Covalent Dimerization of CD28/CTLA-4 and Oligomerization of CD80/CD86 Regulate T Cell Costimulatory Interactions*

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T lymphocyte receptors CD28 and CTLA-4 bind costimulatory molecules CD80 (B7-1) and CD86 (B7-2) on antigen-presenting cells and regulate T cell activation. While distinct functional roles have been ascribed to some costimulatory receptors, little is known about how they interact. To better characterize these interactions, we have used surface plasmon resonance to perform equilibrium and kinetic binding analyses of extracellular fragments of CD28/CTLA-4/CD80/CD86. We show that CTLA-4 and CD28 binding are both characterized by rapid kinetic on-rates and rapid dissociation rates. Native disulfide-linked homodimers of CD28 and CD86 are bound with high avidity and slow dissociation, while CD80/CD86 is bound with low avidity and rapid dissociation. Therefore, covalent dimerization of CTLA-4 is required for its high avidity binding. Oligomerization of CD80/CD86 is also required for high avidity CTLA-4 binding since CTLA-4 binds with low avidity to monomeric CD80. This contrast with the ability of CD80/CD86 on antigen-presenting cells to bind CTLA4Ig with high avidity and predicts their organization as oligomers or clusters that permit multivalent binding. Thus, covalent receptor dimerization and ligand oligomerization are two key features of the CD28/CTLA-4/CD80/CD86 receptor system that control ligand binding and may regulate signal transduction by controlling the duration of receptor occupancy.

Inter cellular interactions between T lymphocytes and antigen-presenting cells (APC) generate T cell costimulatory signals that regulate T cell responses to antigen in an antigen-nonspecific manner (1, 2). A key costimulatory signal(s) is provided by engagement of T cell receptors CD28 and CTLA-4 with B7 molecules on APC (3–6). CD28 and CTLA-4 are both members of the immunoglobulin superfamily, having single variable-like domains in their extracellular regions (7). These molecules share sequence identity, most notably clustered in their CDR3-like regions of the Ig variable-like domain (8). CD80 and CD86 had similar equilibrium properties for binding to CTLA-4 and CD28, although different kinetic properties were noted for binding of these ligands to CTLA-4 (11).

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1 The abbreviations used are: APC, antigen-presenting cell(s); mAb, monoclonal antibody; SPR, surface plasmon resonance; PAGE, polyacrylamide gel electrophoresis; RU, resonance unit(s).

Despite the structural similarities and common binding partners of CD28/CTLA-4 receptors and CD80/CD86 ligands, different functional properties have been attributed to each of these molecules. Engagement of CD28 by monoclonal antibodies (mAbs) or B7 ligands greatly enhances T cell activation (3–6), whereas engagement of CTLA-4 by specific mAbs may suppress T cell proliferation (14–16). Additional evidence that CTLA-4 negatively regulates the immune system comes from studies of CTLA-4-deficient mice, which have a severe lethal lymphoproliferative phenotype (18, 19). Thus, CD28 and CTLA-4 apparently have different functions during an immune response (17). CD80 and CD86 have also been reported to have different functions, with CD86 preferentially stimulating the production of interleukin-4, but not interleukin-2 (20, 21).

The different biological properties of CD28 and CTLA-4 receptors and CD80 and CD86 ligands may emanate from different binding properties or from different patterns of expression. Relative little is known about how these molecules interact. Studies using recombinant soluble immunoglobulin fusion proteins have shown that CTLA-4 binds with higher avidity to CD80 and CD86 than does CD28 (9–12). Amino acid residues responsible for the increased binding activity of recombinant CTLA4Ig were localized to the CDR1 and extended CDR3-like regions of the Ig variable-like domain (8). CD80 and CD86 had similar equilibrium properties for binding to CTLA-4 and CD28, although different kinetic properties were noted for binding of these ligands to CTLA-4 (11).

Despite these studies, many fundamental properties of CD28/CTLA-4/CD80/CD86 interactions remain unknown. For instance, it is not known how the subunit structures of CD28 and CTLA-4 affect their binding properties. While these molecules have two binding sites per homodimer (13), previous studies have shown only single equilibrium constants for CD80/CD86 binding to CD28 and CTLA-4 (9–12). It is also unclear how previous binding determinations were affected by multivalent Ig fusion proteins. Moreover, very little information is available on the binding properties of CD28, as studies on this molecule have been hampered by its weak avidity.

It was therefore of interest to better characterize the binding interactions of CD28/CTLA-4/CD80/CD86. In this study, we have used surface plasmon resonance (SPR) to measure the precise equilibrium and kinetics of binding of defined fragments of CD28/CTLA-4 and CD80/CD86. SPR provides a sensitive means to measure, in real time, binding interactions between biological molecules without problems associated with other techniques (22). Binding of soluble proteins to immobilized ligands is measured by the accumulation of mass on the surface of a sensor chip. SPR can be used to directly measure equilibrium binding constants and also to estimate kinetic association (k\text{on}) and dissociation (k\text{off}) rate constants, which can then be used to calculate equilibrium binding constants.
CD28 and CTLA-4 Binding to CD80 and CD86

The results of our analysis demonstrate previously unknown roles for CD28/CTLA-4 dimerization and CD80/CD86 oligomerization in regulating binding interactions between these molecules.

MATERIALS AND METHODS

**Ig Fusion Proteins and Thrombin Fragments**—Chimeric mAb L6 was obtained from Bristol-Myers Squibb Pharmaceutical Research Institute (Seattle, WA). Anti-CTLA-4 mAb 10A8 and 11D4; anti-CD28 mAb 9.3, and Ig fusion constructs CTLA4Ig, CD28Ig, CD80Ig, and CD86Ig have also been described (11, 23). For preparation of recombinant forms of the extracellular domains of CD28 and CTLA-4, Ig fusion proteins were prepared that contained a synthetic thrombin cleavage site between the extracellular region and the Fc region. Polymerase chain reaction products encoding the extracellular domains of CD28 and CTLA4Ig (9, 10) were digested with HindIII and BclI restriction enzymes and ligated into a HindIII/BamHI-digested Ig expression vector encoding a synthetic thrombin cleavage site 5’ to the hinge region of a human IgG1 Fc domain (24). The resulting thrombin cleavage site-containing fusion constructs were designated CTLA4Ig and CD28Ig, respectively. Ig fusion proteins were expressed and purified, except for COOH-terminal transfected COS cells or stably transfected Chinese hamster ovary cells and purified by affinity chromatography on immobilized protein A-Sepharose (Repligen, Cambridge, MA) (9). The purified recombinant Ig fusion proteins had properties indistinguishable from those of CD28Ig and CTLA4Ig (9, 10). For preparation of the dimeric extracellular domains of these proteins, the fusion proteins were digested with purified bovine thrombin (Armour Pharmaceutical Corp., Kankakee, IL) at a final concentration of 5 units/mg of protein at 37 °C for 40–60 min. The cleaved extracellular domains were purified from other digestion products (13) and are designated CTLA4-tlp and CD28-tlp, respectively. The predicted amino acid sequence of CTLA4-tlp contains residues 1–127 (numbered as in Ref. 8) of CTLA4 fused to a thrombin-cleaved linker peptide (PSDSPGGGGGSGGGLV-GLPPSPPPSPGGGS-). Concentrations of CTLA4-tlp and CD28-tlp were estimated using extinction coefficients of 2.2 and 1.7 ml/mg, respectively; these were experimentally determined by measuring the absorbance of solutions whose protein concentration was determined by amino acid analysis. Molecular weights were calculated using monomeric molecular weights. CTLA4-tlp and CD28-tlp preparations were analyzed by gel permeation chromatography to ensure their homogeneity; all preparations used in this paper contained <2% of high molecular weight aggregate(s). Some preparations of CTLA4-tlp contained excessive amounts of high molecular weight aggregate(s), so these were subjected to further purification by size fractionation by gel permeation chromatography by gel permeation chromatography. CTLA4-tlp and the previously described thrombin fragment, CTLA4-tl (13), were used interchangeably in these experiments since they had identical binding properties. Preparation of CTLA4-tl (a monomeric form of CTLA4) with a cysteine to serine mutation in the position of the interchain disulfide of CTLA-4) and CD86-tlp (a monomeric form of the extracellular domain of CD86) was described previously (13).

**Analytical Techniques**—Two columns were used for gel permeation chromatography. In most experiments, protein fragments were analyzed on a TSK-GEL G3000 SWXL column (Tosohaa, Montgomeryville, PA) equilibrated in phosphate-buffered saline containing 0.02% NaN3 at a flow rate of 1 ml/min. In some experiments, protein fragments were analyzed on a Waters SW 991 column equilibrated with phosphate-buffered saline containing 10 mM Tris-HCl, pH 7.4, and 0.01% NaN3 at a flow rate of 0.35 ml/min. Equivalent results were obtained with both columns. SDS-PAGE was performed on Tris/glycine gels (Novex, San Diego, CA). Gels were stained with Coomassie Blue, and images of wet gels were obtained by digital scanning.

**BIAcore Analysis**—All experiments were run on BIAcore 3000 or BIAcore 2000 biosensors (Pharmacia Biotech AB, Uppsala) at 25 °C. Ligands were immobilized on research-grade CM5 sensor chips (Pharmacia) using standard N-ethyl-N-(dimethylaminopropyl)carbodiimide/N-hydroxysuccinimide coupling (22, 25). Proteins were diluted in 10 mM sodium formate buffer, pH 4.0, and incubated with activated sensor chips times varied to optimize the degree of derivatization for particular experiments. After coupling, excess N-hydroxysuccinimide groups were inactivated with ethanolamine HCl (22, 25). Control chips were either derivatized with chimeric mAb L6 or mock-derivatized by activating and then inactivating with ethanolamine without the addition of protein. Identical results were obtained with both types of control chips. After binding of CTLA-4 or CD28, CD80Ig- and CD86Ig-coated chips were regenerated with a 60-s pulse of 50 mM sodium citrate and 500 mM NaCl, pH 4.0 or 5.0, respectively. Control experiments showed that CD80Ig- and CD86Ig-derivatized surfaces were able to withstand >30 rounds of regeneration without losing binding capacity. Before use, all sensor chips were subjected to several rounds of analyte binding, followed by regeneration to ensure a stable level of derivatization. Mobile phase buffer for immobilization was phosphate-buffered saline, pH 7.4, containing 0.005% P20 surfactant (Pharmacia). For binding assays, 200 μg/ml bovine serum albumin was added to the mobile phase buffer.

**BIAcore Data Analysis**—Sensorgram base lines were normalized to zero response units (RU) prior to analysis. Samples were run over mock-derivatized flow cells to determine background RU values due to bulk refractive index differences between solutions. Equilibrium dissociation constants (KD) were calculated from plots of Rmax versus C, where Rmax is the steady-state response minus the response on a mock-derivatized chip, and C is the molar concentration of analyte. Binding curves were analyzed using commercial nonlinear curve-fitting software (Prism, GraphPAD Software, San Diego, CA). Experimental data were first fit to a model for a single ligand binding to a single receptor (one-site model, i.e. a simple Langmuir system, A + B → AB), and equilibrium dissociation constants (KD = [A][B]/[AB]) were calculated as described by the equation Rmax = C/KD + C. Subsequently, data were fit to the simplest two-site model of ligand binding (i.e. to a receptor having two non-interacting independent binding sites), as described by the equation Rmax = C/KD1 + C + C/KD2 + C. The goodness-of-fit of these two models were analyzed visually by comparison with the experimental data and statistically by an F test of the sums-of-squares. The simpler one-site model was chosen as the best fit unless the two-site model fit significantly better (p < 0.1).

**Association and Dissociation Kinetics**—Association and dissociation analyses were performed using BIAevaluation 2.1 software (Pharmacia). Association rate constants (ka) were calculated in two ways, assuming both homogeneous single-site interactions and parallel two-site interactions. For single-site interactions, ka values were calculated according to the equation R = Rmax(1 − exp(−kt)), where R is the response at a given time, t; Rmax is the steady-state response; t is the time of the start of the injection; and k = dR/dt = −kdC + koff, where C is the concentration of analyte, calculated in terms of monomeric binding sites. For two-site interactions, ka values were calculated according to the equation R = Rmax(1 − exp(−kt2)) + Rmax(1 − exp(−kt1)), for each model, the values of ka were determined from the calculated correlation coefficients (at 70% maximal association) of plots of k versus C.

**Dissociation Data Analysis**—Dissociation data were analyzed according to one-site (AB → A + B) or two-site (AB → A + B) models, and rate constants (kd) were calculated from best fit curves. The one-binding site model was used except when the residues were greater than machine background (2–10 RU, according to machine), in which case the two-binding site model was employed. Half-times of receptor occupancy were calculated using the relationship t1/2 = 0.693/koff.

**RESULTS**

**Preparation of Defined Fragments of the Extracellular Domains of CTLA-4 and CD28**—Accurate measurement of CD28/CTLA-4/CD80/CD86 interactions required preparation of fragments of these molecules with defined valencies. In a previous study (13), we described the preparation and characterization of dimeric and monomeric forms of the extracellular domain of CTLA-4 and a monomeric form of CD86 (CD86-tlp). Preparation of these fragments was accomplished by thrombin cleavage of (presumed) dimeric CTLA4Ig and CD86Ig at a nonclassical cleavage site in the Fc hinge region of these molecules (13). While these fragments were useful, they were difficult to produce in sufficient yield because of inefficient thrombin cleavage. To improve the yield of these extracellular fragments, we re-engineered the CTLA4Ig and CD86Ig fusion proteins such that a synthetic thrombin recognition site was introduced between the extracellular domains of CTLA-4 or CD28 and the Fc domain (see “Materials and Methods”). The resulting CTLA4Ig and CD86Ig fusion proteins had binding properties indistinguishable from those of our original fusion proteins, but...
could be more efficiently cleaved by thrombin. From these new fusion proteins, we prepared fragments of the extracellular domains of CTLA-4 and CD28, termed CTLA-4tp and CD28tp, as described under “Materials and Methods.” CTLA-4tp and CD28tp retained binding activities essentially equivalent to those of the corresponding Ig fusion proteins as indicated by monitoring binding activities during purification. These proteins also retained the ability to bind anti-CTLA-4 and anti-CD28 mAbs (data not shown), indicating that they had native conformations. These fragments were characterized by SDS-PAGE and gel permeation chromatography in the experiments shown in Fig. 1. Under nonreducing conditions, CTLA-4tp migrated at $M_r \sim 55,000$–60,000 when analyzed by either technique; upon reduction, it migrated at $M_r = 25,000$–35,000. CTLA-4tp therefore was a disulfide-linked dimer. CD28tp migrated at $M_r \sim 65,000$ and 45,000 by SDS-PAGE under nonreducing and reducing conditions, respectively. In contrast, during gel permeation chromatography, CD28tp eluted at $M_r \sim 180,000$ and 65,000 under nonreducing and reducing conditions. The elution volume of CD28tp during gel permeation chromatography was therefore greater than would be predicted by SDS-PAGE. This difference may be due to the predicted extensive glycosylation of CD28tp, or it may indicate that CD28tp in solution existed as a tetramer. At present, we cannot distinguish between these possibilities, but to simplify subsequent calculations, we have assumed that CD28tp was dimeric. Neither CTLA-4tp nor CD28tp preparations used for binding measurements had significant amounts of aggregated material present. Monomeric CD86 (CD86t) and monomeric CTLA-4 (CTLA-4C120S, prepared by site-directed mutagenesis of the cysteine residue that mediates the intrachain disulfide bond) were prepared as described (13).

Equilibrium Binding Analysis of CTLA-4 and CD28—We initially used SPR to measure equilibrium binding of CTLA4Ig, CD28Ig, CD80Ig, and CD86Ig fusion proteins using the methods of van der Merwe et al. (26). This procedure was devised for determining the equilibrium binding properties of lymphocyte surface receptors with low binding avidity and rapid kinetic off-rates. CD80Ig and CD86Ig were coupled to sensor chips, and solutions of CTLA4Ig and/or CD28Ig were added to the mobile phase. The levels of derivatization of the chips were adjusted to ensure that steady-state (equilibrium) binding was achieved at low concentrations of analyte. Similar results were obtained at different levels of chip derivatization. Equilibrium binding of CTLA4Ig at a given concentration was determined by comparing the steady-state RU on CD80Ig- or CD86Ig-coated chips with the value obtained on control chimeric mAb L6 or mock-derivatized chips. From this comparison, levels of specific binding were determined at different concentrations. Equilibrium dissociation constants ($K_d$) could then be determined from binding isotherms by curve fitting. Similar results were obtained whether we measured binding of CTLA4Ig to immobilized CD80Ig or CD86Ig or binding of CD80Ig or

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2 J. L. Greene, G. M. Leytze, J. Emswiler, R. Peach, J. Bajorath, W. Cosand, and P. S. Linley, unpublished data.
Equilibrium binding constants were measured directly in experiments performed as described for Figs. 2–4. Shown are means ± S.D. of equilibrium dissociation constants ($K_d$) from two to five independent experiments. $K_d$ values were obtained by curve fitting experimental data to one- and two-site models as described under "Materials and Methods." $p$ values for the statistical improvement in choosing a particular two-site model over a one-site model are given. When a two-site model was chosen ($p < 0.1$), both high avidity ($K_d$) and low avidity ($K_{ds}$) dissociation constants were calculated. The proportion of total binding attributable to the high avidity site is indicated as percent $K_{ds}$. Calculated $K_d$ values were derived from experimentally determined kinetic rate constants (see Table II) using the equation $K_d = k_{on}/k_{off}$. Where two $K_{ds}$ values were determined (see Table II), two values for $K_d$ are given; the calculated percent $K_d$ was determined from the percentage of total binding attributable to the more slowly dissociating (higher avidity binding) component (see Table II).

| Immobilized protein | Analyte            | $p$     | $K_{ds}$ | $K_d$ | $K_{ds}$ | $K_d$ | $K_{ds}$ | $K_d$ |
|---------------------|--------------------|---------|----------|-------|----------|-------|----------|-------|
| CD80lg              | CTLA4X C120S       | 0.786   | 211 ± 34 | NA    | 4 ± 1    | 115 ± 91 | 78 ± 2 | 6 ± 1    | 4 ± 1 |
| CD80lg              | CD80lg-4p         | <0.0001 | 1 ± 1    | NA    | 4 ± 1    | 115 ± 91 | 78 ± 2 | 6 ± 1    | 4 ± 1 |
| CD80lg              | CD80lg-2tp        | 0.091   | 2500 ± 2100 | 41,000 ± 19,000 | 15 ± 12 | 4900 ± 90,000 | 31 ± 8 |
| CD86lg              | CTLA4X C120S       | 1.0     | 2200 ± 970 | NA    | NA      | 2000 ± 8000 | 62 ± 11 |
| CD86lg              | CTLA-4tp          | <0.001  | 4 ± 3    | 510 ± 230 | 58 ± 10 | 4 ± 70 | 62 ± 11 |
| CD86lg              | CD28tp            | <0.001  | 2300 ± 1500 | 37,000 ± 13,000 | 23 ± 6 | 5600 ± 34,000 | 38 ± 4 |

* NA, not applicable (i.e. a one-binding site model fit the data adequately).
10-fold). Finally, in no case did use of the more complex two-site model improve the linear relationship (as judged by correlation coefficient ($r$) and the standard error of measurement) between the $k_s$ values and concentration. For these reasons, we therefore concluded that use of two-site models of association was not justified. We determined a single association rate constant ($k_{on}$) for each combination of proteins (Table II). Monomeric CTLA-4 bound with $k_{on}$ values within 2–6-fold those for dimeric CTLA-4 (for CD80Ig and CD86Ig, respectively). The similarity in these $k_{on}$ values argues that the native conformation of monomeric CTLA-4 was maintained. The rapid $k_{on}$ values determined for CTLA-4 were near the limits of instrument detection.

The apparent dissociation rate constants ($k_{off}$) can be influenced by the rebinding of dissociated analyte. For these determinations, therefore, it was desirable to minimize rebinding by...
derivatizing sensor chips with moderately low levels of CD80 Ig or CD86 Ig and by maximizing analyte concentrations. Dissociation of monomeric and dimeric CTLA-4 and dimeric CD28 was measured from CD80 Ig-derivatized (data not shown) sensor chips. Dissociation curves were fit with one- and two-binding site models (Fig. 6). Dissociation of monomeric CTLA-4 from CD80 Ig (Fig. 6, A and B) and CD86 Ig (data not shown) was approximated by a one-binding site model such that the residuals between the observed and calculated curves were within experimental variation of BIAcore measurements. Analysis of these dissociation curves gave very rapid $k_{off}$ values (Table II), corresponding to half-times of receptor occupancy of $\sim$8 and $\sim$2 s for CD80 Ig and CD86 Ig, respectively. The $k_{off}$ values calculated in this way were in good agreement with those estimated from the y intercept of the $k_{on}$ versus concentration plots used to calculate $k_{on}$ values (Fig. 5).
CD28 and CTLA-4 Binding to CD80 and CD86

### Table II

**Summary of apparent kinetic constants**

| Immobilized protein | Analyte         | $k_{on}$ ($\times 10^3$) | $k_{off}$ ($\times 10^{-3}$) | $K_d$ ($\times 10^{-3}$) | $R^2$ |
|---------------------|-----------------|--------------------------|-----------------------------|--------------------------|-------|
| CD80lg              | CTLA4X120S      | 440 ± 40                 | 91 ± 39                     | NA                       | NA    |
| CD80lg              | CD86lg          | 890 ± 220                | 4 ± 2                      | 90 ± 77                  | 86 ± 3|
| CD86lg              | CD80tp          | 7 ± 0.1                  | 32 ± 34                    | 580 ± 290                | 31 ± 8|
| CD86lg              | CTLA4tp         | 420 ± 130                | NA                        | NA                       |       |
| CD86lg              | CD28tp          | 5 ± 4                    | 93 ± 65                    | 62 ± 11                  |       |
| CD86lg              | CD28tp          | 1300 ± 96                |                            |                          |       |

*NA, not applicable (i.e. a one-binding site model fitted the data adequately). The determined $k_{on}$ and $k_{off}$ values for monomeric CTLA-4 binding to CD80lg and CD86lg, equilibrium binding constants ($K_d$) could be calculated (Table I), which were in good agreement with the experimentally determined $K_d$ values.

Analysis of dissociation curves for dimeric CTLA-4 and CD28 was more complicated (Fig. 6, C and D; E and F, respectively). In neither case did a one-binding site model provide a satisfactory fit to the experimental data (Fig. 6, C and E; F), residuals were greater than the experimental variation, especially in the early phases of the dissociation curves. However, for both dimeric CTLA-4 and CD28, two-binding site models gave a reasonable agreement between the observed and expected binding (Fig. 6, D and F), and residuals were within experimental variation. From these models, two $k_{off}$ values could be calculated (Table II), consistent with the calculation of two equilibrium binding constants ($K_d$) (Table I). From the kinetic constants ($k_{on}$ and $k_{off}$) (Table II), equilibrium binding constants ($K_d$) for CTLA-4tp and CD28tp binding could be calculated (Table I). These, for the most part, agreed well with the experimentally determined values, both in magnitude and in percentage of total binding.

**High Avidity Binding of CTLA-4 Is Due to Rebinding**—Binding of dimeric CTLA-4tp was characterized by two $K_d$ values and two $k_{off}$ values (Tables I and II). The more rapidly dissociating (weaker binding) $k_{off}$ values for dimeric CTLA-4tp approximated the $k_{off}$ value for monomeric CTLA4X120S. Slower dissociation (stronger binding) could indicate rebinding of dimeric CTLA-4 during the dissociation measurements (11). To test this possibility, we measured $k_{off}$ rates of CTLA-4tp from immobilized CD80lg and CD86lg in the presence of increasing amounts of soluble CD80lg (Fig. 7). This would be expected to occupy free CTLA-4tp binding sites and prevent their rebinding to immobilized CD80lg and CD86lg.

As shown in Fig. 7, the rate of dissociation of CTLA-4tp increased in the presence of increasing concentrations of soluble CD80lg. Very high concentrations (>1 mg/ml) of CD80lg were required to produce these effects, probably because of the high density of CD80lg and CD86lg immobilized on the sensor chips. The apparent dissociation constants ($k_{off}$ values determined using a one-binding site model) for CTLA-4tp measured in the presence of increasing competitor increased asymptotically and approached values determined for monomeric CTLA4X120S (Table II). The more slowly dissociating, higher avidity component of dimeric CTLA-4tp binding was therefore reduced when rebinding was blocked. In other experiments, we measured the effects of increasing concentrations of CD80lg on the dissociation of CTLA4X120S and CD28tp from immobilized CD80lg and CD86lg. In contrast to its effects on dissociation from CTLA-4tp, soluble CD80lg did not greatly affect dissociation of these other molecules (~2-fold effect). Thus, rebinding of monomeric CTLA-4 or dimeric CD28 affected dissociation measurements less than it did for dimeric CTLA-4. It should be emphasized that the $k_{off}$ values determined for these molecules in the absence of competition were near the limit of detection, so further increases may not have been possible to measure.

**Effects of CD86 Valency on High Avidity Binding of CTLA-4**—It was also important to determine the effects of valency of CD80/CD86 molecules on CTLA-4 binding avidity. We therefore measured binding of monomeric CD86 to immobilized CTLA4lg. The use of immobilized CTLA4lg was justified by other experiments (data not shown) showing that CTLA4lg gave similar equilibrium and kinetic constants as CTLA-4tp for binding to immobilized CD80lg and CD86lg.

We compared equilibrium binding of monomeric CD86lg and multimeric CD86lg to immobilized CTLA4lg (Table III). For monomeric CD86lg, the data were adequately fit by a one-binding site model, yielding a low affinity $K_d$ of ~1400 nm. This was similar to the $K_d$ measured for binding of monomeric CTLA4X120S to immobilized CD86lg (~2200 nm) (Table II). The association constants ($k_{on}$ and $k_{off}$) for CD86 binding to CTLA4lg were estimated as $3.7 \times 10^5$ M$^{-1}$ s$^{-1}$ and 0.5 s$^{-1}$, which were also very similar to those measured for binding of monomeric CTLA4X120S to immobilized CD86lg (Table II). Thus, both equilibrium and kinetic binding constants for binding of monomeric CD86lg to CTLA4lg reflect low avidity binding. In contrast, binding of multimeric CD86lg gave both high and low avidity equilibrium binding constants ($K_d$ = 7 and 110 nm), which were very similar to those determined for dimeric CTLA-4tp binding to immobilized CD86lg (Table II). Thus, valency of both CTLA-4 and CD86 affects binding avidity.

**DISCUSSION**

Previous studies showed that a covalent CTLA-4 homodimer contained two binding sites for CD80/CD86 molecules, but how these two sites affected binding avidity was not examined (13). Indeed, studies on the binding properties of CTLA-4 and CD28 identified only single avidity classes of binding sites on these molecules (7, 9–12). Here, using the more sensitive techniques of SPR, we show that dimeric CD28 and CTLA-4 show both high and low avidity binding, consistent with their 2:2 binding stoichiometries.

Binding of both monomeric and dimeric CD28 to CD80lg or CD86lg was characterized by very rapid apparent $k_{off}$ values (~0.2–1 $\times 10^6$ M$^{-1}$ s$^{-1}$). It should be mentioned that determination of kinetic on-rates to ligands immobilized in a polymer matrix can lead to underestimation of the true kinetic on-rates (28). The kinetic on-rates for CTLA-4 binding are comparable to those for other T lymphocyte receptor/ligand pairs, such as CD2/CD58 (~4 $\times 10^5$ M$^{-1}$ s$^{-1}$) (26) and T cell receptor-major histocompatibility class I peptide complexes (~2 $\times 10^9$ M$^{-1}$ s$^{-1}$).
The $k_{off}$ values for CD28 binding to CD80Ig and CD86Ig were also very rapid, but were similar to other lymphocyte surface receptors (26, 27). This would predict a short time of receptor occupancy for CD28/CD80/CD86 interactions (half-times of less than $-1$ s for CD28/CD80/CD86, compared with less than or equal to $-6$ s for CD2/CD58 (26) and $-17$ s for T cell receptor/major histocompatibility complex class I peptide interactions (27)). These values for CD28 were calculated from
bivalent (higher avidity) binding constants; monovalent (lower avidity) binding would lead to much shorter occupancy times. Comparison of occupancy times for dimeric CTLA-4 is more difficult because of the effects of receptor valency on apparent dissociation rate. Dimeric CTLA-4 has slower kinetic off-rates compared with CD28, but its time of receptor occupancy is still relatively short (half-time of less than \(180 \text{ s}\)). Monomeric CTLA-4 binding would also lead to a short period of receptor occupancy (less than \(8 \text{ s}\) and \(2 \text{ s}\) for binding to CD80Ig and CD86Ig, respectively), but the physiological relevance of this is unclear since CTLA-4 primarily exists as a disulfide-linked homodimer.

CD80 and CD86 have very similar equilibrium binding properties, despite sharing very little amino acid sequence homology (11). Nevertheless, CD80 and CD86 were reported to deliver qualitatively different costimulatory signals during T cell activation (20, 21). It is therefore of interest to examine the binding properties of these molecules in detail. Certain of the measurements made here confirm measurements made in previous studies (11). For example, the \(K_d\) values for dimeric CTLA-4 binding to CD80Ig and CD86Ig were similar to those reported previously for CTLA4Ig binding. Also, binding of dimeric CTLA-4 to CD80Ig was 3–4-fold higher than to CD86Ig (11). The difference in the kinetic off-rates of monomeric CTLA-4 binding to CD80Ig and CD86Ig (Table II) was similar to a previous estimate made using transfected cells (11). In other instances, however, SPR has permitted comparisons which were not possible in previous studies. Here we provide the first estimates of affinity constants (as opposed to avidity constants) for monomeric CTLA-4 binding to CD80Ig and CD86Ig (\(200 \text{ and } 2200 \text{ nM}\)), which differ by \(10\)-fold. It remains to be determined how different CD80 and CD86 binding properties affect signaling properties of these molecules.

A comparison of the binding properties of CTLA-4 and CD28 is important in light of the apparently different biological functions of these molecules (17). Binding of dimeric CTLA-4 to CD80Ig was 2500- and 270-fold stronger (for \(K_d\) values) compared with dimeric CD28. This difference is greater than that previously determined (9, 10) because of the more than \(10\)-fold larger (indicating weaker binding) \(K_d\) values for high avidity binding of dimeric CD28 measured in the present study. This difference probably arises from aggregation and uncertain valencies of the Ig fusion proteins used previously. Despite the weak binding of dimeric CD28 measured in this study, our analysis predicts that monomeric CD28 would bind even weaker. Binding of dimeric CTLA-4 to CD86Ig was

![Figure 7](http://www.jbc.org/)

**Figure 7.** Slow apparent dissociation of dimeric CTLA-4 is due to rebinding. Shown are the effects of increasing concentrations of soluble CD80Ig on the dissociation of CTLA-4-tp from immobilized CD80Ig (A) or CD86Ig (B). For CD80Ig and CD86Ig, 2200 and 2280 RU were immobilized, respectively. Dissociation measurements were run at 80 \(\mu\text{m/min}\). The upper panels show raw sensorgrams transformed to remove bulk refractive index changes due to the high concentrations of competing CD80Ig. The lower panels show the dissociation rate constant \((k_{off})\) as a function of competitor concentration. The \(k_{off}\) values were determined for the dissociation of 20% of bound CTLA-4-tp, beginning after bulk refractive index changes (arrows).

**Table III**

High avidity binding of CTLA-4 requires multivalent CD86

| Immobilized protein | Analyte     | \(K_{d1}\) | \(K_{d2}\) |
|---------------------|-------------|------------|------------|
| CTLA4Ig             | CD86t       | 1400       | NA*        |
| CTLA4Ig             | CD86Ig      | 7          | 110        |

* NA, not applicable (i.e. data were adequately fit by a one-site model).
570- and 70-fold stronger (for $K_{d1}$ and $K_{d2}$, respectively) compared with CD28.

Rapid kinetic off-rates facilitate the transitory intercellular interactions characteristic of lymphocytes, but present the problem of how to maintain CD80/CD86 ligand occupancy long enough to generate productive signals. We have demonstrated here two characteristics of the CD28/CTLA-4/CD80/CD86 receptor system that would tend to counteract the effect of fast kinetic off-rates and thereby increase the duration of receptor occupancy. The first of these is the influence of the two CD80/CD86-binding sites per pre-existing CD28/CTLA-4 covalent dimer; the second, oligomerization of CD80/CD86 molecules on APC.

We have shown that bivalent CTLA-4 and probably CD28 have different kinetic and equilibrium binding characteristics compared with the monomeric molecules. Bivalently bound CD28/CTLA-4 receptors have a decreased likelihood of dissociating from CD80/CD86 ligands. This decreased dissociation due to bivalent binding would prolong the effective receptor occupancy time.

Oligomerization of CD80 and/or CD86 molecules would also tend to prolong CD28/CTLA-4 receptor occupancy. CD80/CD86 molecules are not known to dimerize (7). However, while (dimeric) CTLA4Ig binds with high avidity to APC, CTLA-4 dimers do not bind with high avidity to monomeric CD80. High avidity binding of CTLA4Ig to APC may be accounted for by oligomerization or clustering of CD80/CD86 molecules on the cell surface. Clusters of CD80/CD86 could either pre-exist or may be ligand (CTLA4Ig)-induced. Available data support the existence of pre-existing clusters of CD80/CD86 molecules since high avidity binding of CTLA4Ig is observed under conditions that discourage ligand-induced clustering (i.e. at low temperature, in the presence of metabolic inhibitors, or on fixed cells). The existence of pre-existing oligomers of CTLA4Ig-binding molecules on human Langerhans cells was also suggested by earlier immunofluorescence microscopy studies (29). Pre-existing oligomers of CD80/CD86 molecules would favor multiple engagements with CD28 and/or CTLA-4 receptors.

Engagement of CD28 and/or CTLA-4 receptors by clustered CD80/CD86 ligands would increase the local receptor concentration and provide for greater receptor occupancy. Despite the concentration-driven increase in receptor occupancy, the rapid dissociation kinetics of CD28/CTLA-4/CD80/CD86 complexes would still result in transiently unoccupied CD80/CD86 molecules. These could then rebind other CD28/CTLA-4 receptors, perhaps allowing engagement of many CD28/CTLA-4 receptors by relatively few CD80/CD86 molecules. A similar process has been suggested for T cell receptor-major histocompatibility class I peptide complexes (30).

Clustering of CD80/CD86 molecules on the APC surface may explain earlier findings that CD28 aggregation by mAbs enhances signaling through this receptor and alters the biochemical nature of signals generated (reviewed in Ref. 31). Clusters of CD80/CD86 molecules could cross-link different CD28/CTLA-4 receptors because of their bivalent binding. Cross-linking would also be favored by the rapid kinetic on- and off-rates of binding. Clustering of CD80/CD86 molecules on APC may therefore be an important control point for the activity of this receptor/ligand system.

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