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*J Immunol* 1998; 161:2700-2707; 
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Two Regions in the CD80 Cytoplasmic Tail Regulate CD80 Redistribution and T Cell Costimulation

Raymond T. Doty* and Edward A. Clark2*†

CD80 is a major T cell costimulatory molecule, delivering signals distinct from those of the CD3/TCR complex, which regulate cytokine and cytokine receptor expression, cell proliferation, and cell viability. CD28 needs to be cross-linked to initiate signals, yet both of its ligands, CD80 and CD86, are expressed as monomers. Previously, we determined the cytoplasmic tail of CD80 is required for CD28-mediated costimulation and subcellular relocalization of CD80 in lymphocytes. In this study, we report that Reh B cell transfectants expressing CD80 with mutations in the cytoplasmic tail region either at 275–278 (RRNE→AAAA, CD80/AA) or serine 284 (S→A, CD80/SA) can bind ligand similar to transfectants expressing wild-type CD80, yet are unable to costimulate T cell proliferation. These mutant CD80 molecules are expressed on the surface of the Reh cells in small clusters or foci indistinguishable from those of wild-type CD80 molecules. However, mutant CD80 molecules unlike wild-type CD80 cannot be readily induced by ligand into caps. Thus, small clusters of CD80 found on APC are insufficient to initiate CD28-mediated signals, and the formation of CD80 caps appears to be a critical factor regulating the initiation of T cell costimulation. A 30-kDa phosphoprotein that associates with the cytoplasmic tail of CD80 in activated cells may play a role in CD80 redistribution and thus CD82-mediated costimulation. These results indicate two distinct regions of the CD80 cytoplasmic tail regulate its costimulatory function, and both regions are required for CD80 function. The Journal of Immunology, 1998, 161: 2700–2707.

Materials and Methods

Abs and cell lines

The CD80-binding mAb BB1 (1), the CD16 mAb FC1 (22), the CD3 mAb 64.1 (23), and the MHC class I mAb W6/32 (24) were purified from ascites before use. CTLA4Ig fusion protein (15) for immunoprecipitations and ligand-binding studies was kindly provided by Dr. P. Linsley (Bristol-Myers Squibb, Seattle, WA). Human IgG from Sigma (St. Louis, MO) was used as a control for CTLA4Ig. Goat anti-mouse IgM-biotin and goat anti-human IgG-biotin were purchased from Jackson ImmunoResearch (West Grove, PA). Streptavidin-Texas Red was purchased from Molecular Probes.
The resulting CD4 plates at a density of 10^5 cells/well or as indicated. Anti-CD3 was added to mitomycin C (Sigma) in RPMI (no serum) for 45 min, then washed three times, and vector-transfected Reh cells (RRc) were described before (21), and CD80 expression) for use in costimulation assays.

CD80 expression) for use in costimulation assays.

lower levels of CD80 on the cell surface (equivalent to levels of mutant CD80 proteins, Grand Island, NY). A subline of R80 was isolated that expresses lower levels of CD80 on the cell surface (equivalent to levels of mutant CD80). The R80 transfectant was treated with 100 µg/ml of active G418 (Life Technologies) before adding Ab or CTLA4Ig to a final concentration of 10 µg/ml. The bound material was precipitated with protein A-Sepharose and washing four times with lysis buffer. The postnuclear fractions were precleared twice with protein A-Sepharose (Pharmacia) before adding Ab or CTLA4Ig to a final concentration of 10 µg/ml. The bound material was precipitated with protein A-Sepharose and washing four times with lysis buffer. The samples were extracted with reverse precipitation buffer (150 mM NaCl, 5 mM MgCl2, 2 mM L-glutamine, 1.8 mM glucose, 1 mM CaCl2, in 10 mM Tris, pH 7.5). The cells were resuspended in POP medium (POP buffer with 2% diazoyl FCS) and incubated at 37°C for 1 h. The cells were washed again, resuspended in POP medium with 1 mCi[^32P]Pi, and incubated at 37°C for 4 h. Then cells were stimulated with PMA (10 ng/ml) and ionomycin (500 ng/ml) for the final 10 min of culture. The cells were washed and resuspended in PBS before being lysed in Nonidet P-40 lysis buffer (0.5% Nonidet P-40, 50 mM Tris, pH 8.0, 150 mM NaCl, and 5 mM EDTA) containing protease and phosphatase inhibitors.

The postnuclear fractions were precleared twice with protein A-Sepharose (Pharmacia) before adding Ab or CTLA4Ig to a final concentration of 10 µg/ml. The bound material was precipitated with protein A-Sepharose and washing four times with lysis buffer. The samples were extracted with reducing sample buffer and run on 9% SDS-PAGE. The gels were fixed and dried before being exposed to x-ray film.

**Results**

The cytoplasmic tail of CD80 is required for T cell costimulation and for Ab-mediated T cell contact-induced CD80 redistribution (21). To identify regions in the cytoplasmic tail of CD80 required for T cell costimulation and for CD80 redistribution, we generated several truncation mutants, and two substitution mutants of CD80. The truncation mutants presented in this study, CD80a16 and CD80a11, are missing the carboxyl-terminal 16 and 11 amino acids of CD80, respectively (see Table I). The cytoplasmic tail of human CD80 contains a tetra peptide motif RRNE at positions 275–278. This sequence is conserved in the cytoplasmic tails of CD80 proteins.

### Table I. Mutant CD80 proteins

<table>
<thead>
<tr>
<th>Construct</th>
<th>BB1</th>
<th>CTLA4Ig</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD80</td>
<td>7.3</td>
<td>8.6</td>
</tr>
<tr>
<td>CD80/Δ16</td>
<td>10.0</td>
<td>9.1</td>
</tr>
<tr>
<td>CD80/Δ11</td>
<td>10.4</td>
<td>12.0</td>
</tr>
<tr>
<td>CD80/4A</td>
<td>12.5</td>
<td>13.8</td>
</tr>
<tr>
<td>CD80/SA</td>
<td>5.0</td>
<td>5.5</td>
</tr>
</tbody>
</table>

*Fluorescence intensity (FI) of wild type and mutant CD80 expression on Reh transfectants in Figure 1 as detected by BB1 or CTLA4Ig.

**Abbreviations used in this paper:** FL, fluorescence intensity; GPI, glycosylphosphatidylinositol.

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(Eugene, OR). Reh cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in RPMI 10 (RPMI 1640 with 10% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin). Wild-type CD80-expressing Reh cells (R80), tailless CD80-expressing Reh cells (R80Δ16), and vector-transfected Reh cells (RRc) were described before (21), and were cultured in RPMI 10 with 250 µg/ml of active G418 (Life Technologies, Grand Island, NY). A subline of R80 was isolated that expresses lower levels of CD80 on the cell surface (equivalent to levels of mutant CD80 expression) for use in costimulation assays.

T cells and proliferation assays

T cells were isolated from normal healthy donors by rosetting with SRBC, followed by hypotonic shock to lyse the SRBC. This Er3 population routinely consisted of about 85% CD3+ T cells, 15% CD16+ NK cells, and less than 1% CD14+ monocytes or CD20+ B cells. Purified CD4+ T cells were isolated by depletion of CD8+ and CD16+ cells on Ab-coated plates. The resulting CD4+ T cell population was routinely greater than 97% CD3+ and 95% CD4+.

Reh transfectants were treated with 100 µg/ml of mitomycin C (Sigma) in RPMI (no serum) for 45 min, then washed three times in RPMI 10 before being plated in triplicate in 96-well flat-bottom plates at a density of 10^5 cells/well or as indicated. Anti-CD3 was added to a final concentration of 1 µg/ml or as indicated. Fifty thousand CD4+ T cells were then added to each well. The cells were pulsed with 0.5 mCi of [3H]thymidine (NEN, Wilmington, DE) for the final 16 h of a 5-day culture before being harvested on glass fiber filter strips and counting the incorporated 3H with a scintillation counter.

**Site-directed mutagenesis and transfections**

Mutant CD80 cDNA constructs were made from the Rc/CD80 plasmid using the U.S.E. mutagenesis kit (Pharmacia, Piscataway, NJ), as before (21). The Rc-Clad primer (5′-AGGCCGGTTTGGCTGGTACGTGATGATGATAAGGACGACGAGG-3′) was used as the selection primer for all of the mutagenesis reactions. The CD80a11 (5′-GAAGAAGGAAATGTAGTTGTTGGAAAGGG-3′), CD80a4A (5′-GAAGAAGGAAATGTAGTTGTTGGAAAGGG-3′), and CD80aSA (5′-GAAGAAGGAAATGTAGTTGTTGGAAAGGG-3′) primers were used as templates to introduce the desired mutations into the Rc/CD80 plasmid. All mutant constructs were sequenced completely to ensure sequence fidelity. The relevant regions of the mutant CD80 molecules are shown in Table I. Reh transfectants were made as before (21) using Lipofectin reagent (Life Technologies). Briefly, 2 × 10^6 Reh cells were incubated in 3 ml of OPTI-MEM (Life Technologies) in a 10-cm tissue culture plate. A total of 3 µg of linear plasmid was diluted in Opti-mem, and 20 µl of Lipofectin was diluted in Opti-mem. The two solutions were mixed and added dropwise to the Reh cells. Eighteen hours later, 10 ml of RPMI 10 was added. Two days later, G418 was added to 750 µg/ml (active concentration). Dead cells were removed as necessary and viable clones were isolated by limiting dilution. Clones were screened and sorted by flow cytometry to obtain those expressing similar levels of CD80.

**Flow cytometry and immunofluorescence microscopy**

Cells were collected and washed once in ice-cold staining buffer (2% FCS in PBS with 0.02% NaN3). Two hundred fifty thousand cells were incubated with either FITC-conjugated BB1 mAb or control FC1 mAb for 40 min on ice. The cells were washed twice, resuspended in 1% paraformaldehyde in PBS, and stored at 4°C until analysis on a FACScan (Becton Dickinson). For CTLA4Ig binding, CD80 transfectants were incubated with either CTLA4Ig or human IgG control at the concentrations indicated for 40 min on ice. The samples were washed twice in staining buffer, and then binding was detected with anti-human IgG-phycoerythrin conjugate and incubated as above. The relative fluorescence intensity (FI) was calculated as before (21) by dividing the mean fluorescence intensity of the sample by that of the control sample; thus, a FI of 1 is background. Percentage of maximal binding was calculated as follows: [(FI of the sample − 1) ÷ (FI of the maximum sample − 1)] × 100%.

Samples were prepared for microscopy as before (21). To test ligand-induced CD80 redistribution samples were incubated with CTLA4Ig, and then detected with biotin-conjugated goat anti-human IgG, followed by streptavidin-Texas Red (Molecular Probes). Samples from each cell line were stained with FC1 or human IgG as controls for BB1 and CTLA4Ig staining, respectively. For each experiment, at least 100 cells were scored for CD80 distribution at each sample time point. The scoring was performed as before (21). Briefly, cells were scored as “diffuse” when CD80 was distributed in random foci; “clustered” when CD80 was no longer in random foci, but was in more than 10 distinct spots; “patched” when CD80 was in 10 or fewer distinct spots; and “capped” when CD80 was distributed in three or fewer spots on the cell surface. Representative fields were collected on a Bio-Rad (Richmond, CA) MRC 1024 laser-scanning confocal microscope fitted to collect both fluorescence and Nomarski images. The fluorescent images were collected in serial sections with the focal plane approximately 0.15 µm apart stepping through the cells. Confocal Assistant software (Bio-Rad) was used to project the serial sections into a single plane for presentation.

**Immunoprecipitations**

Fifty million Reh transfectants were collected and washed once in POP buffer (150 mM NaCl, 5 mM MgCl2, 2 mM L-glutamine, 1.8 mM glucose, 1 mM CaCl2, in 10 mM Tris, pH 7.5). The cells were resuspended in POP medium (POP buffer with 2% dialyzed FCS) and incubated at 37°C for 1 h. The cells were washed again, resuspended in POP medium with 1 mCi[^32P]Pi, and incubated at 37°C for 4 h. Then cells were stimulated with PMA (10 ng/ml) and ionomycin (500 ng/ml) for the final 10 min of culture. The cells were collected and washed once in PBS before being lysed in Nonidet P-40 lysis buffer (0.5% Nonidet P-40, 50 mM Tris, pH 8.0, 150 mM NaCl, and 5 mM EDTA) containing protease and phosphatase inhibitors. The postnuclear fractions were precleared twice with protein A-Sepharose (Pharmacia) before adding Ab or CTLA4Ig to a final concentration of 10 µg/ml. The bound material was precipitated with protein A-Sepharose and washing four times with lysis buffer. The samples were extracted with reducing sample buffer and run on 9% SDS-PAGE. The gels were fixed and dried before being exposed to x-ray film.
CD80 in all species in which CD80 has been cloned to date: rabbit, rat, mouse, and human (2, 25–27), making it a good candidate site to function in CD80 costimulation and capping. Thus, we created a mutant cDNA, CD80/4A, which has these highly conserved residues, RRNE, replaced with four alanines (Table I) and expressed it in Reh cells (R80/4A). Another short region in the cytoplasmic tail of CD80 residues 282–284 is conserved in mouse, rat, and human as RE(S/T), and in rabbit as RLS. While there is no evidence yet demonstrating that the cytoplasmic tail of CD80 can be phosphorylated at the highly conserved serine 284 (S284), the residues surrounding S284 fit the consensus sequences for protein kinase C and cyclic nucleotide-dependent kinases (28–32). The conservation of this region suggests that S284 may be important for CD80 function; to test this possibility, we produced a mutant CD80 cDNA, CD80/SA (Table I), with S284 replaced with alanine.

Mutant CD80 molecules still bind ligand

We transfected Reh cells with mutant CD80 constructs and isolated clones expressing similar surface levels of the mutant CD80 molecules. The wild-type CD80 Reh (R80) described previously had higher levels of CD80 expressed on the surface than the new transfectants; thus, we sorted the original R80 line to isolate a subline expressing lower levels of CD80. As shown in Figure 1 and Table I, all of the transfectants used in these studies express similar levels of CD80, except for the mutant S284 transfectants (R80/SA), which express slightly less CD80 on their surface than the other transfectants (Fig. 1A and Table I). After continued culture, R80/SA expressed CD80 in a more homogeneous unimodal pattern, but it was still expressed at twofold lower levels (data not shown). Vector transfectants were not bound by the CD80-binding mAb BB1 (Fig. 1A), demonstrating that all BB1 binding to the transfectants is dependent upon the presence of the CD80 transgenes.

We found previously that deletion of the cytoplasmic tail of CD80 did not alter binding to either CD28 or CTLA-4 (21). Two groups found that mutations in CD80 that reduce binding avidity to one ligand also have a reduction in binding to the other ligand (6, 7); thus, these results indicate CTLA4Ig binding to mutant CD80 molecules is a good indication of normal binding to both CD28 and CTLA-4. These mutant CD80 molecules were still able to bind ligand in a manner similar to wild-type CD80: as shown in Figure 1B, all of the transfectants bound to CTLA4Ig with similar avidity. CTLA4Ig binding was detected in all transfectants at about 4 ng/ml of CTLA4Ig, and reached maximal binding at about 200 ng/ml. Direct comparison of maximal FI obtained with the CD80 mAb BB1 and CTLA4Ig (Fig. 1, A and B) is presented in Table I. There is no substantial difference between CTLA4Ig and BB1 binding to the wild-type and mutant CD80 molecules; thus, the new mutants presented in this study have no defect in ligand-binding capacity.

Two regions in the CD80 cytoplasmic tail are required for costimulation

We next tested the ability of these transfectants expressing mutant or wild-type CD80 to costimulate T cell proliferation (Fig. 2). Only the R80 cells expressing wild-type CD80 could costimulate T cell proliferation. Maximal T cell proliferation was induced with 10 ng/ml of soluble anti-CD3 and 100,000 Reh transfectants per well. We consistently detected T cell proliferation in cultures with R80 at doses of anti-CD3 as low as 100 pg/ml (Fig. 2A). At low numbers of transfectants per well, only a low level of proliferation was induced, but as the ratio of R80 cells to T cells approached 1:1, a very strong proliferative response was evident, which reached a maximum response with 2:1 R80 cells to T cells (Fig. 2B). The CD80Δ11, CD80Δ11, CD80/4A, and CD80/SA mutants were unable to costimulate T cell proliferation at all cell doses examined. Even at the ratio of 6 Reh cells to 1 T cell, transfectants expressing mutant CD80 were unable to induce significant T cell proliferation, thereby ruling out possible effects due to small differences in the numbers of CD80 molecules present on different transfectants. Thus, residues in at least two regions of the cytoplasmic tail of CD80 are critical for costimulation. Specifically, mutation at two distinct sites, at either the conserved RRNE (275–278) site or at S284, ablated the ability of CD80 molecules to costimulate T cells. Consistent with this, CD80 molecules with the Δ11 mutation, which is missing S284 but retains the RRN (275–277) site, could not costimulate. There was some variation in the ability of T cells from donors to respond to the different transfectants, but generally, T cell proliferation induced by the mutant CD80 transfectants was less than 10% of that induced by R80 (data not shown).

Mutant CD80 molecules have impaired redistribution

We previously found CD80 could relocalize from a diffuse pattern to a clustered pattern independent of the cytoskeleton. Further redistribution into patches and caps consisting of fewer than 10 and 3 distinct spots, respectively, was dependent upon an intact cytoskeleton and the presence of the cytoplasmic tail of CD80 (21). To determine whether CD80 redistribution and the ability to costimulate T cell proliferation were mediated by the same regions in...
the cytoplasmic tail of CD80, we tested whether CD80Δ11, CD80/4A, and CD80/SA expressed on Reh cells undergo Ab-mediated redistribution. As shown in Figure 3, A–D, wild-type and mutant CD80 molecules were localized in foci randomly distributed over the cell surface. There were no obvious differences in the distribution, or the size of the foci of CD80 found on R80, R80Δ16, R80/4A, or R80/SA cells at time 0. After 5 min of exposure to CD80 mAb at 37°C, wild-type CD80 already had undergone significant redistribution, while mutant CD80 molecules had not relocalized as much (Fig. 3, E–H). The kinetics of patching (Fig. 4A) and capping (Fig. 4B) was also delayed in each of the CD80 mutants. After 5 min at 37°C, on average, 20% of the cells had distributed wild-type CD80 into patches and 7% into caps, significantly more than found on either tailless CD80 mutants or the other mutants. Similar differences were evident at 10 min. After 20 min at 37°C, the majority of wild-type CD80 on R80 cells had been relocalized into patches and caps, while CD80Δ16 was still predominately found in clusters (Fig. 3, I and J). CD80/4A and CD80/SA had undergone more redistribution than CD80Δ16, but less than wild-type CD80 (Fig. 3, K and L). Figure 4 shows three distinct patterns of CD80 redistribution. R80Δ16 underwent very little redistribution, which occurred very slowly and led to very few CD80 caps. The other mutants, R80Δ11, R80/4A, and R80/SA, underwent a slightly faster kinetics of redistribution than R80Δ16 cells, but CD80 was redistributed more slowly in these mutant CD80-expressing cells than in wild-type CD80-expressing cells (Fig. 4C). R80Δ11, R80/4A, and R80/SA formed significantly more caps of CD80 than R80Δ16, but did not cap as much as wild type (Fig. 4B).

All of the transfectants expressing mutant or wild-type CD80 constructs initially express the CD80 molecules in foci (Fig. 3, A–D), but only wild-type CD80 could costimulate T cell proliferation (Fig. 2). This demonstrates that the foci of CD80 found on APC are insufficient to aggregate CD28 and directly mediate costimulation, and also shows that the rate of CD80 redistribution is dependent upon the presence of both RRNE (275–278) and S284. Furthermore, even though mutant CD80 molecules could be induced with IgM mAb to redistribute into patches and caps, they could not costimulate T cells (Figs. 2 and 4). This suggests that CD80 redistribution into patches and caps is required, but insufficient for T cell costimulation.

R80Δ11, R80/4A, and R80/SA all underwent significant patching and capping at later time points when cross-linked with IgM mAb, but were unable to induce T cell proliferation. Since CD80 mAb has 10 F(ab’)2 binding sites (decavalent) and the natural ligands for CD80 are normally divalent, we compared the ability of divalent CTLA4Ig vs CD80 mAb to redistribute CD80. Divalent CTLA4Ig redistributed wild-type CD80 into patches similar to that obtained with mAb (Fig. 5A). CTLA4Ig induced some redistribution of CD80/4A and CD80/SA into patches, but was much less effective in inducing patches than decavalent mAb (Figs. 4A and 5A). CTLA4Ig did not induce much capping of these mutant CD80 molecules; however, like IgM mAb, it was able to induce capping of wild-type CD80 molecules to high levels (Fig. 5B). The only difference observed between CTLA4Ig- and BB1-induced capping was the difference in the kinetics of the response (Figs. 4 and 5). This is most likely due to the difference in the valency (2 vs 10 binding sites) between the two reagents. There was no other difference between these different reagents. The little capping observed in mutant CD80 constructs was no more than two- to fourfold higher than that detected with tailless CD80 compared with 20-fold increases in capping with wild-type CD80. Thus, native ligand can induce wild-type CD80 into caps, a critical step for induction of costimulation, but not mutant CD80 molecules. In short, most of the CD80/4A and CD80/SA patching and capping induced by mAb did not occur when native ligand was used (Figs. 4 and 5).

The CD80 cytoplasmic tail associates with a 30-kDa phosphoprotein

Since mutating S284 abrogated the ability of CD80 expressed on Reh cells to costimulate T cell proliferation, we attempted to determine whether S284 could be phosphorylated in intact cells. We labeled R80 cells with 32P and precipitated CD80 from lysates of unstimulated or stimulated cells. We were unable to detect any 32P-labeled CD80, even after stimulating cells with PMA and/or the calcium ionophore, ionomycin, under conditions in which we are clearly able to detect 125I surface-labeled CD80 (data not shown). However, we did detect a specific band approximately 30 kDa in size (pp30) coc precipitating with CD80 from cells stimulated with either ionomycin or ionomycin plus PMA (Fig. 6). This band was not present in CD80 precipitates from cells incubated with media or PMA alone. As we were unable directly to measure CD80 levels present in the precipitates, we used MHC class I precipitates from the same lysates as controls for equivalent labeling and precipitations. The levels of MHC class I present in the precipitations were very similar (Fig. 6), suggesting that the lanes also had equivalent levels of CD80.

Ionomycin stimulation alone was sufficient to induce the association of the 30-kDa phosphoprotein with CD80, but the amount
of pp30 associating with CD80 was enhanced greatly by PMA stimulation. This suggests that the interaction between pp30 and CD80 is regulated by intracellular calcium levels, and may be enhanced by phosphorylation induced by PMA. It is not clear whether pp30 is constitutively associated with CD80 and is phosphorylated after activation of the cells, or whether it only associates with CD80 after it is phosphorylated during B cell activation.

Discussion

Ligand binding is clearly insufficient to initiate signaling through CD28 (21). CD28 needs to be aggregated by ligand to costimulate T cells as F(ab')2 fragments to CD28 block costimulation, and soluble ligand only costimulates if it is first immobilized on plastic (18, 19). Linsley et al. (33) demonstrated that native CTLA-4 homodimers bind two CD86 molecules, and presumably two CD80 monomers. Furthermore, oligomerization of CD80 and CD86 regulates binding to CD28 and CTLA-4 dimers (17). Thus, cells expressing CD80 may be able to regulate T cell costimulation by regulating the subcellular localization of CD80. Greenfield et al. (34) found CD86 expressed on T cells could not costimulate T cells, demonstrating that simply expression of costimulatory molecules does not always correlate with delivery of costimulation.

While mutant CD80 molecules could be redistributed by mAb into patches and caps, they did not form significant numbers of caps in response to native ligand (Figs. 4 and 5). We found five- to ninefold fewer CD80 caps on R80/SA and R80/4A than on wild-type CD80-expressing R80 (Fig. 5 and data not shown). This suggests that CD80 redistribution into caps is required for CD28-mediated costimulation, and that at least two regions of the CD80 cytoplasmic tail are involved in initiating CD28-mediated costimulation. Deletion or mutation of one of these regions alters the rate of CD80 redistribution, and prevents ligand-induced CD80 capping (Fig. 5). Since the BB1 mAb and the ligand CTLA4Ig both induced similar amounts of capped and patched CD80 on each wild-type or mutant CD80 transfectant, the capping defect observed with mutant CD80 molecules is most likely caused by the specific CD80 mutations. The rate of CD80 redistribution may also play a role in costimulation. If a TCR interaction with the APC is of low affinity and the resulting signal is weak and not capable of activating the T cell, costimulatory signals may need to be initiated rapidly so that the T cell does not disengage from the APC before becoming fully activated. Dustin and Springer (35) demonstrated that TCR signals transiently induce high avidity CD11a/18-dependent binding that returns to basal levels at about 30 min after stimulation. Capping of wild-type CD80 occurs well within this time frame, while ligand does not induce caps of the mutant CD80 molecules that are incapable of costimulation. One possibility is that capped CD80 not only induces costimulatory signals, but also plays a role in prolonging APC-T cell contact.

While CD80 is not a glycosylphosphatidylinositol-anchored protein, there are reports describing the costimulatory activity of chimeric CD80 containing a glycosylphosphatidylinositol anchor (GPI-CD80) (36, 37). McHugh et al. (36) found that detergent-purified GPI-CD80 could costimulate when incorporated into cell membranes. They found uneven incorporation of the purified CD80 into cell membranes, suggesting it was aggregated and thereby could cross-link CD28 to costimulate T cells. Brunschwig et al. (37) generated several GPI-CD80 constructs using the CD59 (decay-accelerating factor) transmembrane region as the GPI signal sequence, and also found the GPI-CD80 could costimulate. The GPI form of CD59 is targeted to the basolateral region of cells (38); thus, even though GPI-anchored proteins lack a transmembrane and cytoplasmic tail, these proteins can be targeted to specific locations on cell membranes. Thus, it is likely that GPI-CD80 in these studies is either constitutively aggregated or can be inducibly capped. Expression of tailless CD80 at very high levels was able to circumvent the need for CD80 redistribution and could costimulate T cells, presumably because it is already at a high enough concentration at the contact site (21); thus, overexpression of GPI-CD80 may also have the same effect. This may also explain how fixed transfectants expressing high levels of CD80 are still able to provide costimulation.

CD80/SA was expressed at slightly lower levels on the surface of transfectants compared with other CD80 mutants (Fig. 1A and
While it is difficult to exclude formally that this transfectant was deficient in costimulation because it expressed less extracellular CD80, we have found that the absolute level of surface CD80 on Reh cells generally has little effect on the level of T cell proliferation induced. In at least six different costimulation experiments, we found R80 clones expressing wild-type CD80 with a FI between 6.9 and 8.2 were able to induce high levels of proliferation, 20- to 100-fold higher proliferation than observed with vector transfectants. Furthermore, CD80 CHO transfectants with a FI of 2 were able to induce high levels of T cell proliferation (21). R80/SA cells, having a FI of 8, were unable to induce significant proliferation of T cells, inducing at most a two- to three-fold increase in proliferation compared with controls (Fig. 2 and data not shown). In addition, wild-type CD80-bearing cells costimulated at cell doses of 30,000 R80 per well, while 10-fold more R80/SA cells were still not able to induce T cell proliferation (Fig. 2B). These data are consistent with the hypothesis that CD80 needs to be localized to specific sites to provide costimulation, and that even a very low level of CD80 can provide costimulation if it is properly localized on the APC surface.
Linsley and coworkers have demonstrated that oligomerization of CD80 or CD86 regulates both binding to CD28 and CTLA-4 and signal transduction, and suggested that these foci of CD80 or CD86 present on Langerhans cells are sufficient to aggregate CD28, thus initiating CD28-mediated signals (17, 20, 39). The notion that oligomers of CD80 are required for CD28 aggregation and signaling is consistent with our data. However, CD80 in individual foci on the cell surface is insufficient to generate CD28-mediated signals, as CD80Δ16, CD80/4A, and CD80/SA all express CD80 in foci indistinguishable from wild-type cells (Fig. 3), yet do not costimulate T cells. This suggests that the foci of CD80 may facilitate a higher overall avidity binding to CD28, but do not initiate signaling. One testable possibility is that in order for T cell costimulation to occur, the APC must receive a signal that induces CD80 to redistribute on the APC. In other words, a signal from the T cell back to the APC may be necessary to induce CD80 redistribution and for costimulation to occur (40).

Kupfer and Singer (41) demonstrated that CD11a/18 localizes to the site of APC/T cell contact, and Moingeon et al. (42) demonstrated that CD2, in addition to CD11a/18, is redistributed to the site of cell to cell contact. Based upon these data, we propose that when two molecules of CD80 are cross-linked by binding to a single CD28 dimer, the CD80 molecules are directed to migrate to a site of APC-T cell contact. The CD28 signal may only be initiated when it has been sufficiently aggregated or when it is relocalized to the same site as the cognate TCR complex.

The CD80-pp30 association is induced with ionomycin alone, as indicated, for 10 min. Lysates were incubated with [32P]Pi,Pi and stimulated with media, PMA (10 ng/ml), ionomycin (500 ng/ml), or a combination of PMA and ionomycin, as indicated, for 10 min. Lysates were incubated with anti-CD80 antibody (lanes 1 and 2) or with preimmune serum (lanes 3), respectively, and precipitated with protein A-Sepharose. The bound proteins were extracted with sample buffer and analyzed by SDS-PAGE. The positions of m.w. markers are indicated in kDa.

The pp30 phosphoprotein is not detected in association with CD80 unless cells are first stimulated with a calcium-dependent signal (Fig. 6), and the level of pp30 associating with CD80 is further augmented by PMA. These results suggest a signal to APC that induces increases in intracellular free calcium could change the structure and function of CD80 complexes. Ligating either surface Ig or MHC class II on B cells, unlike PMA, up-regulates levels of both intracellular free calcium and CD80 (44 – 47). Thus, it is possible that during APC-T cell interactions or after B cells take up multivalent Ags through their Ag receptors, calcium is released and pp30 associates with CD80, thereby preparing CD80 for effective costimulation to CD28. In the absence of calcium-dependent signals, such as with CD40 signaling only (44, 48), CD80 expression is induced (40), but it may not be able to effectivley costimulate T cells until MHC class II receptors are engaged by TCR recognition. This would be desirable to prevent stimulation of bystander B cells through CD40 to become competent APC in absence of specific Ag-derived signals.

We favor this model over a direct effect of CD28 binding to CD80 inducing costimulation-dependent competent CD80 complexes, as to date there is no evidence that CD80 ligation can induce changes in cytosolic calcium (E. A. Clark, unpublished observations).

The fact that the CD80/4A and the CD80/SA mutations do not overlap, residues 275–278 and 284, suggests either that the entire cytoplasmic tail is required for a single interaction, or that two distinct interactions may be required for T cell costimulation. Both mutants behave in a similar manner during redistribution, and both have a slower kinetics of Ab-mediated redistribution and fail to enter caps after ligand-induced cross-linking (Figs. 4 and 5); this suggests that both of these sites are utilized in interactions with the cytoskeleton. Even though we cannot detect any phosphorylation of S284, the fact that a single mutation of serine to alanine at this position ablates both ligand-induced CD80 capping and costimulation demonstrates that S284 is a critical residue, and suggests it may be phosphorylated. Thus, we favor the hypothesis that phosphorylation of S284 is necessary for CD80 redistribution and costimulation. Perhaps the two sites are sequentially recognized: RRNE (275–278) as a protein-Docking site, and S284 as a phosphorylation site that regulates protein associations such as pp30 association with CD80.

Note added in proof. In an accompanying paper (49), we report that the BB1 mAb binds to both CD74 and CD80. This finding does not affect the conclusions of our study with CD80 mutants, since all results were confirmed using CTLA4Ig and the binding of BB1 or CTLA4Ig to Reh cells occurred only after expression of CD80.

Acknowledgments
We thank Dr. Peter Linsley for providing the CTLA4Ig used in this study, Mr. Dario Magaletti for assisting with preparations of T cells, and Drs. Che-Leung Law and Stephen Klaus for useful discussions.

References


