Identification of Residues in the V Domain of CD80 (B7-1) Implicated in Functional Interactions with CD28 and CTLA4

By Christine A. Fargeas,* Alemseged Truneh,‡ Manjula Reddy,‡ Mark Hurle,§ Raymond Sweet,‡ and Rafick-Pierre Sékaly*‖¶

*From the *Laboratoire d'Immunologie, Institut de Recherches Cliniques de Montréal, Québec, Canada H2W 1R7; Departments of ‡Molecular Immunology and §Macromolecular Sciences, SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania 19406-2799; ‖Département de Microbiologie et Immunologie, Faculté de Médecine, Université de Montréal, Québec, Canada H3C 3J7; and ¶Department of Microbiology and Immunology, School of Medicine, McGill University, Montréal, Québec, Canada H3A 2B4*

Summary

The CD80 (B7-1) molecule is a 45–60-kD member of the immunoglobulin superfamily that is expressed on a variety of cell types of haematopoietic origin. CD80 can provide a critical costimulatory signal to T cells by interacting with the T cell surface molecule CD28. CD80 also binds to the CD28-related molecule CTLA4, which is expressed on activated T cells. Recently, additional ligands of CD28 and CTLA4 have been described in mice and humans. One of them, CD86 (B-70 or B7-2) was characterized at the molecular level. Although similar in predicted structure to CD80, it is distantly related in amino acid sequence. In this study, human CD80 mutants were generated and tested for their ability to maintain the interaction with CD28 leading to adhesion and enhanced IL-2 production. Two hydrophobic residues in the V-like domain of CD80 were identified as critical for binding to CD28 and are also important for the interaction with CTLA4. These residues are adjacent to the epitope of the BB1 antibody, which inhibits CD28–CD80 interactions. One of these residues, Y87, is conserved in all CD80 and CD86 cloned from various species. These results begin to unravel the structural requirements for binding to CD28 and CTLA4.

During the onset of an immune response, T cells require two distinct signals for efficient activation. The first signal is antigen-specific and is delivered through the TCR upon engagement with the antigenic peptide–MHC complex while the second signal is mediated by the interaction of the T cell surface molecule CD28 with ligands expressed on APC (1, 2). Its interaction with CD80 (B7-1) leads to an increase in cytokine production and T cell proliferation (3). Evidences on the role of CD80/CD28 interaction in antigen-specific responses have accumulated in the recent past (for review see references 4 and 5). This interaction is critical since absence of costimulatory molecules on the surface of APCs leads to the induction in T cells of a state of anergy (reviewed in references 4 and 5). CD80, as expected for a costimulatory molecule, is expressed on different cell types involved in antigen presentation (4). CD80 also has the capacity to interact with CTLA4 (6), a CD28-related molecule expressed primarily after T cell activation (7). This interaction occurs with a 20-fold higher affinity compared to CD80–CD28 interactions, as demonstrated through the use of a soluble form of this molecule consisting of the extracellular domain of CTLA4 fused to the Fc domain of IgG1 (6).

Structurally, human CD80 (huCD80)[1] is a 262-residue type I transmembrane glycoprotein of the Ig superfamily (IgSF) (8). CD80 is predicted to be present at the cell surface as a highly glycosylated single chain molecule with an extracellular region consisting of two Ig-like domains: an NH_2-terminal V-like domain followed by a C-like domain. Murine CD80 (muCD80) shares 44% identity with its human counterpart and recognizes huCD28 as shown by the ability of murine cells expressing CD80 to costimulate human $CD4^+$ T cells (9). Recently, a molecule distantly related to CD80, namely B70 or B7-2, now identified as the CD86 differentiation antigen (10), was shown in both humans and mice to bind CTLA4Ig and to provide costimulatory signals through CD28 (11–13). Like CD80, it is an IgSF member with a single V-like and a single C-like extracellular domain, but human CD80 and B70 (B7-2) only show 24 and 30% amino acid identity in those two domains, respectively.

[1] *Abbreviations used in this paper:* CAM, calcein-AM; hu, human; IgSF, immunoglobulin superfamily; mu, murine.

To define the CD28 recognition site on huCD80, single and multiple substitutions were introduced into predicted exposed sites in the V- and C-like domains with particular emphasis on residues that are conserved between muCD80 and huCD80. The possible role of carbohydrates in this interaction was also investigated by mutations at conserved potential N-linked glycosylation sites. These mutants were assessed for their effects on CD28-mediated adhesion to CD80-transfected L cells and on stimulation of IL-2 production by $CD28^+$ T cells. Our results show that two hydrophobic residues at the base of a distal loop in the V domain of CD80 are critical for the interaction with both CD28 and CTLA4.

Materials and Methods

Antibodies. The anti-human CD80 mAbs L307 (IgG1) (14), 2D10 (IgG1), and BB1 (IgM) (15) were kindly provided by Dr. L.L. Lanier (DNAX Research Institute, Palo Alto, CA), Dr. D. Olive (Institut Paoli Calmette, Marseille, France), and Dr. M. Tremblay (CHUL, Quebec, Canada), respectively. The anti-human CD80 mAb104 (16) was purchased from AMAC (Westbrook, MA). The anti-murine CD80 (B7-1) mAb 16-10A1 (17) was a gift from Dr. H. Reiser (Dana Farber Cancer Institute, Boston, MA). The anti-human CD28 L293 was purchased from Becton Dickinson & Co. (Mountain View, CA).

Cell Culture and Transfection. L cells were grown in DMEM plus 4 mM dextrose (Sigma Chemical Co., St. Louis, MO), essential and nonessential amino acids, 1 mM sodium pyruvate. The Raji B cell line, Jurkat T cell line, as well as the IL-2–dependent T cell line CTLL-2, with the addition of 10 U/ml rIL-2 (Cetus Corp., Emeryville, CA), were maintained in RPMI 1640. All culture media were supplemented with 10% FCS, 2 mM L-glutamine, 10 μM 2-ME (Sigma Chemical Co.), and 20 μg/ml gentamicin (all from GIBCO BRL, Bethesda, MD, unless otherwise stated). L cells were cotransfected with 10 μg of wild-type or mutated huCD80 cDNA and 1 μg of pSVneo plasmid carrying the neomycin resistance gene using the calcium phosphate coprecipitation technique (18). Stable transfectants were selected in 2 mg/ml of G418 (GIBCO BRL). Homogeneous populations of wild-type or mutated CD80-transfected cells expressing comparable levels of the different forms of CD80 were obtained by aseptic cell sorting on a FACStar® Plus (Becton Dickinson).

Flow Cytometry Analysis. Transfected cell populations were stained with the murine anti-huCD80 mAbs L307, 2D10, mAb104, and BB1. FITC-conjugated goat antimurine IgG or FITC-conjugated goat antimurine IgM were used as secondary antibodies. In negative controls, the primary antibody was omitted. Cells were analyzed by flow cytometry on a FACScan® (Becton Dickinson). Live cells were gated by light scatter. A minimum of 10,000 events was accumulated for each histogram. Fluorescence was accumulated on a four-decade log scale.

CD80 cDNA Cloning and Mutagenesis. Total RNA was isolated from the Raji cell line using RNAzol (Cinna/Biotecx Laboratories, Friendswood, TX) and huCD80 cDNA was obtained by reverse-transcription PCR (19). The first-strand cDNA was reverse transcribed using the CD80-specific antisense primer. The oligonucleotides (forward: 5′ CCTAAGCATCTCGAGCCATGGGCCACACAC 3′ and reverse: 5′ GCTTCTGCGGATCCTGTTATACAGGGCGTACAC 3′, containing XhoI and BamHI restriction sites, respectively) were designed to amplify the entire coding sequence (nucleotides 316–1,187) (8). PCR products were subcloned in pBluescriptII SK^- vector and sequenced, using double-strand plasmid as template (20), before subcloning into the eukaryotic expression vector pCDLSRα (21). Site-directed mutagenesis of the human cDNA was performed using the PCR overlap extension technique (22). Briefly, 50 ng of pCDLSRα-CD80 plasmid, 50 pmol of oligonucleotide primers containing the mutations at the positions listed in Table 1, and the 5′ or 3′ oligonucleotide primers used previously to clone the CD80 cDNA were used to generate two PCR fragments with complementary ends that contain the mutated codons. DNA fragments were excised from low melting-temperature agarose gel after electrophoresis of the PCR products. Overlap extension was carried out in low melting-temperature agarose using one tenth of each fragment and only the 5′ or 3′ primer to generate a full-length fragment. Depending on the position of the mutations, a 300-bp XhoI-EcoRV or 580-bp EcoRV-BamHI digestion fragment was subcloned in the pCDLSRα-CD80 plasmid from which the corresponding fragment was removed by digestion with the appropriate restriction enzymes. Oligonucleotides linking the sequence corresponding to the CD80 residues 137–142 to that sequence corresponding to residues 240–245 were used in a similar two-step PCR procedure to generate the deletion mutant ΔC. The 283-bp EcoRV-BamHI digestion fragment was subcloned as described for the other mutants. All PCR-generated cDNA and ligation products were entirely sequenced.

Cell–Cell Adhesion Assay. CD80-transfected L cells (and untransfected as negative control) were seeded in triplicate in flat-bottom 96-well microtiter plates at 5.10^4 cells per well the day before the assay. The next day, $CD28^+$ Jurkat T lymphoma cells were loaded with calcein-AM (CAM) in Dulbecco's PBS containing 0.1% BSA by passive diffusion across viable cells membrane for 1 h, washed three times with PBS, and added to the plates at 4.10^5 cells per well. After 30 min incubation at room temperature, nonadherent Jurkat cells were removed by repeated washes with ice-cold Ca^{2+}, Mg^{2+}-free PBS. The remaining cells were lysed in 2% Triton X-100 and fluorescence was determined at 485–538 nm using a Labsystems Fluorskan II (Flow Laboratories Inc., McLean, VA) or Cytofluor 2300 System (Millipore Corp., Bedford, MA) plate readers. For each sample, the percentage of Jurkat cells binding was calculated as follows: percentage of binding = [amount of fluorescence/amount of fluorescence without washing (total input)] × 100. In mAb-inhibition experiments, muCD80 mAb 16-10A1, huCD80 mAb L307, huCD28 mAb L293, and their respective controls were added to the wells at 10 μg/ml 10 min before the addition of CAM-loaded Jurkat cells.

Functional Assay. Untransfected and transfected L cells were used as costimulators of PHA-activated Jurkat cells. Each assay was performed in triplicate as follows: 5×10^4 Jurkat cells were cocultured with $2{,}500$–10^4 L cells in the presence of 1 μg/ml PHA. This concentration had no effect on the subsequent CTLL proliferation assay used to determine IL-2 production (data not shown). After 20 h, supernatants were collected. In mAb inhibition experiments, cocultures were performed as described above in the presence of the indicated antibodies at 2 or 6 μg/ml. IL-2 production was measured using the IL-2–dependent CTLL-2 line. Briefly, serial twofold dilutions of the coculture supernatants were added to 4,000 IL-2–dependent CTLL-2 cells. These cultures were pulsed with 1 μCi [^{3}H]thymidine for 6 h, 42 h after initiation of the culture. Radioactivity was quantified on a β counter (Betaplate; Pharmacia LKB, Uppsala, Sweden) and counts per minute were converted to IL-2 units using a standard curve of recombinant human IL-2 (Cetus Corp.).

CTLA4-Ig Binding Assay. For each transfectant, 2.10^5 cells were incubated with serial dilutions of CTLA4-Ig (6) in PBS con-

taining 5% FCS for 30 min on ice, washed with PBS-FCS, and incubated subsequently with a saturating concentration of FITC-conjugated goat anti-human IgG mAb (Fc specific) (Caltag Laboratories, San Francisco, CA) for an other 30 min on ice. After washing, the cells were analyzed on a FACScan® as described for flow cytometry analysis.

Results

Generation and Expression of CD80 Mutants. To study the molecular basis of the interaction of CD80 with its counter-receptors CD28 and CTLA4, a panel of 24 CD80 mutants were generated by PCR and stably transfected into mouse L cells. The design of the mutants was initially guided by the alignment of the human CD80 amino acid sequence with its murine homologue (Fig. 1) and by the general structural properties of Ig-like domains (i.e., residues conserved throughout the IgSF that are important to the Ig fold, such as the cysteines in the B and F strands, the tryptophane in the C strand, or the hydrophobic residues two positions ahead of the cysteines, as well as conserved patterns characteristic of the V or C1 set were not substituted) (23). Thus, for example, in the V domain, two stretches of amino acids (63–68 and 84–91) are identical in mouse and huCD80. The first stretch (residues 63–68) contains structural features present in all V-like domains and was therefore not chosen as a target for mutagenesis. The second stretch (positions 84–91) contains residues that diverge among V-like domains, and it was considered as a more likely candidate for mutagenesis. The choice of the residues to be substituted was targeted to the regions of the V- and C-like domains predicted to be exposed based on the structure of related molecules. Since additional ligands for CD28 and CTLA4 (hu- and muCD86) were characterized at the molecular level during the process of this study, this information was integrated into the design of additional mutants. When all sequences are aligned, it appears that among the consensus residues, most of them are critical to the Ig fold and are less likely to contribute directly to the interaction with CD28 or CTLA4 (Fig. 1 *a*). The positions and natures of these mutations are listed in Table 1. Biochemical analysis has shown that both mu- and huCD80 are heavily glycosylated molecules. Mutants were also designed to remove seven out of eight potential glycosylation sites of the CD80 molecule (M7, M8, M10, M13, M16, and M20–M23).

The expression and conformational integrity of CD80 mutants in transfected L cells were first examined by flow cytometry using four mAbs to huCD80. All but five mutants showed good binding to each of the mAbs (Table 1), indicating that the overall structure of the CD80 molecule was not altered by the different substitutions. The most interesting exception was M2 (M77A, D80A) in the CDR2-like region, which completely abrogated BB1 binding while partially affecting the binding of mAb 104. This result suggests that the epi-

a V-like domain

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          35                                *                          73
huCD80    V.IHVT...K  EVK..EVATL  SCGHNVSVEE  LAQTRIYWQK  .....EKKMV
muCD80    -DEQL...S-  S--..DKVL-  P-RY-SPH-D  ESED------  .....HD V

VL        IVMTQSPSSL  SVSPG RVTI  --RASQNG..  ..SSYLA-YQ  QKPGQSP-LL
            b b                b   b b            bbbb        b  b
VH        -QLQESG.GG  LVKPGGSLK-  --KASGFTFT  ..SYYMH-VR  QRPGKGLEWI
                                  ←-------CDR1------->

                          *           *                              118
huCD80    LTMMS..GDM  NIWPEYKNRT  IFD...ITNN  LSIVILALRP  SDEGTYECVV
muCD80    -SVIA..-KL  KV--------  LY-...N-.T  Y-LI--C-VL  --R   S
                          *           *
VL        TY.....-AS  -LASGVPD-F  SGSG..SGTD  FTLT-SSVEA  E-LAT-Y-QQ

VH        GYINPGN ST  -YNEKFKG-F  TITRDKSSST  AYLQLSSLTS  E-TAV-Y-AR
          ←-------CDR2------->                                      ←

                                  139
huCD80    LKYEKDAFKRE  HLAEVTLSVK
muCD80    Q-K-RGTYEVK  ---L-K--I-

VL        WSSSP....LT  FCCCTK-EI-
                       bb b  b
VH        DY-YGS..FDY  WGQGT-VT-S
          CDR3------>
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b C-like domain

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                                                          *      189
huCD80    ADFPTPSISD  FEIPTSNIRR  IICSTSGGFP  EPHLSWLENC  EELNAIINTTV
muCD80    ---S--N-TE  SGN-SADTK-  -T-FA-----  K-RP------  R--PG----I

                          *     *                 *      *          241
huCD80    SQDPETELYA  VSSKLDFNMT  TNHSFMCLIK  YGHLRVNQTF  NWNTTKQEHF  PD
muCD80    -----S---T  I--Q----T-  R--TIK----  --DAH-SED   T EKPPEDP.  --
                          *   *
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Figure 1. Comparison of human and murine CD80 extracellular amino acid sequences. The (*a*) V-like and (*b*) C-like domains are, respectively, 46 and 55% identical. The numbering is that of the human CD80 sequence starting with the first methionine as 1. Residues conserved in four out of five B7 family members appear in boldface in the human CD80 sequence while consensus residues for IgSF members are in italics. Dashes in the other sequences indicate residues identical to human CD80. Predicted N-linked glycosylation sites are marked with asterisks. (*a*) An alignment of the V-like human and mouse CD80 sequences with consensus variable domains from Ig light and heavy chains. Because the alignment between the CD80 sequences and Ig sequences is low (<30% identity), the alignment was performed manually. Special emphasis was given to aligning those residues that are hydrophobic in the CD80 sequences and those that are buried in known Ig structures. Those residues whose side chains have an average of >80% of their surface buried in both Ig domains are indicated by the letter b. The locations of the CDRs in the Ig domains are indicated.

Table 1. *Expression of CD80 Mutants in L Cell Fibroblasts*

	MUTATIONS	REGION	mAb STAINING			
			L307	2D10	104	BB1
		V-like domain				
M1	VEE (56-58) AQQ	CDR1	+++	+++	+++	+++
M2	MSGD (77-80) ASGN	CDR2	++++	+++	++	-
M3	W 84 A	C" - D loop	+++	+++	+++	+++
M4	W 84 F	C" - D loop	+++	+++	+++	+++
M5	E 86 A	C" - D loop	+++	+++	+++	+++
M6	Y 87 A	C" - D loop	+++	+++	+++	+++
M7	YKN (87-89) FKQ	C" - D loop	+++	+++	+++	+++
M8	YKN (87-89) FAQ	C" - D loop	+++	+++	+++	+++
M9	K 88 A	C" - D loop	+++	+++	+++	+++
M10	N 89 A	C" - D loop	+++	+++	+++	+++
M11	I 91 A	C" - D loop	++++	+++	++++	+++
M12	DIT (94-96) NIA	D - E loop	++++	+++	++++	++++
M13	N 98 Q	D - E loop	+++	+++	+++	+++
M14	KYEKDA (120-125) RFSGST	CDR3	+++	+++	+++	+++
M15	H 130 A	G strand	+++	++	+++	+++
		C-like domain				
Delta C	deletion (P 143-F 239)	whole domain	-	-	-	-
M16	N 186 Q	D strand	++	++	+++	+++
M17	SQD (190-192) AAN	D - E loop	+++	+++	+++	+++
M18	ETE (194-196) AAQ	D - E loop	+	+	+	+
M19	H 130 A, Y 198 H	D - E loop	-/+	-/+	-/+	-/+
M20	N 207 Q	E - F loop	+++	+++	+++	++
M21	N 207 Q, T 228 A	E - F loop & G strand	+++	+++	+++	+++
M22	N 207 Q, N 226 A	E - F loop & G strand	++	++	+++	+++
M23	N 211 Q	E - F loop	+++	++	+++	+++

The huCD80 mutants are described, using the one-letter code, by the wild-type sequence followed by the position and the amino acid substitution. Residues conserved in muCD80 and huB70 appear in boldface and underlined, respectively. mAb binding was analyzed by flow cytometry. For each CD80 mutant, staining was scored as relative means of fluorescence compared to wild-type staining with the same antibody at saturating concentrations as follows: −, no staining above background; + + + +, >200%; + + +, 50–200%; + +, 25–50%; +, 5–25%; −/+, <5%.

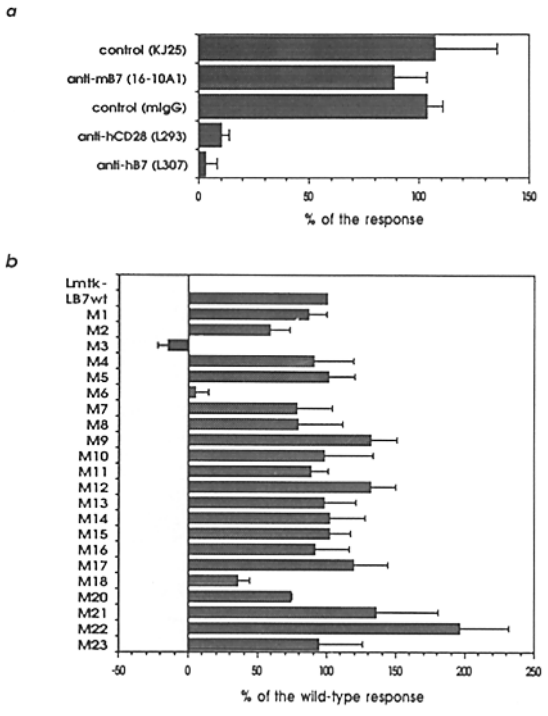

Figure 2. Adhesion interaction of CD80 mutants with huCD28. (*a*) Binding of CD28+ Jurkat cells to huCD80-transfected L cells is specific for CD80–CD28 interaction. Binding of CD28+ cells to wild-type

tope for BB1 includes the CDR2-like region. Mutants M18 and M19 showed poor binding to all mAbs tested, indicating a lower level of expression compared to wild-type CD80. The C-like domain deletion mutant (ΔC) was not detected by any of the mAbs, suggesting that it was not expressed at the cell surface.

Assessment of the CD28 Binding Capacity of CD80 Mutants. The effect of the mutations on binding to CD28 was measured by adhesion of CD28+ CTLA4− human T lym-

CD80+ cells was measured in the presence and absence of mAbs at 10 μg/ml final. The results were normalized, after correction for binding to untransfected L cells, for the percentage of binding obtained in the absence of mAb in each experiment. Percentage of response = (percentage of binding in the presence of mAb − percentage of binding to untransfected cells)/(percentage of binding in the absence of mAb − percentage of binding to untransfected cells) × 100. (*b*) Binding of CD28+ Jurkat cells to huCD80 mutants. The mean ± SD of two to six independent experiments are reported. To pool the results, binding of Jurkat cells to CD80-transfected L cells was corrected for binding to untransfected cells. The results were then normalized for the percentage of binding obtained with the wild-type in each experiment as follows: Percentage of wild-type response = (percentage of mutant binding − percentage of untransfected binding)/(percentage of wild-type binding − percentage of untransfected binding) × 100. For M2, M3, M6, M18, and M22, n = 6, 10, 4, 4, and 5, respectively, wherein n represents the number of independent experiments.

phoma Jurkat cells. Since the CD80–CD28 interaction is independent of divalent cations (24), Ca^{2+}, Mg^{2+}–free PBS was used in the final washing steps to minimize potential interference mediated by integrins. Adhesion to wild-type huCD80 was blocked by mAbs to huCD28 (L293) and huCD80 (L307), while mAbs to muCD80 (16-10A1) or other control mAbs had no effect (Fig. 2 *a*). Only three of the mutations (M3, M6, and M18) significantly reduced adhesion to $CD28^+$ cells. Two independent point mutations W84A (M3) and Y87A (M6) completely abrogated CD28–CD80–mediated cell–cell adhesion (Fig. 2 *b*). These residues lie in the region of the first domain corresponding to the C″ strand and the C″-D loop of the Ig V domain (see Fig. 5). The more conservative substitutions W84F (M4) and Y87F (M8), at either of these residues, retained binding to huCD28. In the C domain, M18 also decreased CD28-mediated cell adhesion; however, the comparable reduction in binding for all CD80 mAbs (Table 1) indicated a poor surface expression of this mutant that would account for the low CD28 binding to L cells transfected with the mutant. Mutant M2, which ablates binding of mAb BB1, retained CD28-dependent adhesion, although only at ~40% of the wild-type CD80. These results, coupled with the ability of BB1 to block stimulation of $CD28^+$ T cells by human CD80-transfected fibroblast (reference 24, and data not shown), suggest close, possibly overlapping, but distinct binding sites for CD28 and BB1-antibody on CD80.

Oligosaccharides contribute significant mass to CD80 (16–24 kD) as also found in several other IgSF members. To examine their possible role in the intermolecular binding to CD28, mutations were introduced at seven of the eight sites of predicted N-linked glycosylation. As shown in Fig. 2 *b*, among the eight mutants that modify putative N-linked glycosylation sites of CD80, M22, which eliminates two sites (N207Q and N226A), consistently resulted in a twofold increase in CD28 binding without affecting cell surface expression. Thus, carbohydrates may play some role in the recognition of CD28.

Mutations in the V Domain of CD80 also Impair the Upregulation of IL-2 Production by PHA-stimulated Jurkat Cells. Concordant results were obtained in a functional assay for costimulation. IL-2 production is triggered in Jurkat T cells using a combination of PHA and CD28 mAbs, where PHA substitutes for TCR mediated signals (25). Coculturing of $CD28^+$ Jurkat cells with CD80-transfected L cells in the presence of a suboptimal concentration of PHA also led to a 6–10-fold enhanced secretion of IL-2, compared to similar cultures with untransfected cells (Fig. 3 *a*). This enhancement was dependent on huCD80, since it was completely inhibited by mAb L307, while isotype controls and mAb 16-10A1 to muCD80 had no effect (Fig. 3 *a*). This functional assay was used to analyze the interaction of CD28 with the panel of CD80 mutants. Each mutant was tested two to six times. The results of a representative experiment are shown in Fig. 3 *b*. Consistent with the results of the adhesion assay, only M3, M6, and M18 significantly reduced the IL-2 response to near background levels (Fig. 3 *b*). The reduced IL-2 production observed with M18 likely reflects its poor expression.

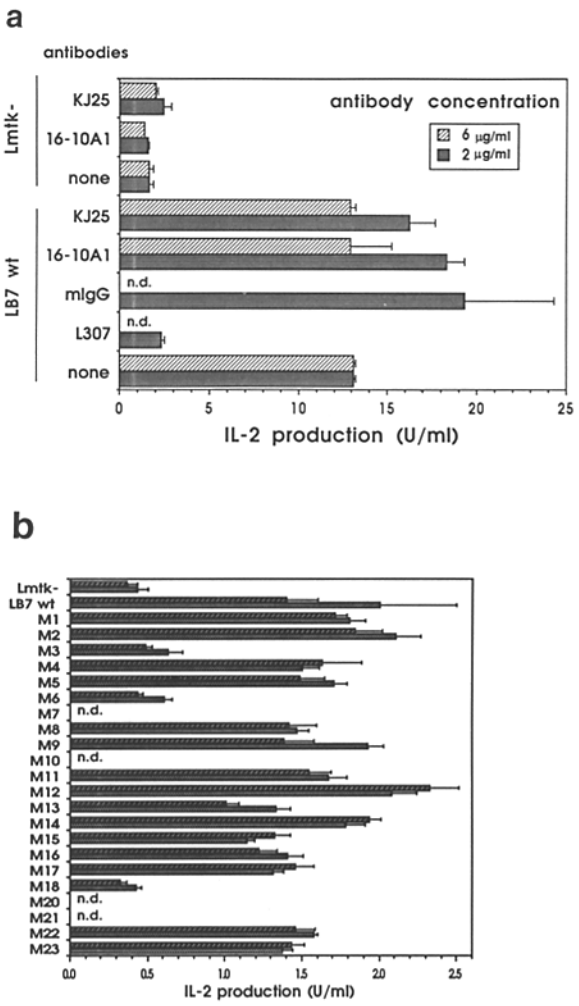

Figure 3. Functional assay of the CD28-CD80 interaction. (*a*) CD80-transfected L cells costimulate IL-2 production by PHA-activated Jurkat cells in a CD28-CD80–dependent manner. 10^4 L cells stably transfected with CD80 (or untransfected $Lmtk^-$ cells as control) were cocultured for 20 h with 5.10^4 PHA-activated Jurkat cells, as described in Materials and Methods, in the presence of the indicated antibodies at 2 or 6 µg/ml. Supernatants were then assayed for IL-2. The means ± of triplicate samples of one out of three representative experiments are shown. (*b*) Functional capacity of CD80 mutants. L cells transfected or not with different forms of CD80 were cocultured with PHA-activated Jurkat cells at a stimulator/responder ratio of 1:20 (*hatched bars*) and 1:5 (*dotted bars*). IL-2 production is indicated as the mean ± SD triplicate samples. Each individual mutant has been tested from 2 to 10 times. One out of six representative experiments is shown.

Mutations of CD80 Abrogating CD28 Binding also Affect CTLA4 Binding. Linsley et al. have shown that a chimeric form of CTLA4-Ig, CTLA-Ig, binds CD80 with a 10–20-fold higher affinity than a similar CD28 chimera (6). Moreover, CTLA4-Ig competes efficiently with CD28 for binding to CD80. The interaction between CTLA4-Ig and the above-described panel of CD80 mutants was analyzed by flow cytometry using a range of concentrations of CTLA4-Ig spanning over three orders of magnitude. Binding of CTLA4-Ig to murine L cells required the expression of the huCD80 molecule, and the level of fluorescence was proportional to the amount of CTLA4-Ig added to $CD80^+$ cells. Mutants that

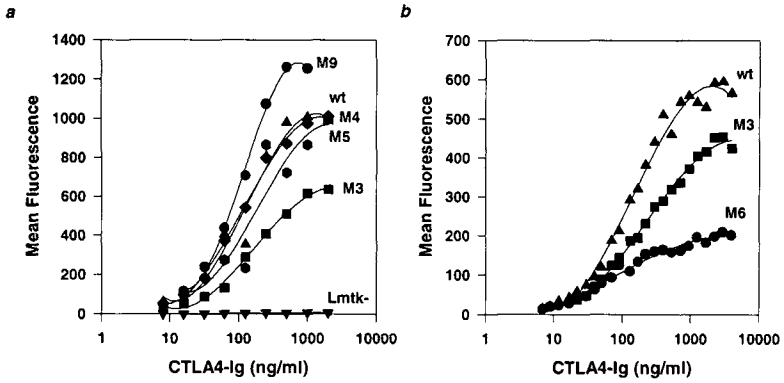

Figure 4. Binding of CTLA4-Ig to CD80 mutants. L cells expressing comparable levels of CD80 wild type, M3, M4, M5, M6, and M9 (and untransfected Lmtk$^-$ cells as negative control) were incubated with increasing amounts of CTLA4-Ig. Bound CTLA-Ig was revealed by FITC-labeled goat anti-human γ chain (Fc specific) and cells were analyzed by FACScan®: two independent experiments are presented in *a* and *b*. Results are expressed as means of green fluorescence (in arbitrary units) plotted against the concentration (nanograms per milliliter) of CTLA4-Ig.

did not have any effect in the adhesion assay or in the functional assay were all capable of binding CTLA4-Ig to levels comparable to the wild-type molecule (Fig. 4 *a* and data not shown). Mutants M3 and M6, however, reproducibly showed reduced binding to CTLA4-Ig, even at the highest concentrations (Fig. 4, *a* and *b*).

Discussion

We have identified a functional region in the V-like domain of human CD80 by assessing the capacity of CD80 mutants to retain costimulatory activity. Three different assays have implicated residues W84 and Y87, which lie in the sequence WPXYKNRT that is conserved across species (mouse, human, and rat) in CD80 (8, 9, 26), in the interaction with CD28 and CTLA4. The nonconservative substitution of alanine at these positions led to a marked decrease in the interaction with both counterreceptors, while the more conservative substitution of phenylalanine had little effect on the same assays. These results suggest that the hydrophobic side chain of residues W84 and Y87 either directly contact CD28 and CTLA4 or are essential in maintaining the local conformation of this binding site region. The finding that key determinants of the BB1 epitope (an mAb inhibiting CD80-dependent T cell responses [3]) lie proximal to the site further argues that this region is involved in recognition of CD28 and CTLA4. Based on alignments of the V-like domain of CD80 with consensus Ig V regions, the WPXYKNRT motif is predicted to lie between the CDR2 loop and the D strand (Fig. 5). If organized as in a typical V domain, this region would include the C″ β strand of the CDR2 region. Interestingly, the CDR2-like region has already been implicated in the interaction between two other members of the IgSF CD2 and CD58 (LFA-3) (27). On CD28 and CTLA4, a hydro-

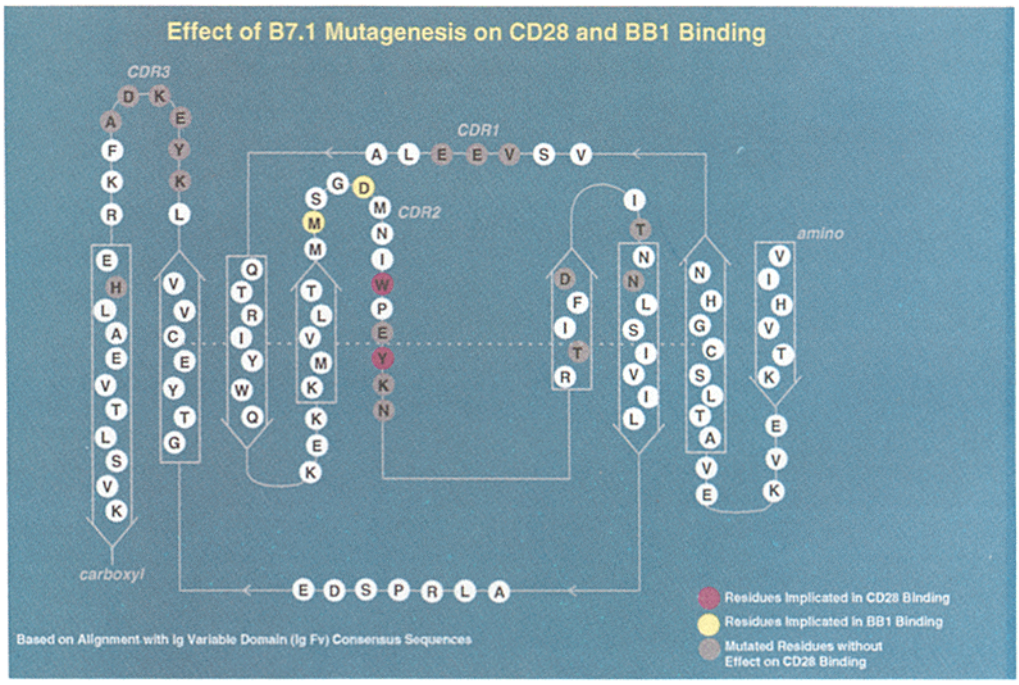

Figure 5. Model of the β strand folding pattern of CD80 V-like domain. The residues implicated in CD28 and CTLA4 binding are indicated in red and are localized to the C″ strand and C″ to D loop. Residues implicated in binding to mAb BB1 appear in yellow. The canonical disulfide bond in Ig domains between the B and F strands is shown as a dashed line. Because of the low homology between CD80 and other V-like domains, it was necessary to manually generate an alignment against consensus V_H and V_L sequences. The structure was thus predicted by placing special emphasis on the location of hydrophobic residues in positions that are buried in analogous Fv structures.

phobic region in the CDR3, encompassing a MYPPPY motif that is conserved among both molecules and across species (28), is critical for the interaction with CD80 (reference 29 and Truneh, A., manuscript in preparation). Moreover, interactions between proline-rich motifs and hydrophobic residues have been involved in binding of Src homology 3 regions to their targets (30). Finally, hydrophobic contacts have also been observed or suggested for the interaction of other IgSF molecules with their protein ligands (31–34).

Although the sensitivity of the two assays used to assess CD28 and CTLA4 binding capacity of the mutants might differ and do not allow direct comparison, the differential read-out that was obtained toward both counterreceptors raises the possibility that additional residues of CD80 might be critical to the interaction with CTLA4. In fact, contrary to CD28 binding, which was totally abrogated, even when using 20-fold excess of responder cells, the alanine substitution at residues 84 and 87, retained a low level of binding to CTLA4-Ig. This result indicates that other residues may be involved in recognition of CTLA4 and possibly contribute to the higher affinity of CD80 for the CTLA4-Ig molecule. This hypothesis would be consistent with the recent finding that the CDR1 of CTLA4 was responsible for the difference in affinity toward CD80 (29) when compared with CD28. CTLA4 could thus contact other regions of CD80 through its CDR1.

In the CD80 region we identify here as critical for binding, Y87 is conserved among all members of the B7 family, including hu- and muCD86, whereas W84 is not (13). The MYPPPY region of CD28 mentioned above appears to be also important for binding to the more recently described ligand CD86 (B7-2, B70), indicating that the interactions of CD28 with its ligand(s) on B cells probably involve conserved residues (Truneh, A., manuscript in preparation). CD80 and CD86 bind with similar avidities to both CD28 and CTLA4, although the dissociation of CTLA4 from CD86 occurs faster than from CD80 (35). It will thus be of interest to determine whether these residues have a common role in recognition of CD28 and CTLA4 by CD86.

The role of the C domain of CD80 has been addressed in our experiments and in two recent studies (36, 37). Our attempt to address this question by deleting the C domain did not lead to conclusive results since this construct was not expressed in repeated transfections. Using an alternative spliced form of muCD80 that lacks exon 3 encoding for the CD80 C domain, two groups have reported conflicting results as to the capacity of the truncated molecule to interact with CD28 and CTLA4. Inobe et al. (36) demonstrate that both molecules have high affinity interactions with the truncated CD80. Interestingly, CTLA4-Ig binding activity was reduced by opposition, suggesting that CTLA4 has additional requirements (possibly the C domain) for high affinity binding to B7 molecules. In contrast, Guo et al. (37) show that this truncated form of CD80 does not bind CD28 and CTLA4. Moreover, mutations at conserved residues within the C domain also fail to bind both CD80 ligands. It is noteworthy, however, that four out of the six mutants generated in this study involve prolines known to be critical for the structural integrity of molecules. These results are difficult to reconcile with our own. Indeed, we have generated mutants in the amino acid stretch SDQXXXELY located in the C-like domain and pointed to as of potential importance by Freeman et al. (9). These residues are conserved among CD80 and CD86 across species (8–13, 26). Three mutants of the present study were altered in this sequence. M17 had no effect on function, while M18 and M19 drastically reduced cell surface expression of CD80 as measured with all our panel of mAbs. These results suggest that this region of the molecule could be involved in other functions of the molecule such as dimerization or interaction with other cell surface molecules. Moreover, as previously mentioned, antibodies that affect the CD80–CD28/CTLA4 interaction map to the V domain. Further studies will be required to determine the role of the C domain in recognition of CD28 and CTLA4. Thus, while our results provide direct evidence for the involvement of the NH_2-terminal, V-like domain of CD80 in its interaction with CD28 and CTLA4, the contribution of the C-like domain can not be ruled out. A more definitive resolution of the interactions between CD80/B70 and CD28/CTLA4 may await structural analysis.

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Address correspondence to Rafick-Pierre Sekaly, Laboratoire d'Immunologie, Institut de Recherches Cliniques de Montréal, 110 Avenue des Pins Ouest, Montréal, Québec, Canada H2W 1R7.

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