 Levine, B. L., Ueda, Y., Craighead, N., Huang, M. L. and June, C. H. 1995. CD80 ligands CD80 (B7-1) and CD86 (B7-2) induce long-term autocrine growth of CD4+ T cells and induce similar patterns of cytokine secretion in vitro. Int. Immunol. 7:891.
B7-2 IgV domain is co-stimulatory for T lymphocytes

A. and Peach, R. 1994. Human B7-1 (CD80) and B7-2 (CD86) bind with similar avidities but distinct kinetics to CD28 and CTLA-4 receptors. *Immunology* 1:793.


