Chimeric Co-stimulatory Molecules That Selectively Act through CD28 or CTLA-4 on Human T Cells*

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CD28 and CTLA-4 (CD152) play a pivotal role in the regulation of T cell activation. Upon ligation by CD80 (B7–1) or CD86 (B7–2), CD28 induces T cell proliferation, cytokine production, and effector functions, whereas CTLA-4 signaling inhibits expansion of activated T cells and induces tolerance. Therefore, we hypothesized that co-stimulatory molecules that preferentially bind CD28 or CTLA-4 would have dramatically altered biological properties. We describe directed molecular evolution of CD80 genes derived from human, orangutan, rhesus monkey, baboon, cat, cow, and rabbit by DNA shuffling and screening. In contrast to wild-type CD80, the evolved co-stimulatory molecules, termed CD28-binding protein (CD28BP) and CTLA-4-binding protein (CTLA-4BP), selectively bind to CD28 or CTLA-4, respectively. Furthermore, CD28BP has improved capacity to induce human T cell proliferation and interferon-γ production compared with wild-type CD80. In contrast, CTLA-4BP inhibited human mixed leukocyte reaction (MLR) and enhanced interleukin 10 production in MLR, supporting a role for CTLA-4BP in inducing T cell anergy and tolerance. In addition, co-stimulation of purified human T cells was significantly suppressed when CTLA-4BP was cotransfected with either CD80 or CD28BP. The amino acid sequences of CD28BP and CTLA-4BP were 61 and 96% identical with that of human CD80 and provide insight into the residues that are critical in the ligand binding. These molecules provide a new approach to characterization of CD28 and CTLA-4 signals and to manipulation of the T cell response.

T cell response is dependent on complex integration of antigen-specific and nonspecific extracellular and intracellular signals. The specificity of the response is determined by recognition of an antigene peptide in the context of major histocompatibility complex on the plasma membrane of antigen-presenting cells, such as monocytes, dendritic cells, Langerhans cells, or primed B cells. However, a second signal, mediated by co-stimulatory molecules expressed on antigen-presenting cells, is required for induction of a complete T cell response (1–3). The most extensively studied co-stimulatory molecules are CD80 (B7–1) and CD86 (B7–2), which bind the CD28 and CTLA-4 (CD152) ligands expressed on CD4+ and CD8+ T cells. Additional co-stimulatory molecule-ligand pairs such as ICOS-B7-H and PD1-PDL have recently been identified, further illustrating the complexity of the signals that regulate the function of T cells (1–3). Co-stimulatory signal in addition to the T cell receptor signal is a prerequisite for induction of optimal T activation, proliferation, cytokine production, and effector functions.

The functional characterization of CD80, CD86, CD28, and CTLA-4 has also illustrated the delicate balance between positive and negative signals mediated through CD28 and CTLA-4, respectively. Upon ligation by CD80 or CD86, CD28 synergizes with T cell receptor signaling to induce activation of both CD4+ and CD8+ T cells (4–7), whereas CTLA-4 ligation inhibits expansion of activated T cells and induces tolerance (7–11). The positive and negative signals mediated through CD28 and CTLA-4 in vivo were clearly demonstrated in mice lacking these molecules. CD28-deficient T cells have strongly impaired capacity to respond to antigen stimulus in vivo (4, 5, 12, 13), whereas CTLA-4-deficient mice demonstrate excessive T cell growth, resulting in lethal lympho-proliferative disease within weeks after birth (8, 14). Moreover, although recognition of specific antigen in the absence of co-stimulation results in anergy in vitro (15, 16), CTLA-4 signaling also plays an important role in inducing and maintaining anergy and tolerance in vivo (7, 9–11).

It appears that a number of diverse B7 variants capable of binding to their two ligands have existed during natural evolution. This notion is supported by the fact that CD80 and CD86 genes appear to be results of a gene duplication (17), yet they currently only share ~25% of their amino acids (3, 18, 19). In addition, the severe phenotype of CD28- and CTLA-4-deficient mice (4, 5, 8, 12–14) and the structurally conserved ligand binding sites of CD80 and CD86 (20–24) suggest that natural evolution has strongly favored the binding of B7 molecules to both of their respective ligands. Directed molecular evolution by DNA shuffling followed by screening has previously been successful in evolving, for example, subtilisin, interferon α, viruses, and bacteria (25–31). We hypothesized that directed molecular evolution of diverse mammalian CD80 genes by DNA shuffling combined with the appropriate screening procedures would enable the generation of chimeric molecules that preferentially bind and signal through either CD28 or CTLA-4. cDNAs encoding human, orangutan, rhesus monkey, baboon, cat, cow, and rabbit CD80 genes were subjected to DNA shuffling, and libraries of shuffled genes were generated. After two rounds of DNA shuffling and screening, chimeric genes with dramatically altered ligand binding and functional properties were identified. These new molecules, termed CD28-binding...
protein (CD28BP)\(^1\) and CTLA-4-binding protein (CTLA-4BP), help in further dissecting the mechanisms by which CD28 and CTLA-4 trigger their biological effects, and they provide a new approach to manipulation of the T cell response.

**Experimental Procedures**

**Isolation of Mammalian cDNAs and Generation of Libraries—**CD80 genes derived from human, rhesus monkey, baboon, orangutan, cow, cat, and rabbit were cloned by the reverse transcriptase-PCR method. Cell lines Raji, PBLT, LCL6864, and 28CB-1 were used as the source of messenger or total RNA for human (Homo sapiens), orangutan (Pongo pygmaeus), rhesus monkey (Macaca mulatta), and baboon (Papio hamadryas), respectively. Peripheral blood was used as the source of messenger or total RNA for cat (Felis catus), cow (Bos taurus), and rabbit (Oryctolagus cuniculus sub-species domestica) CD80 cDNA preparation. Peripheral blood mononuclear cells (PBMC) were isolated from RBC gradient separation. PBMC were activated for 48 h with medium containing 5 μg/ml lipopolysaccharide, 0.25 μg/ml Pockeweed mitogen, and phytohemagglutinin.

mRNA or total RNA was isolated using FastTrack 2.0 mRNA isolation kit (Invitrogen) or RNAGents Total RNA Isolation System kit (Promega, Madison, WI), respectively. Primers used to clone CD80 cDNA were designed based on published human and rabbit CD80 genes (32–34), and they contained a BamHI site 5’ of the start codon and a RpiI site 3’ of the stop codon. Reverse transcription was performed using the cDNA cycle kit (Invitrogen). DNA shuffling was performed as described previously (25–27). The shuffled sequences were digested with BamHI and Asp-718 and gel-purified, and the resulting chimeric genes were cloned into a pcDNA3.1 expression vector (Invitrogen).

The FLAG sequence (DYKDDDDK) was inserted at the junction separating the signal peptide and mature coding region of the human CD80 (hCD80) and CD28BP clones using the ExSite PCR site-directed mutagenesis kit (Strategene, San Diego, CA). Mutagenesis primers were designed with the FLAG sequence flanked by 24 nucleotides of signal and mature sequence specific to each clone.

**DNA Purification and Transfections—**Escherichia coli strain DH10B (Invitrogen) was transformed with Maxiprep DNA that was generated from the shuffled libraries, and the transformed cells were plated overnight. Individual colonies were picked from the libraries and inoculated into 96-well blocks containing 1.2 ml of Terrific Broth-amp (50 g/ml). The cultures were grown for 20 h at 37 °C, and plasmid DNA was purified using the Biorobot (Qiagen, Valencia, CA).

293 or COS-7 cells were transfected with the plasmids encoding chimeric genes using Superfect (Qiagen) or LipofectAMINE (Invitrogen) according to manufacturer’s instructions. Transfections were performed in tissue culture flasks (Falcon, Franklin Lakes, NJ) containing 60–80% confluent 293 or COS-7 cells according to the manufacturer’s instructions. The cells were incubated for 5–7 h at 37 °C in a humidified incubator containing 5% CO\(_2\). An equal volume of Dulbecco’s modified Eagle’s medium, 20% fetal calf serum was added to the flask and incubated overnight. Cells were trypsinized, replated, and incubated for an additional 24 h. The cells were then removed from plastic using EDTA treatment and counted, and expression of the transgenes was confirmed by flow cytometry.

**Protein Conjugation, Flow Cytometry, and Ligand Binding Analysis—**Soluble (s) CD28-Ig and sCTLA-4-Ig fusion proteins (R&D Systems, Minneapolis, MN) were conjugated with biotin (Pierce) and fluorescein isothiocyanate (FITC) (Molecular Probes, Eugene, OR), respectively. Molar ratios of 1.38 for CD28-Ig/FITC and 1.35 for sCTLA-4-Ig/biotin were used during the conjugation. Proteins at 1–3 mg/ml were dissolved in 0.1 M carbonate buffer for FITC conjugation and 0.1 M sodium bicarbonate buffer for biotin conjugation. FITC or biotin at 2 mg/ml in Me\(_2\)SO was added to dialyzed protein, incubated at 25 °C in the dark for 2 h, and then dialyzed with phosphate-buffered saline overnight to exchange buffers.

The transfected cells (293 or COS-7) were first incubated with biotin-conjugated sCD28BP for 15 min at 4°C, the cells were washed twice with PBS and incubated with streptavidin-phycoerythrin (PE) (Pharmingen) for 15 min. The cells were washed twice and resuspended in medium with 5 μg/ml propidium iodide. The cells were analyzed using FACS Calibur flow cytometer and CellQuest software (BD Biosciences, San Jose, CA). Anti-hCD80 monoclonal antibodies (mAbs; Pharmingen) were used as positive controls for biotin and fluorescein transfections.

**Library Screening—**Cell sorting was used to enrich the libraries for clones with altered ligand binding properties and was performed using FACS Vantage SE cell sorter (BD Biosciences). Transfected cells were stained with sCD28-Ig and sCTLA-4-Ig as described above, and plasmids were recovered from the sorted cells by Hirt preparation. Briefly, the transformed cells were incubated in 20 μl of Hirt’s solution (0.6% SDS, 10 mM EDTA) for 30 min, 100 μl of 0.5 M NaCl was then added to the lysate, and the lysate was subsequently incubated overnight. The DNA was isolated by phenol/chloroform extraction, ethanol-precipitated, and resuspended in 10 μl of TE buffer. The isolated plasmids were used to transform DH10B E. coli, and the transformed cells were plated on LB agar plates. All colonies were harvested and combined, and plasmid DNA was isolated using the Qiagen Maxiprep kit.

To screen individual shuffled clones, 293 cells were plated in 96-well plates and transfected with DNA isolated from the sorted cells as described above. The 293 cells were plated at a density of 2 x 10^4 cells/well the day before transfection. Plated 293 cells were washed once with 200 μl of phosphate-buffered saline, and 50 μl of OptiMEM was then added/well. The next day 20 μl of DNA was added to OptiMEM, and 50 μl was plated/well in 96-well U-bottom plates. 50 μl of OptiMEM with LipofectAMINE (Invitrogen) at 0.03 μl/1 μl was added to each well containing diluted miniprep DNA. The mixture was incubated for 15 min at room temperature, and 20 μl/well of the mixture was added to wells containing 293 cells in 50 μl of OptiMEM. The cells were incubated at 37 °C for 5–7 h, 70 μl of Dulbecco’s modified Eagle’s medium, 20% fetal calf serum, replaced in sterile V-bottom plates, and incubated overnight. Ligand binding was subsequently analyzed by flow cytometry as described above.

**Cell Purification and Cultures—**Peripheral blood was obtained from healthy blood donors as standard buffy coat preparations collected at Stanford Medical School Blood Center (Palo Alto, CA). PBMC were isolated by centrifugation over Histopaque-1077 (Sigma). T cells were purified either by staining the cells with anti-human CD2 mAbs (Pharmingen) and sorting CD2- cells using a FACS Vantage SE or removing cells that stained with mAbs specific for CD4, CD20, CD56, and CD16 (Pharmingen) by magnetic beads (Dynal, Lake Success, NY).

The purity of the cells was 96–99% when analyzed by staining with anti-CD3 mAbs. 5 x 10^4 purified T cells were cultured in triplicate in the presence of irradiated (5000 rads) transfectants and soluble anti-human CD3 mAbs (5 μg/ml) in flat-bottomed 96-well plates (VWR, West Chester, PA) at 37 °C in a humidified atmosphere containing 5% CO\(_2\) in Yssel’s medium (IMDM enriched with human transferrin (20 μg/ml; BioWhittaker, Walkersville, MD), insulin (5 μg/ml; Sigma), linoleic acid (2 μg/ml; Sigma), oleic acid (2 μg/ml; Sigma), palmitic acid (2 μg/ml; Sigma), bovine serum albumin (0.25% w/v; Sigma), and 2-amino ethanol (1.8 μg/ml; Sigma)) supplemented with 10% fetal calf serum for a total of 3 days. 1 μCi/well of [3H]thymidine (Amersham Biosciences) was added for the last 8 h of the cultures, and the cells were harvested onto filter paper by a cell harvester (Tomtec, Hamden, CT). [3H]Thymidine incorporation was measured using a MicroBeta scintillation counter (Wallac, Turku, Finland).

**Analytical Techniques**

**Rabbit and Human CD80**

Mixed leukocyte reaction (MLR) was performed using irradiated PBMC as stimulator cells and allogeneic PBMC as responders. Stimulator cells were irradiated (2500 rads) and co-cultured with allogeneic PBMC (5 x 10^5 in 96-well flat-bottomed microtiter plates (VWR) at 1:1 ratio for a total of 5 days. Transfectants expressing CD28BP, CTLA-4BP, or hCD80 were added to these cultures at increasing cell numbers as indicated in the text. [3H]Thymidine incorporation was measured as described above for purified T cells.

**Analysis of Cytokine Levels in the Culture Supernatants—**Supernatants of cell cultures were collected after 48 h and stored at –80 °C until they were analyzed for the presence of cytokines. Cytokine levels were determined using cytokine enzyme-linked immunosorbent assay. IL-10 and IFN-γ levels were determined using commercially available kits, and the assays were performed according to the manufacturer’s instructions (R&D Systems).

**Generation of mAbs Specific for CD28BP—**Balf/c mice were immunized a total of 3 times, with the last dose administered by the intravenous route. Injections were given 2 weeks apart, and 3 days after the
last immunization, single cell spleen suspensions were prepared. The cells were fused with Sp2/0 cells (ATCC CRL-1581), essentially as previously described by Ozato and Sachs (35). Briefly, 3% dextan (Sigma) was added to the cell mixture, and after 5 min at room temperature, the cells were centrifuged and resuspended first in 1 ml of PBS/15% Roche Applied Science, Indianapolis, IN) and then in 20 ml of serum-free Dulbecco’s modified Eagle’s medium. The cells were centrifuged, resuspended in selection medium containing 2 µg/ml azaserine (Sigma), and cultured in 96-well flat bottom plates (Costar). The hybridomas were screened by flow cytometry using 293 cells stably transfected with CD28BP.

RESULTS
DNA Shuffling of Mammalian CD80 cDNAs—mRNA encoding human (H. sapiens), orangutan (P. pygmaeus), rhesus monkey (M. mulatta), baboon (P. hamadryas), feline (F. catus), bovine (B. taurus), and rabbit (O. cuniculus sub-species domesticus) CD80s were isolated from the respective species. The corresponding cDNAs were subjected to DNA shuffling to generate libraries of chimeric genes. Sequencing of randomly selected clones indicated that 12 of 12 clones comprised fragments from at least 2 of the starting genes, illustrating efficient chimerism. To address the functionality of the shuffled chimeras, the ligand binding properties of randomly selected clones were analyzed. A large fraction of the shuffled, chimeric molecules exhibited binding to either sCD28-Ig or sCTLA-4-Ig, which is likely to be attributable to the fact that all starting genes encoded functionally and structurally related molecules (57–99% amino acid sequence identity). Less than 10% of the randomly selected chimeras demonstrated no binding to either sCD28-Ig or sCTLA-4-Ig (data not shown), indicating high functional fitness of proteins generated by DNA shuffling of natural mammalian genes.

First Round Screening of Shuffled Libraries—Flow cytometry-based cell sorting was used to screen the libraries for clones with increased or decreased relative binding to sCD28-Ig and sCTLA-4-Ig. Subsequent analysis of 1000 individual clones recovered from the sorted cells identified a number of clones with altered ligand binding profiles relative to hCD80. Four clones with preferential binding to sCD28-Ig were subjected to more detailed analysis, and reduced binding to sCTLA-4-Ig was observed in at least two separate experiments, whereas the binding to sCD28-Ig remained intact (data not shown). However, although the level of binding of these clones to sCTLA-4-Ig was reduced, it was still detectable (data not shown). On the other hand, preferential binding to sCTLA-4-Ig was observed in at least 15 clones of the 1000 individual clones analyzed by flow cytometry (data not shown). Again, however, the loss of binding to sCD28-Ig was only partial.

Second Round of DNA Shuffling and Screening—As in natural evolution, DNA shuffling followed by screening allows the selection of the desired phenotypes to new rounds of breeding. We selected four clones with the most biased binding to sCD28 and five clones with preferential binding to CTLA-4 for a second round of DNA shuffling. The two groups of genes were shuffled in parallel experiments, and two separate libraries were generated. Subsequent screening of 1000 individual clones from both libraries identified clones that exhibited strongly biased binding to sCD28-Ig or sCTLA-4-Ig. The two clones exhibiting the most skewed binding to CD28 or CTLA-4 were named CD28-binding protein (CD28BP) and CTLA-4-binding protein (CTLA-4BP), respectively. As shown in the Fig. 1, transfectants expressing CD28BP, CTLA-4BP, or hCD80 and stained under identical conditions demonstrated dramatically altered ligand binding profiles. The molecules illustrated in Fig. 1 were not unique solutions in that the second round of breeding resulted in at least 14 and 19 clones with different amino acid sequences that exhibited further biased binding to sCD28-Ig or sCTLA-4-Ig, respectively, as compared with the clones selected from the first round of breeding (data not shown).

Sequence Analysis of CD28BP and CTLA-4BP—The sequence analysis of CD28BP indicated that it was a chimera derived from the human, baboon, bovine, and rabbit sequences (Fig. 2A). The remaining 13 clones selected from the second round of breeding that displayed preferential binding to CD28 shared 74–99% amino acid sequence identity with CD28BP, further illustrating the diversity of sequences that exhibited the given properties. The nucleotide and amino acid sequence identities of CD28BP with hCD80 were 73 and 61%, respectively. Interestingly, all of the 14 different clones selected from the second round of evolution demonstrating preferential binding to CD28 contained a substitution of valine for isoleucine at amino acid position 49 of the mature sequence (data not shown).

CTLA-4BP contained sequences corresponding to the human, baboon, rhesus monkey, and bovine CD80 genes (Fig. 2B), and the nucleotide and amino acid sequences of CTLA-4BP were 97 and 96% identical with those of hCD80, respectively. In addition, CTLA-4BP contained three amino acid mutations that were not derived from any of the starting genes (Fig. 2B). The remaining 18 clones with preferential binding to sCTLA-4-Ig were 95–99% identical with CTLA-4BP. Interestingly, Tyr-31 was replaced by histidine in 14 of the 19 selected clones with preferential binding to CTLA-4, whereas all 14 clones with preferential binding to CD28 retained the tyrosine at the corresponding position.

Analysis of Individual Ligand Binding to CD28BP, CTLA-4BP, and CD80 Molecules Encoded by the Starting Genes—Staining of transfectants individually with varying concentrations of sCD28-Ig or sCTLA-4-Ig indicated that CD28BP transfectants bound sCD28-Ig at higher levels than transfectants expressing hCD80, whereas sCTLA-4-Ig binding was significantly reduced, yet detectable (Fig. 3). On the other hand, little or no binding of sCD28-Ig to CTLA-4BP transfectants was detected even at high concentrations, whereas the same transfectants did bind sCTLA-4-Ig, although at somewhat lower levels than the transfectants expressing hCD80 (Fig. 4).
lack of binding of sCD28-Ig to CTLA-4BP was not due to the lack of expression, as determined by binding of anti-hCD80 mAbs to the transfectants (Fig. 4, C and D).

mAbs specific for hCD80 could not be used to analyze expression of CD28BP due to no or minimal cross-reactivity of the mAbs (data not shown). Therefore, to analyze whether increased expression of CD28BP on the transfected cells might have contributed to the improved binding of sCD28-Ig, we fused a FLAG tag to hCD80 and the shuffled clones, and the binding of a FLAG-specific mAb M2 to the corresponding transfectants was studied. No difference in the binding of anti-FLAG mAbs to transfectants expressing CD28BP or hCD80 was observed. In seven separate transient transfections, the mean fluorescence intensity of 293 cells transfected with CD28BP-FLAG and hCD80-FLAG was 52 ± 12 and 55 ± 12, respectively (mean ± S.E.). Although no significant difference in the expression of the FLAG constructs was observed, significantly improved binding of sCD28-Ig was also observed using the FLAG constructs (Figs. 3, A and B), strongly suggesting that the improved binding of sCD28-Ig by CD28BP was due to improved affinity rather than improved expression of CD28BP.

We also analyzed the ligand binding properties of all of the starting genes by two-color analysis using sCD28-Ig and sCTLA-4-Ig to illustrate that CD28BP and CTLA-4BP showed
Evidence of truly novel properties. 293 cells transfected with human, rhesus monkey, orangutan, and baboon CD80 genes exhibited essentially identical binding profiles, whereas feline CD80 showed little or no binding to sCD28-Ig or sCTLA-4-Ig (data not shown). Bovine and rabbit CD80 transfectants demonstrated sCD28-Ig binding that was in the same range as that observed for human and primate CD80, but the level of binding to sCTLA-4-Ig was somewhat lower. However, the binding level of sCTLA-4-Ig to CD28BP was consistently less than that of any of the starting genes (the mean fluorescence intensities of sCTLA-4-Ig binding to human (n = 4), bovine (n = 4), rabbit CD80 (n = 2), and CD82BP (n = 2) were 247, 102, 205 and 30, respectively). In other words, CD28BP and CTLA-4BP displayed properties that were unique as compared with any of the starting genes.

Improved Co-stimulation of Human T Cells by CD28BP—To investigate the functional properties of CD28BP, we first studied the effects of transient transfectants expressing CD28BP or hCD80 on proliferation of purified human T cells cultured in the presence of anti-CD3 mAbs. Transient transfectants expressing CD28BP induced greatly increased proliferation of human T cells when compared with cells transfected with hCD80 (Fig. 5A). Approximately 10-fold fewer transient transfectants expressing CD28BP than those expressing hCD80 were required to obtain a similar level of human T cell proliferation. Moreover, the level of cpm observed in response to CD28BP transfectants was ~3-fold higher than those induced by equal numbers of hCD80 transfectants (Fig. 5A).

As described above using FLAG-tagged CD28BP and hCD80 constructs, no difference in expression levels of CD28BP was observed on transient transfectants when compared with expression of hCD80, supporting the notion that the increased proliferation resulted from the altered ligand binding. We also tested the transfectants expressing these FLAG-tagged molecules in the T cell co-culture experiments, but these constructs demonstrated strongly reduced capacity to co-stimulate human T cells (less than 30% of the responses induced by the non-tagged molecules, data not shown), which was to be expected since the FLAG epitope was inserted between the signal peptide and the membrane distal V domain. Nevertheless, in two separate experiments, more than 3-fold higher T cell proliferative response was observed in the presence of CD28BP-FLAG than hCD80-FLAG transfectants (data not shown), further indicating that expression level differences did not explain the stronger co-stimulus provided by CD28BP.

We also studied the effects of stable transfectants of CD28BP and hCD80 on human T cells activated in the presence of anti-CD3 mAbs. The co-stimulation induced by the stable transfectants was generally higher than that induced by the transient transfectants, which is most likely due to the fact that all the stable, in contrast to the transient, transfectants express the transgene. Nevertheless, CD28BP transfectants again induced stronger T cell co-stimulation than hCD80 transfectants (Fig. 5B). We also studied the effects of these transfectants on IFN-γ secretion under similar culture conditions. As shown in Fig. 5C, production of IFN-γ in response to transfectants expressing CD28BP was higher than that induced by hCD80 transfectants, further supporting a more potent T cell co-stimulation by CD28BP than hCD80. Given that the FLAG tag interferes with the function of the molecules, comparison of expression levels of CD28BP and hCD80 on the stable transfectants could only be done using indirect measurements. However, based on mean fluorescence intensity in flow cytometry assays, there was no difference in the level of sCD28BP-Ig binding (Fig. 3C) to stable CD28BP and hCD80 transfectants when using high, saturating concentrations of sCD28BP-Ig (100 μg/ml), supporting the notion that the number of molecules on the surface of both transfectants was comparable and further indicating a more potent co-stimulation by CD28BP than hCD80.

CTLA-4BP Does Not Co-stimulate Human T Cells but Inhibits MLR—CTLA-4BP lacked the capacity to co-stimulate human T cells cultured in the presence of soluble anti-CD3 mAbs (Fig. 6). No measurable co-stimulation of human T cells was induced by either transient (Fig. 6) or stable (data not shown) CTLA-4BP transfectants, whereas hCD80 transfectants and

Fig. 5. Co-stimulation of purified human T cells by CD28BP and hCD80. 293 cells were transiently (A) or stably (B) transfected with CD28BP, hCD80, or a control vector, and the irradiated transfectants were co-cultured with purified human T cells (5 × 10^5/well) and anti-CD3 mAbs (5 μg/ml). A and B, T cell proliferation was measured by adding 1 μCi/well of [3H]thymidine for the last 8 h of the cultures. The cells were then harvested, and [3H]thymidine incorporation was measured using scintillation counting. The data represent mean ± S.E. of cpm obtained in three (A) or six (B) independent experiments using transient or stable transfectants, respectively. C, irradiated stable transfectants expressing CD28BP or hCD80 and negative control cells transfected with a vector lacking the insert were co-cultured with purified human T cells, and the levels of IFN-γ production were measured after a culture period of 48 h. A representative experiment is shown; similar data were obtained in three other experiments.
particularly CD28BP transfectants induced potent co-stimulation under identical culture conditions (Fig. 6). The lack of co-stimulation was not due to lack of expression because efficient expression of CTLA-4BP on the surface of the transfectants was observed using anti-CD80 mAbs (Fig. 4C). In fact, only 2 of the 19 selected clones with preferential binding to CTLA-4 had the capacity to induce a T cell response that was more than 10% that induced by hCD80 (data not shown), illustrating that the lack of sCD28-Ig binding to these clones correlated with the lack of signaling through CD28.

To further analyze the effects of CTLA-4BP, stable transfectants expressing CTLA-4BP were generated and added to cultures of human MLR. As shown in Fig. 7A, CTLA-4BP transfectants inhibited T cell proliferation induced in MLR in a dose-dependent manner, whereas hCD80 transfectants had no effect (Fig. 7A). Moreover, CTLA-4BP transfectants enhanced IL-10 production in MLR, whereas IFN-γ production was reduced as compared with hCD80 or control transfectants (Fig. 7, B and C), supporting the notion that CTLA-4BP has dramatically altered biological function as compared with hCD80.

**CTLA-4BP Inhibits Co-stimulation Provided by hCD80 or CD28BP**—To directly address the function of CTLA-4BP on co-stimulation provided by hCD80 or CD28BP, plasmids encoding CTLA-4BP were transiently cotransfected with plasmids encoding hCD80 or CD28BP into 293 cells, and the transfected cells were subsequently co-cultured with anti-CD3 mAbs and purified T cells isolated from three different donors. Mean ± S.E. cpm induced by hCD80 and hCD80 + CTLA-4BP in the three experiments was 34452 ± 9362 and 13166 ± 3212, respectively. In addition, Fig. 8A illustrates the level of T cell proliferation in a representative experiment when CTLA-4BP was cotransfected with both hCD80 and CD28BP, and the effect of CTLA-4BP cotransfection on the expression level of CD28BP also was simultaneously studied. We have recently generated mAbs specific for CD28BP, and these Abs were used to analyze the effect of CTLA-4BP cotransfection on the expression level of the transgenes. Anti-hCD80 mAbs cross-react with CTLA-4BP but not with CD28BP (Fig. 8, B and C). Most of the transfected cells were double-positive for CTLA-4BP and CD28BP (Fig. 8, B and C), but CTLA-4BP had no inhibitory effect on the expression level of CD28BP (Fig 8, D and E), yet a significant reduction in T cell co-stimulation was observed (Fig. 8A). These data further indicate that CTLA-4BP down-regulates the function of human T cells and that CTLA-4BP can counteract the co-stimulus provided by hCD80 or CD28BP.

**DISCUSSION**

We describe the generation of novel co-stimulatory molecules, CD28BP and CTLA-4BP, that selective bind to and signal through CD28 or CTLA-4. These unique ligand binding profiles resulted in functional properties that are dramatically different from those of hCD80. CD28BP has improved the capacity to activate human T cells as compared with hCD80, whereas CTLA-4BP induces no co-stimulation of human T cells and inhibits T cell response in MLR.

B7 co-stimulatory molecules have improved the efficacy of gene- and cell-based vaccines in animal models (37–40) and have shown promising results in pre-clinical tumor models as soluble immunotherapeutics (41). Our data indicate increased binding of sCD28-Ig to transfectants expressing CD28BP as compared with those expressing hCD80, and the improved ligand binding was also associated with enhanced co-stimulation of human T cells. The increased binding of sCD28-Ig cannot be explained by higher expression levels of CD28BP because FLAG-tagged constructs demonstrated equal expression.

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**Fig. 6. Effects of CTLA-4BP on purified human T cells.** Purified T cells were co-cultured in the presence of soluble anti-CD3 mAbs (5 μg/ml) and transient transfectants expressing hCD80, CD28BP, CTLA-4BP, or a vector control lacking the insert. T cell proliferation was measured by adding 1 μCi/well of [3H]thymidine for the last 8 h of the 72 h cultures. The cells were then harvested, and [3H]thymidine incorporation was measured using scintillation counting. A representative of three experiments is shown.

**Fig. 7. Effects of CTLA-4BP on proliferation and cytokine production in human MLR.** MLR was performed by co-culturing irradiated (2500 rads) PBMC as stimulator cells with allogeneic PBMC (1 × 10^6/well) in 96-well flat-bottomed microwell plates (VWR) at a 1:1 ratio for a total of 5 days. A, increasing numbers of 293 cells stably transfected with CTLA-4BP (●), hCD80 (○) or a control vector without an insert (△) were added to the MLR cultures as indicated in the figure. The data represent mean ± S.E. of cpm obtained in six separate MLR cultures, each performed using 4–6 replicate wells. B and C, MLR was cultured in the presence of 25,000 irradiated 293 cells stably transfected with hCD80, CTLA-4BP, or a control vector without an insert. IFN-γ and IL-10 levels were measured by enzyme-linked immunosorbent assay after an MLR culture period of 48 h. Six independent experiments were performed, and the values obtained within one experiment are connected by a solid line. The levels of IFN-γ were significantly increased (p < 0.05), and those of IL-10 significantly decreased (p < 0.01) in cultures of CTLA-4BP expressing transfectants as compared with hCD80 or vector control transfectants (paired Student’s t test).
levels and because binding to sCTLA-4-Ig was simultaneously decreased. The maximum level of T cell proliferation and IFN-γ production induced by CD28BP transfectants also increased as compared with those observed in the presence of hCD80 transfectants. Again, the improved T cell proliferation could not be attributed to improved expression levels of CD28BP because FLAG-tagged constructs analyzed using anti-FLAG mAbs demonstrated comparable expression levels on these transient transfectants. Although the FLAG epitope was found to interfere with the function of both CD28BP and hCD80, CD28BP induced at least 3-fold higher T cell proliferative response than hCD80 also when analyzed using anti-CD28BP mAbs and flow cytometry. Cells transfected with CD28BP alone or together with CTLA-4BP were shown in gray histograms. 293 cells transfected with empty control vector and stained under identical conditions are shown as controls (open histograms). The CD28BP transfectants shown in D–E were also used to co-stimulate the T cells shown in A.

CTLA-4BP exhibited functional properties not shared by hCD80 in that CTLA-4BP inhibited MLR and IFN-γ production, whereas it enhanced IL-10 secretion. CTLA-4BP also inhibited T cell co-stimulation induced by hCD80 or CD28BP. The inhibitory effects of CTLA-4BP were not due to interference in expression levels, because a significant reduction in the level of T cell proliferation was observed, whereas cotransfection of CTLA-4BP had no suppressive effect on the expression levels when analyzed by flow cytometry. These data imply that CTLA-4BP inhibits T cell function through signaling via CTLA-4 and suggest that the effects of CTLA-4BP are mediated at the immunological synapse where the interactions between CD80 and CD28/CTLA-4 have previously been shown to take place (42, 43). Induction of IL-10 by CTLA-4BP is of particular interest because of the role of IL-10 in inducing anergy and regulating the function of professional antigen-presenting cells. Both IL-10 and IL-10 homologue encoded by Epstein-Barr virus inhibit antigen presentation, cytokine production, and accessory cell function of monocytes and dendritic cells (44–46). IL-10 also prevents antigen-specific activation and proliferation of T cells, it is produced at high levels by anergic and regulatory T cells, and it induces anergy and tolerance in vitro and in vivo.
Moreover, human IgE synthesis, which is dependent on IL-4 and IL-13 (53, 54), can be blocked by IL-10 via monocyte-dependent mechanisms (45, 50). Additional studies will be useful to further characterize the biological effects of CTLA-4BP in vitro and in vivo. Nevertheless, the present results are consistent with data demonstrating inhibition of T cell response in mice by a mouse CD80 mutant selectively binding to CTLA-4 (55). Moreover, given the data supporting a role for CTLA-4 and IL-10 in inducing and maintaining immunological tolerance, our results suggest that CTLA-4BP may be beneficial in down-regulating the function of specific T cells in allergy and autoimmune diseases.

The sequence information of the chimeric CD28BP and CTLA-4BP provides new insight into the residues that are involved in the ligand binding of B7 co-stimulatory molecules. Sequencing analysis revealed that all of the 14 different clones selected from the second round of evolution, which demonstrated preferential binding to CD28, contained a substitution of valine for isoleucine at amino acid position 49 of the mature sequence. Ile-49 does not appear to be directly involved in the interaction of CD80 with CTLA-4 (23), but substitution I49A was previously shown to completely abolish binding of CD80 to both CD28 and CTLA-4, illustrating the importance of this residue in the ligand binding (20). Although the I49V substitution is considered a relatively conservative replacement, our data suggest that substitutions derived from naturally existing genes via DNA shuffling (e.g. in this case I49V from the bovine CD80) provide improved means to screen for altered functional properties in proteins. Interestingly, bovine CD28 is the only exception among CD28 and CTLA-4 sequences analyzed from 12 different species in which the heptapeptide MYPPPY and Gly-66 are not fully conserved (i.e. MYPPPY is replaced by LYPPPY, and Gly 66 is replaced by valine) (22). Mutations in the MYPPPY sequence generally lead to reduced binding to both CD80 and CD86 (21, 22), and all of these residues with the exception of Pro-101 are in direct contact with CD80 (23). These data suggest that the differences in the bovine CD80-B7 interactions and corresponding sequences contributed to the sequence diversity that also benefited the in vitro evolution of CD28BP described here.

In 14 of the 19 selected clones with preferential binding to CTLA-4, Tyr-31 was replaced by histidine, whereas all 14 clones with preferential binding to CD28 retained the tyrosine at the corresponding position. Interestingly, Tyr-31 in human CD86 is substituted by phenylalanine without any apparent change in the ligand binding affinities (18, 19, 24), whereas the Y31A substitution completely abolishes the binding of hCD80 to both CD28 and CTLA-4 (20). The present data demonstrate that the Y31H substitution permits retention of the interaction with CTLA-4, at least when present in the context of the shuffled CD28BP sequence, whereas this mutation appears to contribute to the loss of binding to CD28. On the other hand, the WPXYNNT motif between the CDR2 loop and the D strand of CD80, which has also been implicated in the interaction with CD28 and CTLA-4 (56) and is conserved across species, was retained in both CD28BP and CTLA-4BP, supporting the notion that this region contributes to binding of CD80 to both of its ligands. Nevertheless, more information of the three-dimensional structures of CD28BP and CTLA-4BP will be useful to further characterize the residues and structures that contribute to the preferential binding of these molecules to the two ligands.

The present results also emphasize the complexity of protein structures and receptor-ligand interactions and illustrate the difficulty in predicting the residues within and outside ligand binding sites that most significantly affect the properties of the proteins. In contrast to the present results, mutations of hCD80 that were previously designed based on structural information and predicted receptor binding sites generally had equivalent effects on binding to CD28 and CTLA-4 (20). The theoretical number of different protein sequences that can be generated by recombining the amino acids naturally present in any given position in the seven different mammalian CD80 molecules used in this study is $4 \times 10^{72}$. This is far more than can be tested in any laboratory, but the current data show that the frequency of functional shuffled variants with altered ligand binding properties is high enough that the desired phenotype can be rapidly identified by selectively designed screening procedures. Thus, appropriate screening of the sequences provided by recombination of known mammalian genes is an effective means to identify chimeric sequences having novel functional properties without detailed knowledge of the receptor binding sites or structures of the proteins.

In summary, the present study demonstrates the generation of novel chimeric co-stimulatory molecules that selectively bind to and signal through CD28 or CTLA-4. CD28BP and CTLA-4BP may help in further dissecting the mechanisms by which CD28 and CTLA-4 trigger positive and negative signals to T cells, respectively. The chimeric co-stimulatory molecules described here, CD28BP and CTLA-4BP, exhibit novel functional properties and provide a unique means to characterize and modulate CD28 and CTLA-4 signaling pathways in T cells.

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