Complementarity Determining Region 1 (CDR1)- and CDR3-analogous Regions in CTLA-4 and CD28 Determine the Binding to B7-1

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Summary

T cell surface receptors CD28 and CTLA-4 are homologous members of the immunoglobulin superfamily (IgSF), each comprising a single V-like extracellular domain. CD28 and CTLA-4 bind to the B7-1 and B7-2 counter-receptors on antigen presenting cells (APCs), thereby triggering a costimulatory pathway important for optimal T cell activation in vitro and in vivo. Soluble forms of CD28 and CTLA-4 in which the V-like extracellular domains were fused to Ig constant domains (CD28Ig and CTLA4Ig), have been used to study their interactions with B7-1 and B7-2, with CTLA4Ig binding B7-1 more strongly than CD28Ig ($\sim$20-fold higher avidity). We have now, by site-specific and homologue mutagenesis, identified regions in CTLA4Ig important for strong binding to B7-1. A hexapeptide motif (MYPPPY) in the complementarity determining region 3 (CDR3)–like region is fully conserved in all CD28 and CTLA-4 family members. Alanine scanning mutagenesis through the motif in CTLA4Ig and at selected residues in CD28Ig reduced or abolished binding to B7-1. Chimeric molecules HS4, HS4-A, and HS4-B were constructed in which CDR3-like regions of CTLA-4, COOH-terminally extended to include nonconserved residues, were grafted onto CD28Ig. These homologue mutants showed stronger binding to B7-1 than did CD28Ig. Grafting of the CDR1-like region of CTLA-4, which is not conserved in CD28 and is predicted to be spatially adjacent to CDR3, into HS4 and HS4-A, resulted in chimeric molecules (HS7 and HS8) which bound B7-1 even better. Inclusion of the CDR2-like domain of CTLA-4 into HS7 and HS8 did not further increase binding. Thus, the MYPPPY motifs of CTLA4Ig and CD28Ig are important for their binding to B7-1, but the increased strength of this binding by CTLA4Ig is mediated by nonconserved residues in the CDR1- and CDR3-analogous regions.

For T cells to respond to an antigenic stimulus, multiple activation signals are required from the APC (1, 2). An antigen-specific signal occurs when the TCR binds to antigenic peptides presented by MHC molecules. An important nonspecific costimulatory signal is delivered to the T cell when B7-related receptors on APCs bind to CD28 and/or CTLA-4. There are at least two homologous B7 family members found on APCs, B7-1 (also called B7 or CD80) and B7-2, both of which can deliver costimulatory signals to T cells via CD28 and/or CTLA-4 (3–7). Costimulation through CD28 or CTLA-4 is essential for T cell activation since a soluble Ig fusion protein of CTLA-4 (CTLA4Ig) blocks T cell–dependent immune responses in vitro and in vivo (5, 8, 9). Failure to deliver this second signal may lead to clonal inactivation or T cell anergy (10–12).

CD28 and CTLA-4 both contain a single Ig V-like extracellular ligand binding domain which share $\sim$25% sequence homology (13–15). The B7 ligands are also members of the Ig superfamily (IgSF)[1] but have, in contrast to CD28 and CTLA-4, two Ig domains in their extracellular region, an NH_2-terminal V-like domain and a C-like domain (3, 6, 7). CTLA4Ig binds to B7-1 with $\sim$20 fold higher avidity than a corresponding Ig fusion of CD28 (5, 16) but the molecular details of how CD28 and CTLA-4 interact with B7-1/B7-2 are not known. In particular, it is not known what determines the different binding affinities of these receptors for their counter-receptors. Using Ig fusion proteins of CTLA-4 and CD28, we have analyzed regions of these molecules that are critical for their interaction with B7-1. The results of site-specific and homologue scanning mutagenesis experiments combined with an approximate three-dimensional model of the CTLA-4 extracellular domain suggest an important role

of residues in the CDR3- and CDR-1-analogous regions in CTLA-4 for the binding to B7-1.

Materials and Methods

Cell Culture. Stably transfected B7-1[+] Chinese hamster ovary (CHO) cells were described previously (16). Cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 0.2 mM proline, and 1 μM methotrexate. COS cells were grown in DMEM supplemented with 10% FBS.

mAbs and Fusion Proteins. Anti-CTLA-4 mAbs 7F8 and 10A8 (17), and anti-CD28 mAb 9.3 (18) have been previously described. CD28Ig was prepared in transiently transfected COS cells (16), whereas CTLA4Ig was prepared in stably transfected CHO cells (8). B7-1mIg fusion protein was prepared by transfecting COS cells with plasmid cDNA encoding the extracellular domain of human B7-1 genetically fused to cDNA encoding a mouse IgG2a Fc region (Brady, W. and P. S. Linsley, unpublished observations). COS cell conditioned media was used as a source for Ig fusion proteins.

CTLA4Ig and CD28Ig Site-directed Mutant Expression Plasmids. Site-directed mutagenesis was performed on cDNA encoding a soluble chimeric form of CTLA-4 (CTLA4Ig) in which the extracellular domain of CTLA-4 was genetically fused to the hinge and C regions of a human IgG H chain (5). Using the PCR, alanine residues were introduced at each position of the conserved hexapeptide 99MYPPPY104. PCR forward primers were synthesized encoding the desired mutation and the naturally occurring SacI restriction enzyme site immediately preceding the codon encoding amino acid M99. A single reverse primer corresponding to the hinge region of a human IgG1 molecule was also used in the PCR reactions where CTLA4Ig cDNA was used as the template. The PCR products were digested with SacI and BclI (previously engineered into the junction site of cDNA encoding the extracellular domain of CTLA-4 and the hinge region of the human IgG), and in a three-way ligation fused to PCR-amplified human IgG H chain cDNA encoding the hinge and C regions (digested with BclI and XbaI), and SacI/XbaI-cut CDM8-CTLA-4 plasmid.

CD28Ig site-directed mutants were prepared by encoding the desired mutation in overlapping oligonucleotide primers and generating the mutants by PCR (19). Using a CD28Ig plasmid as the template, two mutants were prepared encoding the mutations P103A and Y104A from the CD28 99MYPPPY104 hexapeptide. Other flanking PCR primers synthesized for these reactions were designed to allow the final PCR products to be digested with HindIII and XbaI and ligated into HindIII/XbaI-digested CDM8.

PCR conditions consisted of 6 min at 94°C followed by 25 cycles of 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C. Taq polymerase and reaction conditions were used as suggested by the vendor (Perkin Elmer Cetus, Emeryville, CA). To confirm that the desired mutations had been inserted and to verify the absence of secondary mutations, each site-directed mutant was sequenced by the dideoxy chain termination/extension reaction with Sequenase reagents used according to the manufacturer's recommendations (United States Biochemical Corp., Cleveland, OH). Plasmids were transfected into COS cells (20) and the conditioned media was used as a source for Ig fusion proteins.

CTLA4Ig Homologue Mutant Expression Plasmids. 14 homologue scan mutant plasmids were designed to introduce CTLA-4 cDNA sequences into CD28Ig while, at the same time, deleting the equivalent region from CD28. Similarly, two mutant plasmids (HS3 and HS6) were designed to introduce CD28 sequences into CTLA4Ig. Each cDNA construct was genetically linked to cDNA encoding

the hinge and C regions of a human IgG1 in order to make soluble chimeras. HS1, HS2, HS5, and HS6 were constructed using a two-step PCR by initially amplifying appropriate regions of cDNA then, using flanking 5' and 3' PCR primers encoding HindIII and BclI restriction sites respectively, fragments whose sequences overlapped were fused together and amplified (19). PCR products were digested with HindIII and BclI and ligated to HindIII/XbaI-cut CDM8 along with a BclI/XbaI-digested PCR fragment encoding the human IgG H chain hinge and C region. HS3 and HS4 hybrids were prepared by exchanging SacI/XbaI-cut fragments of CD28Ig and CTLA4Ig. A SacI restriction site was present in CTLA4Ig and engineered into CD28Ig at the same relative position by mutating V98L using overlapping PCR primer-directed mutagenesis (19). This substitution did not affect the ability of the mutant CD28Ig to bind B7-1 relative to wild-type CD28Ig. HS4-A and HS4-B were also constructed by overlapping PCR primer-directed mutagenesis using HS4 as a template. PCR products were digested with SacI and BclI and ligated into similarly digested HS4-CDM8. HS7, HS8, and HS9 were prepared by replacing a 5' HindIII/HpaI fragment of HS4, HS4-A, and HS4-B, respectively, with the equivalent cDNA fragment similarly digested from HS5, thus introducing the CDR1-like loop of CTLA-4 into those CD28Ig hybrids already containing the CTLA-4 CDR3-like regions. HS10-HS14 were prepared by overlapping PCR primer-directed mutagenesis in which the CD28 CDR2-like loop of previously prepared homologue mutants was replaced with the homologous loop from CTLA4Ig. Thus, HS4 served as a template to make HS10, HS7 to make HS11, HS4-A to make HS12, HS8 to make HS13, and CD28Ig to make HS14. All mutants were sequenced by the dideoxy chain termination/extension reaction. Plasmids encoding each of the mutants were transfected into COS cells and the resulting culture media used as a source for soluble Ig fusion proteins.

Enzyme Immunoassays. To quantitate Ig fusion proteins in culture media, microtiter plates (Immulon2; Dynatech Labs., Inc., Chantilly, VA) were coated with 0.5 μg/ml goat anti–human IgG (Jackson ImmunoResearch Labs., Inc., West Chester, PA) for 16 h at 4°C. Wells were blocked for 1 h with specimen diluent (Genetic Systems, Seattle, WA), then washed with PBS containing 0.05% Tween 20 (PBS-Tween). COS cell culture media containing fusion protein was added and incubated for 1 h at 22°C. Known concentrations of CTLA4Ig were also added to separate wells on each plate. After washing, horseradish peroxidase (HRP)–conjugated goat anti–human IgG (Tago, Inc., Burlingame, CA) diluted 1:12,000, was added and incubated for 1 h at 22°C. Wells were then washed and incubated with 3,3',5,5' tetramethylbenzidine (TMB) substrate (Genetic Systems) for 15 min before stopping the reaction by the addition of 1 N H_2SO_4. Absorbance was measured at dual wavelengths of 450 and 630 nm on a microtiter plate reader (Genetic Systems). Concentrations of mutant fusion proteins were determined by comparison with the linear range of a standard curve of known concentrations of CTLA4Ig. Triplicate determinations were made and replicates differed from the mean by <10%. Experiments were repeated at least twice. The ability of CTLA4Ig point mutants, and CTLA4Ig/CD28Ig homologue mutants to bind B7-1 was measured using stably transfected B7-1^{+} CHO cells. Round bottom, tissue culture–treated, 96-well microtiter plates (Corning Inc., Corning, NY) were seeded with B7-1^{+} CHO cells at 10^3 cells/well. 2 d later, the confluent cells were fixed in 95% ethanol, washed with PBS-Tween, and mutant fusion proteins added and incubated for 1 h at 22°C. After washing, HRP-conjugated goat anti–human IgG (Tago Inc.) was added followed by TMB substrate and absorbances read. Site-directed mutants of CD28Ig were assayed for their ability to bind to recombinant B7-1mIg fusion

protein coated on microtiter plates at 5 μg/ml for 16 h at 4°C. Wells were blocked, washed with PBS-Tween, and COS cell culture media added and incubated for 1 h at 22°C. Known concentrations of CD28Ig were also added to separate wells on each plate. After washing, HRP-conjugated goat anti–human IgG (Tago Inc.) was added followed by TMB substrate and absorbances read.

Immunoprecipitation and Western Blot Analysis. CTLA4Ig/CD28Ig homologue scan mutant fusion proteins present in culture media were adsorbed to protein A–Sepharose by overnight incubation at 4°C. Beads were washed twice with PBS before addition of SDS PAGE sample buffer. The eluted proteins were electrophoresed in 8% SDS polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were blocked for 16 h with specimen diluent (Genetic Systems), washed in PBS-Tween, and incubated with HRP-conjugated goat anti–human IgG (Tago Inc.) diluted 1:10,000. Blots were washed and developed using the enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham, Arlington Heights, IL) as specified by the manufacturers.

CTLA-4 Molecular Model. An approximate three-dimensional model of the CTLA-4 extracellular domain was generated based on the conservation of consensus residues of IgSF V-like domains. Using such consensus residues as "anchor points" for sequence alignments, CTLA-4 residues were assigned to the A, B, C, C', C", D, E, F, G strands of an Ig V fold (21) and the connecting loop regions. The CTLA-4 model was built (Insight II, Discover, molecular modeling and mechanics programs, respectively, Biosym Technologies, Inc., San Diego, CA, 1993) using the VH chain of HyHEL-5 (22) as template structure. Side chain replacements and loop conformations were approximated using conformational searching (23). Several versions of the model with modified assignments of residues to β-strands or loops were tested using three-dimensional profile analysis (24) in order to improve the overall alignment of the CTLA-4 extracellular region sequence with an IgSF V fold.

Results

Construction and Binding Activity of CTLA4Ig and CD28Ig Point Mutants. A sequence alignment of the extracellular domain of various homologues of CTLA-4 and CD28 is shown

in Fig. 1. Residues referred to in this study are numbered according to the alignment in Fig. 1. Conserved residues are scattered throughout the extracellular domains of these proteins. A number of these are consensus residues of the IgSF rather than residues unique to the CTLA-4/CD28 family (25). The most notable conservation of amino acids that are not IgSF consensus residues is the hexapeptide MYPPPY sequence motif located in the CDR3-like loop of both CTLA-4 and CD28. This sequence conservation suggests a role for this region in the interaction with B7-related molecules. To test this hypothesis, we performed site-directed mutagenesis on soluble Ig fusions of CTLA-4 and CD28 and assessed the ability of these soluble mutants to bind B7-1. Alanine-scanning mutations were introduced into the MYPPPY region of CTLA4Ig using PCR oligonucleotide primer-directed mutagenesis. Similarly, two alanine mutations were introduced into the CD28Ig MYPPPY motif. The concentrations of mutant Ig fusion proteins in serum-free COS cell culture media were determined by an Ig quantitation assay. The structural integrity of mutant fusion proteins was examined by assessing their ability to bind mAbs specific to CTLA-4 (7F8 and 10A8) (17) or CD28 (9.3) (18) in an indirect immunoassay. mAbs 7F8 and 10A8 bound to each of the CTLA4Ig mutants to a level similar to CTLA4Ig, indicating that the epitope(s) for these mAbs were unaffected by the mutations. mAb 9.3 failed to bind to the CD28Ig mutant P103A but bound weakly to Y104A, suggesting that the MYPPPY hexapeptide was required for the epitope of this mAb.

The ability of each CTLA4Ig mutant to bind to B7-1 transfected CHO cells was determined by an indirect cell binding immunoassay, and the binding of CD28Ig mutants to B7-1Ig was assessed by an indirect enzyme immunoassay. Mutagenesis of each residue of the CTLA4Ig MYPPPY motif to Ala had a profound effect on binding to B7-1 (Fig. 2 *A* and Table 1). Mutants Y100A and P102A bound to B7-1 but considerably less than wild-type CTLA4Ig. In contrast, mutants
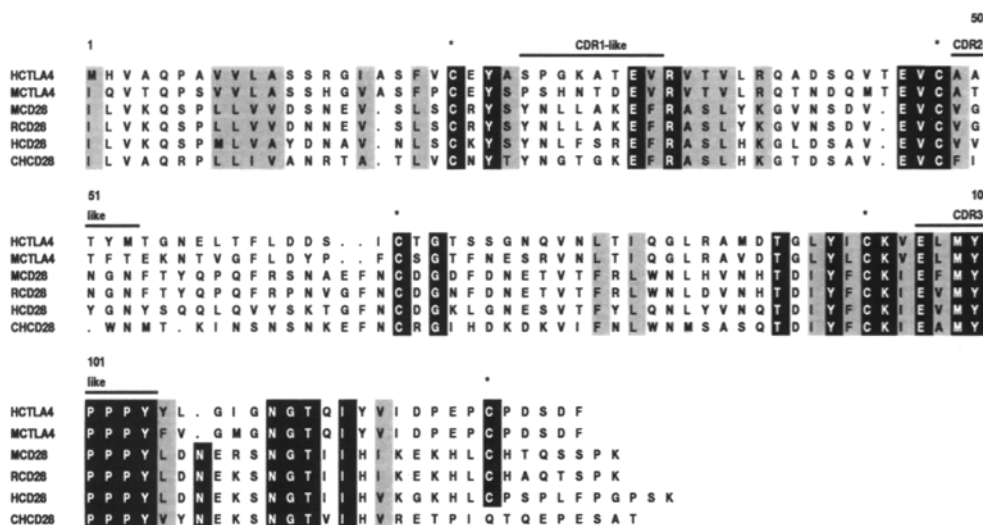

Figure 1. Sequence alignment of the extracellular regions of CTLA-4/CD28 family members. Sequences are aligned from the predicted NH2 terminus of mature human CTLA-4 (15) and numbering is based on this alignment. The predicted CDR-analogous regions are shown. Dark and stippled boxed areas highlight completely conserved and conservatively substituted residues, respectively. Asterisks denote cysteine residues. These sequence data are available from EMBL/GenBank/DDBJ under the following accession numbers: human CTLA-4 (*HCTLA4*), L15006; murine CTLA-4 (*MCTLA4*), X05719; murine CD28 (*MCD28*), M34563; rat CD28 (*RCD28*), X55288; human CD28 (*HCD28*), J02988; and chicken CD28 (*CHCD28*), X67915.

A

A450 (x 1000)

1200

1000

800

600

400

200

0

● CTLA4Ig
■ Y100A
▲ P101A
○ P102A
□ Y104A

0.0001   0.0010   0.0100   0.1000   1.0000

Fusion protein (µg/ml)

B

A450 (x 1000)

1400

1200

1000

800

600

400

200

0

● CD28Ig
■ P103A
▲ Y104A

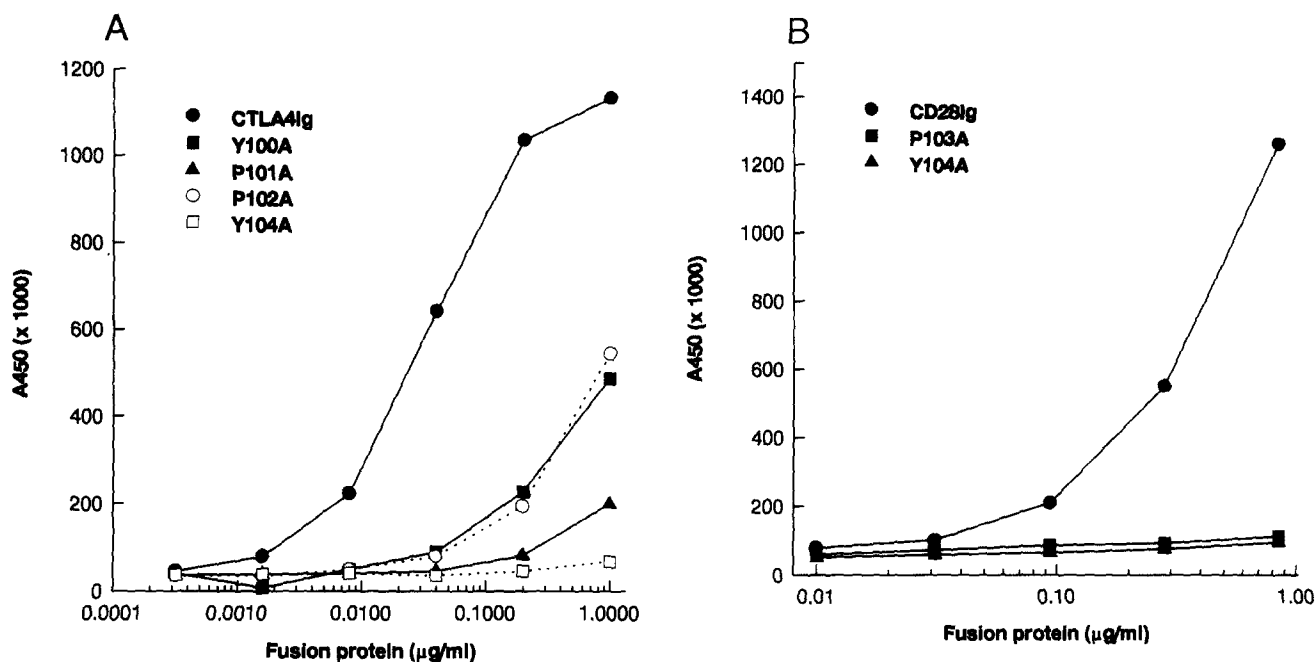0.01   0.10   1.00

Fusion protein (µg/ml)

Figure 2. Mutations in the MYPPPY motif of CTLA4Ig and CD28Ig disrupt binding to B7-1. Site-directed mutant fusion proteins were produced in transiently transfected COS cells, quantitated and tested for their ability to bind to B7-1. (A) CTLA4Ig mutants binding to stably transfected, ethanol-fixed B7-1+ CHO cells. (B) CD28Ig mutants binding to B7-1Ig immobilized on 96-well plates. Fusion protein quantitations were determined as described in Materials and Methods. Binding data are expressed as the average of duplicate determinations and replicates differed from the mean by <10%. The data are representative of at least two experiments.

M99A, P101A, P103A, and Y104A showed an almost complete loss of binding. Equivalent results were obtained when B7-1 transfected CHO cells were incubated with mutant CTLA4Ig fusion protein, followed by anti–human Ig-FITC, and binding was measured using flow cytometry. CD28Ig MYPPPY mutants P103A and Y104A did not bind detectably to recombinant B7-1mIg immobilized on plastic wells (Fig. 2 b). These results demonstrate a critical role for the MYPPPY motif in both CTLA4Ig and CD28Ig binding to B7-1.

Characterization of CTLA-4/CD28 Homologue Mutant Proteins. Since the MYPPPY motif is common to both CTLA-4 and CD28, it cannot account for the observed differences in B7-1 binding to CTLA4Ig and CD28Ig. To assess the contribution of less conserved residues in CTLA4Ig to B7-1 binding, a series of homologue scan mutants were prepared in which regions of CD28Ig were replaced with the homologous regions from CTLA4Ig. Those hybrid mutants that bound more strongly to B7-1 than CD28Ig would indicate that the CTLA-4 region(s) included in the hybrid played a specific role in binding. Regions adjacent to the MYPPPY hexapeptide in the CDR3-like loop as well as the CDR1- and CDR2-like loops of CTLA-4, were tested. 16 hybrid cDNA constructs were prepared (Fig. 3 and Table 2), sequenced, transfected into COS cells, and tested for binding activity. In an indirect immunoassay, most homologue mutant proteins bound anti-CD28 mAb 9.3 to a level similar to wild-type CD28Ig, indicating that these proteins had maintained structural integrity. The electrophoretic mobility of

the hybrid fusion proteins was compared by SDS-PAGE, including Western blotting analysis (Fig. 4). Under nonreducing conditions, the chimeric proteins migrated as species with relative molecular masses between ∼100 and ∼150 kD, with some proteins having an additional species with relative molecular masses between ∼50 and 70 kD. Under reducing conditions, each hybrid fusion protein migrated with a molecular mass between that of CTLA4Ig (∼50 kD) and CD28Ig (∼65 kD) (data not shown). These fusion proteins exist as mixtures of monomers and disulphide-linked dimers despite mutagenesis of the cysteine residues in the hinge region of the Fc (5). Since four of the conserved cysteine residues in CTLA-4 and CD28 are likely to form intrachain disulphide bonds (see below, Fig. 6), dimerization of the fusion proteins was probably due to cysteine 123 in CTLA-4Ig. This is supported by a recent report (26) of a chicken CD28 homologue that lacks this cysteine and also does not form disulfide linked homodimers during SDS-PAGE.

Binding of CTLA4Ig/CD28Ig Homologue Mutant Proteins to B7-1. Hybrid fusion proteins were tested for their ability to bind to B7-1 by the indirect cell binding immunoassay (Table 1). Under these conditions, the binding of CD28Ig to B7-1 CHO cells is not easily detectable at fusion protein concentrations <1.0 µg/ml (Fig. 5 A). Hybrids HS1 and HS3 showed no detectable binding to B7-1 (Table 1) but the hybrids HS2 and HS4 bound approximately two and a half orders of magnitude better than CD28Ig (Fig. 5 A), ∼10% of wild-type CTLA4Ig binding (Table 1). Hybrids HSA4-A and HS4-B did not bind to B7-1 as well as had HS4 but still bound

Table 1. *Relative Specific Binding Activities of CTLA4Ig and Homologue Scan Mutants*

Mutant	Percentage of B7-1 binding activity
CTLA4Ig	100
CD28Ig	<0.1
CTLA4Ig	
M99A	<0.1
Y100A	3
P101A	<1
P102A	3
P103A	<0.1
Y104A	<0.1
HS1	<0.1
HS2	10
HS3	<0.1
HS4	12
HS4-A	5
HS4-B	5
HS5	<0.1
HS6	10
HS7	30
HS8	32
HS9	5
HS10	17
HS11	13
HS12	5
HS13	7
HS14	<0.1

Specific binding activities were determined for each of the indicated fusion proteins. The concentration of a fusion protein required to give an arbitrary A$_{450}$ the same as that found for CTLA4Ig was determined from the linear region of binding curves (see Figs. 2 A and 5 A) and expressed as a percentage of specific binding activity of CTLA4Ig. Values represent the average of three determinations from each binding curve; replicates differed from the mean by <20%. Values are representative of four experiments.

Figure 3. Maps of CTLA4Ig/CD28Ig homologue scan mutants. Conserved Ig-like cysteine residues (C) at positions 21 and 94 and the membrane proximal cysteine 123 are shown, as is the position of the MYPPPY motif. (*Open areas*) CD28 sequence; (*filled areas*) CTLA-4 sequence; (*stippled areas*) beginning of IgG Fc (also refer to Table 2).

greater than 20-fold better than CD28Ig (Fig. 5, *A* and *B*, and Table 1). These results demonstrated that the COOH-terminally extended region of the CDR3-like loop of CTLA-4 contributed to B7-1 binding.

To test whether the CDR1-like loop of CTLA-4 was also important for B7-1 binding, mutant HS6 was prepared (Fig. 3). This hybrid bound B7-1 comparable to HS4 (Fig. 5 *a*), whereas the reciprocal hybrid, HS5, did not bind B7-1 (Table 1). Thus, the CDR1-like region of CTLA-4 also played a role in B7-1 binding but this region alone could not sustain strong binding. Several hybrids were constructed to test whether the CDR1-like loop of CTLA-4 could support strong binding associated with the COOH-terminally extended
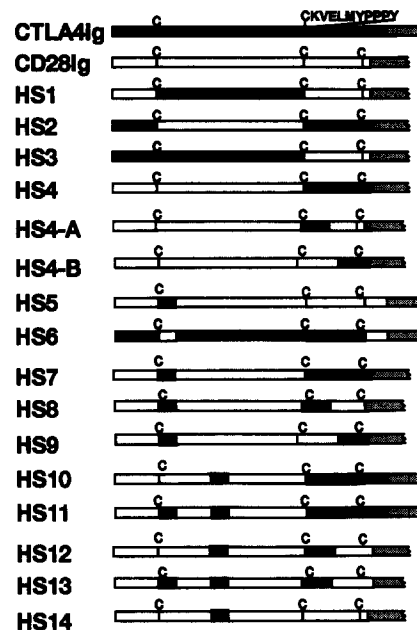
CDR3-analogous region of CTLA-4 (Fig. 3). Hybrid HS7 showed a nearly threefold increase in binding to B7-1 relative to HS4, representing ~30% of wild-type CTLA4Ig binding (Table 1). Similarly, hybrid HS8 bound at ~32% of wild-type binding (Fig. 5 *A* and Table 2), ~sixfold better than HS4-A. However, grafting the CTLA-4 CDR1-like region onto HS4-B to make the hybrid HS9 did not improve binding to B7-1 (Table 1). These results demonstrated that the CDR1-like loop could augment strong binding to B7-1 but that residues immediately adjacent to the MYPPPY motif were necessary for CDR1 augmentation.

Another series of homologue mutants was constructed to assess the role of the CDR2-like region of CTLA-4 in binding to B7-1. Hybrids HS10 and HS12 did not bind B7-1 better than HS4 or HS4-A (Fig. 5 *b*), whereas HS14 did not bind measurably to B7-1 (Table 1). The presence of all three CDR-like regions of CTLA-4 in hybrids HS11 and HS13 did not augment binding to B7-1 (Table 1). Thus, in contrast to the CDR1- and CDR3-like regions, the CDR2-like region does not significantly contribute to B7-1 binding.

CTLA-4 Model. Fig. 6 shows a schematic representation of a molecular model of the extracellular domain of a CTLA-4 monomer. Three-dimensional profile analysis indicated that the CTLA-4 sequence is, overall, consistent with an Ig V-fold. Due to the low sequence identity to any IgSF V-like molecule with known three-dimensional structure, the "resolution" of the model is limited, with some of the structural features of the CTLA-4 V-like domain, including the conformations of the CDR-like regions, only approximated. Residue Val117 represents the last amino acid of the CTLA4 Ig-
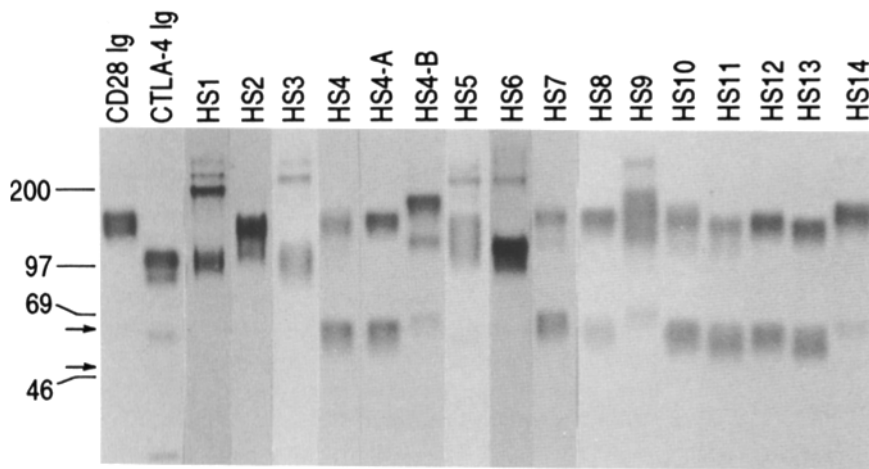
Table 2. *CTLA4Ig/CD28Ig Homologue Mutant Junction Sequences*

Mutant				
HS1	-21CKYasp26-		-94ckvEVM99-	-123CPSDQE-
HS2	-19fvcKYS24-		-94CKIeIm99-	-123cpdDQE-
HS3			-94ckvELM99-	-123CPSDQE-
HS4			-94CKIeIm99-	-123cpdDQE-
HS4-A			-94CKIeIm99-	-113tqiHVKGKHLCPSDQE-
HS4-B				-113TIlyvidpepcpdDQE-
HS5	-21CKYaspgkateFRA34-			-123CPSDQE-
HS6	-21ceySYNLFSREvrv34-			-123cpdDQE-
HS7	-21CKYaspgkateFRA34-		-94ckIeIm99-	-123cpdDQE-
HS8	-21CKYaspgkateFRA34-		-94CKIeIm99-	-113tqiHVKGKHLCPSDQE-
HS9	-21CKYaspgkateFRA34-			-113TIlyvidpepcpdDQE-
HS10		-47VCVatymtgneLQV60-	-94CKIeIm99-	-123cpdDQE-
HS11	-21CKYaspgkateFRA34--	47VCVatymtgneLQV60-	-94CKIeIm99-	-123cpdDQE-
HS12		-47VCVatymtgneLQV60-	-94CKIeIm99-	-113tqiHVKGKHLCPSDQE-
HS13	-21CKYaspgkateFRA34-	-47VCVatymtgneLQV60-	-94CKIeIm99-	-113tqiHVKGKHLCPSDQE-
HS14		-47VCVatymtgneLQV60-	-94CKIeIm99-	-123CPSDQE-

Junction sequences of the CTLA4Ig/CD28Ig hybrid fusion proteins. Amino acids are denoted by their single letter code with those in uppercase being CD28 residues, those in lowercase being CTLA-4 residues, and those in bold upper case being human IgG1 residues. Amino acids are numbered as in Fig. 1.

Figure 4. SDS-PAGE analysis of CTLA4Ig/CD28Ig homologue mutant fusion proteins. Aliquots of medium from transfected COS cells containing equivalent amounts of Ig fusion protein (0.4 μg as determined by immunoassay) were subjected to immunoprecipitation, SDS-PAGE under nonreducing conditions, and Western blotting analysis as described in Materials and Methods. (*Left*) Relative molecular mass of protein standards. (*Upper* and *lower* arrows) Relative mobilities under reducing conditions of CD28Ig and CTLA4Ig monomers, respectively.

like domain. The conformation of the region between Val117 and the membrane-proximal Cys123, which is predicted to form the CTLA-4 homodimeric disulfide bond (15), is uncertain and schematically represented in extended conformation. Although our data show that CTLA-4 is a disulfide-linked homodimer (17), we currently have no information on how the monomer subunits interact. The CTLA-4 model illustrates the Ig-like disulfide bond between residues Cys21 and Cys94 and also suggests the presence of an additional (non-Ig) disulfide bond between residues Cys48 and Cys68. Two possible N-linked glycosylation sites in CTLA-4 map to solvent-exposed positions of the Ig β-strand framework regions. The assignment of CTLA-4 residues to CDR-like

regions is shown in Fig. 1. As is generally true for an Ig V-like structure, this model shows that the CDR1-like loop is spatially adjacent to the CDR3-like region but that the CDR2-analogous loop is more distant. This suggests how residues within the CDR1-analogous region modulate the effects of the CDR3-analogous region on CTLA-4Ig binding, as shown by binding activities of homologue scan mutants.

Discussion

We have demonstrated that the MYPPPY hexapeptide is required for binding of CTLA4Ig and CD28Ig to B7-1. This hexapeptide represents the most notable sequence motif
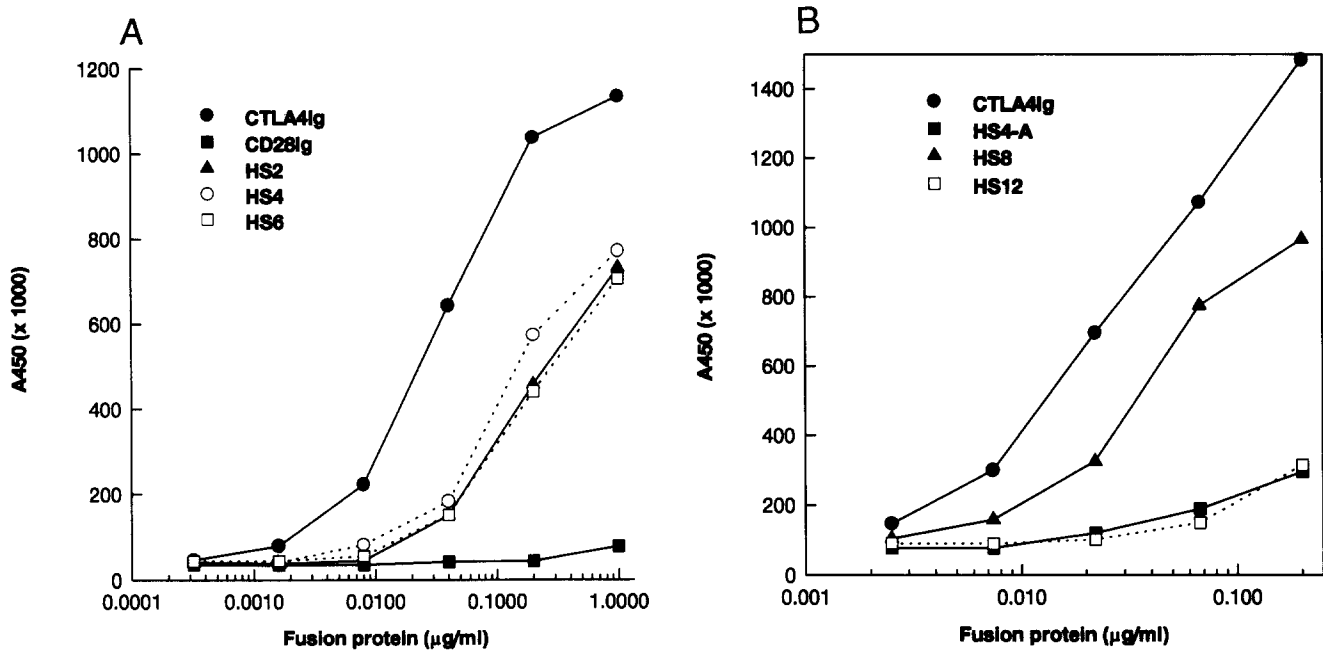

Figure 5. CTLA4Ig/CD28Ig homologue mutant fusion proteins demonstrate involvement of CDR-analogous regions in strong binding to B7-1 CHO cells. Mutants were assayed as described in Fig. 2 *A*. (*A*) Demonstration of the role of the CDR3-analogous region of CTLA4Ig in strong binding to B7-1. (*B*) Demonstration of the role of the CDR1-analogous region of CTLA4Ig in augmenting strong binding to B7-1. Data are expressed as the average of duplicate determinations and differed from the mean by <10%. The data are representative of at least four experiments.
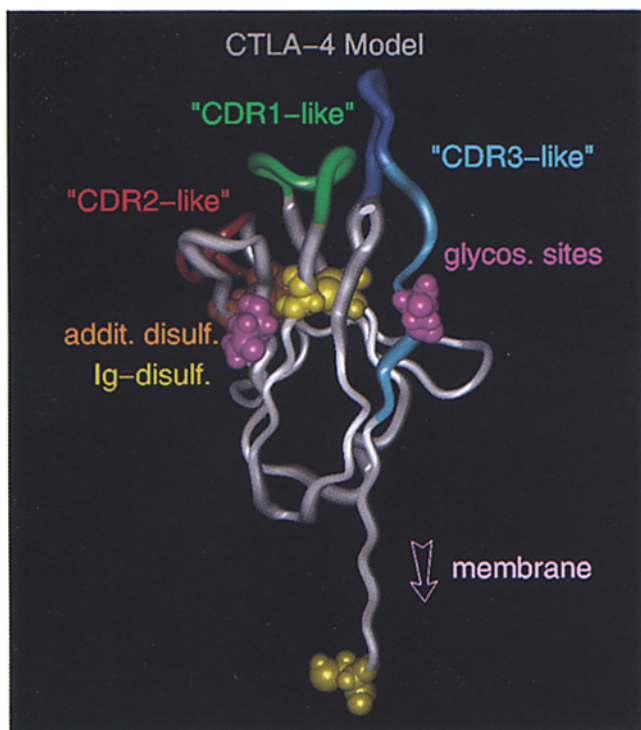
Figure 6. Approximate molecular model of monomeric CTLA-4 Ig V-like extracellular domain. The model was constructed as described in Materials and Methods. Highlighted are the three CDR-analogous regions; CDR1-like (*green*), CDR2-like (*red*) and COOH-terminally extended CDR3-like (*blue*) with the MYPPPY hexapeptide colored in dark blue. Cysteine residues involved in disulfide bonding are shown (*orange* and *yellow*), as are potential N-linked glycosylation sites (*magenta*).

common to CTLA-4 and CD28. Alignment of CTLA-4 and CD28 on an Ig-V fold shows that the MYPPPY motif maps to the CDR3-like loop region in these molecules and is, therefore, predicted to be solvent exposed. The MYPPPY motif is distinctly hydrophobic and conformationally constrained due to three adjacent proline residues. The rigorous conservation of this unusual motif within the CTLA-4/CD28 family suggested a functional significance (15) which we have demonstrated by site-directed mutagenesis analysis.

Since both CTLA4Ig and CD28Ig have identical MYPPPY motifs, other elements in the extracellular domain of CTLA4Ig must account for its stronger avidity in binding to B7-1. We have shown by homologue scan mutagenesis that the COOH-terminally extended CDR3-analogous region in CTLA-4 is important in B7-1 binding. This region has very little sequence conservation in the CTLA-4/CD28 family. CTLA-4 contains a number of hydrophobic residues, but the equivalent region in CD28 features several charged residues and is one residue longer (Fig. 1). These differences presumably account for the increased binding ability of soluble hybrids bearing the COOH-terminally extended CDR3-like region of CTLA-4. Due to the method of construction, hybrids including HS4 and HS4-A have a leucine at position 98 instead of valine seen in CD28. In control experiments, this change was not responsible for improved binding.

Another nonconserved region in CTLA-4 which contributes to B7-1 binding is the CDR1-like loop. The sequence of the CDR1-like loop is highly variable in the CTLA-4/CD28 family (Fig. 1). Homologue scan mutagenesis experiments showed that the CDR1-like loop of CTLA4Ig further enhanced the effects of the CDR3-analogous region. In contrast, inclusion of the CTLA-4 CDR2-like region either alone or in combination with the CDR3-like region did not further improve binding. It will be important to determine whether similar CDR-mediated molecular interactions characterize the binding between CTLA-4/CD28 and the recently described B7-2 molecule. B7-1 and B7-2 are only ~25% homologous but both molecules bind to CTLA4Ig and CD28Ig and deliver costimulatory signals to T cells. Binding studies utilizing site-directed and homologue scan mutants described here should establish whether these functionally similar molecules bind similarly to their common receptors.

Hybrid molecules which included the CTLA-4 CDR3- and CDR1-like regions did not fully restore wild-type CTLA4Ig binding activity. This suggests that other residues in CTLA-4 also contribute to binding. Alternatively, the levels of full binding activity may indicate that hybrid molecules bear structural perturbations that reduced binding activity, though this possibility seems unlikely as mAb 9.3 binding to most of the hybrids was comparable with wild-type CD28Ig (data not shown).

The indirect binding assays we have employed in these studies show a greater difference in binding activity between CTLA4Ig and CD28Ig than was reported for the avidity constants of the molecules (5). The relationship of activity in indirect binding assays to binding avidity is unclear. Indirect binding is measured in a two-step assay and therefore involves more than one equilibrium reaction. Also, the length of time during second-step incubations would tend to favor detection of binding reactions having slow off rates. Attempts to relate the binding differences reported here to equilibrium or kinetic constants are in progress.

CD28 exists as a homodimer (13). Our data indicate that CTLA-4 is also a homodimer (24), although a recent report by Lindsten et al. (27) suggested that CTLA-4 monomers rather than homodimers were present on the T cell surface. The structural basis for CTLA-4 dimerization is unclear since, like CD28, it does not display the conserved β-bulge sequence motifs that mediate Fv-like dimerization (28–30). It is also unclear how dimerization affects binding avidity and how many binding sites there are per homodimer. Despite repeated efforts, we have been unable to express monomeric soluble forms of either CTLA-4 or CD28. In preliminary experiments we have expressed a soluble form of the extracellular domain of CTLA-4 without the Fc tag. Preparations of this molecule, when run on SDS-PAGE, consist largely of disulfide-linked homodimers and, upon size exclusion column chromatography, migrate with a molecular weight consistent with that of a homodimer. This suggests that homodimerization is an essential property of CTLA-4. It will be important to determine how dimerization of CTLA-4 and CD28 affect B7 binding.

Fusion protein oligomerization might contribute to B7-1 binding by homologue scan mutants, but we think this is unlikely. We have observed no direct correlation between the degree of fusion protein aggregation and binding ability. We previously reported that CTLA4Ig and CD28Ig form higher molecular weight aggregates in solution with the lower avidity CD28Ig existing as larger species (5, 16). Preliminary experiments with size exclusion chromatography showed that homologue scan mutants also existed as oligomers, but that the degree of oligomerization did not correlate with binding activity. Equivalent results were obtained when the binding assays for the homologue scan mutants HS4, HS7-8, and HS10-11 were reversed such that purified hybrid fusion pro-tein was immobilized and assayed for soluble B7-1mIg binding. Immobilization would be expected to neutralize effects on binding of hybrid fusion protein oligomerization. Finally, the specific activity of B7-1 binding was constant for different sized oligomers of fusion protein HS7 size fractionated on a TSK G3000SW column (TosoHaas Corporation, Montgomeryville, PA). Thus, the relative abilities of homologue scan mutants to bind B7-1 were not dependent upon protein oligomerization.

The role of CDR-like regions in mediating the strong binding interaction between CTLA4Ig and B7-1 is in some ways analogous to the use of hypervariable regions by antibodies when binding to antigen. CDR-analogous loops of other IgSF members, or parts of these loops, are also important binding determinants in the CD8–MHC class I interaction (31, 32) and in the CD4–MHC class II interaction (33, 34). More recently, a peptide mimetic of the CDR3-like loop of CD4 was shown to inhibit the pathological role of CD4^{+} helper T cells in experimental allergic encephalomyelitis (35). Results presented here suggest that this approach should be evaluated as a means of obtaining peptide inhibitors of the molecular interaction between CTLA-4/CD28 family members and B7-related molecules.

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