Mutational Analysis and an Alternatively Spliced Product of B7 Defines Its CD28/CTLA4-binding Site on Immunoglobulin C-like Domain

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Summary

Costimulatory molecules B7 and B7-2 interact with T cell surface receptors CD28/CTLA4 and deliver a costimulatory signal essential for T cell growth. However, the structure basis of this interaction is not known. B7 and B7-2 are members of immunoglobulin (Ig) superfamily and their extracellular portion consists of an IgV- and IgC-like domain. Here we report that a naturally occurring, alternatively spliced form of B7 reveals that exon 3-encoded IgC domain is essential for CD28/CTLA4 binding. Mutational analysis of B7 demonstrates a critical role of several amino acids around loops between strands B and C and D and E, for binding CTLA4/CD28. These amino acids are clustered to form a single binding site centered at 201Y. A comparison of the effects of mutations on the binding of CD28 and CTLA4 reveals that CD28 and CTLA4 binds to the same site on B7. These results have important implications on the role of CTLA4 and CD28 in T cell costimulation. The structure of the CD28/CTLA4-binding site also provides valuable information for immune intervention targeted at the B7/B7-2-CD28/CTLA4 interactions.

Activation of T cells requires two types of biological signals: signal 1 is delivered by interaction between the TCR and MHC-peptide complex, whereas signal 2 is called the costimulatory signal (1–6). Recent studies from various laboratories indicate that signal 1 determines the specificity of T cell activation, whereas signal 2 determines the fate of T cells. Cells that receive both signals 1 and 2 clonally expand and differentiate into effector cells. In contrast, T cells that receive signal 1 alone are either functionally inactivated (anergized) and/or undergo programmed cell death (7–11).

Several interactions have been described that are involved in T cell costimulation (12–19). Accumulating evidence strongly suggests that the interaction between CD28/CTLA4 on T cells with their ligands B7 and/or B7-2 appears to be most important. First, anti-CD28 mAbs can augment T cell proliferation and prevent the induction of clonal anergy (20–23). Second, blocking B7 and B7-2 inhibits T cell proliferation and induces clonal anergy (11–24). APCs from mice with a targeted mutation of B7 gene show a significantly reduced costimulatory activity (25). Third, transfection of B7 or B7-2, the natural ligands for CD28 and CTLA4-Ig confers costimulatory activity into the recipient cells (12, 15, 16, 26). In several tumor models tested, transfection with B7 increases tumor immunogenicity and leads a T cell–mediated rejection of tumors (27–30).

B7 and B7-2 are members of the Ig super family (15–17, 31). The extracellular portion consists of an IgV-like domain and an IgC-like domain. Murine B7 gene contains 5 exons encoding, respectively, signal peptide, IgV-like domain, IgC-like domain, transmembrane domain, and cytoplasmic domain. B7 and B7-2 bind to CD28 (32) and, with a higher affinity, to a less abundant receptor CTLA4 (33). It has been suggested that CD28 and CTL4 may transduce qualitatively different signals when engaged by B7 and/or B7-2 (34). As a first step to understand the mechanism of T cell costimulation mediated by B7/B7-2-CD28/CTLA4 interaction, we set out to determine the structural basis of these interactions. Here we report the B7-binding site for CD28 and CTLA4 as defined by a naturally occurring, alternatively spliced B7 and by site-directed mutagenesis.

Materials and Methods

Experimental Animals, Cell Lines, and mAbs

Male CBA/CaJ mice were purchased from the Jackson Laboratory (Bar Harbor, ME) as donors of lymphocytes. B cell lymphoma CRC-2, provided by Dr. Thorbecke (New York University Medical Center) (35), CH27, and M12 were cultured in RPMI medium containing 5% of FCS. Anti-B7 mAb 16.10A (36) was kindly
Construction of the Fusion Proteins CD28Ig and CTLA4Ig

Fusion proteins CTLA4Ig and CD28Ig, comprised, respectively, the extracellular domains of murine either CTLA4 or CD28 and Fc portion of murine IgG2a, were generated according to a described procedure (33). Briefly, DNA encoding the extracellular region of murine CD28 was amplified by PCR using the synthetic oligonucleotide CACAAGCTTGGCATCGTTGCTTGGACT as a forward primer, GGGCTCTCGATACAGTGCAATCCGGGCGATGTC as reverse primer, and cDNA from Con-A-activated murine spleen T cells as template. The product of this PCR reaction was digested with HindIII and BclI and ligated together with a Bcl/XbaI cleaved DNA fragment encoding the hinge, CH2 and CH3 domains of murine IgG2a as a reverse primer. The latter fragment was amplified from cDNA prepared from a murine IgG2a hybridoma using the oligonucleotide GACCTGATCAGCGAGCCCAGAGGGCCCAACA as forward primer, and GACCGAGTCTAGATTTTTCCTGATCAGAAG as reverse primer. Ligation products were cloned into the CD8 mammalian expression plasmid (Invitrogen, San Diego, CA) and the construction was verified by DNA sequence analysis. The encoded molecule comprises protein 1–161 of murine CD28 fused to the hinge region of murine heavy chain Cy2a. Similarly, the extracellular domain of CD28 (AA 1–150) was amplified from CD28 cDNA (37) kindly provided by Dr. J. Allison (University of California, Berkeley, CA), using CCACAA-GCTTCAAGTGGACACCTACCGTACTG as forward primer and TGTGGGCCCTCTGGGCTCGAGCTTAGGAGATGACTG as reverse primer. The PCR product was digested with ApaI. The IgG2a fragment was prepared by digesting CTLA4Ig construct with ApaI and XbaI. The CD28 fragment and the Ig fragment were ligated into pCDM8 vector digested with HindIII plus XbaI. The final construct was verified by DNA sequence analysis. The fusion proteins were prepared from CHO cells stably transfected with either CD28Ig or CTLA4Ig construct. The CHO cell supernatants were concentrated 10 times and used for this study. The supernatants contain 10 times the excess of fusion proteins required for saturating binding against wild-type B7 in flow cytometry.

Construction of B7-HSA, B7IgV-HSA, and B7IgC-HSA

All fusion proteins were generated by three piece ligation including HindIII plus XhoI-digested B7-fragments, XhoI + XbaI-digested HSA fragment and HindIII + XbaI-digested pCDM8 vector. The B7 and HSA fragments are generated as follows: B7. The extracellular portion of B7, including the signal peptide, IgV and IgC domain, were amplified by PCR, using GCTCGAAGCTTGGCATCGTTGCTTGGACT as forward primer, and GTCAGCCATCTCGAGTTTTTCCAGGATTGAAGT as reverse primer. The final PCR product encodes the signal peptide and exon 3 encoded B7 IgC domain (M1-D37 + A143-K236).

Construction of B7-IgV.

The B7IgV construct was generated from B7IgC-HSA by digesting pCDM8-B7 with BgII and KpnI. The B7IgV domain was amplified from cDNA prepared from a murine IgG2a hybridoma using the oligonucleotide GCTCGAAGCTTGGCATCGTTGCTTGGACT as a forward primer and GAGTTTTTCCAGGATTGAAGT as a reverse primer. The final PCR product encodes the signal peptide, B7IgV domain plus 15 AA from B7IgC domain as spacer.

Construction of B7-IgC.

First, the signal sequence of B7 (B7SP) was amplified with GCTCGAAGCTTGGCATCGTTGCTTGGACT as reverse primer and GAGTTTTTCCAGGATTGAAGT as a reverse primer, and exon 3 sequence of B7 (B7E3) was amplified with GTGCTTCCAGATGCTGAATCTCTCTACTACCCCAAAC as a forward primer and GTCAGCCATCTCGAGTTTTTCCAGGATTGAAGT as a reverse primer. A mixture of B7SP and B7E3 fragments were used as template, GCTCGAAGCTTGGCATCGTTGCTTGGACT as a forward primer and GTCAAGCCATCTCGAGTTTTTCCAGGATTGAAGT as a reverse primer. The final PCR product encodes the signal peptide and exon 3 encoded B7 IgC domain (M1-D37 + A143-K236).

HSA. The HSA fragment that contains all HSA protein sequence except signal peptide was amplified using GAAAAACCTCAGAAGATGAAACCAAAATCTGGTGCC as forward primer and CCAAGAAGATGAAACCAAAATCTGGTGCC as reverse primer. The final PCR product encodes the signal peptide and exon 3 encoded B7 IgC domain (M1-D37 + A143-K236).
### Table 1. Summary of Amino Acid Substitutions and the Binding Characteristics of the B7 Mutants

| Mutants | Nucleic acid(s) | Amino acid* | Anti-B7 | CD28Ig | CTLA4Ig |
|---------|----------------|-------------|---------|--------|---------|
| **Experiment 1** | | | | | |
| B7 | TTGGAA>CTCGAG | No change | 224 | 47(100) | 649(100) |
| C165G | TGC>GGC | C165>G | 109 | 0(0) | 30(10) |
| Loop BC | | | | | |
| P172A | CCA>GCT | P172>A | 113 | 2(7) | 8(3) |
| K173E | AAG>GAG | K173>E | 271 | 44(77) | 772(98) |
| P174A | CCT>GCT | P174>A | 191 | 0(0) | 61(11) |
| R175S | CGC>AGC | R175>S | 276 | 15(26) | 334(42) |
| S177A | TCT>GCT | S177>A | 294 | 40(63) | 476(56) |
| L179V | TTG>GTG | L179>V | 234 | 12(18) | 274(40) |
| E180D | GAA>GAC | E180>D | 259 | 38(68) | 622(83) |
| LE/VD | TTGGAA>CTCGAG | L179E180>VD | 188 | 16(40) | 281(51) |
| Loop DE | | | | | |
| QD/LE | CAGGAT>CTCGAG | Q194D195>LE | 132 | 3(11) | 103(26) |
| QDP/LEER | CAGGATCCT>CTCGAGCGT | Q194D195P196>LEER | 102 | 1(3) | 42(15) |
| QDP/LEH | CAGGATCCT>CTCGAGCAT | Q194D195P196>LEH | 93 | 1(3) | 36(13) |
| LY/VD | TTGTAC>GTCGAC | L200V201>VD | 105 | 0(0) | 0(0) |
| **Experiment 2** | | | | | |
| B7 | No change | No change | 134 | 73(100) | 235(100) |
| Loop DE | | | | | |
| E199A | GAA>GCA | E199>A | 97 | 16(31) | 108(63) |
| L200A | TTG>GCG | L200>A | 105 | 28(53) | 142(85) |
| Y201A | TAC>GCG | Y201>A | 50 | 1(3) | 0(0) |

* The amino acid sequences of murine B7 IgC domain are shown below with the conserved amino acid in bold. Underlined are the locations of $\beta$-sheet strands based on the Ig superfamily.

| ADFSTPNGSNPSADTTRITFCASSGFPKPRFSWLENGRELPGINTTSQDPESELYTISQSLDFNWTRNHTKCLIKYGDAH |
| VSEDFTWKEKPED | |

$^{1}$ The data shown are MF of transfected COS cells after being stained with either anti-B7 mAb 10.16A.1 or chimeric molecules CD28Ig or CTLA4Ig, with that of controls (staining in the absence of first step-reagents) subtracted. Numbers in the parentheses are % wild-type B7 binding after normalizing the cell surface expression of B7 mutants as described in experimental procedure. Each staining has been repeated for at least three times.

66 h of culture, the cells were pulsed with 1 $\mu$Ci/well of $[^{3}H]$TbR for additional 6 h. T cell proliferation was measured by the cpm of the incorporated $[^{3}H]$TbR. The data shown were means of duplicates with variation <15%.

**Flow Cytometry**

Mock-transfected COS cells, or COS cells transfected with either wild-type or mutant B7 were used between 72 and 96 h after transfection. The antibodies used were: anti-B7 mAbs 10.16A.1, 3A12, 7A5 (18), anti-HSA antibody M1/69 (43), fusion protein CD28Ig, and CTLA4Ig. The second step reagents used were FITC-labeled, goat anti–mouse IgG, goat anti–hamster IgG, and mouse anti–rat IgG. All of these second step reagents give essentially identical background staining in the absence of primary antibody, therefore only one control is shown in each experiment. Details of flow cytometry have been described (44). Data shown in Table 1 are mean fluorescence (MF) obtained by flow cytometry with that of the controls (in the absence of first step reagents) subtracted.

1 Abbreviation used in this paper: MF, mean fluorescence.
The numbers in parentheses are the percent wild-type B7 binding after normalizing the cell surface expression of individual mutant B7 based on binding of an anti-B7 mAb 10.16A.1, that binds the IgV domain regardless of mutation in the IgC domain. Wild-type B7 binding percentage was calculated by the following formula: % wild-type B7 binding = $100 \times \frac{x}{y}$, where $x = \frac{\text{MF}_{\text{B7}}}{{\text{MF}_{\text{B7}}}}$, when mutant molecules are used, and $y$ is the same ratio when the wild-type B7 molecule is used.

Results

A Naturally Occurring, Alternatively Spliced B7 Reveals a Critical Role of B7 IgC Domain in Binding CD28 and CTLA4. In the process of analyzing the B7 expression in a number of different cell lines by PCR, we have observed two major forms of B7 cDNA: a longer form of 930 bp and a shorter form of ~650 bp (Fig. 1 a). Cloning and cDNA sequencing revealed that the larger band is B7, whereas the shorter band is a truncated B7 with a deletion from base 428 to base 699 (number begins at the start codon), which is the entire sequence of exon 3 (Fig. 1 b). Thus this shorter form of B7 is a product from an alternative splicing of B7. As the only extracellular portion of the protein encoded by this alternatively spliced B7 gene is the IgV domain, we call it B7IgV.

To test whether B7IgV contains CD28/CTLA4-binding sites, we first amplified various forms of B7 mRNA by RT-PCR. First-strand cDNA prepared with random hexamer primers from RNA of either spleen or B leukemic cell lines CH27, CRCS, or M12 were amplified using B7 and GAPDH forward and reverse primers that should amplify the open reading frame of the full-length B7 (12). The PCR products were analyzed using either a full-length B7 open reading frame probe or a GAPDH probe (12). The short B7 cDNA is an alternatively spliced form of B7. The junctional sequence of the full-length B7 that is identical to the sequence of the DNA in the higher molecular weight band (top) is a predicted junctional sequence of an alternatively spliced form of B7, B7IgV, that is identical to the sequence of the DNA in the lower molecular weight band (bottom). Analysis of the binding of B7IgV, B7 to anti-B7 mAb 7A5, and fusion proteins CD28lg and CTLA4lg reveals comparable expression of B7IgV is detected with two other anti-B7 mAbs 3A12 and 10.16A.1 (data not shown). Mutation 165C>G eliminates CD28 binding and reduces CTLA4lg binding by ~10-fold.
we generated fusion proteins that consist of murine IgG2a Fc portion and extracellular domains of either murine CD28 (CD28Ig) or murine CTLA4 (CTLA4Ig). We then used these fusion proteins and a panel of anti-B7 mAbs to monitor the cell surface expression of B7 and B7IgV on COS cells transiently transfected with these genes. Whereas the B7 binds anti-B7 mAbs (Fig. 1c and data not shown) CD28Ig and CTLA4Ig, B7IgV binds anti-B7 mAbs but not CTLA4Ig and CD28Ig (Fig. 1c). The lack of binding to CTLA4 was not due to poor cell surface expression of the B7IgV, because CTLA4Ig binding to wild-type B7 is better than the anti-B7 mAbs used. Thus the structure encoded by exon 3, which is the B7IgC domain, is involved in binding CD28/CTLA4. Consistent with this notion, a mutation of 165C>G that destroys the disulfate chain in the IgC domain, eliminates B7 binding to CD28Ig and reduces its binding to CTLA4Ig by 10-fold (Fig. 1d, Table 1).

Site-directed Mutagenesis Defines the Roles of the Conserved Residues within the B7IgC Domains for CD28/CTLA4 Binding.

So far, four molecules that bind CD28/CTLA4 have been identified, namely human B7, human B7-2, murine B7, and murine B7-2 (15–17, 26, 31). As these four molecules cross-react with both human and murine CD28/CTLA4, we reasoned that the structure involved in binding CD28/CTLA4 should be conserved among these four molecules. Previous analysis of B7 sequence homology (15–17, 26, 31) revealed that 17 of the 98 amino acids in the IgC domain are conserved. Among them, two are cysteines at positions 165 and 219. All 11 conserved amino acids between two cysteines are clustered at or near two loops, if we use the structure of IgC-domain as a reference (Table 1 legend). One is the loop between strands B and C (loop B-C), and the other between strand D and E (loop D-E). This analysis suggests that these two regions are involved in binding to CD28/CTLA4. To further define the CD28/CTLA4-binding sites, we generated a series of B7 mutants by site-directed mutagenesis and tested their binding to CD28Ig and CTLA4Ig. Binding to anti-B7 mAbs was used as an indicator for cell surface expression of B7 mutants. As shown in Table 1 and Fig. 2, mutants 172P>A and 174P>A fail to bind CD28Ig, and their binding to CTLA4Ig is reduced by 10-fold. In contrast, mutations of 173K>E, 177S>R, and 180E>D have little effect on B7 binding to CD28Ig/CTLA4Ig. Mutations 175R>S, 179L>V reduced CD28Ig binding by four- to fivefold, and CTLA4Ig binding by twofold. These results indicate that amino acids at the B-C loop are involved in binding CTLA4/CD28.

Similarly, amino acids in the D-E loop are critical for CD28/CTLA4 binding. As shown in Fig. 3 and Table 1, mutation 194Q>L195D>E reduces CD28 binding by 10-fold and CTLA4Ig binding by fivefold. An additional mutation in this region 194Q>L195D>E196P>H, or 194Q>L195D>E196P>R eliminates CD28-binding site and reduces CTLA4Ig binding by 10-fold. The most drastic effect is caused by mutation 200L>V201Y>D, that leads to elimination of binding to both CTLA4Ig and CD28Ig. This result demonstrates that one or both of these amino acids is essential for binding CD28/CTLA4Ig. To test this, we replaced, one by one, three amino acids 199E, 200L, and 201Y with A and tested the effect of these mutations on B7 binding to CD28 and CTLA4. As shown in Fig. 4, replacement of either 199E or 200L by A does not significantly reduce the binding of B7 to CD28 and CTLA4, suggesting that these two amino acids are not critical for CD28/CTLA4Ig binding. In contrast, mutation 201Y>A eliminated the binding of both CD28 and CTLA4, suggesting that the 201Y plays a critical role in binding CD28 and CTLA4.

The results of the site-directed mutagenesis reveal that all mutations that affect CD28Ig binding affect CTLA4Ig binding. Interestingly, the effects of mutations on CD28 and CTLA4 binding follow a grossly similar hierarchy (Table 1), although CTLA4Ig binding is generally more resistant to mutations. These results strongly suggest that CTLA4 and CD28 have the same binding site on B7.

An important issue is whether IgC domain contains all
the necessary information for binding CD28/CTLA4. To address this issue, we deleted the IgV domain and transfected the B7IgC into COS cells. No binding is detected in COS cells transfected with the B7IgC (data not shown). However, as no antibody against this domain is available, it is equally possible that this domain is not stable enough to give cell surface expression. We therefore constructed a chimeric molecule that consists of the B7IgC domain and the heat-stable antigen extracellular domain. As shown in Fig. 5, whereas the chimeric molecules are expressed on the cell surface as demonstrated by its binding to anti-HSA mAb, they do not bind CD28Ig and CTLA4Ig. These results can be interpreted as evidence that B7IgV domain may play a role in the CTLA4/CD28 binding. However, it should be stressed that as the overall structure of the IgC domain cannot be ascertained at this stage, it is equally possible that the role of B7IgV is to allow correct folding of the CD28/CTLA4 binding sites.

Binding to CD28/CTLA4 Is Essential for the Costimulatory Activity of B7. To determine whether binding to CD28/CTLA4 was necessary for the costimulatory activity of B7, we compared the costimulatory activity of wild-type B7 that binds CD28/CTLA4 with the B7 mutants that have lost the ability to bind CD28/CTLA4. As shown in Fig. 6 a, wild-type B7 costimulates T cell proliferative responses to anti-CD3 mAb, whereas the alternatively spliced form of B7 (B7IgV) fails to do so. Because anti-B7 mAb 7A5 binds B7IgV rather

Figure 3. Effects of two representative mutations in DE loop on CD28/CTLA4Ig binding. See Fig. 2 legend for details.

Figure 4. The central role of 201Y in CD28/CTLA4 binding. COS cells transfected with either wild-type B7 or B7-201Y>A mutant were compared for their binding to anti-B7 mAb 7A5 or fusion proteins.

Figure 5. B7IgC-HSA fails to bind CTLA4Ig and CD28Ig. B7IgC-HSA were constructed as described in Materials and Methods. COS cells were transiently transfected with either B7-HSA or B7IgC-HSA. 3 d after transfection, the transfected COS cells were analyzed for the expression of the HSA (M1/69), B7 (7A5) epitopes or CD28/CTLA4Ig-binding sites.
Figure 6. B7IgV cannot costimulate proliferation of CD4 T cells. COS cells were transfected with either FcR or with FcR plus either wild-type or mutant B7 molecules and used to stimulate proliferation of CD4 T cells in the presence of anti-CD3 for 72 h. (a) Proliferation of CD4 T cells using COS cells transfected with FcR or FcR plus either wild-type B7 or B7IgV. (b) Costimulatory activity of B7-HSA and B7IgV-HSA for CD4 T cells. COS cells transfected with either FcR or FcR in conjunction with either B7-HSA or B7IgV-HSA were treated with mitomycin C and used as accessory cells. T cell proliferation has been repeated for at least three times. (c) Expression and binding characteristics of the B7-HSA or B7IgV-HSA.

Discussion

We have reported here that a naturally occurring, alternatively spliced B7 that lacks IgC domain has lost the CD28/CTLA4-Ig-binding sites. Site-directed mutagenesis based on homology analysis revealed several conserved amino acids within the IgC-like domains are involved in binding CD28 and CTLA4. To determine whether these amino acids can be clustered in the three-dimensional structure, we have mapped the residues of our mutagenesis study to an available Fab C-domain structure (3FAB, reference 46). There are a few x-ray protein structures available from the Brookhaven Protein Data Bank that contain IgC-like domains. Supposition of these structures shows that the loops B-E and D-E form a localized region towards one end...
Figure 7. Costimulatory activity of B7 requires CD28/CTLA4-binding site. COS cells were transfected with either FcR alone or FcR plus wild-type B7 or B7 mutants. The costimulatory activity of the COS cells were determined by proliferation of CD4 T cells to anti-CD3 mAb. Data shown are representative of two independent experiments.

Figure 8. Three-dimensional model of B7 IgC domain based on the x-ray structure of Ig Fab fragment. Only the 201Y side chain is shown explicitly, the rest of the molecule is represented by Coz trace. Residues that upon mutation significantly reduce CD28/CTLA4 binding are labeled (~), all of them are within 7Å of 201Y.
to the IgV domains, the effect of several anti-IgV antibodies can be explained by steric hindrance. Second, the IgC domains have not been shown to bind CD28/CTLA4 in the absence of IgV domain. Third, Inobe et al. (47) reported that a stable cell line thought to be transfected with the B7lgV domain can bind CD28 and CTLA4. However, as no characterization of the stable transfecant has been done to verify the gene product, it is hard to reconcile the results with our study.

An important conclusion that can be derived from the results of our mutational analysis is that CD28 and CTLA4 bind to the same site on the B7lgC domain. Thus, all mutations that affect CD28 binding affect CTLA4 binding. More strikingly, the effects of mutations on CD28 and CTLA4 binding follow a grossly similar hierarchy (Table 1), although CTLA4Ig binding is generally more resistant to mutations, consistent with an earlier finding that CTLA4 has a 17-fold higher affinity for B7 (33). This conclusion has an important implication on the mechanism of costimulation by B7. As CD28 and CTLA4 have different cytoplasmic domains, they may transduce qualitatively different signals (34). As CD28 and CTLA4 do not form heterodimers (33), and because they are located at distinct patches on the T cell surface as detected by confocal microscopy (34), the simplest mechanism that allows direct interaction of CD28-mediated signaling machinery with that mediated by CTLA4 would be a cross-linking of the two molecules by B7 or B7-2. By necessity, this model implies that CTLA4 and CD28 have distinct binding sites on B7 to allow cross-linking by a single B7 molecule. Our results that CTLA4 and CD28 bind B7 at the same sites do not support this model. We favor an alternative hypothesis that CTLA4 and CD28 bind different B7 molecules and transduce distinct biological signals. The facts that CTLA4 is expressed after T cell activation and that CTLA4 has a higher affinity for B7 or B7-2 suggests that CD28–B7/B7-2 interaction is likely to dominate at the induction phase of immune responses, whereas CTLA4–B7/B7-2 interaction is likely to dominate effector phase of immune responses. Our previous study (30) demonstrated that B7 can play an important role at both the induction and effector phases of T cell responses, thus raising an interesting possibility that CD28–B7 interaction may deliver a signal necessary for induction of T cell responses, whereas CTLA4–B7 interaction may deliver a signal for effector phase of T cell responses. Experiments are underway to test this hypothesis.

Whereas it is known that antibody stimulation through CD28 transduces the costimulatory signal for T cells, and that B7 binds CD28 and delivers the costimulatory activity, it is still formally possible that B7 costimulate T cells via other yet unidentified receptors on T cells. Our results demonstrate that B7 mutants that lose their binding to CD28 and CTLA4 also lose their costimulatory activity. These results demonstrate that B7 costimulate T cells by binding CD28/CTLA4.

Finally, recent studies have demonstrated that immune intervention targeted at the CD28/CTLA4–B7/B7-2 interaction have a vast potential in transplantation (48, 49) and tumor therapy (27–30). The structure of B7-binding sites for CD28/CTLA4 defined in this study provides valuable information for immunotherapy.

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