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The Role of the Cytoplasmic Tail of Antigen-Presenting Cell Surface Molecule CD80 in Delivery of T Cell Costimulation

By

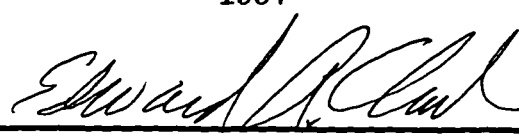
Raymond Thomas Doty

A dissertation submitted in partial fulfillment
of the requirements for the degree of

Doctor of Philosophy

University of Washington

1997

Approved by 

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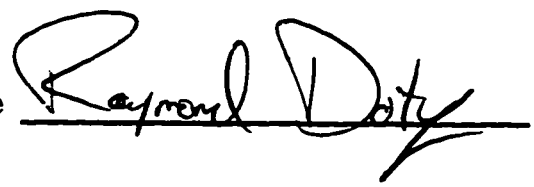
Signature 

Date 30 MAY '97

University of Washington

Abstract

The Role of the Cytoplasmic Tail of Antigen-Presenting Cell Surface Molecule CD80 in Delivery of T Cell Costimulation

by Raymond Thomas Doty

Chairperson of the Supervisory Committee: Professor Edward A. Clark
Departments of Microbiology and Immunology

CD80 is a highly glycosylated surface protein of 45-60 kDa found on activated B cells, T cells, monocytes, and dendritic cells. Based upon the CD80 cDNA sequence, the molecular weight of the protein core is 30 kDa. The remaining 15-30 kDa of the molecular weight is N-linked carbohydrate attached at eight possible sites in the extracellular region. CD80, and its structural and functional homolog CD86, both bind to the T cell molecules CD28 and CTLA-4. CD28 is found on resting and activated T cells, and is a major costimulatory molecule in the immune response. Ligation of CD28 increases both cytokine gene transcription and mRNA stability, and up-regulates cytokine receptor expression. CTLA-4 is expressed on T cells and B cells only after activation. Its role in T cell immunity is less clear than that of CD28. Initially, antibodies to CTLA-4 were shown to enhance CD3- and CD28-mediated T cell proliferation, but it has become clear recently that CTLA-4 is a major negative regulatory protein, limiting T cell expansion. CD28 crosslinking is necessary to provide costimulation; thus, monomeric CD80 or CD86 should not be able to induce CD28-mediated costimulation. We found that cells expressing a mutant form of CD80 lacking the cytoplasmic tail are incapable of inducing CD28-mediated

signals, and do not undergo actin-dependent antibody-mediated redistribution. Furthermore, we found that there are at least two sites in the cytoplasmic tail of CD80 which are required for normal CD80 redistribution and CD28-mediated costimulation. Chimeric CD80 molecules containing the transmembrane and cytoplasmic domains of CD2 or CD54 can induce CD28-dependent T cell proliferation. CD2 and CD54 interact with the tubulin-based and the actin-based cytoskeleton, respectively. These results suggest there is no requirement for CD80 to interact specifically with the actin-dependent cytoskeleton to induce costimulation. We identified a protein of approximately 30 kDa that interacts with the cytoplasmic tail of CD80 in cells treated with calcium ionophore and phorbol esters. The interaction of CD80 with this protein may regulate the redistribution of CD80 on antigen-presenting cells and thus regulate the ability of CD80 to induce CD28-mediated costimulation.

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List of Abbreviations

Abs	antibodies
APC	antigen presenting cell
ATCC	American Tissue Culture Collection
CCB	cytochalasin B
CHO	Chinese hamster ovary
CTL	cytotoxic T lymphocyte
Da	Daltons
DC	dendritic cells
DMEM-10	DMEM medium containing 10% FCS, 10 U/ml penicillin, and 10 μg/ml streptomycin
EAE	experimental allergic encephalomyelitis
Er	sheep erythrocyte rosette
FCS	fetal calf serum
FDC	follicular dendritic cells
FI	fluorescence intensity
FITC	fluorescein isothiocyanate
GPI	glycosylphosphatidylinositol-anchored
hr	hour
IFN	interferon
Ig	immunoglobulin
mAbs	monoclonal antibodies
MLR	mixed lymphocyte reaction
MMC	mitomycin C
m.w.	molecular weight
PBL	peripheral blood lymphocyte
PBS	phosphate-buffered saline
PE	phycoerythrin
PMA	phorbol myristate acetate
RPMI-10	RPMI 1640 medium containing 10% FCS, 10 U/ml penicillin, and 10 μg/ml streptomycin
SRBC	sheep red blood cells
UTR	untranslated region

Acknowledgments

I would like to thank the members of the Clark Lab, past and present, with whom I have had the opportunity to work closely. In particular I would like to thank Kevin Draves and Geraldine Shu who did everything in their power to keep the lab running smoothly. I would like to thank Corrine Leprince and Svetlana Sidorenko for their guidance with immunochemistry early in these studies, and Kristin Brevik Andersson for her friendship and camaraderie. Of all the people in the lab I enjoyed working with, Che-Leung Law stood out in every way. He was always available to discuss experiments, which frequently led to much more esoteric discussions of science in general. He always reminded me of the big picture, not only in science, but in life. He is a genuinely nice person who is a great role model and friend. Most of all, I would like to thank my advisor, Ed Clark for his ideas, support, and encouragement–without which I would not have been able to pursue this project through to the end. In addition to those in the lab, I owe my parents a debt of gratitude for their endless support and encouragement throughout my entire life. And finally, I would like to extend my deepest appreciation to my wife, Dr. Sharon Lafferty Doty, who always provided support, encouragement, love, and understanding throughout all the ups and downs of my graduate studies.

Chapter 1: Introduction

Initiation of the immune response

Bretscher and Cohn (1970) proposed the two signal model of immune activation which provided critical insight into immune regulation; however, it is an over simplification of the molecular interactions involved in immune regulation. According to this model, signal one is the antigen specific signal, and is required to initiate either T cell or B cell responses. The second signal would be provided by a separate receptor on the B or T cell, and is required for the antigen specific signal to lead to a productive immune response. In the absence of the second signal, the primary signal through the antigen receptor would not lead to a productive immune response. *In vitro* studies tend to support this model, in which T cell proliferation can be induced only by the combination of antibodies to CD3 and CD28. B cell proliferation and Ig secretion can be induced *in vitro* by antibodies to CD40 in conjunction with cytokines (Rousset *et al.*, 1991). However, *in vivo* studies reveal the induction of an immune response is far more complex than indicated in the two signal model of immune regulation. Studies of mice with defects in the CD40–CD40 ligand pathway are immunocompromised and do not form germinal centers in response to immunization (Xu *et al.*, 1994). Mice deficient in CD28–CD80/86 interactions have a similar defect (Ferguson *et al.*, 1996; Borriello *et al.*, 1997). This suggests that multiple molecular interactions are required for normal immune responses, and that signals back into both the antigen-presenting cell

(APC) and the cognate T cell are required for normal cell activation and immune responses. In other words, there is a reciprocal dialog between the B cell and the T cell (Clark and Ledbetter, 1994). Furthermore, this demonstrates that interfering independently with multiple different molecular interactions results in very similar effects.

CD28 is found on resting and activated T cells, and is a major costimulatory molecule in the immune response (reviewed by Linsley and Ledbetter, 1993). Ligation of CD28 enhances both cytokine gene transcription and mRNA stability, and up-regulates cytokine receptor expression (Fraser *et al.*, 1991; Lindsten *et al.*, 1989; Ledbetter *et al.*, 1990; Linsley *et al.*, 1991a). CD28 is one of a few molecules which have been described to provide costimulation to T cells. Heat stable antigen (HSA), CD2, CD54, 4-1BB, and the newly defined CDw150 (SLAM/IPO3) molecules all have been described to enhance T cell responses (Liu *et al.*, 1992a; Liu *et al.*, 1992b; Koyasu *et al.*, 1990; Boussiotis *et al.*, 1994; Damle *et al.*, 1992b; Hurtado *et al.*, 1997; Cocks *et al.*, 1995; Sidorenko and Clark, 1993; Aversa *et al.*, 1997). Any one of these accessory molecules may play major roles in the absence of CD28 signals, as CD28-deficient mice are capable of generating an immune response to specific antigen (Shahinian *et al.*, 1993). CD28 deficient mice were able to generate relatively normal cytotoxic T lymphocyte (CTL) responses. The IgM response to antigen in CD28-deficient mice was also relatively normal, but IgG antibody

production was significantly lower than in normal mice. This suggests other molecules can provide costimulation, but the outcome will be qualitatively different.

The most compelling evidence for CD28 providing critical costimulation signals is that blocking CD28 binding to its ligands can inhibit mixed lymphocyte reaction (MLR) responses, T cell-dependent antibody secretion, graft rejection, and autoimmune diseases (Damle *et al.*, 1988; Tan *et al.*, 1993; Van Gool *et al.*, 1994; Linsley *et al.*, 1992b; Lenschow *et al.*, 1992; Pearson *et al.*, 1994; Sayegh *et al.*, 1995; Lenschow *et al.*, 1995; Kuchroo *et al.*, 1995; Perrin *et al.*, 1995). CD80 is a ligand for CD28 and plays a major role in costimulation and regulation of humoral and cellular immunity. Boussiotis *et al.* (1994) found that CD80 expression on artificial allogeneic APC was sufficient to prevent anergy in alloreactive T cell clones. They were able to break this tolerance not by CD80-mediated costimulation, but by culture with IL-2 and challenge with CD58-expressing allogeneic APC. This suggests that the prevention of tolerance and the reversal of tolerance are not mediated by the same molecules.

Linsley *et al.* (1992b) found that blocking CD28-mediated costimulation *in vivo* reduced both the number of antigen-specific B cells and T cell-dependent splenocyte proliferation. Interestingly, rechallenge with antigen in the absence of CD28 blocking revealed, *in vivo*, the inhibition of proliferation was not long-lived, as was found with T cell clones. Possible explanations for these results

are that newly developed naive B and T cells mediate a primary immune response generated during the secondary challenge, or other accessory molecules, such as CD58, expressed on APC subsequently break tolerance induced in the absence of CD28-mediated costimulation. As an alternative explanation, *in vivo*, T cell recognition of antigen may be insufficient to initiate an immune response in the absence of costimulation, thus the T cells are not tolerized and respond as naive T cells in subsequent stimulations.

Cytotoxic T cell responses are also directly affected by CD28-mediated costimulation. Introduction of CD80 into non-immunogenic tumors can induce a tumor-specific immune response and resulting in the elimination of the tumor (Chen *et al.*, 1992; Townsend and Allison, 1993). After the initial induction of the anti-tumor response, CD80-mediated costimulation may not be required for killing, as $CD80^-$ parental tumors can subsequently be rejected (Chen *et al.*, 1992; Townsend and Allison, 1993). However, not all tumors can be rejected simply by introducing CD80 (Chen *et al.*, 1994), suggesting other molecules are involved in CTL mediated killing of some or all target cells. This also demonstrates that there is not just a single costimulatory molecule, but multiple molecules are required for and involved in T cell activation.

Down regulation of the immune response

While the majority of the accessory molecules studied enhance immune responses, there is an increasing number of molecules identified which dampen or stop an immune response. CD95 (fas) is a molecule found on the surface of

activated B and T cells, which when ligated with antibody or its natural ligand, induces apoptosis (Nagata and Golstein, 1995). This is useful to prevent an immune response in areas where such a response may cause serious and irreparable damage such as in the eye (Griffith *et al.*, 1995). Cytotoxic T cell activation molecule-4 (CTLA-4) is expressed on T cells and B cells only after activation, and its role in T cell immunity is less clear than that of CD28 (Brunet *et al.*, 1987; Kuiper *et al.*, 1995). Initially CTLA-4 was shown to enhance CD3- and CD28-mediated T cell proliferation (Linsley *et al.*, 1992a), but recently it has become clear that CTLA-4 is a major regulatory protein that seems to limit T cell proliferation (Walunas *et al.*, 1994; Gribben *et al.*, 1995). CTLA-4 deficient mice die at a very early age from a lymphoproliferative disease, illustrating its critical role in limiting T cell proliferation (Tivol *et al.*, 1995; Waterhouse *et al.*, 1995). Despite the role that CTLA-4 plays in limiting T cell proliferation, we cannot yet rule out the possibility that it sends a positive or growth promoting signal to the cells expressing it under some circumstances (Linsley, 1995). CTLA-4 limits the expansion of activated T cells by inhibiting proliferation or inducing apoptosis when it is engaged by CD80 or CD86 (Walunas *et al.*, 1994; Tivol *et al.*, 1995; Gribben *et al.*, 1995). This makes sense, as after the majority of the antigen has been cleared by an immune response, the large number of T and B cells specific for the antigen generated during the response need to be eliminated, only leaving behind small numbers of long-lived

memory cells. Not only is it a waste of resources to generate and maintain B and T cells specific for an antigen which no longer exists in significant amounts, but inflammatory cytokines from these activated cells can damage tissues. Proteins or other antigens leaked from damaged cells may themselves elicit an anti-self immune response which can be deleterious. Experimental allergic encephalomyelitis (EAE) is an autoimmune disease mediated by T cells which are reactive to components of the nervous system such as myelin basic protein (Martin, *et al.*, 1992). Expression of CD80 in addition to high levels of MHC Class II molecules are sufficient to induce the destruction of the β cells in the pancreas, a condition similar to autoimmune diabetes (Guerder, *et al.*, 1994). While the actual precipitating event is unknown, autoimmune diabetes and EAE are examples of autoimmune diseases caused by the immune system responding to self antigens which normally do not elicit an immune response.

The role of CD80 and CD86 in the immune response

By using both positive costimulatory signals and negative regulatory signals, the immune system rapidly expands the antigen-specific response, then just as rapidly, removes the cells which are no longer needed, leaving only a small population of long-lived memory cells. Both CD80 and CD86 play a major role in the expansion and the removal of T cells by interacting with both CD28 and CTLA-4. While the differences between CD28 and CTLA-4 are evident, the different roles of CD80 and CD86 in an immune response are less clear. CD86 is able to costimulate T cell proliferation to similar levels as does CD80 (Azuma

et al., 1993a; Freeman *et al.*, 1993a; Freeman *et al.*, 1993c). There have been several reports suggesting that CD80 and CD86 mediate unique effects on T cells. Freeman *et al.* (1995) found that CD86 expressing transfectants induced $CD4^+$ T cells to secrete IL-4 in addition to IL-2 and IFN-γ, while CD80 transfectants induced T cells to secrete IL-2 and γ-IFN, but not IL-4. On the other hand, June and coworkers (Levine *et al.*, 1995) found that CD80 or CD86 transfected CHO cells induced T cells to secrete similar levels of IL-2, IL-4, γ-IFN, TNF-α, and GM-CSF. Lanier *et al.* (1995), also found CD80 and CD86 had similar effects on induction T cell proliferation, cytokine secretion, and the generation of CTL. Recently Schweitzer *et al.* (1997) examined the function of APC derived from mice selectively lacking either CD80 or CD86. They found that both $CD80^{-/-}$ and $CD86^{-/-}$ APC were able to induce IL-2, IL-4, and γ-IFN. The conditions of stimulation such as peptide concentration, T cell number, and APC number were the key factors which had the major impact on the profiles of cytokines secreted, while the presence of CD80 versus CD86 had only minor effects.

In contrast to the *in vitro* studies, *in vivo* studies strongly suggest CD80 and CD86 play unique roles in immunity and the induction of autoimmune diseases. Borriello *et al.* (1997), found B cells in mice deficient in CD86 expression were unable to undergo class switching or form germinal centers in response to intravenous antigen in the absence of adjuvant, while B cells in mice

deficient in CD80 expression did undergo significant class switching and germinal center formation. However, antigen administered in adjuvant, or by subcutaneous or intraperitoneal routes of administration revealed only slight differences in the kinetics of B cell class switching in CD80 deficient versus CD86 deficient mice. Blocking CD86 binding to ligand reduced the incidence of diabetes in NOD mice, while blocking CD80 binding to ligand accelerated the onset of the disease (Lenschow *et al.*, 1995). Kuchroo *et al.* (1995), found that CD80 mAbs reduced the severity of EAE while CD86 mAbs exacerbated disease.

As both EAE and diabetes in NOD mice are Th1-mediated diseases, these findings argue against a direct correlation between CD80 or CD86 costimulation and the development of Th1 or Th2 responses. Thus the differences observed in the previous studies are not a result of differential signal transduction through CD28 (or CTLA-4) upon ligation with CD80 or CD86. The differences in the cytokine secretion profiles observed in different *in vitro* systems probably reflect cell concentrations or quantitative differences in the amount of antigen. The differences observed in the *in vivo* studies are more likely due to the absence of CD28-mediated costimulation at discrete stages or times during the immune responses when either CD80 or CD86 is predominately expressed, thus mediating unique effects without mediating unique signals through CD28 or CTLA-4.

Expression of CD80 and CD86

Once a T cell has matured and entered the periphery, it does not directly distinguish self from nonself peptides. The APC provides both the primary (antigen-specific) and the secondary (costimulatory) signals to naive T cells. Thus the APC must regulate the expression of costimulatory molecules so it provides costimulation to naive T cells only in conjunction with non-self peptides, and presents self peptides without costimulation. APC do not directly distinguish self from non-self, but they up-regulate costimulatory signals in response to prior stimulation or T cell contact, such as with activated B cells, or in response to cytokines present in damaged tissues, such as dendritic cells (DC). DC are potent APC which express high levels of MHC class II and costimulatory molecules such as CD80 and CD86 in response to cytokines present in inflamed tissues (Steinman, 1991). Activated B cells also express costimulatory molecules and are competent APC for naive T cells (Croft *et al.*, 1992; Cassell and Schwartz, 1994).

The CD80 protein was initially found on activated B cell blasts, B lymphoblastoid cell lines, many Burkitt's lymphomas, and other lymphomas (Yokochi *et al.*, 1982; Freedman *et al.*, 1987). Since the original characterization of CD80 expression, it has been reported to be on many non B-cells, including HTLV-1-transformed T cells, activated T cells, γ-IFN stimulated monocytes, blood DC, and Langerhans cells (Vallé *et al.*, 1990; Azuma *et al.*, 1993b; Freedman *et al.*, 1991; Young *et al.*, 1992; Symington *et al.*, 1993). CD80 can be

induced on resting B cells by a number of *in vitro* methods. Crosslinking the antigen receptor, IgM, or MHC Class II induces CD80 expression on resting B cells (White *et al.*, 1991; Koulova *et al.*, 1991; Nabavi *et al.*, 1992; Watts *et al.*, 1993). In addition to signals directly involved in antigen recognition, Ranheim and Kipps (1993) found that CD40 and IL-4 together could induce CD80 expression on purified B cells and chronic lymphocytic leukemia B cells.

Expression of CD86 on APC *in vitro* parallels expression of CD80 with a few differences. Anti-Ig induces CD86 and CD80 with similar kinetics, but CD86 is induced to much higher levels than CD80 (Hathcock *et al.*, 1994). Lenschow *et al.* (1994), found anti-Ig-induced CD80 expression was extremely low in their hands, and they proposed that CD86 is induced faster than CD80; however, their kinetic analysis does not strongly support this. Discounting the extremely low levels of CD80 compared to CD86, the kinetics of induction looks very similar. Isolated human and murine Langerhans cells express both CD80 and CD86 after *in vitro* culture (Rattis *et al.*, 1996; Kawamura and Furue, 1995). IL-4 but not IL-2 enhances both CD80 and CD86 expression on Langerhans cells. γ-IFN has no effect on CD86 expression but inhibits CD80 up-regulation about 40% on Langerhans cells, while IL-10 inhibits up-regulation of both CD80 and CD86 about 50% (Kawamura and Furue, 1995). Expression of CD80 and CD86 on activated murine macrophages is regulated in a similar manner as on Langerhans cells: γ-IFN enhances CD86 expression and inhibits

CD80 expression (Hathcock *et al.*, 1994). CD80 expression on human blood DC is not affected by IL-10, but CD86 expression is down-regulated about ten-fold (Buelens *et al.*, 1995). This suggests CD80 and CD86 are independently regulated but none of the cytokines tested can completely prevent the expression of CD80 or CD86, nor are they absolutely necessary for the induction of either CD80 or CD86 on B cells, macrophages, Langerhans cells, or DC *in vitro*. Interestingly, Prabhu Das *et al.* (1995) found CD80 and CD86 were reciprocally expressed on murine T cell clones after antigen stimulation. CD86 but not CD80 was expressed on resting T cell clones, while CD80 but not CD86 was expressed after activation.

In situ analysis of CD80 and CD86 expression reveals they are independently regulated *in vivo*. CD80 is expressed on the majority of cells of dendritic origin and monocytic cells present in inflamed tissues but not on those cell types in normal non-inflamed tissues (Vandenberghe *et al.*, 1993). Vyth-Dreese *et al.* (1995) found CD80 preferentially expressed on centroblasts in the dark zone of germinal centers, and at low levels on centrocytes in the basal light zone, but not in the apical light zone. They found CD86 expressed preferentially on centrocytes in the apical light zone of germinal centers and in decreasing amounts through the basal light zone, but not on centroblasts in the dark zone. This recipricol expression is similar to what was found on T cell clones (Prabhu Das *et al.*, 1995), and suggests that even though CD80 and CD86 have similar

functions and regulation of expression *in vitro*, they have distinct regulation and function *in vivo*, particularly on B cells.

The distinct germinal center localization of CD80 and CD86 identified by Vyth-Dreese *et al.* (1995) provides a model for *in vivo* regulation of CD80 and CD86 expression and provides insight into the unique roles they may play. The absence of either molecule on cells in the follicular mantle zone, or on non-inflamed tissues supports a model in which these molecules are only expressed on activated APC, and the act of isolating Langerhans cells is probably responsible for CD80 found on these cells immediately after isolation (Rattis *et al.*, 1996). After primary activation in T cell zones, B cells migrate into the dark zone of a germinal center and undergo very rapid proliferation as $CD80^+$, $CD86^-$ centroblasts. While it is not known if CD86 is transiently expressed immediately after B cell activation, it is clearly lost by the time the activated B cell has entered the germinal center reaction as a centroblast. After many rounds of proliferation and clonal expansion, $CD80^+$ centroblasts migrate out of the dark zone, becoming non cycling centrocytes in the marginal light zone where they can interact with antigen on follicular dendritic cells (FDC). At this point, in the marginal light zone, these centrocytes have already begun the shift from CD80 to CD86 expression. The antigen signal provided by iccosomes on FDC presumably rescues these centrocytes from immediate cell death. Thus, they survive and migrate into the apical light zone as $CD86^+$, $CD80^-$ centrocytes which can present antigen to follicular T cells and receive CD40-dependent

signals required for long term survival (reviewed in MacLennan, 1994). This pattern of expression is similar to that found on T cell clones. After antigen stimulation the proliferating T cells become $CD80^+$ and $CD86^-$, reverting to $CD86^+$,$CD80^-$ after they have rested (Prabhu Das *et al.*, 1995).

Characterization of the CD80 protein.

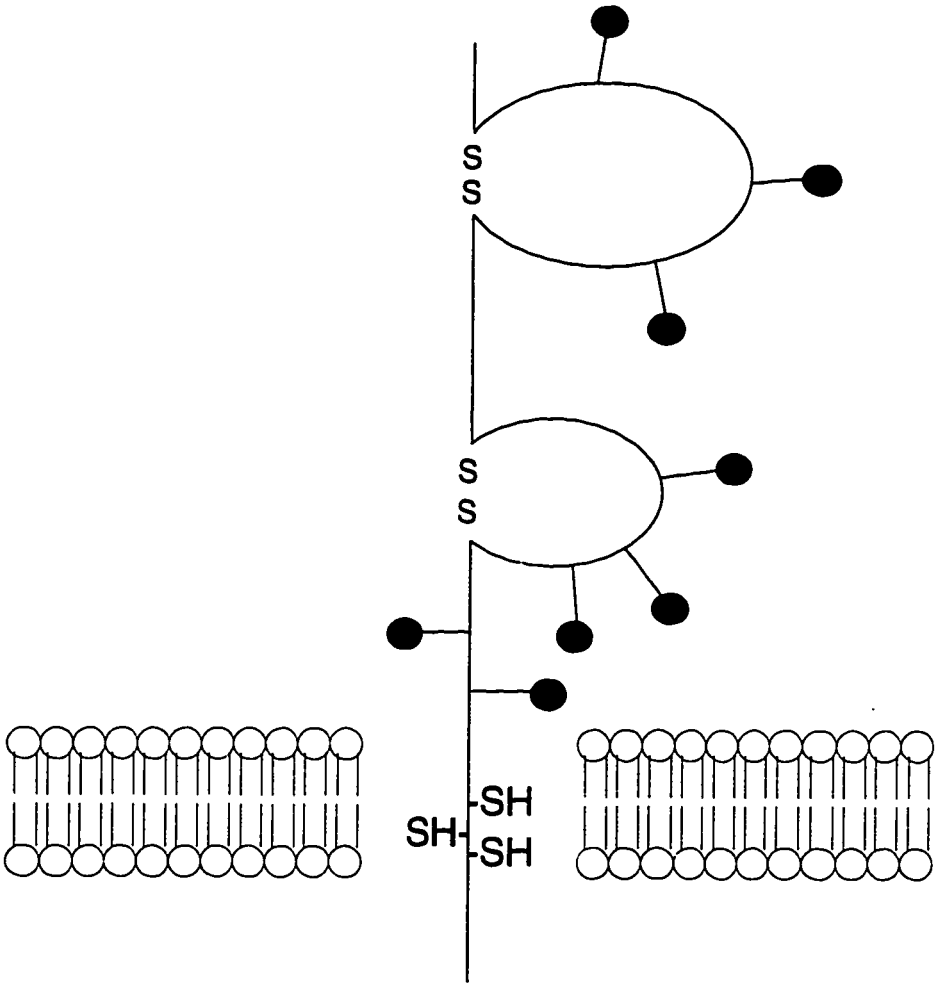
CD80 was first identified as a protein expressed on B lymphoblasts recognized by the mAb BB1 (Yokochi *et al.*, 1982). Nadler and coworkers independently identified a molecule recognized by the mAb B7, which had a very similar, but distinct pattern of reactivity (Freedman *et al.*, 1987). The B7 mAb bound the $CD80^-$ Ramos cell line while BB1 did not, and BB1 bound to the BALM-4 cell line while B7 did not. Furthermore, biochemical characterization of the B7 Ag identified it as a broad band of approximately 60 kDa molecular weight, and BB1 was reported to bind to a protein of 37 kDa (Freedman *et al.*, 1987; Clark *et al.*, 1986). Because of these differences, Nadler and coworkers conjectured that these mAbs recognize two distinct proteins. However, after they isolated and cloned a cDNA from the B cell line Raji and expressed it in transfectants, they found these transfectants were bound by both the B7 and BB1 mAbs, demonstrating that both mAbs recognize the same protein (Freeman *et al.*, 1989). It is unclear exactly why there are slightly different reactivities between these two mAbs. Both B7 and BB1 mAbs are IgM antibodies and are relatively low affinity antibodies. They may cross-react with different proteins,

or some cells may express CD80 in a manner which one but not the other mAb may bind, perhaps dependent upon particular glycosylation patterns. There have been some studies suggesting that the BB1 mAb recognizes a distinct molecule from that bound by the B7 mAb (Boussiotis *et al.*, 1993; Nickoloff *et al.*, 1993), but there have not been any definitive biochemical studies demonstrating this.

CD80 is a monomeric type I surface protein and a member of the Ig supergene family (Fig. 1.1). Although the protein core of CD80 is 30 kDa, it is predominately expressed as a 45-65 kDa glycoprotein, indicating CD80 must be highly glycosylated. There are eight potential N-linked glycosylation sites on CD80 and two potential sites for O-linked glycosylation. Treating CD80 precipitates with endoglycosidase F, which removes N-linked glycosylation, reveals that the difference between the observed and expected molecular weights are most likely due to only N-linked glycosylation.

The extracellular region consists of two Ig domains: an N-terminal, membrane distal IgV (Variable)-like domain; and a membrane proximal, IgC (Constant)-like domain (Freeman *et al.*, 1989). Linsley and coworkers modeled the CD80 Ig domains on the crystal structure for the IgV and IgC domains to characterize the ligand binding regions (Linsley *et al.*, 1994b; Bajorath *et al.*, 1994; Peach *et al.*, 1995). These studies revealed that the ligand binding residues of CD80 exist on one face of the Ig domain while the asparagine residues potentially involved in N-linked glycosylation are on the opposite face.

Figure 1.1. CD80 protein and gene structure.
CD80 protein diagram indicating the two Ig domains, relative position of N-linked glycosylation sites (•—), and cysteine residues present in the transmembrane region (-SH). Below is the structure of the *CD80* gene locus with the known exons of human *CD80* numbered. Exon 1 contains the 5' UTR; exon 2 contains more 5' UTR, the protein initiation site, and signal sequence; exons 3 and 4 encode the IgV-like and IgC-like domains, respectively; exon 5 encodes the transmembrane spanning region; and exon 6 encodes the cytoplasmic tail, 3' UTR, and the poly-A site. The gray box located downstream from exon 6 represents the alternatively spliced exon 7 which can be used instead of exon 6 and is found in some murine CD80 transcripts, but not identified in the human *CD80* gene locus.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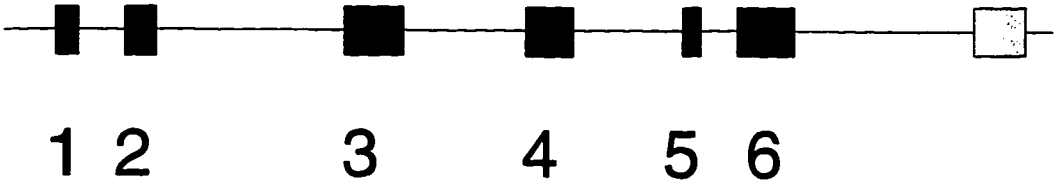
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These observations suggest that the carbohydrate moieties are not directly involved in ligand binding, but may stabilize the structure of CD80 aiding in its binding to its ligands, CD28 and CTLA-4.

The transmembrane region of human CD80 contains three cysteine residues which may interact with other proteins or specific lipid moieties. Murine, rat, and rabbit CD80 also have cysteine residues in the transmembrane region, suggesting that these moieties may be important in CD80 function as they are usually not found in transmembrane regions (Freeman *et al.*, 1991; Judge *et al.*, 1995; Isono and Seto, 1995). The cytoplasmic tail of human CD80 is relatively short (19 amino acids) and 9 of the residues are arginine. The high concentration of arginine in the cytoplasmic tail of CD80 suggests that either arginine itself is important in CD80 function, or that charges provided by arginine are important. There are two short regions in the cytoplasmic tail of CD80 conserved across species, corresponding to positions (in human CD80) 275-278, RRNE, and 282-284, RE(S/T). The first sequence is conserved across all 4 species in which *CD80* has been sequenced: human, mouse, rat, and rabbit (Freeman *et al.*, 1989; Freeman *et al.*, 1991; Judge *et al.*, 1995; Isono and Seto, 1995). This region is similar to the calmodulin binding site identified in the polymeric Ig receptor, RRNV, identified by Chapin *et al.* (1996). The second region is RLS in rabbit CD80, RET in mouse and rat CD80, and RES in human CD80. This serine is a potential target for protein kinase C and the cyclic

nucleotide (cAMP and cGMP) -dependent kinases (Feramisco *et al.*, 1980; Glass and Smith, 1983; Kishimoto *et al.*, 1985; Glass *et al.*, 1986; Woodgett *et al.*, 1986). We have not yet detected calmodulin binding to the RRNE sequence, nor have we detected inducible phosphorylation of S284. However, we have found that these specific residues are critical for T cell costimulation through CD28 (Chapter 3).

The short cytoplasmic tail of CD80 suggests it may not initiate signal transduction back into APC. However, several reports suggest the length of the cytoplasmic tail does not necessarily correlate with the ability of a surface protein to signal into a cell. For example, membrane IgM has a cytoplasmic tail of 3 amino acids yet it is clearly able to transduce signals into B cells expressing it. Membrane IgM associates with Igα and Igβ; thus, when it is ligated, these associated proteins initiate signal transduction (reviewed in Cambier *et al.*, 1994). Hirokawa *et al.* (1996) have recently provided some evidence for direct signal transduction to the APC by CD80. Crosslinking CD80 on the B lymphoma line Raji induced tyrosine phosphorylation of several proteins, inhibited growth, and induced morphological changes in the cells. While a non-binding antibody did not have any effect, these results were very similar to those obtained with CD29 crosslinking, suggesting these effects may not be specific for CD80 signaling.

Characterization of the CD80 gene family

When Freeman *et al.* (1989) cloned and characterized the CD80 cDNA, they found that most cells expressing CD80 had multiple CD80 RNA transcripts, 1.7, 2.9, 4.2, and 10 kb in length. The cDNA isolated was 1.4 kb in length, and probably corresponds to the 1.7 kb transcript. The roles of the alternative transcripts remain unknown. They may simply reflect incompletely processed mRNA. It is possible that one or more of these species reflects an alternatively spliced form of CD80 with distinct regulation and function. There have been two alternatively spliced variants of murine CD80 mRNA identified which encode functional proteins (Inobe *et al.*, 1994; Borriello *et al.*, 1994). The first variant encodes CD80 lacking the membrane proximal IgC-like domain. It still binds to CD28 and CTLA-4, but it does not bind to CTLA-4 as well as does full length CD80. Interestingly, it did not stimulate resting T cells as well as does the full length CD80 molecule, although it was able to induce levels of proliferation in activated T cells similar to those induced by full length CD80 (Inobe *et al.*, 1996). The second naturally occurring variant of murine CD80 contains an alternatively spliced cytoplasmic tail, using an exon located 7 kb beyond the exon for the first of the alternatively spliced cytoplasmic tails. The internal structures of both human and murine CD80 gene loci are typical of Ig supergene family members (see Fig. 1.1); each domain is encoded on a separate exon which allows for inclusion or exclusion of individual domains without

disrupting the reading frame (Hood *et al.*, 1985; Selvakumar *et al.*, 1992; Selvakumar *et al.*, 1993).

Linsley and coworkers identified CTLA-4 as a ligand for CD80, and engineered CTLA4Ig as a soluble form of CTLA-4. CTLA4Ig, was far more potent than the CD80 mAb BB1 at inhibiting T cell proliferation and T cell-induced Ig secretion from B cells (Linsley *et al.*, 1991b). While the ability of CTLA4Ig to inhibit costimulation could be attributed solely to the fact that its avidity for ligand is higher than that of the antibodies used, that turned out to only be part of the story. Late in 1993, several groups characterized and cloned a second costimulatory molecule, CD86, also called B7-2 or B70. Wu *et al.* (1993) identified a ligand for CTLA-4 that could costimulate T cell proliferation independently of CD80. Freeman *et al.* (1993b) reported a 70% decrease in T cell proliferation in response to alloantigen in mice deficient in CD80 expression; however, even though these mice were lacking CD80, their spleen cells still elicited a costimulatory response that could be blocked by CTLA4Ig. This suggested the expression of another CD28/CTLA-4 ligand in CD80-deficient mice. Freeman and coworkers isolated both murine and human CD86 cDNA while Azuma *et al.* independently cloned the human counterpart (Freeman *et al.*, 1993a; Freeman *et al.*, 1993c; Azuma *et al.*, 1993a).

Comparisons between the CD80 and CD86 proteins reveal an overall identity of about 24%, with 30% identity in the IgC-like domain and 24%

identity in the IgV-like domain. The cytoplasmic tails share only 17% similarity, and the cytoplasmic tail of CD80 is 19 amino acids long, while that of CD86 is 62 amino acids long. Furthermore, the conserved regions in mouse, human, rat, and rabbit CD80 discussed above are not present in CD86, suggesting CD80 and CD86 may have unique signaling properties. Linsley *et al.* (1994a) found CD80 binding to CD28 and CTLA-4 was about 2 or 3-fold higher than that of CD86. This is most likely due to a higher off rate for CD86. They also found that at sub-optimal conditions, CD80 was about 3-fold more effective than CD86 on a per cell basis at inducing T cell proliferation. This was reflective of the relative avidity of CD80 and CD86 for CD28, and suggests that CD86 needs to be expressed at slightly higher levels than CD80 to provide equivalent costimulation.

Linsley and coworkers (Linsley *et al.*, 1994b; Bajorath *et al.*, 1994) aligned human and murine CD80 and CD86, and identified common motifs shared among all four proteins. They found there were many additional consensus sites shared among CD80 gene family members which were not conserved in other Ig supergene family members. In the IgV-like domain alone, there are 40 consensus residues in the CD80 gene family while only 8 of those are in the Ig supergene family consensus motif. They found three additional proteins in the Swiss Protein Database that contain the CD80 gene family conserved residues: bovine butrophilin, a component of the milk fat globule membrane; myelin/oligodendrocyte glycoprotein, a component of the myelin sheath; and B-

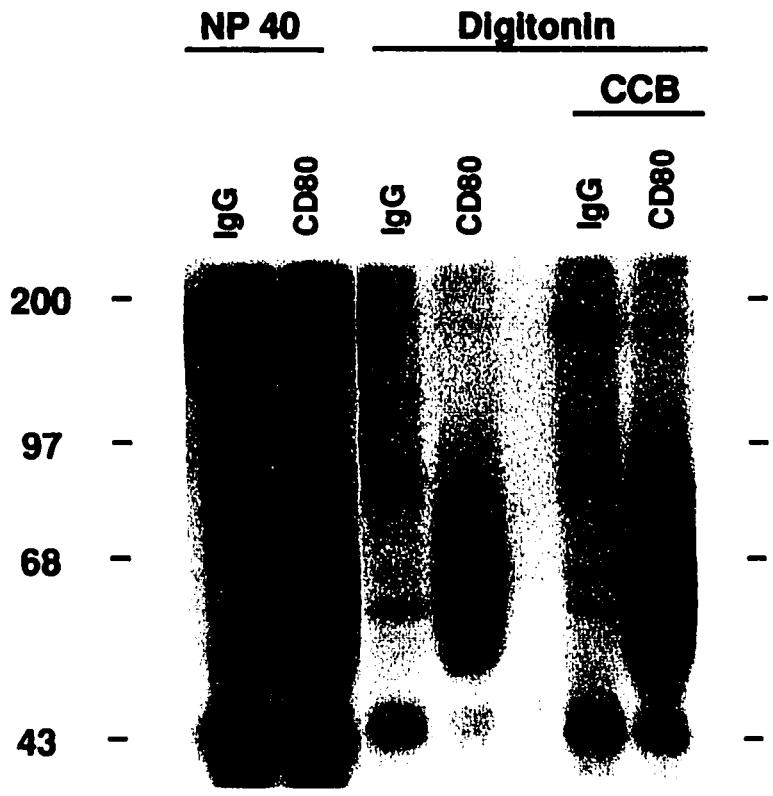
G, a chicken MHC molecule (Jack and Mather, 1990; Steinman, 1993; Kaufman and Salomonsen, 1992). Allogeneic B-G molecules enhance antibody responses to other molecules present on chicken red blood cells, and induce higher and faster antibody titers to themselves (Kaufman and Salomonsen, 1992). This suggests that there may be some functional relationship between CD80 and these distantly related but structurally similar molecules. These proteins related to the IgV-like domain suggest CD80 and CD86 are members of an ancient subfamily within the Ig supergene family. The IgC-like domain in the CD80 gene family contains 27 conserved residues including those found in the Ig supergene family. No other proteins with these consensus residues have been identified yet.

Rationale and pilot studies

While characterizing CD80 expressed on B cells, we found that CD80 was poorly recovered in lysates from cells solubilized in digitonin compared to NP-40 (Fig. 1.2). Digitonin is a mild detergent which does not disrupt the cytoskeleton effectively. Thus, most of the cytoskeleton and associated proteins precipitate with the nuclear fraction. We also found that cytochalasin B (CCB), which disrupts the actin-based cytoskeleton (MacLean-Fletcher and Pollard, 1980), increased the solubility of CD80 in digitonin lysates, thus suggesting CD80 may interact with the actin-based cytoskeleton (Fig. 1.2).

Crosslinking CD28 can induce increases in free cytoplasmic calcium from both intracellular and extracellular stores (Ledbetter *et al.*, 1987). It also in

Figure 1.2. CD80 associates with the actin-based cytoskeleton.
CESS B cells were collected and incubated in RPMI with or without 10 μg/ml CCB then surface labeled with ^{125}I and lysed in either NP40 or digitonin lysis buffer as indicated. The lysates were incubated with either human IgG or CTLA4Ig as indicated, then precipitated with protein A sepharose and washed. The approximate mw are indicated in kDa.


NP 40
Digitonin
CCB
IgG
CD80
IgG
CD80
IgG
CD80
200
97
68
43

induces expression of the IL-2Rα chain (CD25), and enhances T cell proliferation in response to IL-2. Furthermore, higher order aggregates of CD28 mAbs induced more IL-2 mRNA and CD25 expression than non-aggregated mAbs (Ledbetter *et al.*, 1990). Fab fragments of CD28 mAbs inhibit T cell proliferation in mixed lymphocyte reactions (Damle *et al.*, 1988). Thus, CD28 molecules need to be aggregated on the cell surface to provide costimulation. Since CD80 is expressed as a monomer, it cannot directly aggregate CD28 (Freeman *et al.*, 1989). Thus, CD80 interacting with the cytoskeleton may provide the necessary framework to aggregate CD28 and initiate costimulatory signals.

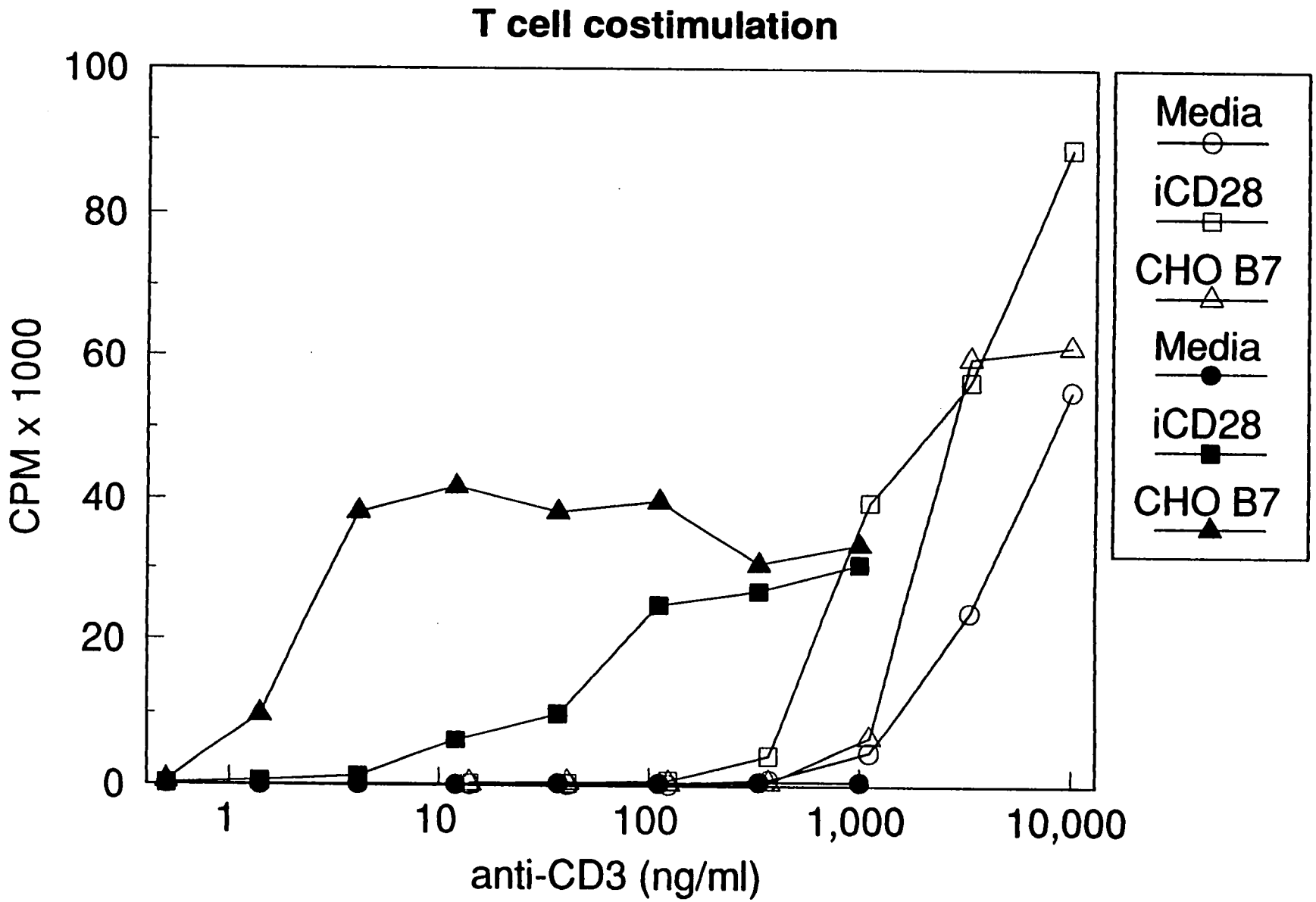
These observations allowed me to formulate the hypothesis that the cytoplasmic tail of CD80 is required for providing costimulation to T cells through CD28. The specific aims to address this hypothesis were:

1. To compare the ability of tailless CD80 molecules with that of wildtype CD80 molecules expressed on APC to costimulate T cells.
2. To determine the region(s) of the cytoplasmic tail of CD80 required for costimulation and thus CD28 aggregation.
3. To identify molecules which interact with CD80 and may regulate costimulation.

There are numerous assays to study costimulation but they require immobilizing anti-CD28 or CD80 recombinant globulins on plastic. These methods would not have allowed me to study how monomeric CD80 molecules expressed on cells can induce CD28-dependent costimulation. To develop a

sensitive assay to measure T cell costimulation delivered by native CD80 expressed on cells, I compared the ability of insoluble CD28 mAbs and CD80 expressed on cells to costimulate T cell proliferation in conjunction with either immobilized or soluble CD3 mAbs (Fig. 1.3). What was clear in this pilot experiment was that soluble CD3 mAbs were unable to induce T cell proliferation alone, and that addition of either immobilized CD28 or CD80-expressing cells induced T cell proliferation at 100 to 1000 fold lower doses than did the highest dose of CD3 mAb alone. Furthermore, CD80 expressed on cells induced T cell proliferation to low doses of anti-CD3 almost in an all or none fashion, suggesting that there is a threshold, that when crossed, induces T cells to respond maximally. The immobilized CD3 mAbs were able to induce high T cell proliferation even in the absence of costimulation, and were only enhanced about 10 fold with costimulation through CD28, making immobilized anti-CD3 an undesirable primary stimulus to measure costimulation. Using this sensitive costimulation assay, I was able to pursue the specific aims of this study.

Figure 1.3. Costimulation of CD3-mediated T cell proliferation.
Ten-thousand freshly isolated peripheral blood Er^+ T cells were stimulated with anti-CD3 either immobilized on plastic (○, □, or △) or soluble (●, ■, or ▲) at the concentrations indicated. Costimulation was provided by media (○ or ●), 10 μg/ml immobilized anti-CD28 (iCD28) (□ or ■), or 10,000 mitomycin C treated CD80-transfected CHO cells (△ or ▲). Proliferation was measured by [^{3}H]thymidine uptake during the final 18 hr of a 4 day culture.


T cell costimulation
100
80
60
40
20
0
CPM x 1000
1
10
100
1,000
10,000
anti-CD3 (ng/ml)
Media
iCD28
CHO B7
Media
iCD28
CHO B7

Chapter 2: Subcellular localization of CD80 receptors is dependent upon an intact cytoplasmic tail and is required for CD28-dependent T cell costimulation.

Abstract

CD28 provides a major costimulatory signal to T cells when it is cross-linked with mAb, immobilized recombinant ligand (CD80Ig or CD86Ig), or ligand-bearing cells but not when it is bound by specific Fab fragments or monomeric ligand. We wanted to determine how CD80 could crosslink CD28 since CD80 is expressed as a monomer on the surface of APC. We found that CD80 may interact with the actin-based cytoskeleton. To test if the interaction of CD80 with the cytochalasin B-sensitive cytoskeleton was necessary for T cell costimulation through CD28, we constructed a tailless form of CD80 and generated stable transfectants of CHO epithelial cells and Reh B cells expressing either the tailless or wildtype CD80 molecules. Unlike control cells expressing wildtype CD80, the tailless CD80 transfectants expressing equivalent levels of surface CD80 were not able to provide a costimulatory signal for anti-CD3 induced T cell proliferation, up-regulation of CD25 (IL-2Rα) expression, or the induction of IL-2 secretion. Thus, the cytoplasmic tail of CD80 apparently is required to signal T cells. Confocal microscopic studies revealed that wildtype CD80 and tailless CD80 have different patterns of subcellular distribution in both epithelial and lymphoid cells. Furthermore, T cell contact induces more patching and capping of CD80 in wildtype CD80-expressing cells than in tailless CD80-expressing cells. This suggests that the

cytoplasmic region of CD80 functions to localize CD80 in complexes required for effective T cell costimulation.

Introduction

CD80 (B7-1/BB1) is a 45 to 65 kDa cell surface protein expressed on activated B cells, monocytes, and DC (Yokochi *et al.*, 1982; Freeman *et al.*, 1989; Freedman *et al.*, 1991; Young *et al.*, 1992). It is a ligand for the T cell costimulatory molecule CD28 and for CTLA-4, a cell surface molecule expressed on activated T cells (Linsley *et al.*, 1990; Linsley *et al.*, 1991b). While CD28 provides a costimulatory signal to T cells in conjunction with TCR/CD3 stimulation that increases both T cell proliferation and cytokine secretion (reviewed in Linsley and Ledbetter, 1993), CTLA-4 appears to play a critical role down modulating T cell proliferation (Tivol *et al.*, 1995; Waterhouse *et al.*, 1995; Krummel and Allison, 1995; Walunas *et al.*, 1994; Gribben *et al.*, 1995). Fab fragments of CD28 mAb do not promote T cell activation, and in fact inhibit APC-derived signals through CD28 (Damle *et al.*, 1988). Thus, crosslinking or aggregating CD28 molecules is necessary for costimulation.

CD80 and its homolog CD86 are expressed as monomers on the surfaces of APC (Freeman *et al.*, 1989; Azuma *et al.*, 1993a; Freeman *et al.*, 1993c). Linsley *et al.* (1991a) found that soluble CD80 monomers do not costimulate T cell proliferation; thus it is unlikely that monomeric CD80 can crosslink CD28 to provide T cell costimulation. In this study, we found that CD80 associates

with a cytochalasin B (CCB) sensitive component of the cytoskeleton, which may provide the necessary framework to crosslink CD28. To determine if this CD80-cytoskeleton interaction was necessary and sufficient for T cell costimulation through CD28, we generated mutant forms of CD80 which lack the cytoplasmic tail or which have the cytoplasmic tail and transmembrane domain of CD80 replaced with those of either CD2, CD54, or CD86. We then tested whether these molecules could costimulate T cell growth. To further characterize the role that the cytoplasmic tail of CD80 plays in T cell costimulation, we examined the distribution of wildtype and tailless CD80 on the surface of cells and found that the cytoplasmic tail of CD80 influences its subcellular localization, in both antibody-mediated and T cell contact-mediated redistribution, and the ability of CD80 to costimulate T cell growth.

Materials and Methods

Antibodies and cell lines.

The purified CD80 mAb L310 was kindly provided by Dr. L. Lanier. The CD80 mAb, BB1 (IgM; Yokochi *et al.*, 1982), CD16 mAb, FC1 (IgM; Ledbetter et al., 1985), CD3 mAb, 64.1 (IgG_{2a}; Hansen et al., 1984), CD28 mAb, 9.3 (IgG_{2a}; Hansen *et al.*, 1980), or control mAbs, P3X (IgG_1; ATCC) and P1.17 (IgG_{2a}; ATCC) were all purified from ascites before use. CD25 and CD69 mAbs were from Becton Dickinson Immunocytometry Systems (San Jose, CA). Goat anti-mouse IgG-FITC conjugate, goat anti-mouse IgM-biotin conjugate and goat anti-human IgG-PE conjugate were from Jackson Immunoresearch (West Grove, PA).

Strepavidin-Texas Red was from Molecular Probes, Inc. (Eugene, OR). CTLA4Ig and CD28Ig with human IgG Fc (Linsley *et al.*, 1991b; Linsley *et al.*, 1991a) were kindly provided by Dr. P. Linsley (Bristol-Myers Squibb; Seattle WA). CTLA4mIg with murine IgG_1 Fc was produced in our lab, as described below, and was used for flow cytometry and for blocking T cell proliferation. Reh cells and CHO cells were from ATCC and were maintained in RPMI-10 (RPMI 1640 with 10% FCS, 10 U/ml each of penicillin and streptomycin) or DMEM-10, respectively. The CTLL-2 cells were kindly provided by Dr. D. Morris and were maintained in RPMI-10 with 50 μM 2-ME and 10 ng/ml of recombinate IL-2 provided by Dr. H. Ochs. The CHO transfectants were made by electroporation with 10 μg of linear plasmid using the manufacturers protocol (BioRad, Richmond CA). One to two days after the electroporation, the remaining viable cells were selected and maintained with 750 μg/ml (active concentration) of G418 (Gibco, Grand Island, NY) and clones were isolated for analysis. The Reh transfectants were made using Lipofectin reagent (Gibco). Two million Reh were transfected with 5 μg of DNA and 20 μl of Lipofectin reagent. After 2 days, G418 was added to 750 μg/ml, then one to two weeks later viable cells were plated in a 48-well plate and sublines were screened for expression of CD80 via flow cytometry using a FACScan (Becton Dickinson). At least two different isolates from each transfection were tested in the T cell proliferation assays with the same results.

PCR and chimeric cDNA constructs.

The CD80 cDNA (pB7) was kindly provided by G. Freeman (Freeman *et al.*, 1989). The CD80 cDNA was excised from the pB7 plasmid and inserted into the pRc/CMV expression plasmid (Invitrogen Corporation, San Diego, CA). The tailless CD80 cDNA was constructed using site directed mutagenesis (U.S.E. mutagenesis kit; Pharmacia, Piscataway, NJ) to insert stop codons at positions 273 and 274, thus deleting the carboxyl terminal 16 amino acids. The mutagenesis primers and all of the primers used in PCR are listed in Table I. The chimeric CD80 constructs were made in three steps: first, the region of the cDNA which encodes for the extracellular portion of CD80 was isolated by PCR and cloned into pBluescriptII SK+ (Stratagene, La Jolla, CA). The reverse primer contained a *Bam*HI restriction site which introduces the two amino acids G,S after position 242. Second, the cDNA regions which encode for the transmembrane and cytoplasmic tails of CD54 and CD86 were isolated by reverse transcription of RNA from activated T and B cells provided by S. Klaus, followed by PCR. The transmembrane and cytoplasmic tail of CD2 was isolated by PCR from a CD2 cDNA provided by K. B. Andersson (Pilon *et al.*, 1991). The forward primers all contained *Bam*HI sites, and the reverse primers all contained *Xba*I sites. These PCR products were all subcloned into pBluescript. The third step was to ligate the two cloned constructs together and clone them into pRc/CMV. The CTLA4mIg construct was made using reverse transcription

Table I. Primers used in chapter 2.

Construct	Primer	Sequence[a]
CD80 tailless	pRc-ClaI	AGGCGTTTTGCGCTG**ATCGAT**GATGTACGGGCCAGA
	CD80Δ16	GCCCCAAGATGCAGA<u>TAGTGA</u>AGGAGGAATGAG
CD80ECD[b]	T7	AATACGACTGAGTATAG
	CD80R	CTC**GGATCC**ATCAGGAAAATGCTCTTGC
CD2TMCT[c]	CD2F	GAC**GGATCC**GACATCTATCTCATCATTGGC
	CD2R	GCG**TCTAGA**TTAATTAGAGGAAGGGGAC
CD54TMCT[c]	CD54F	CAC**GGATCC**GAGATTGTCATCATCACTG
	CD54R	GTG**TCTAGA**GGTTCAGGGAGGCGTGGC
CD86TMCT[c]	CD86F	CAG**GGATCC**CACATTCCTTGGATTACAG
	CD86R	CGC**TCTAGA**TTAAAAACATGTATCACTTTTGTCGC

[a] All primers are written 5' to 3', stop codons are underlined and restriction enzyme recognition sequences are bold.
[b] This construct contains only the extracellular domain of CD80 which is used in the generation of the chimeric constructs.
[c] These constructs only contain the transmembrane and cytoplasmic tail regions of the molecule of interest, and are used to generate the chimeric constructs.

of RNA from activated T and B cells followed by PCR to amplify the extracellular region. This region was inserted into the pBluescript/MuFc plasmid kindly provided by D. Cosman (Immunex, Seattle, Wa). The entire coding region was then subcloned into the pRc/CMV expression vector. The CTLA4mIg recombinate globulin was produced as described by Linsley *et al.* (1991b). All constructs were sequenced to confirm sequence fidelity.

Flow cytometry and immunofluorescence microscopy.

Adherent cells were detached with versene buffer and washed once before being resuspended in cold staining buffer (2% FCS in PBS with 0.02% NaN_3). Two hundred thousand cells were resuspended in 50 µl with the primary mAb at saturating concentrations. After 40 min on ice, the cells were washed twice with PBS and either resuspended in 50 µl staining buffer with secondary Ab and incubated as above, or fixed in 1% paraformaldehyde in PBS. Five thousand events were analyzed on a FACScan for each sample. For soluble ligand binding studies, CD80 transfectants were incubated with either CTLA4Ig or CD28Ig at the concentrations indicated. The fusion proteins were detected with goat anti-human IgG-PE conjugate. The fluorescence intensity (FI) was calculated by dividing the mean fluorescence intensity of the sample by that of the control; thus a FI of 1 is background. Percent of maximal binding was calculated as follows: [(FI of the sample - 1) ÷ (FI of maximum sample -1)] × 100%.

Samples were prepared for microscopy in a similar manner, except there was no azide present in the buffers used for antibody-mediated capping. The

samples were washed after the primary mAb, and incubated with goat anti-mouse IgM-biotin conjugate for 30 min, washed, then incubated with strepavidin-Texas Red for 30 min before being transferred to 37°C for the time indicated. For those experiments using the actin-destabilizing drug CCB (MacLean-Fletcher and Pollard, 1980, Sigma, St. Louis, MO), it was added to 10 μg/ml just prior to the 37°C incubation. The cells were then washed with ice cold staining buffer and fixed as above. The cells were then mounted on slides and sealed with a cover slip before being analyzed on a fluorescence microscope. At least 100 cells were analyzed for each sample to quantify the subcellular distribution of CD80. Representative samples were analyzed on a laser scanning confocal microscope (BioRad MRC 600) at the W. M. Keck Center for Advanced Studies in Neural Signaling (Univ. of Wa., Seattle, WA). Composite figures were generated using Adobe Photoshop. T cell-induced CD80 redistribution was performed by mixing 10^6 Reh transfectants with 3 x 10^6 $CD4^+$ T cells with 1 μg/ml soluble anti-CD3 in 0.5 ml at 37°C for the times indicated. The cells were then collected, stained, and analyzed as above for microscopy.

T cell activation and proliferation assays.

$CD4^+$ T cells were isolated from blood of normal donors as Er^+ PBL and the plastic adherent cells and $CD8^+$ cells were removed by panning at room temperature, the preparations were usually greater than 97% $CD3^+$, and greater than 95% $CD4^+$. When we performed the plastic adherent cell and $CD8^+$ cell depletion at 37°C as performed by Damle and Doyle (1989) or Schwinzer *et al.*

(1992), we find significant T cell proliferation in the absence of costimulation, thus we chose to perform these steps at room temperature to minimize this background. The CHO transfectants were removed from the flasks with versene and washed once before being incubated with 100 μg/ml mitomycin C (Sigma) in RPMI (no serum) at 37°C for 45 min. The CHO transfectants were then washed 3 times in RPMI-10 before being plated in to 96-well flat bottom plates at 50,000 cells per well (a confluent monolayer), unless otherwise indicated. After the CHO transfectants attached to the plates, anti-CD3 was added to a final concentration of 1 μg/ml or as indicated, then 50,000 T cells were added to each well. The cultures were pulsed with 0.5 μCi of [^{3}H]thymidine (NEN, Wilmington, DE) for the final 15 to 18 hrs of a 5 day incubation, unless otherwise indicated; then the cells were harvested and incorporated ^{3}H was counted in a liquid scintillation counter. Reh transfectants were treated as above except that 10^5 cells were added to each well, unless otherwise indicated. In experiments for flow cytometry and cytokine secretion analysis, 10^6 T cells were activated in 6-well plates with a confluent monolayer of the CHO transfectants and 1 μg/ml soluble anti-CD3 in 5 ml of RPMI-10. After 24 hr, the T cells were removed from the monolayer and stained for CD25 and CD69 expression, and the supernatants were collected and stored at -20°C until they were analyzed for IL-2 levels using the CTLL-2 cell assay (Gillis *et al.*, 1978). Ten thousand CTLL-2 cells were added to three fold series dilutions of cell culture supernatant and incubated 24 hrs. The cultures were pulsed with 0.5

μCi of [^{3}H]thymidine for the final 6 hrs of the culture before being harvested and analyzed as above.

Results

Tailless CD80 cannot costimulate T cells.

Soluble Fab fragments to CD28 and monomeric CD80 fusion protein do not costimulate T cells (Damle *et al.*, 1988; Linsley *et al.*, 1991a). Crosslinking CD28 with intact bivalent or immobilized mAb is sufficient to provide costimulation (Linsley and Ledbetter, 1993). CD80 is expressed almost exclusively as a monomer on APC surfaces (Freeman *et al.*, 1989; Linsley *et al.*, 1991b); thus it is likely CD80 needs to be clustered on the cell surface in order to crosslink CD28. Since CD80 can be induced to cap on B cell lines (Ehlin-Henriksson *et al.*, 1983) and immunoprecipitation analysis of CD80 in the presence and absence of CCB suggests that CD80 may interact with the actin-based cytoskeleton (see Fig. 1.2), we wanted to determine if the cytoplasmic tail of CD80 is required for T cell costimulation through CD28.

Using site-specific mutagenesis we generated a mutant form of CD80 which lacks the 16 carboxyl terminal amino acids. Immunoprecipitation analysis revealed that the protein core of tailless CD80, as expected, was approximately 2 kD smaller than that of wildtype CD80 (data not shown). With soluble anti-CD3 mAb, T cells did not proliferate in the absence of a costimulatory signal, which in our system was provided by CD80 expressing cell lines. We then compared the ability of CHO transfectants expressing

approximately equivalent amounts of either wildtype or tailless CD80 (Fig. 2.1A) to costimulate T cell proliferation. Tailless CD80 CHO, unlike CHO cells expressing equivalent levels of wildtype CD80, were unable to induce any T cell proliferation above that of the vector CHO transfectant control cells (Fig. 2.1B). We found that at all doses of anti-CD3, including 10 μg/ml (data not shown), there was no T cell proliferation in cultures with tailless CD80 CHO. We titered the number of CHO cells and found that 10^4 to 5 x 10^4 cells per well provided optimal costimulation (Fig. 2.1C).

In addition, we generated transfectants of the CD80 negative B cell line Reh that express either wildtype or tailless CD80 at high levels (Fig. 2.2A). Even though they expressed high levels of tailless CD80, two independent Reh transfectants were unable to costimulate T cell proliferation while Reh cells expressing wildtype CD80 induced good T cell proliferation (Fig. 2.2B). Optimal T cell proliferation occurred when CD3 mAb was at a concentration of 10 ng/ml, and higher doses of CD3 mAb had no significant effect. We titered the number of Reh transfectants from 3 x 10^3 to 3 x 10^5 cells per well, and chose 10^5 cells per well for subsequent experiment (Fig. 2.2C). Since similar results were obtained with either epithelial or lymphoid transfectants and with several different CD80 tailless transfectants, these results were not attributable to a phenomenon specific to a particular type of cell or transfectant. While soluble CD3 mAb can activate purified T cells allowing them to respond to cytokines, CD3 mAb alone does not induce T cell proliferation unless Fc receptor-bearing cells are present

Figure 2.1. Tailless CD80 CHO do not costimulate T cell proliferation.
CHO cells transfected with the CD80 constructs indicated were stained for the surface expression of CD80. (A) The cells were stained with either control mouse immunoglobulin (· · · · · ·) or anti-CD80 (———), then the samples were then analyzed by flow cytometry as described in materials and methods. T cell costimulation required the cytoplasmic tail of CD80. (B) 5 x 10^4 $CD4^+$ T cells were incubated with 10^4 MMC treated CHO Vector (—), wildtype CD80 CHO (●), or tailless CD80 CHO (■) and anti-CD3 at the concentrations indicated for 5 days. (C) 5 x 10^4 $CD4^+$ T cells were incubated with anti-CD3 at 1 μg/ml and either CHO Vector (—), wildtype CD80 CHO (●), or tailless CD80 CHO (■) at the cell numbers indicated. The cultures were pulsed with 0.5 mCi of [^{3}H]thymidine for the final 18 hrs of a 5 day incubation. An additional tailless CD80 CHO clone was tested with the same result. These data are representative of 21 similar experiments.

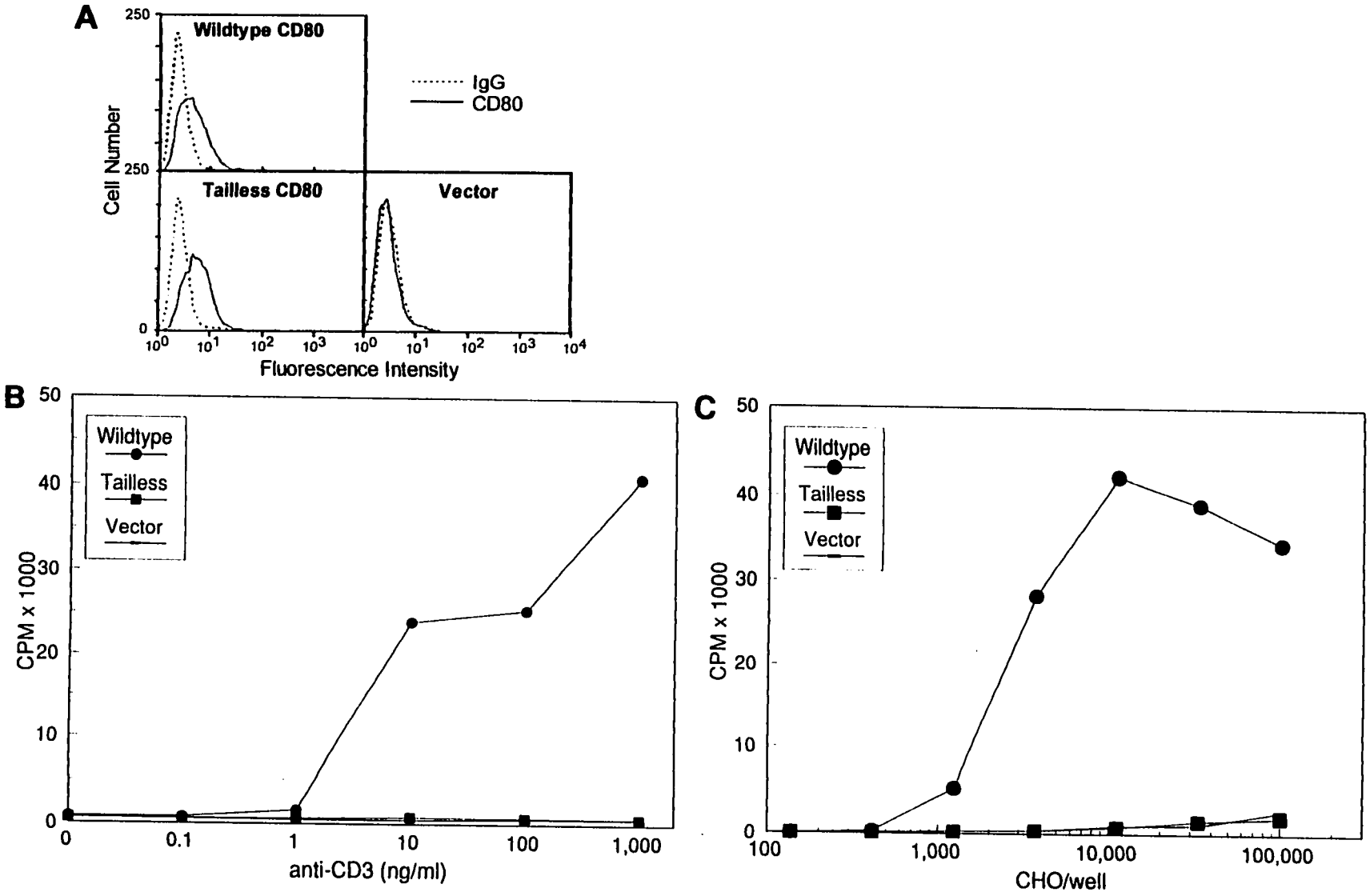
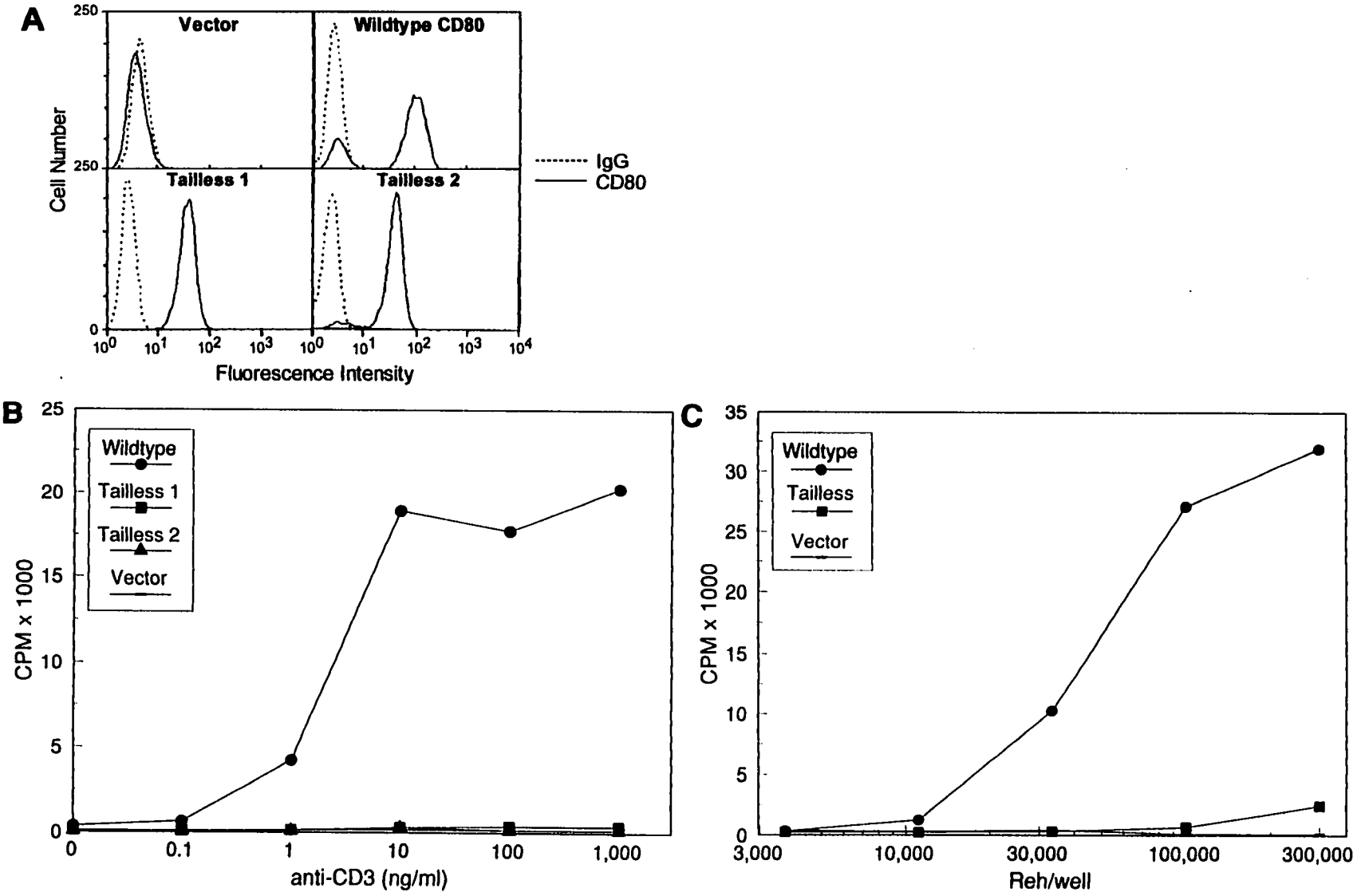

A
Wildtype CD80
Tailless CD80
Vector
IgG
CD80
Cell Number
250
250
0
10^0 10^1 10^2 10^3 10^0 10^1 10^2 10^3 10^4
Fluorescence Intensity
B
Wildtype
Tailless
Vector
CPM x 1000
50
40
30
20
10
0
0 0.1 1 10 100 1,000
anti-CD3 (ng/ml)
C
Wildtype
Tailless
Vector
CPM x 1000
50
40
30
20
10
0
100 1,000 10,000 100,000
CHO/well

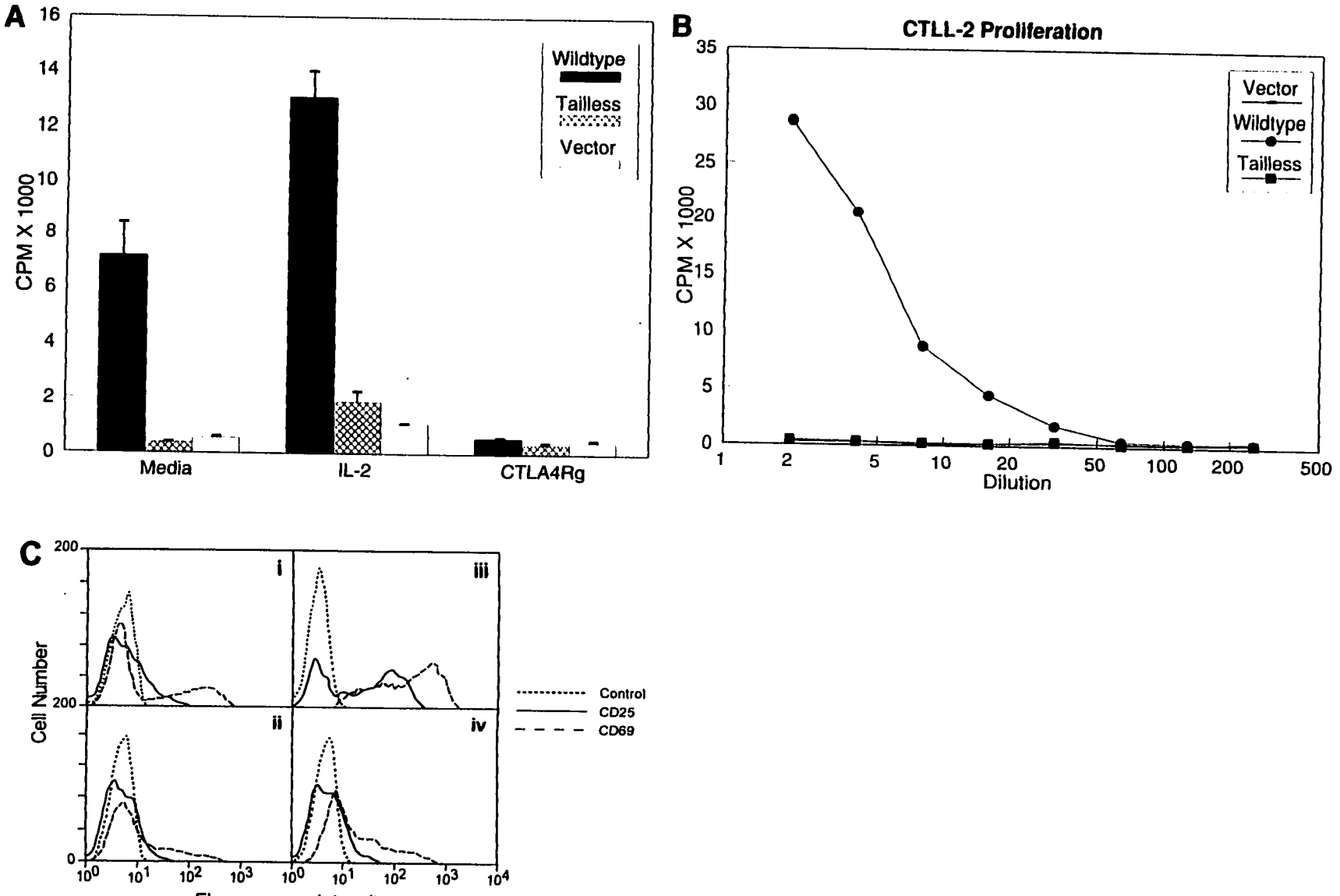
Figure 2.2. Tailless CD80 Reh transfectants do not costimulate T cell proliferation.
Reh cells transfected with either wildtype or tailless CD80 or vector alone were stained for the expression of CD80. (A) The cells were stained with either control mouse immunoglobulin (· · · · · ·) or anti-CD80 (———) and then analyzed by flow cytometry as described above. (B) 5 x 10^4 $CD4^+$ T cells were incubated with 10^5 MMC treated Reh vector (—), wildtype CD80 Reh (●), or two sublines of tailless CD80 Reh (■ and ▲) and anti-CD3 at the concentrations indicated. (C) 5 x 10^4 $CD4^+$ T cells were T cells were incubated with anti-CD3 at 1 μg/ml and either Reh vector (—), wildtype CD80 Reh (●), or tailless CD80 Reh (■) at the cell numbers indicated. The cultures were harvested after a 5 day incubation as described above. These data are representative of 11 similar experiments.


A
Vector
Wildtype CD80
Tailless 1
Tailless 2
Cell Number
250
250
0
IgG
CD80
Fluorescence Intensity
B
Wildtype
Tailless 1
Tailless 2
Vector
CPM x 1000
anti-CD3 (ng/ml)
C
Wildtype
Tailless
Vector
CPM x 1000
Reh/well

to crosslink the mAb (Schwinzer *et al.*, 1992; Damle and Doyle, 1989; Peltz *et al.*, 1988). We detected no T cell proliferation in the absence of costimulation, so our system appears to be Fc-receptor independent (Figs. 2.1 and 2.2).

To test if wildtype CD80 CHO cells were in fact inducing CD28-dependent T cell costimulation, we added CTLA4Ig, an inhibitor of the CD28 pathway (Linsley *et al.*, 1991b) to T cell cultures (Fig. 2.3A). As expected, adding CTLA4Ig completely abolished T cell proliferation. The addition of IL-2 to the cultures enhanced T cell proliferation about 50% in the presence of wildtype CD80 CHO, and two to four-fold in the presence of tailless CD80 or vector transfected CHO cells depending upon the T cell donor (Fig. 2.3A and data not shown). While soluble CD3 mAb alone can induce responsiveness to IL-2 in some systems (Tsoukas *et al.*, 1985; Palacios, 1985), Wakasugi *et al.* (1985) found that highly purified T cells activated with soluble CD3 mAb expressed low levels of CD25 but were not able to proliferate in response to exogenous IL-2 unless monocytes were added back to the culture. This is a result similar to our results with purified $CD4^+$ T cells in the absence of costimulation. The addition of IL-2 to the cultures was not able to restore the T cell proliferation induced with tailless CD80 CHO to the level of T cells in the cultures with wildtype CD80 CHO. This suggests that T cells cultured with the tailless CD80 CHO, unlike those with wildtype CD80 CHO, did not express high levels of IL-2 receptors, and they may not secrete IL-2. Figures 2.3B and C illustrate that this is indeed the case: only T cells cultured with both anti-CD3 and wildtype CD80

Figure 2.3. Tailless CD80 CHO do not up-regulate CD25, CD69, or IL-2 secretion.
(A) 5 x 10^4 $CD4^+$ T cells were incubated with 10^4 MMC treated wildtype CD80 CHO (solid bars), tailless CD80 CHO (cross-hatched bars), or vector CHO transfectants (empty bars) and 1 μg/ml anti-CD3 alone, or anti-CD3 with either 10 ng/ml IL-2 or 10 μg/ml CTLA4mIg as indicated. The cells were pulsed with 0.5 mCi of [^{3}H]thymidine for the last 15 hrs of culture before being harvested and counted as before. Six-well culture plates were seeded with 5 x 10^6 T cells/well either alone or with 1 μg/ml anti-CD3 or with anti-CD3 and 10^6 of either CHO Vector, wildtype CD80 CHO, or tailless CD80 CHO and incubated overnight. (B) The supernatants from the cultures of T cells with CHO Vector (—), wildtype CD80 CHO (●), or tailless CD80 CHO (■) were collected and frozen until assaying for IL-2. Supernatants were diluted as indicated in triplicate and 2 x 10^4 CTLL-2 cells were added to each well. The cells were pulsed with 0.5 mCi of [^{3}H]thymidine for the last 6 hrs of a 24 hr culture. (C) The T cells were recovered from overnight cultures with either anti-CD3 alone (I), or anti-CD3 and vector CHO (ii), CD80 CHO (iii), or tailless CD80 CHO (iv), and analyzed for the expression of CD25 (———) and CD69 (– – – –) by flow cytometry. The dotted line indicates staining with a control mAb. Panel (A) is representative of 3 experiments, (B) and (C) are each one of two experiments with similar results.


A
CPM X 1000
Wildtype
Tailless
Vector
Media
IL-2
CTLA4Rg
B
CTLL-2 Proliferation
CPM X 1000
Vector
Wildtype
Tailless
Dilution
C
Cell Number
i
ii
iii
iv
Control
CD25
CD69
Fluorescence Intensity

CHO, but not tailless CD80 or vector CHO, secreted IL-2 and expressed high levels of CD25.

Supernatants from T cell cultures stimulated with anti-CD3 and wildtype CD80 CHO, tailless CD80 CHO, or vector CHO were screened for relative IL-2 levels using the CTLL-2 assay (Fig. 2.3B). Only the supernatants from the T cells stimulated with anti-CD3 and wildtype CD80 CHO induced CTLL-2 cell proliferation. In addition, only T cells cultured with wildtype CD80 CHO and not tailless CD80 CHO expressed high levels of CD25 (Fig. 2.3C). Soluble anti-CD3 alone induced low levels of CD25 on some cells which was not altered by culture with tailless CD80 CHO or vector transfected CHO cells. This explains why exogenous IL-2 is only able to slightly enhance T cell proliferation in cultures without wildtype CD80-mediated costimulation. Similarly, CD3 mAb alone induced the expression of the activation marker CD69 on some cells, and CD69 was induced on all T cells with wildtype but not tailless CD80 CHO (Fig. 2.3C). Thus, although a primary stimulus through CD3 was sufficient to induce CD69 and CD25 expression on a small portion of the cells, signaling through CD28 was necessary to induce T cell secretion of IL-2, expression of CD25 and CD69 on the majority of the cells, and proliferation in this system. Furthermore, the signal through CD28 required an intact CD80 cytoplasmic tail.

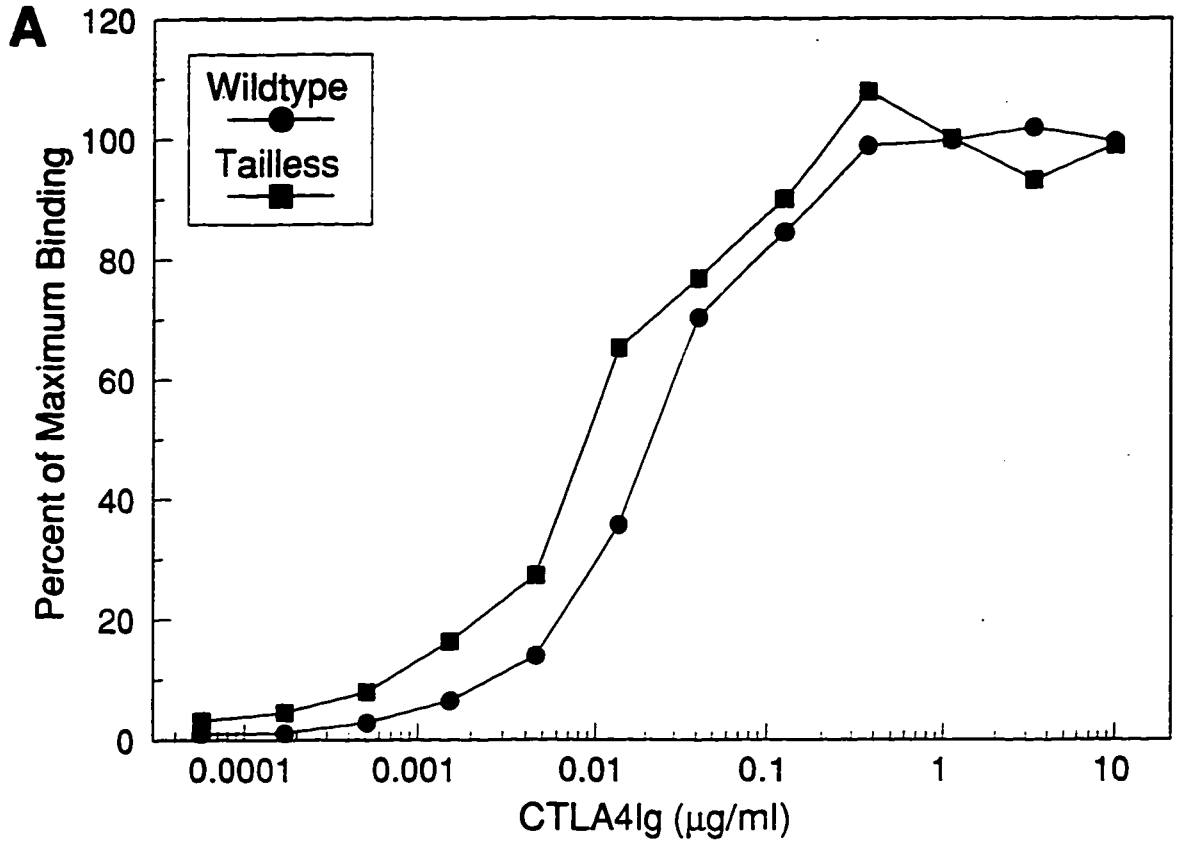
Wildtype and tailless CD80-expressing cells bind ligand equivalently.

In order to determine if tailless CD80 still efficiently bound its ligands, CTLA-4 and CD28, we performed binding assays with both CTLA4Ig and CD28Ig (Fig. 2.4). Figure 2.4A demonstrates that CTLA4Ig bound maximally to both wildtype CD80 Reh and tailless CD80 Reh at 370 ng/ml. CTLA4Ig binding to tailless CD80 and wildtype CD80 was 50% of maximum at 8.8 and 21 ng/ml respectively-about a three-fold difference. About three-fold less tailless CD80 was expressed on Reh cells than wildtype CD80 (Fig. 2.2C), which is consistent with these binding studies. Similar results were obtained with CHO transfectants (data not shown). CD28Ig bound equally well to both CD80 transfectants with nearly identical avidity (Fig. 2.4B), but in this case a concentration of CD28Ig capable of saturating either wildtype CD80 Reh or tailless CD80 Reh was not achieved. CD28Ig binding to both wildtype and tailless CD80 Reh was detected at 1 μg/ml with a FI of 1.25 fold over background and increased to 1.65 and 1.5, respectively at 30 μg/ml.

Cytoplasmic tails of other adhesion molecules can substitute for the CD80 cytoplasmic tail.

In order to test whether there is a specific requirement for the cytoplasmic tail of CD80 to costimulate T cell proliferation, we constructed chimeric CD80 molecules containing the extracellular region of CD80 and the transmembrane and cytoplasmic tails of either CD54 (CD80/54), CD2 (CD80/2), or CD86 (CD80/86) (see Table II); these were then expressed in CHO cells at

Figure 2.4. Wildtype and tailless CD80 bind to ligand equivalently.
Wildtype (●) and tailless (■) transfected Reh cells were washed and incubated with decreasing amounts of CTLA4Ig (A) or CD28Ig (B) as indicated for 40 min at 4°C. The fusion proteins were detected with an anti-human IgG-PE and analyzed by flow cytometry. Maximum binding of CTLA4Ig was calculated by averaging the FI of the four highest doses of CTLA4Ig.


A
Wildtype
Tailless
Percent of Maximum Binding
120
100
80
60
40
20
0
0.0001
0.001
0.01
0.1
1
10
CTLA4Ig (µg/ml)

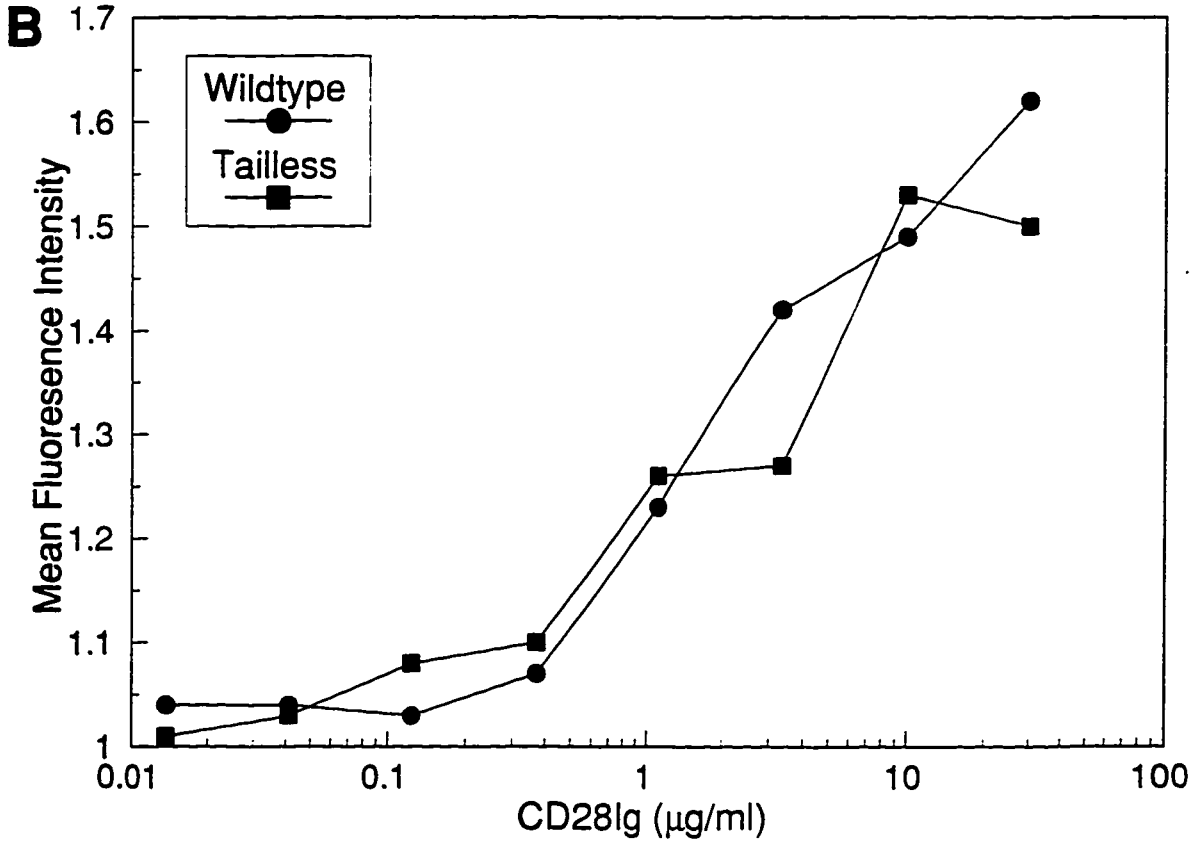

B
Wildtype
Tailless
Mean Fluoresence Intensity
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1
0.01
0.1
1
10
100
CD28Ig (µg/ml)

Table II. Mutant and chimeric CD80 protein sequences.

Construct	AA sequence[a]
CD80	TTKQEHFPDNLLPSWAITLISVNGIFVICCLTYCFAPRCRERRRNERLRRESVRPV
CD80 tailless	TTKQEHFPDNLLPSWAITLISVNGIFVICCLTYCFAPRCR
CD80/54	TTKQEHFPD*GS*EIVIITVVAAAVIMGTAGLSTYLYNRQRKIKKYRLQQAQKGTP...
CD80/86	TTKQEHFPD*GSH*IPWITAVLPTVIICVMVFCLILWKWKKKKRPRNSYKCGTNTM...
CD80/2	TTKQEHFPD*GS*DIYLIIGICGGGSLLMVFVALLVFYITKRKKQRSRRNDEELET...

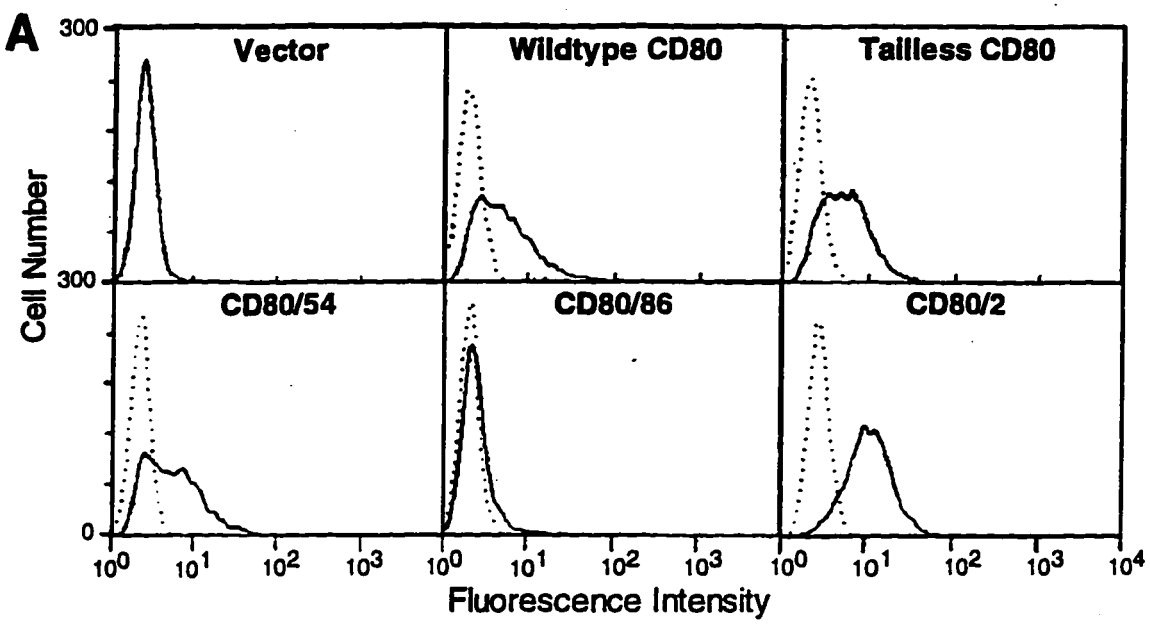
[a] The first threonine is position 233 according to Freeman *et al.* (1989). The putative transmembrane domains are underlined (according to Freeman *et al.*, 1989; Simmons *et al.*, 1988; Azuma *et al.*, 1993a; Seed and Aruffo, 1987), residues introduced during construction are italicized.

equivalent levels (Fig. 2.5A). All three chimeric CD80 receptors costimulated T cell proliferation as well as wildtype CD80 expressed at equivalent levels on CHO cells (Fig. 2.5B). While CD54 interacts with the actin-based cytoskeleton, CD2 interacts with tubulin-based cytoskeleton (Carpén *et al.*, 1992; Offringa and Bierer, 1993); thus, these results suggest that there is not a requirement that CD80 interact specifically with the actin-based cytoskeleton to function. There were some minor differences among the chimeric receptors in their ability to costimulate. Most notable was the consistent observation that the CD80/86 chimera was about two-fold more potent in driving T cell proliferation by day 4 than wildtype CD80. This suggests that despite the ability of all three chimeric receptors to costimulate, the specific transmembrane and cytoplasmic tail regions may provide different quantitative signals.

The cytoplasmic tail of CD80 affects its surface distribution.

Ehlin-Henriksson *et al.* (1983) demonstrated that CD80 can be induced to cap on B cell lines, so we tested whether or not tailless CD80 could cap on CHO cells. Immunofluorescence microscopy of CD80 CHO revealed that neither wildtype nor tailless CD80 could be induced to cap on CHO in a time dependent manner (data not shown). However, wildtype and tailless CD80 had quite different patterns of subcellular localization (Table III). Wildtype CD80 was primarily located in a few large patches while tailless CD80 was located in small foci, randomly or clustered, on the cell surface. Fixing the cells before or after staining, or after incubation at 37°C did not significantly alter the distribution

Figure 2.5. The transmembrane and cytoplasmic tails of CD2, CD54, and CD86 can replace the cytoplasmic tail of CD80.
(A) Wildtype, tailless, and chimeric CD80 CHO cells were collected and stained for expression of CD80 as in Figure 2.2. (B) At the same time cells were treated with MMC then plated 5 x 10^4 cells per well in 96 well plates. After the CHO transfectants formed a monolayer, anti-CD3 was added to 1 μg/ml, and 5 x 10^4 $CD4^+$ T cells were added. The plates were incubated for 3, 4, or 5 days and pulsed with 0.5 μCi of [^{3}-H]thymidine for the final 18 hrs before being harvested and counted. Two clones from each transfection were tested with similar results. Similar stimulation was also obtained when various transfectants were plated at lower densities.


A
Vector
Wildtype CD80
Tailless CD80
CD80/54
CD80/86
CD80/2
Cell Number
300
300
0
10^0 10^1 10^2 10^3 10^0 10^1 10^2 10^3 10^0 10^1 10^2 10^3 10^4
Fluorescence Intensity

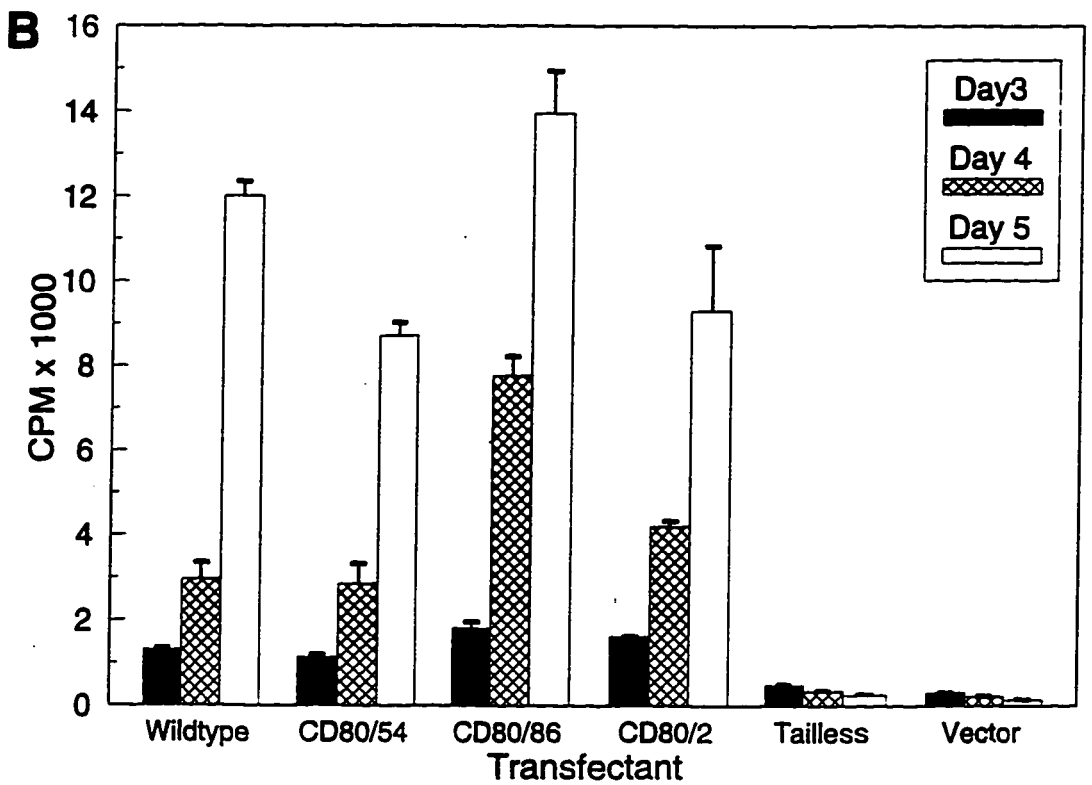

B
CPM x 1000
0
2
4
6
8
10
12
14
16
Day3
Day 4
Day 5
Wildtype
CD80/54
CD80/86
CD80/2
Tailless
Vector
Transfectant

Table III. Subcellular distribution of CD80 in CHO cells.

Distribution[a]	Expt. 1		Expt. 2	
	wildtype CD80	tailless CD80	wildtype CD80	tailless CD80
diffuse	6.2[b]	6.9	9.2	6.8
clustered	18.7	89.6	5.7	93.1
patched	53.0	0	69.0	0
capped	22.9	3.4	16.1	0

[a] Cells were stained for fluorescence microscopy on ice as described in materials and methods, fixed then mounted on slides for analysis. Diffuse: foci of CD80 are randomly and evenly distributed over the entire cell surface; clustered: discrete foci of CD80 not evenly distributed over the cell surface and not yet as large as patches; patched: CD80 localized in fewer than 10 distinct spots on the cell surface; capped: CD80 located in fewer than 3 large spots typically located in one region of the cell.
[b] Percent of cells; at least 100 cells were analyzed in each group.

of CD80 (wildtype or tailless) on CHO cells, suggesting the subcellular localization of CD80 on CHO cells is very stable, and it is dependent upon the CD80 cytoplasmic tail. Unlike wildtype CD80 on CHO cells, Symington *et al.* (1993) found that CD80/86 on Langerhans cells is localized in small foci, not patches, suggesting that CD80 may be regulated differently in adherent epithelial cells than in hematopoietic cells.

Since CD80 is not constitutively patched on lymphocytes (Ehlin-Henriksson *et al.*, 1983), we wanted to test whether tailless or wildtype CD80 differed in their ability to cap on lymphocytes (Fig. 2.6 and 2.7). Figure 2.6 illustrates the four major patterns of distribution of CD80 on Reh lymphoid cells after crosslinking with mAb. CD80 is initially expressed in small foci, randomly distributed over the entire cell surface (Fig. 2.6A), in the same manner as described by Symington *et al.* (1993) for Langerhans cells. After crosslinking with mAb, CD80 is rapidly redistributed to clusters which are unevenly distributed on the cell surface (Fig. 2.6B), and subsequently CD80 patches and caps (Fig. 2.6C and D, respectively). Caps are subsequently shed into the media upon continued incubation at 37°C (data not shown). Wildtype CD80, but not tailless CD80, underwent significant redistribution after ligation with mAb (Fig. 2.7). While the redistribution of CD80 from diffuse spots to clusters occurred very rapidly, it was not dependent upon the presence of an intact actin-cytoskeleton since it occurs in the presence of CCB (Fig. 2.7A and C). The formation of patches and caps of CD80 occurred more slowly, and did require an

Figure 2.6. Wildtype CD80 undergoes antibody induced capping.
Reh cells transfected with wildtype CD80 were stained for fluorescent microscopy and incubated at 37°C for 0, 10, 20, or 30 minutes (A-D, respectively) before being fixed and mounted on slides. The inset in panel D is a transmitted light micrograph of the cell displayed in the rest of the panel. The images were collected on a BioRad MRC 600 confocal microscope with a final magnification of 1200x. (A-D) represent diffuse, clustered, patched, and capped staining patterns respectively.

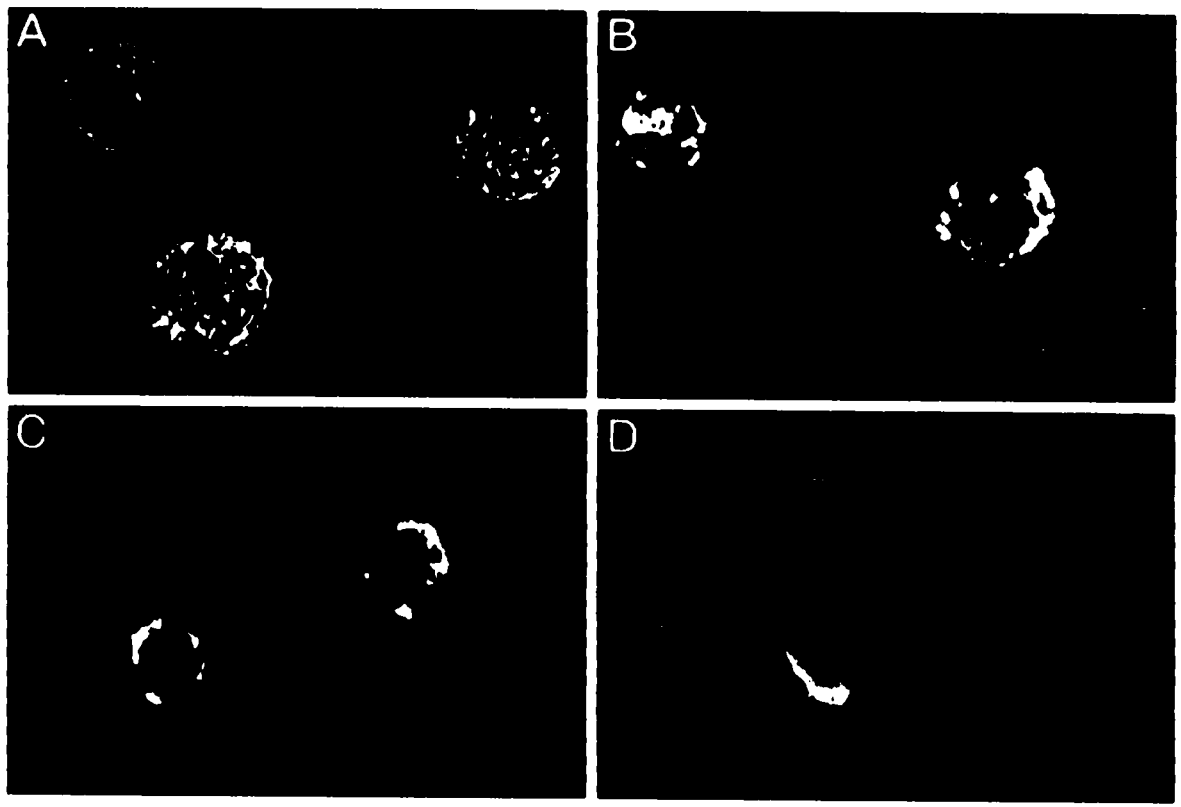
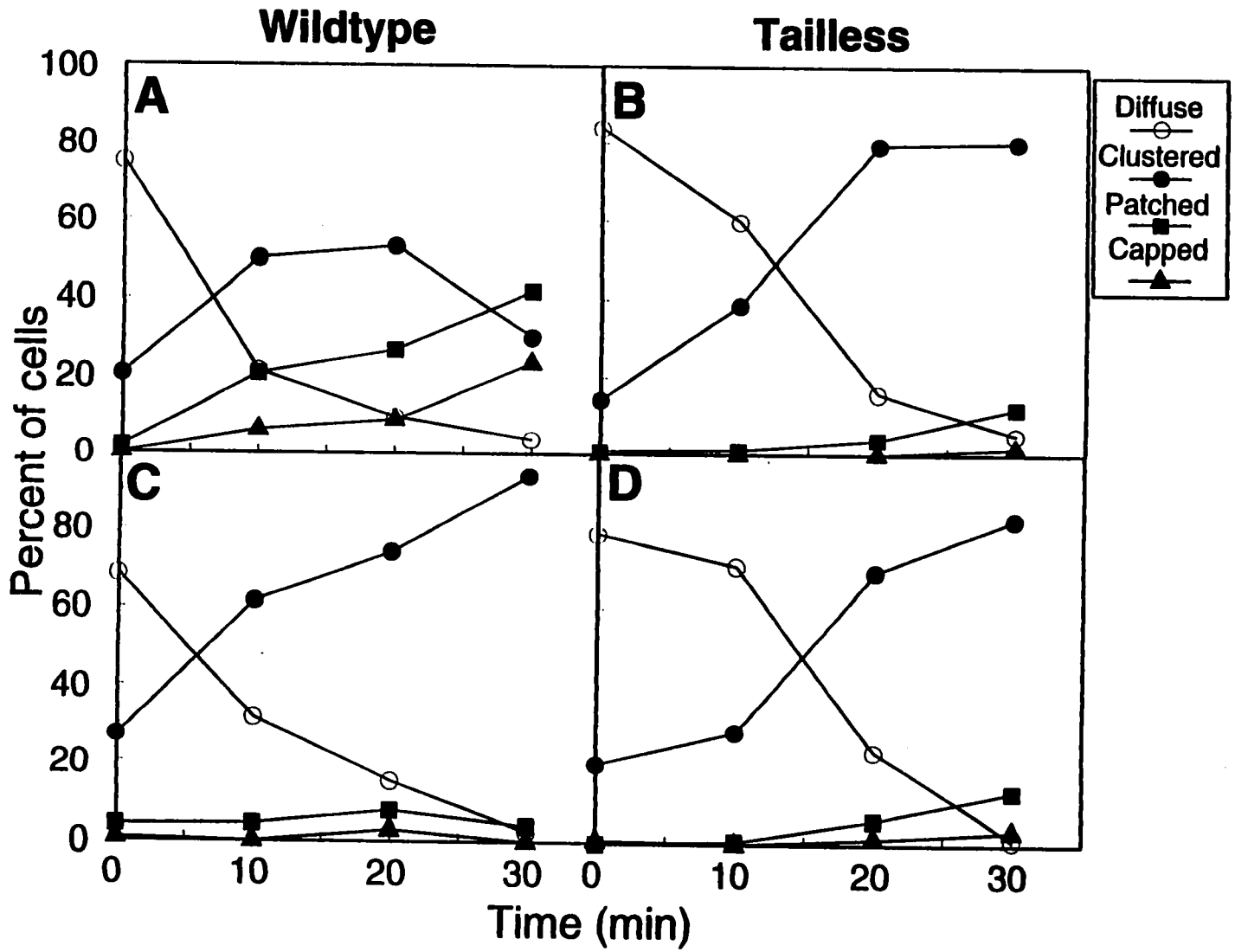

A
B
C
D

Figure 2.7. CD80 patching and capping requires the cytoplasmic tail and is CCB sensitive. Wildtype CD80 Reh (A and C) or tailless CD80 Reh (B and D) were stained for fluorescent microscopy of CD80 as described in the methods then incubated alone (A and B) or with 10 μg/ml CCB (C and D) for the time indicated. The samples were then fixed and mounted on slides for analysis. At least 100 cells were analyzed for each sample and were scored as diffuse (○), clustered (●), patched (■), or capped (▲) as described in Figure 2.6. Capping data without CCB is representative of 4 experiments, while that with CCB is representative of two experiments.


Wildtype
Tailless
A
B
C
D
Diffuse
Clustered
Patched
Capped
Percent of cells
100
80
60
40
20
0
0
10
20
30
Time (min)

intact actin-cytoskeleton, *i.e*, it was inhibited by CCB (Fig. 2.7A and C). The, patching and capping of CD80 not only required an intact actin-cytoskeleton but also required the presence of the cytoplasmic tail of CD80: no significant patching or capping was detected in tailless CD80 Reh transfectants (Fig. 2.7B). As expected, treating tailless CD80 Reh with CCB had no effect on CD80 redistribution (Fig. 2.7B and D). The percent of cells with wildtype CD80 patched or capped did not increase beyond 30 min. This was most likely because caps of CD80 are shed, leaving behind cells stripped of CD80 (R.T.Doty, unpublished observation), and thus not counted.

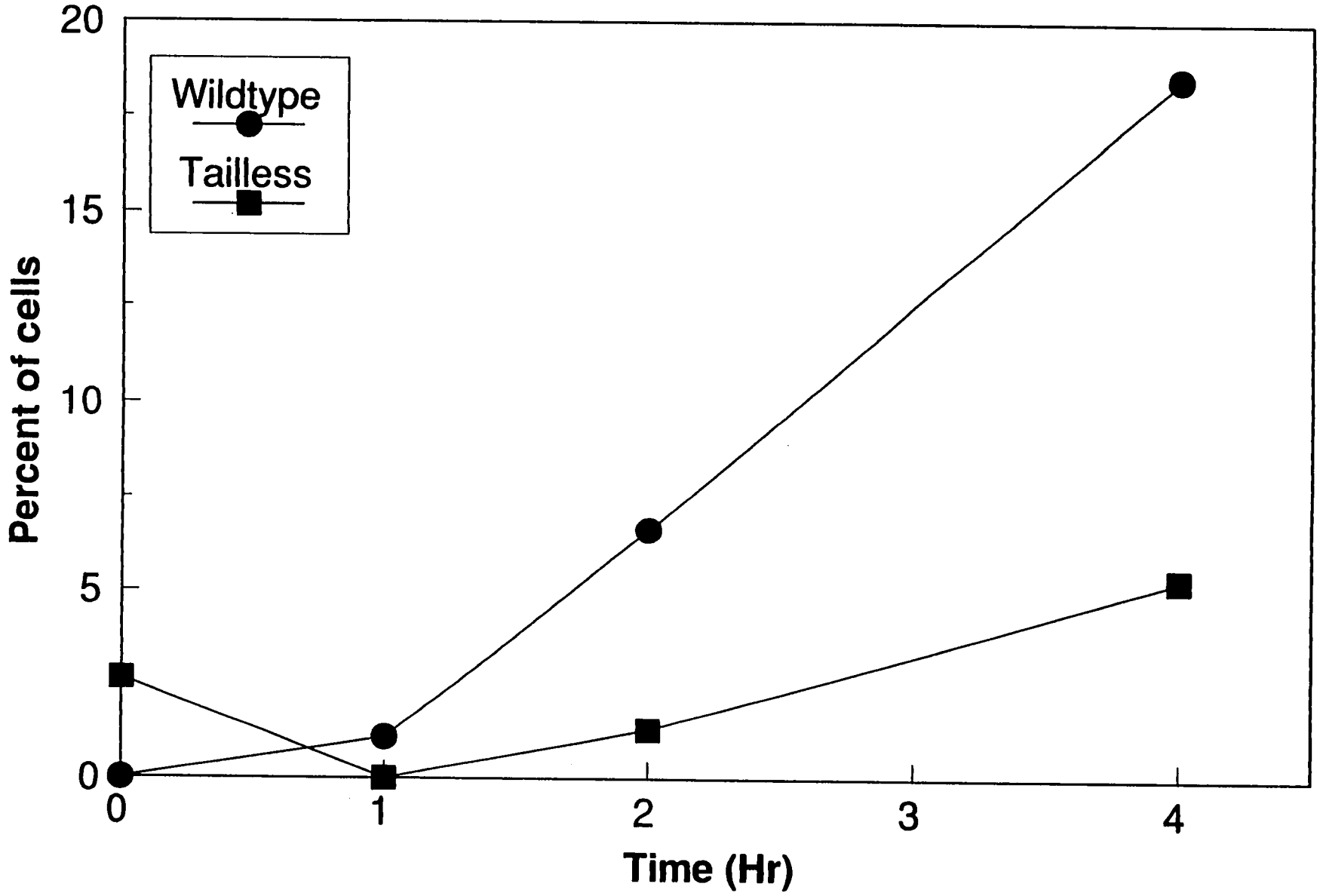
T cell contact induces CD80 redistribution.

Kupfer and Singer (1989) demonstrated that T cell contact induced CD11a/18 redistribution in APC to sites of contact. In order to determine if similar redistribution occurs with CD80, we set up similar experiments (Fig. 2.8). Nearly 20% of the wildtype CD80 Reh cells had CD80 in patches or caps after a 4 hr coculture with T cells. This was about four times the number of cells with patches or caps of CD80 found with tailless CD80 Reh cells treated the same way. These results suggest that CD80 is redistributed during T cell-APC contact, and that the cytoplasmic tail of CD80 plays a role in facilitating T cell contact-mediated redistribution of CD80.

Discussion

CD28 is a major costimulatory molecule for T cell activation and proliferation (Linsley and Ledbetter, 1993). The molecular basis for CD28 and

Figure 2.8. T cell induced CD80 patching and capping.
10^6 wildtype CD80 Reh or tailless CD80 Reh cells were mixed with 3 x 10^6 $CD4^+$ T cells and 1 μg/ml soluble anti-CD3 in 0.5 ml media in a 48-well plate for the times indicated. The cells were collected and stained for microscopy. The cells were mounted on slides and analyzed for surface distribution of CD80 as described in Figure 2.6. The percent of wildtype CD80 Reh (●) or tailless CD80 Reh (■) cells with patches or caps of CD80 are presented as a percent of total cells. Representative of 3 experiments with similar results.


Wildtype
Tailless
Percent of cells
20
15
10
5
0
0
1
2
3
4
Time (Hr)

CTLA-4 interactions with their ligands CD80 and CD86 are beginning to be understood (Peach *et al.*, 1994; Peach *et al.*, 1995; Fargeas *et al.*, 1995). Here we have found that a mutation deleting most of the cytoplasmic tail of CD80 and which does not affect binding of CD80 to CTLA-4 or CD28, abolishes the ability of CD80 to costimulate T cells through CD28. The loss of function of tailless CD80 could not be attributed to a loss of binding or an alteration of ligand binding properties as DeLisser *et al.* (1994) found with a CD31 tailless mutant: wildtype and tailless CD80 CHO were bound equally well by CTLA4Ig and CD28Ig (Fig. 2.4).

The ability of CD28 to costimulate depends upon receptor crosslinking: Damle *et al.* (1988) found that Fab to CD28 inhibit CD28-dependent T cell proliferation mediated by intact APC. Linsley *et al.* (1991a) found that monomeric CD80 fusion protein could drive the proliferation of T cells only if it was immobilized on plastic. Furthermore, Ledbetter *et al.* (1990) demonstrated that while CD28 Fab could not costimulate T cells, intact bivalent CD28 mAb could provide some costimulation which was further increased upon aggregation. As CD80 and CD86 are found as monomers on the surface of APC (Freeman *et al.*, 1989; Linsley *et al.*, 1991a; Azuma *et al.*, 1993a; Hathcock *et al.*, 1993), they are not directly able to crosslink CD28. This suggested that CD80 itself needs to be aggregated on the APC to costimulate T cells. Tailless CD80 is present in foci on Reh cells just like wildtype CD80, yet it does not costimulate

T cell proliferation. This suggests that the foci of CD80 described by Symington *et al.* (1993), and identified in this study, are insufficient to trigger CD28.

Norton *et al.* (1992) demonstrated that CD80 was sufficient to enable the $CD80^-$ B cell line NALM-6 to induce allogeneic T cell proliferation. In contrast, CD80 Reh clearly was not able to induce an allogeneic MLR even though it expresses MHC Class II. Reh, unlike NALM-6, expresses very low levels of the adhesion molecules CD18 and CD54 (FI of 1.5 and 2.6 respectively, R.T.Doty, unpublished observations) which are critical for T cell-APC adhesion and costimulate T cell proliferation (Simmons *et al.*, 1988; Van Seventer *et al.*, 1990). Since the cells used in our studies did not express these other critical adhesion molecules, we were able to examine which regions of CD80 expressed on intact cells were required for CD28-mediated costimulation independent of these other possible signaling pathways.

A major difference between wildtype and tailless CD80 was that they were distributed differently on the cell surface of CHO transfectants; furthermore, the tailless CD80 unlike wildtype CD80 did not cap on lymphoid cells. CHO cell transfectants even without CD80 ligation expressed wildtype CD80 in patches frequently located at sites of cell-to-cell contact (data not shown). In contrast, Reh lymphoid cells expressed wildtype CD80 in a diffuse manner, but upon crosslinking, CD80 redistributed into patches and caps on these cells in a CCB-dependent manner (Fig. 2.7). Both of these artificial APC were able to provide costimulation to T cells. CHO and Reh cells expressed

tailless CD80 in a diffuse or clustered pattern which could not be significantly changed after crosslinking CD80. When CD80 was distributed diffusely or in small clusters, unlike when it was in patches or caps, it could not costimulate T cells. We isolated a clone of CHO cells expressing greater than forty-fold higher levels of tailless CD80 than the wildtype or mutant CD80 transfectants used in this study. CD80 on these cells was distributed over the entire cell surface in a confluent layer and these CD80 bright cells were able to costimulate T cells (R.T. Doty, unpublished observations). This suggests that CD80 normally must be localized to specific sites of cell-to-cell contact for T cell costimulation, but if enough CD80 is expressed on the cell surface, it may be able to costimulate without specific subcellular localization.

The highly charged cytoplasmic tail of CD80 required for redistribution and costimulation resembles the cytoplasmic tail of CD54 (Freeman *et al.*, 1989; Carpén *et al.*, 1992). Carpén *et al.* (1992) identified a positively charged region in the cytoplasmic tail of CD54 located proximal to the membrane region which is essential for the interaction of CD54 with α-actinin. Removing the highly charged cytoplasmic tail of CD80 prevents it from redistributing and from costimulating T cell proliferation. The CD54 cytoplasmic tail can substitute for the CD80 cytoplasmic tail (Fig. 2.5), thus it is possible that the CCB sensitive component required for CD80 redistribution is part of the actin-associated cytoskeleton. Studies to identify these components are currently in progress.

Even though tailless CD80 can no longer redistribute, it is still located in discrete foci on the cell surface of both CHO and Reh cells. This suggests that there may be two regions of CD80 involved in regulating subcellular distribution: one region may map within the cytoplasmic tail and function in the redistribution of CD80 after ligation; the other region, outside of the cytoplasmic tail, perhaps within the transmembrane domain, may function to maintain CD80 in small foci. Studies are in progress to define more precisely the amino acid residues in CD80 required for clustering, capping, and costimulation.

Recently two reports described the construction and function of glycosylphosphatidylinositol-anchored (GPI-) CD80 molecules (McHugh *et al.*, 1995; Brunschwig *et al.*, 1995). McHugh *et al.* (1995) isolated detergent purified GPI-CD80 from CHO transfectants, incorporated them into cell membranes and demonstrated that they could provide a costimulatory signal. Good, but uneven, incorporation of the purified CD80 into cell membranes was achieved, suggesting that the incorporated GPI-CD80 was aggregated and thereby could crosslink CD28 to costimulate T cells. Brunchwig *et al.* (1995) 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 (Zurzolo *et al.*, 1993), suggesting that even though GPI anchored proteins lack a transmembrane and cytoplasmic tail these proteins can be targeted to

specific locations on cell membranes. Thus, these results with GPI-anchored CD80 are not necessarily incompatible with our findings.

Both CD11a/18 and associated talin co-localize to sites of T cell-B cell contact along with the TCR and CD4 after the T cell contacts the B cell APC (Kupfer and Singer, 1989). Before contact, these molecules are spread diffusely throughout the cell surface. They remain at the contact sites until the B cell divides and migrates away. Rosenman *et al.* (1993) have demonstrated that both CD11a/18 and CD44 could unidirectionally induce CD2 to cap. These data suggest that the subcellular localization of adhesion molecules is crucial for T cell-epithelial and T cell-APC interactions, and is tightly regulated, results consistent with and supporting our data that improperly localized CD80 cannot costimulate T cells. T cell contact with CD80 Reh induced caps and patches (Fig. 2.8); thus CD80 may also localize to sites of T cell-APC interaction via a process facilitated by the cytoplasmic tail of CD80.

Currently, two models can explain how CD80 is induced to redistribute and thereby costimulate T cells. One model is that CD80 ligation may transduce a signal back into the APC, which then reorganizes the surface expression of CD80. Another possibility is that an other receptor on the APC such as MHC Class II or CD40, which up-regulate the expression of CD80 (Koulova *et al.*, 1991; Ranheim and Kipps, 1993), may transduce a signal back into the cell which initiates the redistribution of CD80.

Chapter 3: Multiple sites in the CD80 cytoplasmic tail regulate CD80 redistribution and T cell costimulation.

Abstract

CD28 is a major T cell costimulatory molecule, delivering signals distinct from those of the CD3/TCR complex and which regulates cytokine and cytokine receptor expression, cell proliferation, and cell viability. To initiate signals, CD28 needs to be crosslinked by one of its ligands, yet both of its ligands, CD80 and CD86, are normally expressed as monomers. In chapter 2, we determined the cytoplasmic tail of CD80 is required for CD28-mediated costimulation and subcellular relocalization of CD80 in lymphocytes. Next, we will describe specific mutations of residues in the cytoplasmic tail of CD80 that are conserved across species, and the effect of these mutations on redistribution of CD80 and CD80-dependent T cell proliferation. Reh B cell transfectants expressing CD80 with mutations in RRNE 275-278 or S 284 can bind ligand similar to transfectants expressing wildtype CD80 molecules, but are unable to costimulate T cell proliferation. The mutant CD80 molecules are expressed on the surface of the Reh cells in foci indistinguishable from those of wildtype CD80 molecules. However, mutant CD80 molecules are unable to undergo capping and patching at the same rate as wildtype CD80. Thus the foci of CD80 found on APC are insufficient to initiate CD28-mediated signals, and the rate of CD80 redistribution may be critical for the initiation of costimulation. A 30 kDa

phosphoprotein which associates with the cytoplasmic tail of CD80 in activated cells may play a role in CD80 redistribution and CD28-mediated costimulation.

Introduction

CD80 is a monomeric surface protein and a major T cell costimulatory ligand found on activated APC (Yokochi *et al.*, 1982; Freeman *et al.*, 1989; Linsley *et al.*, 1990; Freedman *et al.*, 1991; Young *et al.*, 1992). The extracellular region of CD80 consists of two highly glycosylated Ig-like extracellular domains (Freeman *et al.*, 1989), which have defined regions for binding its ligands, CD28 or CTLA-4, on T cells (Peach *et al.*, 1995; Fargeas *et al.*, 1995). CD28 provides critical costimulatory signals to activated T cells increasing cytokine production and cell proliferation (reviewed in Linsley and Ledbetter, 1993), while CTLA-4 may mediate growth inhibitory signals (Tivol *et al.*, 1995; Walunas *et al.*, 1994). Early studies investigating the effect of CTLA-4 ligation suggested a role similar to CD28 signaling, but recent studies have suggested that CTLA-4 may play a role in counteracting CD28 effects (Linsley *et al.*, 1992a; Krummel and Allison, 1995; Waterhouse *et al.*, 1995).

The same regions of CD80 are involved in binding to both CD28 and CTLA-4, but CD80 binds to CTLA-4 with about a 20-fold higher avidity (Peach *et al.*, 1995; Linsley *et al.*, 1991b; van der Merwe *et al.*, 1997). This suggests that low levels of CTLA-4 expressed on the cell surface can compete for CD80 binding even when CD28 is expressed at much higher levels. Recently, Greene

et al. (1996) demonstrated that two molecules of CD80 bind to a single dimer of CD28 and CTLA-4, and that the high avidity binding of CD80 to CD28 and CTLA-4 was most likely representative of two CD80 monomers binding to CD28 or CTLA-4 (Linsley *et al.*, 1991a; Linsley *et al.*, 1991b).

Monoclonal Fab to CD28 do not signal, and in fact, inhibit CD28-mediated proliferation of T cells (Damle *et al.*, 1988). Furthermore, Linsley *et al.* (1991a) demonstrated that monomeric B7Ig (CD80Ig) needs to be immobilized to costimulate T cell proliferation. Thus, two CD80 monomers can bind to a single CD28 or CTLA-4 dimer, but this binding is insufficient to deliver a signal as multiple CD28 molecules must be aggregated or crosslinked to induce T cell proliferation. Symington *et al.* (1993), found CD80 existed in foci on the cell surface of Langerhans cells, and postulated that these foci of CD80 were sufficient to aggregate CD28 and induce costimulation. We found similar foci of CD80 on CD80-transfected B cells and on a $CD80^+$ B lymphoblastoid cell line. However, although mutant CD80 molecules lacking most of the cytoplasmic tail could still bind CD28 and CTLA-4 and form foci, these molecules could not costimulate T cell proliferation and cytokine production (Doty and Clark, 1996). This suggested the foci of CD80 found on APC are insufficient to aggregate CD28 and induce CD28-mediated signals.

We also found that these tailless CD80 molecules cannot undergo antibody-induced cytoskeleton-dependent redistribution and capping. In this study we examined the role of conserved residues in the cytoplasmic tail of CD80

in antibody-mediated redistribution of CD80 and CD80-induced T cell costimulation. Furthermore, we identified a 30 kDa phosphoprotein that associates with the cytoplasmic tail of CD80 after cell activation, which may play a role in CD80 function.

Materials and Methods

Antibodies and cell lines.

The CD80 mAb, BB1 (Yokochi *et al.*, 1982), the CD16 mAb, FC1 (Ledbetter *et al.*, 1985), the CD3 mAb, 64.1 (Hansen *et al.*, 1984), and the MHC Class I mAb, W6/32 (Barnstable *et al.*, 1978) were produced in our lab and purified before use. CTLA4Ig fusion protein (Linsley *et al.*, 1991b) for immunoprecipitations 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 was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Strepavidin-Texas Red was from Molecular Probes, Inc. (Eugene, OR). Reh cells were from ATCC and cultured in RPMI-10. Wildtype CD80 expressing Reh cells (R80), tailless CD80 expressing Reh cells (R80Δ16), and vector transfected Reh cells (RRc) were described before (Doty and Clark, 1996), 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 for use in costimulation assays.

T cells and proliferation assays.

T cells were isolated from normal healthy donors by rosetting with AET treated SRBC, followed by hypotonic shock to lyse the SRBC. This Er^+ population routinely consisted of about 85% $CD3^+$ T cells, 15% $CD16^+$ NK cells, and less than 1% $CD14^+$ monocytes or $CD20^+$ B cells. $CD4^+$ T cells were isolated by depletion of $CD8^+$ and $CD16^+$ cells on antibody-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 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 100,000/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 [^{3}H]thymidine (NEN, Wilmington, DE) for the final 16 hr of a 5 day culture before being harvested on glass fiber filter strips and the incorporated ^{3}H counted in a scintillation counter.

Site directed mutagenesis and transfections.

The mutant CD80 cDNA constructs were made from the Rc/CD80 plasmid using the U.S.E. mutagenesis kit (Pharmacia, Piscataway, NJ) as before (Doty and Clark, 1996). The primers used in the site directed mutagenesis reactions are listed in Table IV. The Rc-ClaI primer was used as the selection primer for all of the mutagenesis reactions. The CD80Δ11, CD80/4A, and CD80/SA primers were used as templates to introduce the desired mutations into the

Table IV. Primers used in chapter 3.

Primer	Primer Sequence[a]
CD80Δ11	GAAGGAGGAATTGATAGTTGAGAAGGG
CD80/4A	GATGCAGAGAGAGAGCGGCCGCAGCAAGATTGAGAAGGG
CD80/SA	GATTGAGAAGGGAAGCTGTACGCCCTGTA
Rc-ClaI	AGGCGTTTTGCGCTGATCGATGATGTACGGGCCAGA

[a] All primers are written 5' to 3', changes from the native sequences are underlined.

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 V. Reh transfectants were made as before (Doty and Clark, 1996) using Lipofectin reagent (Life Technologies). Briefly, 2 x 10^6 Reh cells were incubated in 3 ml of Opti-mem (Life Technologies) in a 10-cm tissue culture plate. Five micrograms of linear plasmid diluted in Opti-mem was mixed with 20 µl of Lipofectin diluted in Opti-mem and added drop wise to the Reh cells. Eighteen hr 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 then isolated. Clones were screened by flow cytometry to identify 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% NaN_3). 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 and 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 the anti-human IgG-PE conjugate incubated as above. The relative fluorescence intensity (FI) was calculated as before (Doty and Clark, 1996) by

Table V. Mutant CD80 protein sequences.

Construct	AA Sequence[a]
CD80	TTKQEHFPDNLLPSWAITLISVNGIFVICCLTYCFAPRCRERRRNERLRRESVRPV
CD80Δ16	TTKQEHFPDNLLPSWAITLISVNGIFVICCLTYCFAPRCR
CD80Δ11	TTKQEHFPDNLLPSWAITLISVNGIFVICCLTYCFAPRCRERRRN
CD80/4A	TTKQEHFPDNLLPSWAITLISVNGIFVICCLTYCFAPRCRER**AAAA**RLRRESVRPV
CD80/SA	TTKQEHFPDNLLPSWAITLISVNGIFVICCLTYCFAPRCRERRRNERLRRE**A**VRPV

[a] The first threonine is position 233 and the putative transmembrane domain is underlined according to Freeman *et al.* (1989). Residues introduced during construction are in bold.

dividing the mean fluorescence intensity of the sample by that of the control sample; thus, a FI of 1 is background. Percent 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 (Doty and Clark, 1996). Briefly; the cells were incubated in azide free staining media with the primary mAb for 30 min, washed, incubated with the goat anti-mouse IgM-biotin for 30 min, washed, incubated with strepavidin-Texas Red, washed and transferred to 37°C for the times indicated. The capping reaction was stopped by diluting the sample four-fold into ice cold PBS with 0.1% NaN_3 and washing the cells again. The cells were immediately fixed in 4% sucrose, 4% paraformaldehyde in PBS then mounted on slides for analysis. At least 100 cells were scored for CD80 distribution at each sample time point. The scoring was performed as before (Doty and Clark, 1996). 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; capped when CD80 was distributed in 3 or fewer spots on the cell surface. Representative fields were collected on a Bio-Rad 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 mm 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 MgCl, 2 mM L-glutamine, 1.8 mM glucose, 1 mM $CaCl_2$, 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 hr. The cells were washed again and resuspended in POP medium with 1 mCi $[^{32}P]H_3PO_4$, and incubated at 37°C for 4 hr. The cells were stimulated with PMA (10 ng/ml) and ionomycin (500 ng/ml) for the final 10 minutes of culture. The cells were collected and washed once in PBS before being lysed in NP-40 lysis buffer (0.5% NP-40, 50 mM Tris pH 8, 150 mM NaCl, 5 mM EDTA) containing protease and phosphatase inhibitors. The post-nuclear fractions were pre-cleared 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 washed 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 antibody-mediated or T cell contact-induced CD80 redistribution (Doty and Clark, 1996). An intact cytoskeleton is required for mAb-mediated CD80

redistribution as cytochalasin B prevented CD80 redistribution into patches and caps. This suggests that the cytoplasmic tail of CD80 interacts either directly or indirectly with the cytoskeleton. 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 here, CD80Δ16 and CD80Δ11 are missing the carboxyl-terminal 16 and 11 amino acids of CD80, respectively (Table V). The cytoplasmic tail of human CD80 contains the tetra peptide motif RRNE at positions 275-278. This sequence is conserved in the cytoplasmic tails of CD80 in all species in which CD80 has been cloned to date: rabbit, rat, mouse, and human (Isono and Seto, 1995; Judge *et al.*, 1995; Freeman *et al.*, 1991; Freeman *et al.*, 1989). Because of this high level of conservation, this region is a good candidate site to function in CD80 costimulation and capping. To test whether this sequence is required for T cell costimulation or CD80 capping, we created a mutant, CD80/4A, which has these highly conserved residues, RRNE, replaced with 4 alanines (Table V) and expressed it in Reh cells (R80/4A). Another short region in the cytoplasmic tail of CD80 which is conserved in mouse, rat, and human: residues 282-284, RE(S/T), and in rabbit is RLS. While there is no evidence yet demonstrating the cytoplasmic tail of CD80 can be phosphorylated at highly conserved S284, the residues surrounding S284 fit the consensus sequences for protein kinase C and cyclic nucleotide dependent kinases (Feramisco *et al.*, 1980; Glass and Smith, 1983; Kishimoto *et al.*, 1985; Glass *et*

al., 1986; Woodgett *et al.*, 1986). The conservation of this region suggests that S284 may be important for CD80 function; to test this possibility we produced a mutant CD80 molecule, CD80/SA, (Table V) with S284 replaced with alanine and expressed it on Reh cells (R80/SA).

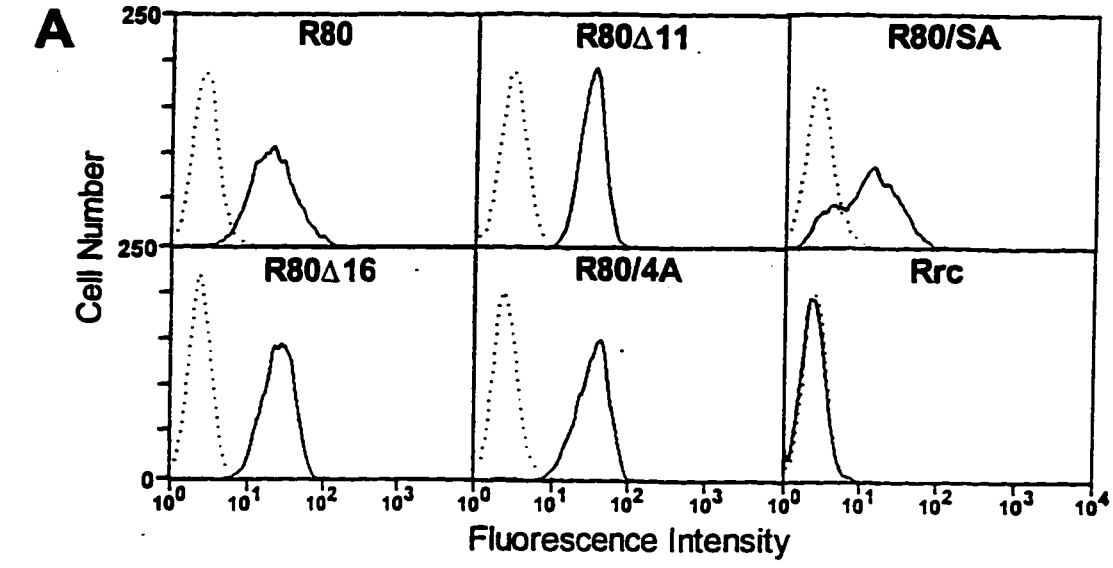
Mutant CD80 molecules still bind ligand.

We transfected Reh cells with mutant CD80 constructs and isolated clones expressing similar surface levels of the mutant CD80 molecules. The wildtype 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 3.1, all of the transfectants used in these studies express similar levels of CD80, except for R80/SA, which expresses slightly less CD80 on its surface than the other transfectants (Fig. 3.1A). These mutant CD80 molecules were still able to bind ligand in a manner similar to wildtype CD80: as shown in Figure 3.1B, all 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 around 200 ng/ml.

The entire CD80 cytoplasmic tail is required for costimulation.

We next tested the ability of these transfectants expressing mutant or wildtype CD80 to costimulate T cell proliferation (Fig. 3.2). Only the R80 cells expressing wildtype CD80 could costimulate T cell proliferation. Maximal T cell proliferation was induced with 10 ng/ml of soluble anti-CD3 and 100,000 Reh

Figure 3.1. Analysis of CD80 transfectants.
Reh transfectants were collected and stained with (A) either control Ig (· · · · · ·) or anti-CD80 (———), or (B) either CTLA4Ig or control IgG followed by goat anti-human IgG-PE conjugate. The samples were analyzed by flow cytometry and are presented as FACS profiles (A) or as percent maximal binding versus CTLA4Ig dose (B) as described in the materials and methods.


A
R80
R80Δ11
R80/SA
R80Δ16
R80/4A
Rrc
Cell Number
250
250
0
Fluorescence Intensity

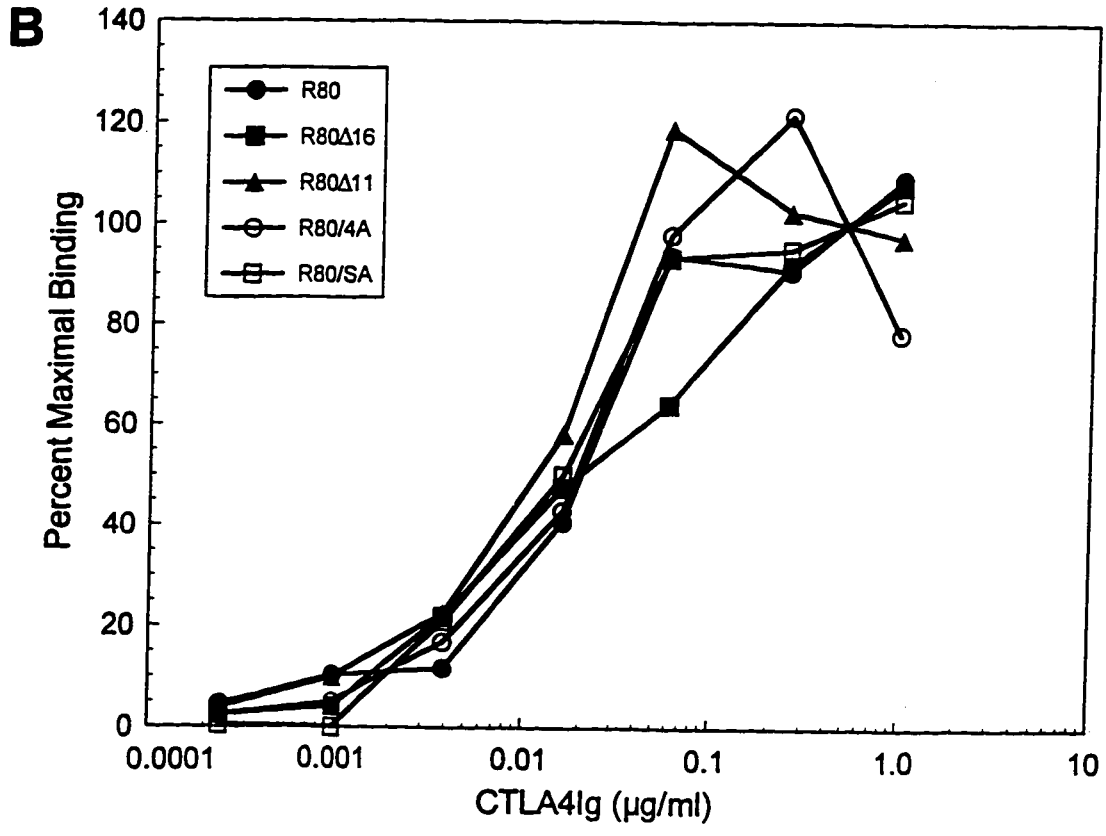
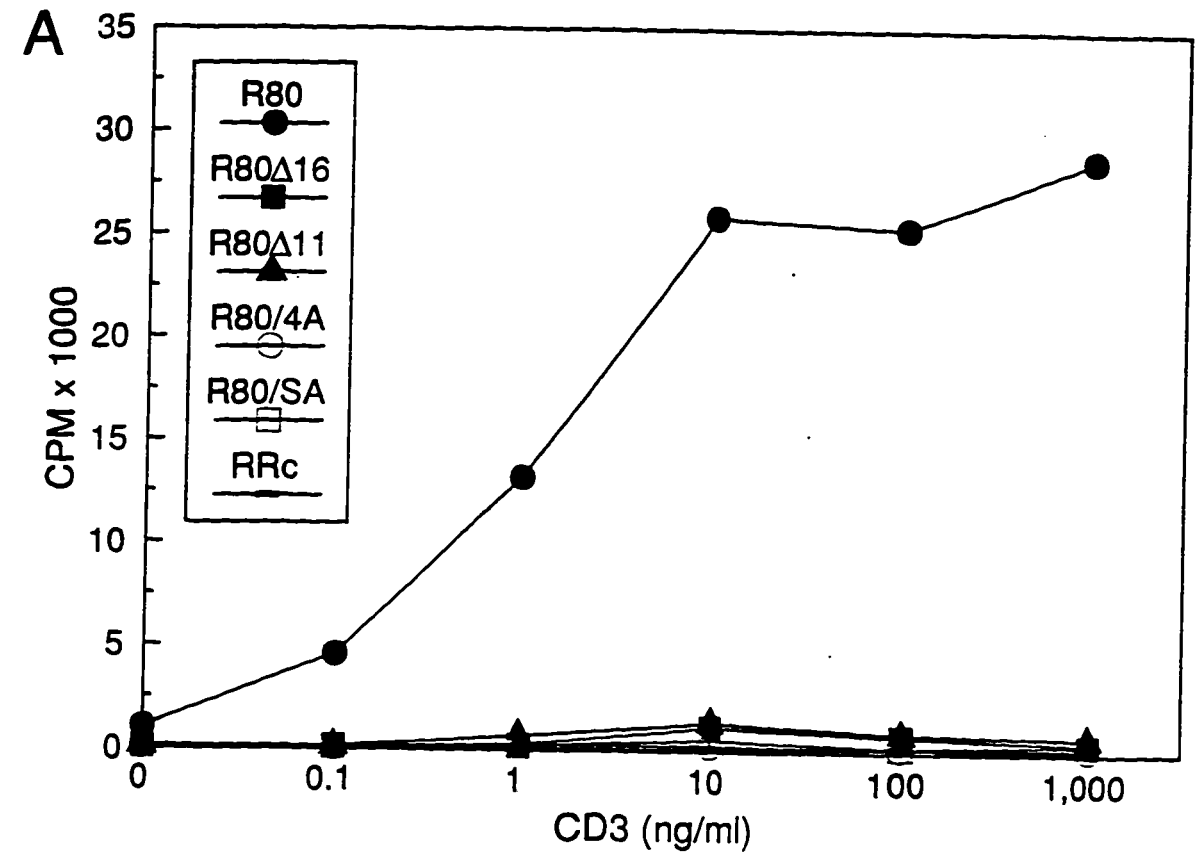
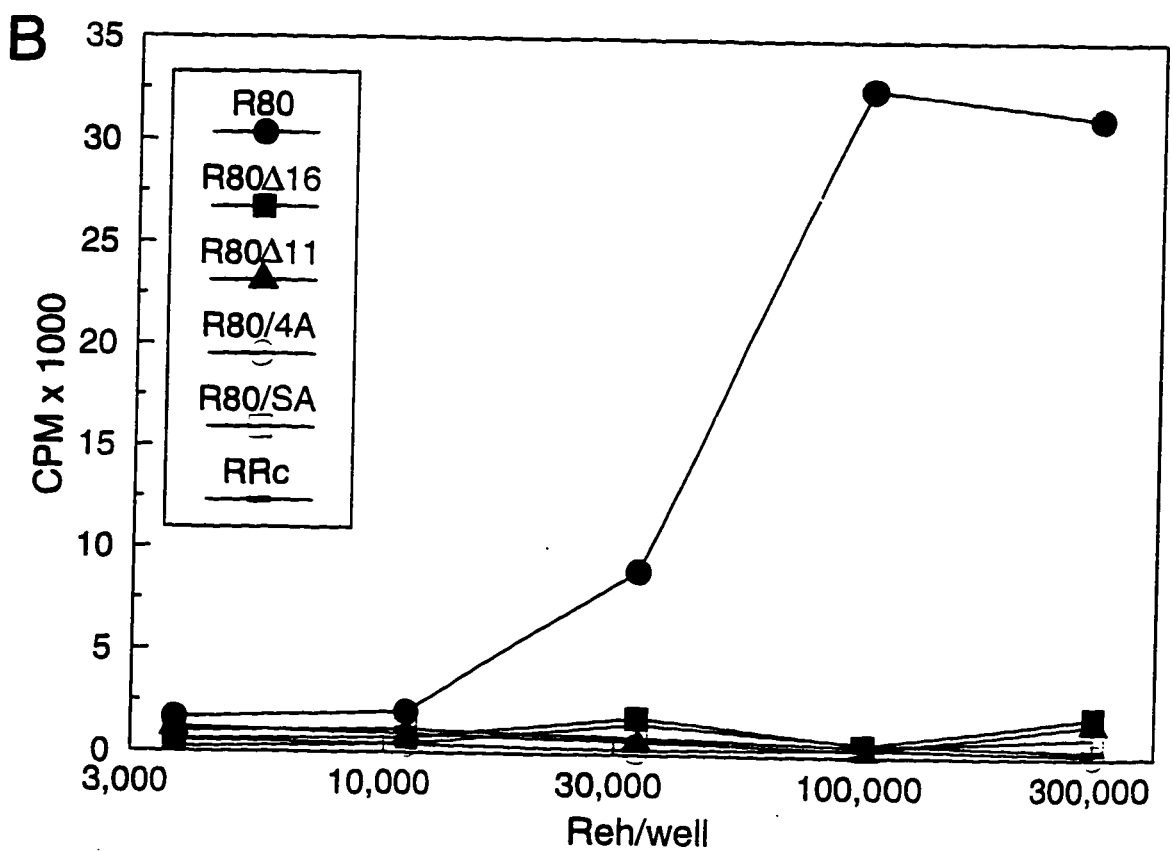

B
R80
R80Δ16
R80Δ11
R80/4A
R80/SA
Percent Maximal Binding
CTLA4Ig (μg/ml)

Figure 3.2. Costimulation with mutant CD80 transfectants. Mitomycin C treated transfectants were plated at a density of 100,000 per well with anti-CD3 at the concentrations indicated (A), or transfectants were added at the concentration indicated with 1 μg/ml anti-CD3 (B). Fifty thousand $CD4^+$ T cells were added to each well. T cell proliferation was assayed on day 5 and is presented as cpm of the incorporated label.


A
35
30
25
20
15
10
5
0
CPM x 1000
R80
R80Δ16
R80Δ11
R80/4A
R80/SA
RRc
0
0.1
1
10
100
1,000
CD3 (ng/ml)


B
35
30
25
20
15
10
5
0
CPM x 1000
R80
R80Δ16
R80Δ11
R80/4A
R80/SA
RRc
3,000
10,000
30,000
100,000
300,000
Reh/well

transfectants per well. We could detect T cell proliferation in cultures with R80 at doses of anti-CD3 between 100 pg/ml and 1 ng/ml (Fig. 3.2). At low numbers of transfectants per well only a low level of proliferation was induced, but as the ratio of R80 cells:T cell approached 1:1, a very strong proliferative response was evident which reached a maximum response with 2:1 R80 cells to T cells. The CD80Δ16, CD80Δ11, CD80/4A, and CD80/SA mutants were unable to costimulate T cell proliferation at all cell doses examined (Fig. 3.2B). Even at the ratio of 6 Reh cells to 1 T cell, transfectants expressing mutant CD80 were unable to induce significant T cell proliferation. Thus, residues throughout 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 serine 284 ablated the ability of CD80 molecules to costimulate T cells. 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 (Doty and Clark, 1996). To determine if 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 antibody-mediated redistribution. As shown in Figure 3.3 (panels A-D), wildtype and mutant CD80 molecules are localized in foci randomly distributed over the cell surface. There are no obvious difference in the distribution, or the size of the foci of CD80 found on R80, R80Δ16, R80/4A, or R80/SA cells. After 5 min of exposure to CD80 mAb wildtype CD80 already had undergone significant redistribution, while mutant CD80 molecules had not relocalized as much (Fig. 3.3E-H). The kinetics of patching (Fig. 3.4A) and capping (Fig. 3.4B) was also delayed in each of the CD80 mutants. After 5 min at 37°C 20% of the cells had redistributed wildtype 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 minutes. After 20 min at 37°C the majority of wildtype CD80 on R80 cells had been relocalized into patches and caps while CD80Δ16 was still predominately found in clusters (Fig. 3.3I-J). CD80/4A and CD80/SA had underwent more redistribution than CD80Δ16, but less than wildtype CD80 (Fig. 3.3K-L). Figure 3.4 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, which have more of the native cytoplasmic tail of CD80 underwent a slightly faster kinetics of redistribution, but CD80 was redistributed more slowly in these mutant CD80 expressing cells than in wildtype CD80 expressing cells (Fig.

Figure 3.3. Mutant CD80 cellular localization.
R80 (A, E, I) R80Δ16 (B, F, J), R80/4A (C, G, K) or R80/SA (D, H, L) transfectants were labeled with BB1 and detected as described in the materials and methods. The labeled cells were incubated at 37°C for 0 (A-D), 5 (E-H), or 20 (I-L) min before being fixed and mounted on slides. Images were collected on a laser scanning confocal microscope. A 10 μm scale bar is presented in panel A.

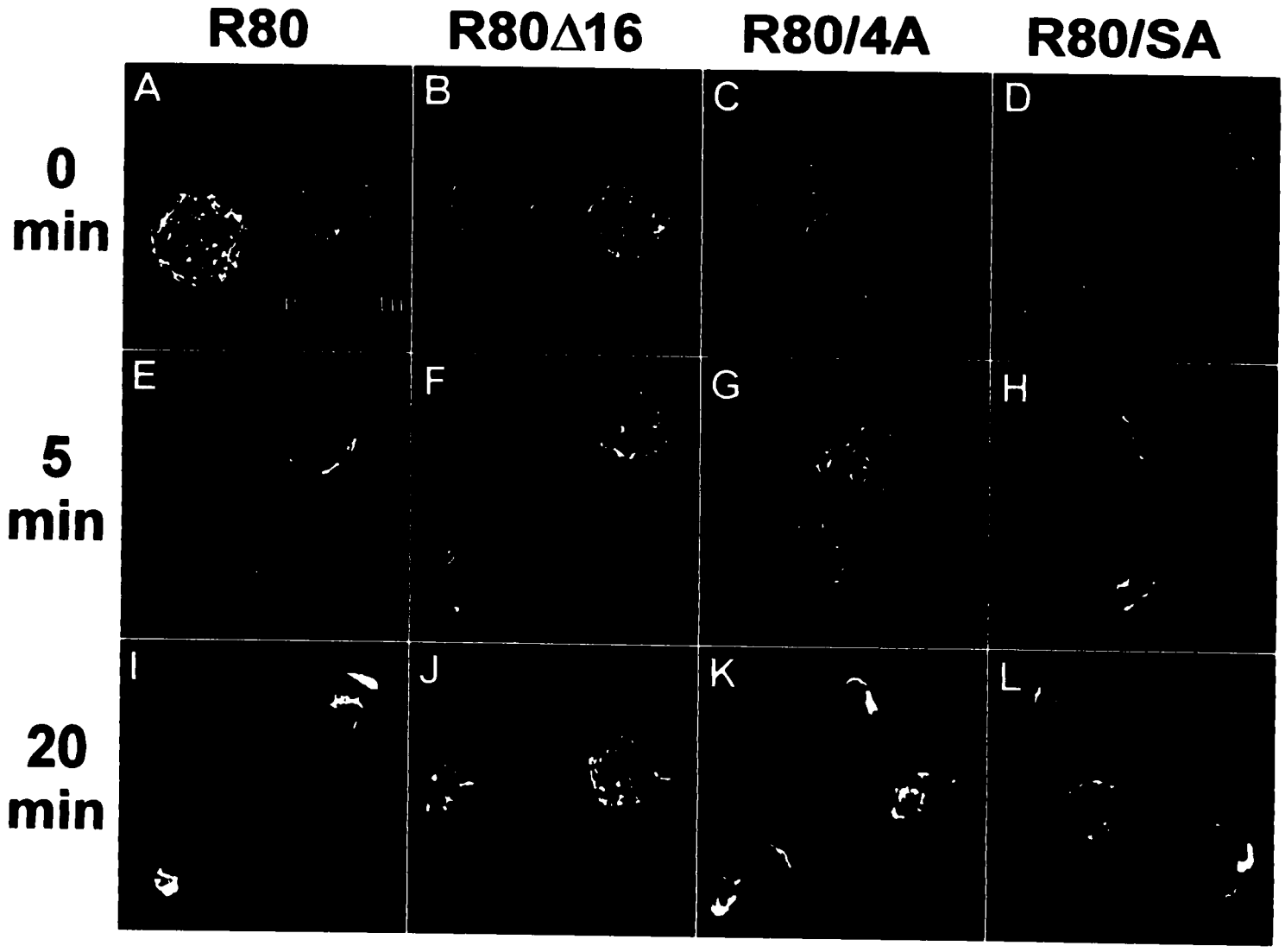
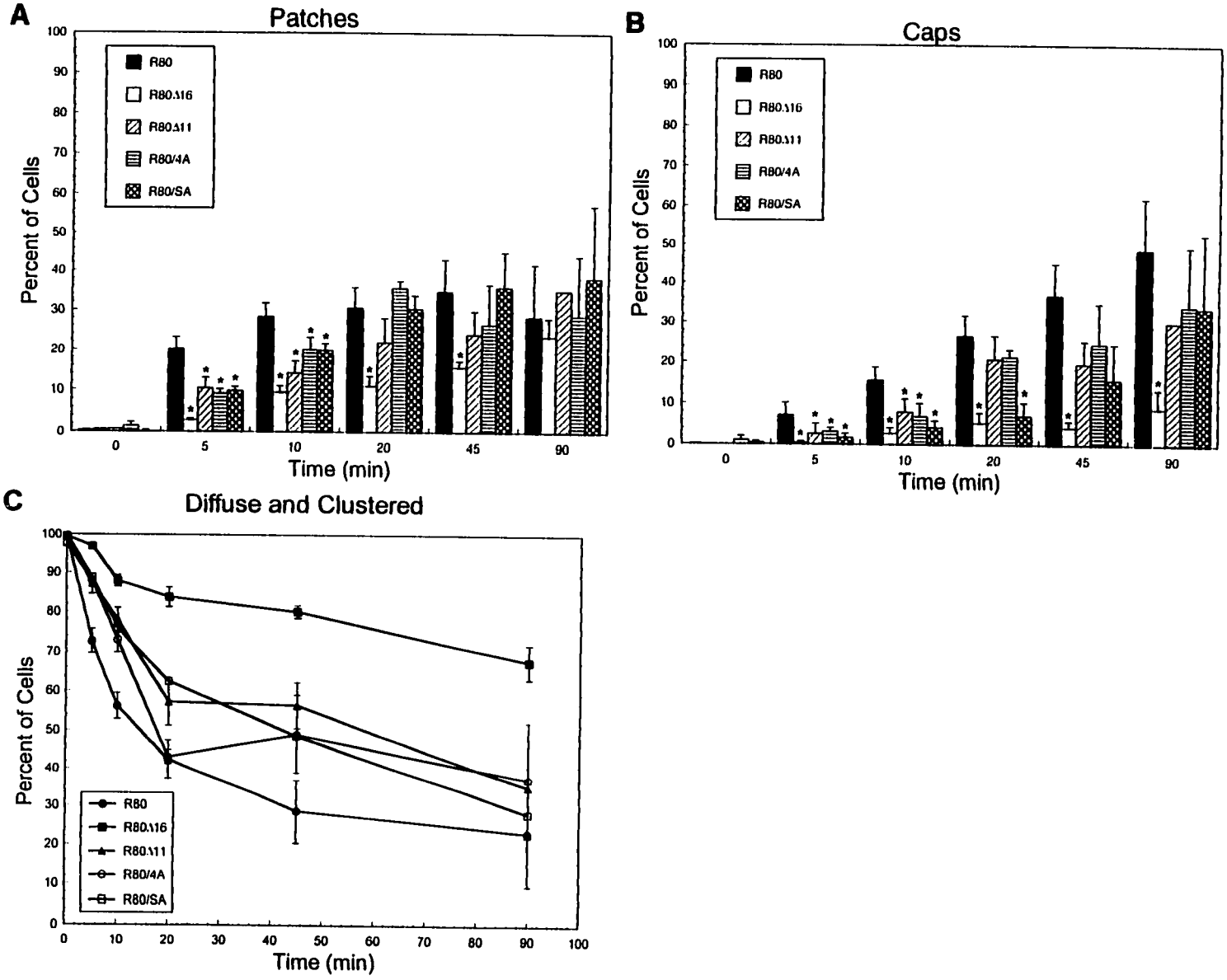

R80
R80Δ16
R80/4A
R80/SA
0 min
5 min
20 min
A
B
C
D
E
F
G
H
I
J
K
L

Figure 3.4. Kinetics of mutant CD80 redistribution.
Transfectants were labeled as described in the materials and methods then transferred to 37°C for the times indicated before being fixed and mounted on slides for analysis. The CD80 distribution was scored as described in the materials and methods. The percent of cells with CD80 patches (A), CD80 caps(B), and CD80 in clusters or diffuse (C) are presented. The means plus standard error values are indicated. The means were calculated from 7 experiments for R80 and R80Δ16, from 4, 3, and 2 experiments for R80Δ11, R80/4A, and R80/SA, respectively. Samples significantly different than wildtype, $p<0.005$, are indicated (*).


A
Patches
Percent of Cells
Time (min)
R80
R80.Δ16
R80.Δ11
R80/4A
R80/SA
B
Caps
Percent of Cells
Time (min)
R80
R80.Δ16
R80.Δ11
R80/4A
R80/SA
C
Diffuse and Clustered
Percent of Cells
Time (min)
R80
R80.Δ16
R80.Δ11
R80/4A
R80/SA

3.4C). R80Δ11, R80/4A, and R80/SA formed significantly more caps of CD80 than R80Δ16, but did not cap as much as wildtype (Fig. 3.4B).

All the transfectants expressing mutant or wildtype CD80 constructs initially express the CD80 molecules in foci (Fig. 3.3A-D), but only wildtype CD80 could costimulate T cell proliferation (Fig. 3.2). This demonstrates that the foci of CD80 found on APC are insufficient to aggregate CD28 and mediate costimulation, and that the rate of CD80 redistribution is dependent upon the presence of both RRNE (275-278) and serine 284. Furthermore, even though mutant CD80 molecules underwent some degree of redistribution into patches and caps, they could not costimulate T cells (Figs 3.2 and 3.4). This suggests that while CD80 redistribution into patches and caps may be required for T cell costimulation, it is insufficient for costimulation of T cell proliferation.

The CD80 cytoplasmic tail associates with a 30 kDa phosphoprotein.

Since mutating serine 284 abrogated the ability of CD80 expressed on Reh cells to costimulate T cell proliferation, we attempted to determine whether serine 284 could be phosphorylated in intact cells. We labeled R80 cells with ^{32}P and precipitated CD80 from lysates of unstimulated or stimulated cells. However, we were unable to detect any ^{32}P-labeled CD80, even after stimulating cells with PMA and/or the calcium ionophore, ionomycin, under conditions where we are clearly able to detect ^{125}I surface labeled CD80 (data not shown). However, we did detect a specific band approximately 30 kDa in size (pp30) coprecipitating with CD80 from cells stimulated with either ionomycin or

ionomycin plus PMA (Fig. 3.5). 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. 3.5), suggesting that the levels of CD80 precipitated from each sample under the same conditions were also similar.

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 greatly enhanced by PMA stimulation. This suggests that the interaction between pp30 and CD80 is regulated by intracellular calcium levels. We next tested if the association of pp30 with CD80 correlated with the ability of CD80 to costimulate T cell proliferation, (Fig. 3.6). The cytoplasmic tail of CD80 is required to precipitate pp30, as pp30 was not present in a precipitation from R80Δ16 cells stimulated with PMA and ionomycin while it was present in precipitations of CD80 from R80 cells (Fig. 3.6). We monitored MHC Class I precipitates as in Figure 3.5 to ensure equal loading among the samples (data not shown).

Discussion

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 B cell activation. Since the CD80-pp30 association is induced with

Figure 3.5. CD80 associates with a 30 kDa phosphoprotein.
R80 cells were labeled with [^{32}-P]H_3PO_4 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-MHC class I, human IgG, or CTLA4Ig as indicated and precipitated with protein A-sepharose. The bound proteins were extracted with sample buffer and analyzed by SDS-PAGE. The positions of molecular weight markers are indicated in kDa.

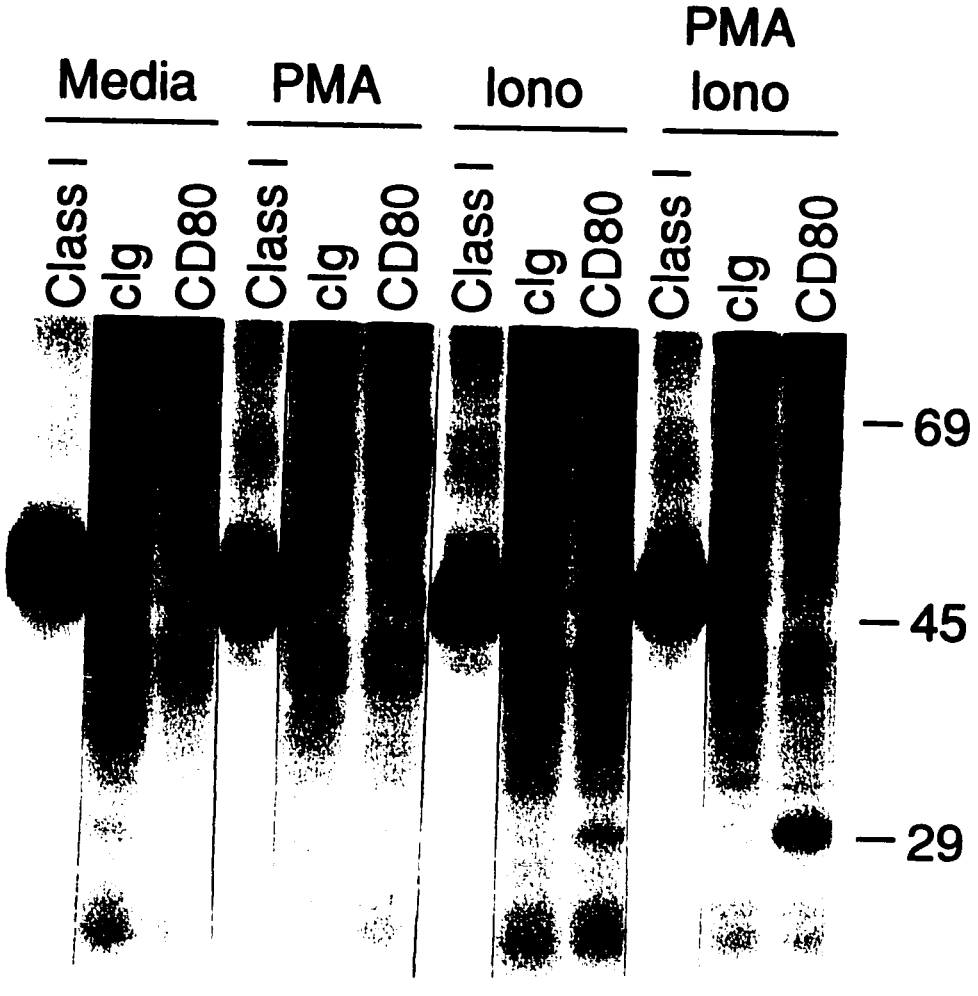
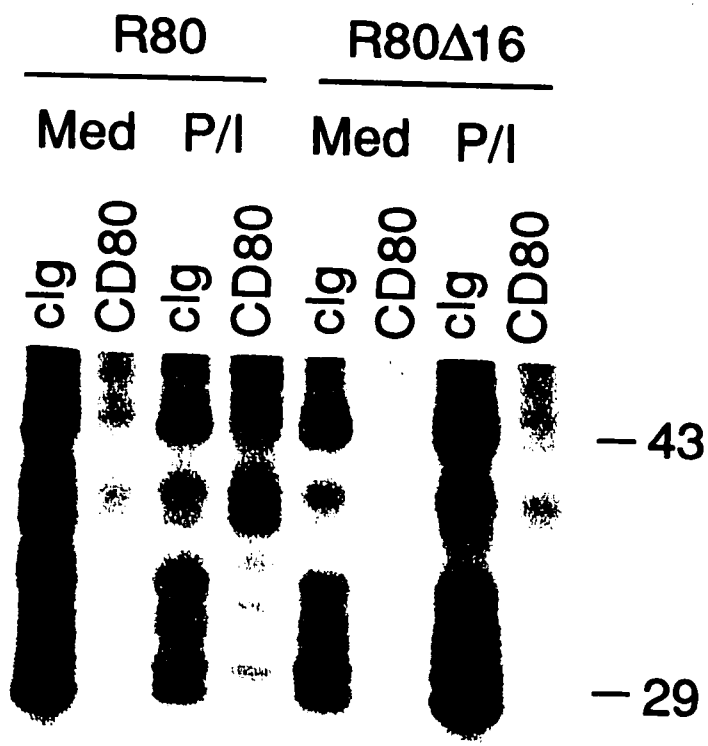

Media
PMA
Iono
PMA
Iono
Class I
clg
CD80
Class I
clg
CD80
Class I
clg
CD80
Class I
clg
CD80
69
45
29

Figure 3.6. The cytoplasmic tail of CD80 associates with pp30.
R80, R80Δ16, and R80/4A cells were labeled as above and stimulated with media or PMA and ionomycin as before. Lysates were incubated with anti-MHC Class I, human IgG, or CTLA4Ig as indicated and precipitated with protein A-sepharose. The bound proteins were analyzed by SDS-PAGE. The positions of molecular weight markers are indicated in kDa.


R80
R80Δ16
Med
P/I
Med
P/I
clg
CD80
clg
CD80
clg
CD80
clg
CD80
43
29

ionomycin alone but not PMA alone, calcium appears to play a critical role in regulating this interaction, while PMA sensitive kinases enhance this association. The CD80 cytoplasmic tail region RRNE 275-278 is similar to the calmodulin binding region of the polymeric Ig receptor RRNV. While calmodulin, a 17 kDa protein, is not pp30, it may regulate the association of CD80 with pp30 since it is a calcium dependent interaction. We have not detected any kinase activity in CD80 precipitates, suggesting that pp30 is not a kinase itself. Recently Hirokawa *et al.* (1996) found a 32 kDa protein which is inducibly and transiently phosphorylated on tyrosine after CD80 crosslinking. It is possible that this 32 kDa protein and pp30 are the same protein and that pp30 association with CD80 is necessary for CD80 redistribution and costimulation.

Ligand binding to CD28 is clearly insufficient to initiate signaling (Doty and Clark, 1996). CD28 needs to be aggregated by ligand to costimulate T cells proliferation as Fab fragments to CD28 block costimulation and soluble ligand only costimulates if it is first immobilized on plastic (Damle *et al.*, 1988; Linsley *et al.*, 1991a). Linsley *et al.* (1995b) demonstrated that the native CTLA-4 homodimer binds two CD86 molecules, and presumably two CD80 monomers. Furthermore, oligomerization of CD80 and CD86 regulate binding to CD28 and CTLA-4 dimers (Greene *et al.*, 1996). Thus, cells expressing CD80 may be able to regulate the ability of CD80 to costimulate T cells by regulating the subcellular localization of CD80. Masten *et al.* (1997) found that lung DC

express high levels of CD86, but blocking CD86 alone was unable to inhibit MLR responses. Furthermore, Greenfield *et al.* (1997) found CD86 expressed on T cells could not costimulate T cells demonstrating that expression of costimulatory molecules does not always correlate with costimulation. This suggests that cells expressing costimulatory molecules may regulate their function independently of their expression on the cell surface.

When we examined the ability of mutant CD80 molecules to undergo antibody-mediated redistribution, we found they were not able to redistribute as rapidly as does wildtype CD80, but they were able eventually to redistribute to similar levels as wildtype CD80 (Fig. 3.4). This suggests that CD80 redistribution is required, but insufficient, for CD28 mediated costimulation, and the rate of CD80 redistribution may be critical for costimulation. Furthermore, the entire CD80 cytoplasmic tail is required for initiating CD28-mediated costimulation, yet deletion or mutation of many residues of the CD80 cytoplasmic tail while altering the rate of CD80 redistribution had little effect on the ability of CD80 to enter eventually into caps or patches. This is most evident by comparing the R80Δ16 and R80Δ11 mutants. Neither mutant costimulates T cell proliferation, but CD80Δ11 eventually reaches similar levels of patches and caps as wildtype CD80 while CD80Δ16 eventually reaches similar levels of patches, but not caps (Fig. 3.4).

CD80/SA was expressed at slightly lower levels on the surface of transfectants compared to other CD80 mutants (Fig. 3.1A). 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 had little effect on the level of T cell proliferation induced. Different Reh transfectants expressing wildtype CD80 on the surface over a ten-fold range, all induced T cell proliferation to similar levels (R.T.Doty, unpublished observations). CD80-transfected CHO cells expressing very low levels of CD80 also can costimulate T cell proliferation to a similar level as Reh transfectants expressing high levels of CD80 (Doty and Clark, 1996). 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. Thus for the CD80/SA mutant, we are left with the possibility that phosphorylation of S284 is necessary for CD80 redistribution and costimulation, even though we are unable to detect it.

Linsley and coworkers have demonstrated that oligomerization of CD80 or CD86 regulates both binding to CD28 and CTLA-4 and signal transduction, and suggest that the foci of CD80 present on Langerhans cells are sufficient to aggregate CD28 thus initiating CD28-mediated signals (Greene *et al.*, 1996; Symington *et al.*, 1993; Linsley *et al.*, 1995a). The notion that oligomers of CD80 are required for CD28 aggregation and signaling is consistent with our data. However, just having CD80 present in individual foci on the cell surface clearly is insufficient to generate CD28-mediated signals as CD80Δ16, CD80/4A, and

CD80/SA all express CD80 in foci indistinguishable from wildtype cells (Fig. 3.3), yet they cannot costimulate T cell proliferation. This suggests that the foci of CD80 present on APC allow high avidity binding to CD28, but do not initiate signaling. To reconcile these data, we proposed a model that CD80 needs to be actively redistributed on the APC in order to costimulate T cell proliferation. In other words, a signal from the T cell back into the APC is necessary for costimulation to occur (Clark and Ledbetter, 1994). The ability of CD80 to redistribute into functional structures, found in patches and caps, was dependent upon the cytoplasmic tail of CD80. It is not clearly understood why tailless CD80 on the cell surface cannot costimulate while a fusion protein of CD80, B7Ig, which lacks the cytoplasmic tail of CD80, can costimulate if it is immobilized on plastic (Linsley *et al.*, 1991a).

Kupfer and Singer (1989) demonstrated that CD11a/18 localizes to the site of APC/T cell contact, and Moingeon *et al.* (1991) 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 crosslinked 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 relocated to the same site as the cognate TCR complex. The pp30 phosphoprotein is not detected in association with CD80 unless cells are first stimulated with a calcium dependent signal (Fig. 3.5), and the level of pp30 associating with CD80 is further augmented by

PMA. These results suggest that a calcium-dependent signal to APC may normally change the structure and function of CD80 complexes. Ligating either surface immunoglobulin or MHC class II on B cells, unlike PMA, both up-regulates levels of intracellular free calcium and the expression of CD80 (Lane *et al.*, 1990; Freedman *et al.*, 1987; Koulova *et al.*, 1991, Nabavi *et al.*, 1992). Thus, it is possible that during APC-T cell interactions or after B cells take up multivalent antigens through their antigen 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 (Berberich *et al.*, 1994), CD80 may not be able to effectively costimulate T cells until MHC Class II receptors are engaged by T cell receptor recognition. This would be desirable to prevent stimulation of bystander B cells through CD40 to become competent APC in absence of specific antigen-derived signals. We favor this model over a direct effect of CD28 binding to CD80 inducing costimulation-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 unique interactions are required for T cell costimulation. Both mutants behave in a similar manner during redistribution, and both have a slower kinetics of redistribution (Fig. 3.4),

suggesting that residues spread throughout the cytoplasmic tail are recognized in a single interaction with the cytoskeleton. Perhaps RRNE (275-278) is a protein docking site while S 284 is a phosphorylation site which regulates protein association. Consistent with this is that mutations of specific amino acids alter the kinetics of redistribution of CD80, most likely caused by a lower avidity between CD80 and a component of the cytoskeleton, possibly pp30.

Borriello *et al.* (1994) identified an alternative spliced form of murine CD80 mRNA. This transcript encodes for CD80 which contains an alternative cytoplasmic tail. It is interesting to speculate that the usage of alternative cytoplasmic tails of CD80 may be able to regulate costimulation. In CD80 transfected CHO epithelial cells, CD80 is constitutively patched at sites of cell-to-cell contact (Doty and Clark, 1996). An alternate form of CD80 may prevent CD80 from being expressed in a constitutive 'active' location on certain cell types. Currently there is no evidence for an alternatively spliced form of human CD80, but we cannot exclude this possibility as there are multiple mRNA transcripts for human CD80 (Freeman *et al.*, 1989), thus it is possible that one or more of these encode an alternative form of CD80.

Chapter 4: CTLA-4 may bind to CD11a/18 (LFA-1)

Abstract

CTLA-4 and CD28 are both ligands for CD80 and CD86, molecules found on APC. A soluble immunoglobulin fusion protein of CTLA-4 (CTLA4Ig) has a higher affinity for CD80 than does CD28Ig. CTLA4Ig *in vitro* blocks the binding of CD28 to CD80 and to CD86, and inhibits costimulation of T cell activation. *In vivo*, CTLA4Ig inhibits T cell-dependent responses. Here we report that although CTLA4Ig and CD28Ig both immunoprecipitate a 55 to 100 kD glycoprotein from $CD80^+$ CHO cells, CTLA4Ig, but not CD28Ig, immunoprecipitated additional proteins of 100 kD and 180 kD in size from several B cell lines and normal, activated B cells. Sequential immunoprecipitation demonstrated that the additional proteins recognized by CTLA4Ig were the CD11a/18 (LFA-1) heterodimer. Subsequently, CTLA4Ig was found to bind to and to immunoprecipitate CD11a/18 from $CD80^-$ cells, demonstrating that the binding of CTLA4Ig to CD11a/18 does not require concurrent binding to CD80. CTLA4Ig was able to inhibit CD18-dependent lymphocyte aggregation. While not all preparations of CTLA4Ig were able to bind CD11a/18, these results do suggest that CTLA-4 may play a role in the immune response independent of its binding to CD80 and CD86. Using a recombinant CTLA-4/CD28 fusion protein, we mapped the region of CTLA-4 responsible for binding to CD11a/18 to the extracellular Ig-like domain.

Introduction

CTLA-4 was originally identified as an mRNA expressed in activated T cells and on the basis of its deduced protein sequence, was thought to be a cell surface receptor (Brunet *et al.*, 1987; Dariavach *et al.*, 1988). Both human and murine *CTLA-4* genes map to the same chromosomal region as the human and murine *CD28* loci (Harper *et al.*, 1991), and CD28 and CTLA-4 share about 30% sequence identity, suggesting they may have a similar role in T cell activation. CD28, a T cell surface protein expressed on most T cells, has been demonstrated to play a significant role in T cell activation by increasing cytokine production, particularly IL-2, thus promoting T cell proliferation. CD28 mAbs synergize with sub-optimal doses of anti-CD3 to induce T cell proliferation and IL-2 expression (reviewed in Linsley and Ledbetter, 1993). CTLA-4 is expressed on the surface of activated T cells concurrently with CD28 expression (Linsley *et al.*, 1992a). Although mAbs to CTLA-4 can enhance stimulation of activated T cells by CD28 mAbs (Linsley *et al.*, 1992a), CTLA-4 plays a major role in limiting T cell proliferation (Tivol *et al.*, 1995; Waterhouse *et al.*, 1995).

Both CD28 and CTLA-4 are ligands for the lymphocyte activation molecules CD80 (Linsley *et al.*, 1990; Linsley *et al.*, 1991a; Linsley *et al.*, 1991b) and CD86 (Azuma *et al.*, 1993a; Hathcock *et al.*, 1993; Freeman *et al.*, 1993b; Freeman *et al.*, 1993c). CD80 was first identified as a B cell-associated activation molecule (Yokochi *et al.*, 1982; Freeman *et al.*, 1989), but is also

expressed on DC, activated monocytes, Langerhans cells, HTLV-1 transformed, and chronically stimulated T cells (Young *et al.*, 1992; Freedman *et al.*, 1991; Symington *et al.*, 1993; Vallé *et al.*, 1990; Azuma *et al.*, 1993b). Using mAbs or CTLA4Ig, several groups have found that CD28 (and possibly CTLA-4) interactions with CD80 have significant effects on both T cells and B cells. T cell hyporesponsiveness, or anergy, is induced when T cells are activated in the absence of CD80 costimulation (Gimmi *et al.*, 1993; Tan *et al.*, 1993), and tumor rejection is induced by transfecting poorly immunogenic tumor cells with the B7 gene (Townsend and Allison, 1993; Chen *et al.*, 1992). CD86 has been identified as an early activation antigen found on activated B cells, activated T cells, γ-IFN activated monocytes (Azuma *et al.*, 1993a); however, it is also found on resting B cells and monocytes (Azuma *et al.*, 1993a; Hathcock *et al.*, 1993). Interfering with the interaction of CD28 with its ligands dramatically reduces B cell Ig secretion (Damle *et al.*, 1991; Shahinian *et al.*, 1993); however, it is unclear whether this inhibition is due to a direct signal to the B cell through a B cell ligand, or caused by interfering with CD28-dependent T cell cytokine production.

In addition to the costimulation signal through CD28, it has been reported that crosslinking CD11a/18 (LFA-1) with either of its ligands, CD54 (ICAM-1) or CD102 (ICAM-2), on T cells also provides a costimulatory signal (Van Seventer *et al.*, 1990; Damle *et al.*, 1992a). Furthermore, Moy and Brian

(1992) found that APC stimulated through CD11a/18 are more efficient at inducing T cell proliferation, thus suggesting that crosslinking CD11a/18 can affect both T cell and APC function. In this report we demonstrate that CD11a/18 may be a ligand for CTLA-4. CTLA-4 can bind to $CD80^-$ cells and thus can bind to CD11a/18 independent of binding to CD80. Furthermore, we found that a soluble form of CTLA-4 can interfere with CD11a/18 binding to other ligands involved in cell adhesion. CTLA-4 binding to CD11a/18 is not solely dependent upon the amino acid sequence of CTLA-4 since not every preparation of CTLA4Ig was able to bind to CD11a/18. Thus, glycosylation or other post-translational modifications regulate CTLA-4 binding CD11a/18.

Materials and Methods

Antibodies and recombinant globulins.

The mAbs used in this study included the CD40 mAb, G28-5 (Clark and Ledbetter, 1986), the CD18 mAb, 60.3 (Beatty *et al.*, 1983), the CD8 mAb, G10-1 (Ledbetter et al., 1985), the CD11a mAb, TS1/22 (Sanchez-Madrid *et al.*, 1982), and the mouse IgG control mAb, P1.17 (ATCC). All mAbs were purified from ascites before use. The CD80 mAbs, L307 (Azuma *et al.*, 1992) and L310 were provided by Drs. David Buck and Doug Kawahara at Becton Dickinson Advanced Cell Biology (San Jose, CA). Purified human IgG was purchased from Sigma Immunochemicals (St. Louis, MO). The recombinant globulins CD28Ig and CTLA4Ig and the CTLA4Ig/CD28Ig domain swap chimera DS2 containing the CD28 Ig-like extracellular domain in place of the Ig-like domain of CTLA4Ig

(see Fig. 4.3A) were prepared as described before (Linsley *et al.*, 1991a; Linsley *et al.*, 1991b; Peach *et al.*, 1994). Biotinylated mAbs and fusion proteins were prepared as described (Coligan et al., 1991). Monoclonal Abs were conjugated to CNBr-activated sepharose 4B according to manufacturer's protocol (Pharmacia Fine Chemicals, Piscataway, NJ). Recombinant human IL-4 was kindly provided by Dr. Ken Grabstein (Immunex, Seattle, WA).

Cells, cell activation, and cell aggregation studies.

CESS, an EBV-transformed surface IgG^+ B cell line (Muraguchi *et al.*, 1981), and Ramos and Daudi, two IgM^+ Burkitt's lymphoma lines, were all obtained from the ATCC and maintained in log phase in RPMI-10. Hut 78, a T cell leukemia line, was obtained from the ATCC and maintained in RPMI-10 with 2-ME. The CD80 CHO cells were maintained in DMEM-10 with L-proline and methotrexate as described before (Linsley *et al.*, 1991a). CG is a $CD18^-$ B lymphoblastoid cell line isolated from a patient with lymphocyte adhesion deficiency and was kindly provided by Dr. Hans Ochs (Beatty *et al.*, 1984). Human mononuclear cells were isolated from tonsils as described previously (Clark and Ledbetter, 1986) using Lymphoprep (Pharmacia). T cells were removed by rosetting with SRBC followed by another Lymphoprep treatment. The $CDw32^+$ L cells were provided by Dr. K. Moore (DNAX, Palo Alto, CA) and were maintained as described (Peltz *et al.*, 1988). Where indicated, normal B cells were activated as described (Banchereau *et al.*, 1991); briefly, a non-

confluent monolayer of CDw32 L cells was treated with 100 μg/ml mitomycin C (Sigma Chemical Company, St. Louis, MO) for 45 min at 37°C and washed 3 times, 2 x 10^8 B cells were added in 50 ml of RPMI-10 and the cells were then cultured for 48 hr with 1 μg/ml anti-CD40 and 10 U/ml recombinant human IL-4. Aggregation of Daudi cells was induced with PMA as described (Barrett *et al.*, 1991); briefly, the cells were washed and resuspended in RPMI-10, 0.15 x 10^6 cells were added (200 μl/well) into 96-well flat-bottomed plates for the time indicated. PMA was added to a final concentration of 3.2 ng/ml, EDTA and EGTA (Sigma) were used at 5 mM each, and mAbs, IgG, or fusion proteins were all used at 10 μg/ml.

Iodination, immunoprecipitation, and electrophoresis.

Cells were washed twice in PBS, resuspended at 5 x 10^7 to 2 x 10^8 cells/ml, then iodinated in 0.5 ml aliquots as described (Leprince *et al.*, 1993). Briefly, the cells were incubated with 30 μg of lactoperoxidase (Sigma) and 1 mCi of $Na^{125}I$ (ICN, Irvine, CA), followed by increasing doses of H_2O_2, and stopped by the addition of excess cold NaI. The cells were washed three times and lysed in NP-40 lysis buffer (0.5% NP-40, 50 mM Tris pH 8.0, 150 mM NaCl, 5 mM EDTA, and a set of protease inhibitors) for 30 min. The lysates were centrifuged for 5 min to precipitate large cellular debris, and the supernatant was applied to a PD-10 sephadex G25M desalting column (Pharmacia) to remove unbound iodine. The resulting lysates were precleared three times either with protein A-sepharose (Pharmacia) or with sepharose-conjugated

mAbs, as described in the figure legends, before being used for precipitation analysis. Routinely 5 x 10^6 cell equivalents were precipitated with 10 μg/ml mAb or recombinant globulin followed by protein A-sepharose (or protein G-sepharose for the murine IgG_1 isotypes) for each lane. The samples were washed four times with NP-40 wash buffer (0.5% NP-40, 50 mM Tris pH 8.3, 0.45 M NaCl, and 0.1% SDS). The samples were extracted with reducing sample buffer and then run on 10% SDS PAGE. The gels were fixed, dried, and exposed to film for 3 hr to 2 weeks.

Flow cytometry.

Cells were washed once and resuspended at 10^7 cells/ml in PBS containing 2% FCS and 0.02% NaN_3. Unless otherwise noted, for indirect immunofluorescence the primary mAb was used at a final concentration of 10 μg/ml, and the second step reagents, either strepavidin-PE, goat anti-human IgG-FITC, or goat anti-mouse IgG-FITC (all from Tago Immunologicals, Camarillo, CA), were used at a final dilution of 1:80. Each step was incubated on ice for 40 min before being washed twice. The samples were fixed in 1% paraformaldehyde in PBS before being analyzed on a FACScan with a four-decade log amplifier (Becton Dickinson Immunocytometry Sys., Mountain View, CA). Competitive binding assays were performed by incubating the cells with saturating amounts of the blocking mAbs, as indicated, on ice for 15 to 30 min before adding biotin-conjugated CTLA4Ig. Fluorescence intensity (FI) was calculated by dividing the mean fluorescence intensity of the sample by that of

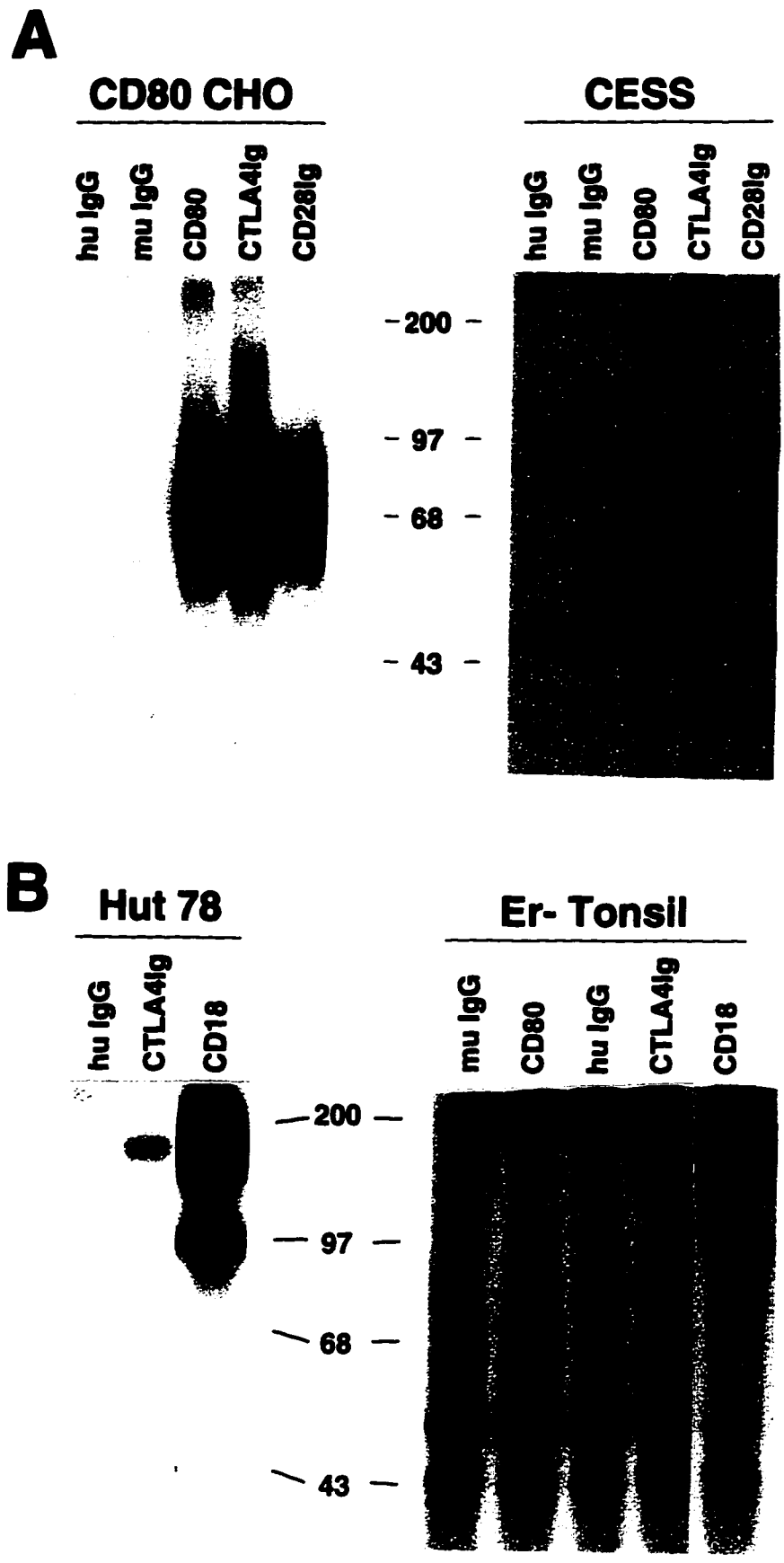
the control Ab. The effect of EDTA/EGTA on CTLA4Ig binding was determined by the addition of 5 mM each of EDTA and EGTA for 30 min prior to the addition of CTLA4Ig, followed by indirect immunofluorescence as described above.

Results

CTLA4Ig can immunoprecipitate surface molecules in addition to CD80.

Initially, we observed that the CD80-specific mAb, L307, could completely inhibit CTLA4Ig binding to CD80 CHO cells, but not to CESS cells (data not shown). This suggested CTLA4Ig might bind to additional ligands on CESS cells. To address this possibility, we compared surface molecules precipitated by CD80 mAb L307, CTLA4Ig, or CD28Ig from the surface of CHO CD80 transfectants and CESS cells. All three reagents were able to precipitate CD80 from CD80 CHO and CESS (Fig. 4.1A). CD28Ig was less effective at precipitating CD80 from both cell lines, probably because it has a lower avidity than CTLA4Ig for CD80 (Linsley *et al.*, 1991a; Linsley *et al.*, 1991b). CTLA4Ig precipitated two additional proteins from CESS cells. These proteins, whose approximate molecular weights are 100 and 180 kD, were not detected in the CD80 CHO lysate. Even after very long exposures to improve the detection of CD80 in the CD28Ig lane, these two additional proteins were not detectable in either the CD28Ig or L307 precipitates from CESS. These proteins still bound to the CTLA4Ig fusion protein after lysates were washed with NP-40 wash buffer containing 0.1% SDS (Fig. 4.1), and after the precipitates had been

Figure 4.1. CTLA4Ig binds to a p180/100 protein complex on human lymphocytes.
(A) NP-40 lysates of surface ^{125}I-labeled CD80 CHO or CESS cells were precipitated with either the control antibodies, human IgG or murine IgG, anti-CD80, CTLA4Ig, or CD28Ig as indicated. (B) NP-40 lysates of surface ^{125}I-labeled Hut 78 or anti-CD40 and IL-4 activated Er^- tonsillar B cells were precipitated with either the control antibodies, anti-CD80, anti-CD18, or CTLA4Ig as indicated. Molecular weight standards are indicated in kDa.


A
CD80 CHO
hu IgG
mu IgG
CD80
CTLA4Ig
CD28Ig
CESS
hu IgG
mu IgG
CD80
CTLA4Ig
CD28Ig
200
97
68
43
B
Hut 78
hu IgG
CTLA4Ig
CD18
Er- Tonsil
mu IgG
CD80
hu IgG
CTLA4Ig
CD18
200
97
68
43

washed successively in high salt and low salt containing wash buffers (data not shown), suggesting that CTLA4Ig directly interacts with these additional proteins.

The broad CD80 bands suggest that it is probably heterogeneously glycosylated at one or more of the eight potential N-linked glycosylation sites (Freeman *et al.*, 1989). With the CD80 CHO cells expressing forms that range in size from about 55 kD to over 100 kD (mean 70 kD), and CESS expressing forms that range from about 50 kD to 90 kD (mean 60 kD). L307, CTLA4Ig, and CD28Ig all precipitated the same sized CD80 bands in a given cell line. After we treated CD80 precipitates from CD80 CHO with endoglycosidase F and then ran the samples on SDS-PAGE, three major bands were present: 32 kD, 30 kD, and 25 kD in size, all of which contain the same protein core as determined by V8 protease mapping (data not shown). Since the molecular weight of the protein core is approximately 30 kD (Freeman *et al.*, 1989), the additional bands are most likely due to partial protein degradation.

We next tested whether CTLA4Ig precipitated the p180/100 proteins from other $CD80^+$ B cell lines and normal activated B cells. To determine whether CTLA4Ig could precipitate the p180/100 proteins from normal B cells, we first activated the B cells to induce the expression of CD80 using anti-CD40 and IL-4 (Banchereau *et al.*, 1991; Ranheim and Kipps, 1993) since CD80 is not present on normal resting B cells (Yokochi *et al.*, 1982; Freedman *et al.*, 1987). CTLA4Ig precipitated both CD80 and the p180/100 proteins from the surface of activated

tonsillar B cells (Fig. 4.1B), demonstrating that the binding of CTLA4Ig to the additional proteins was not simply restricted to transformed B cell lines. Both mAb L307 and CTLA4Ig precipitated a broad CD80 band from the activated B cells as seen previously in cell lines. We also tested four other B cell lines (SKW6.4, Raji, T5-1, and PM) and found that CTLA4Ig precipitated both CD80 and the p180/100 proteins from each line, although the amount of p180/100 proteins detected was variable, depending upon the line used (data not shown). The PM line had the lowest amount of detectable p180/100 proteins, and was difficult to see even on films with long exposures.

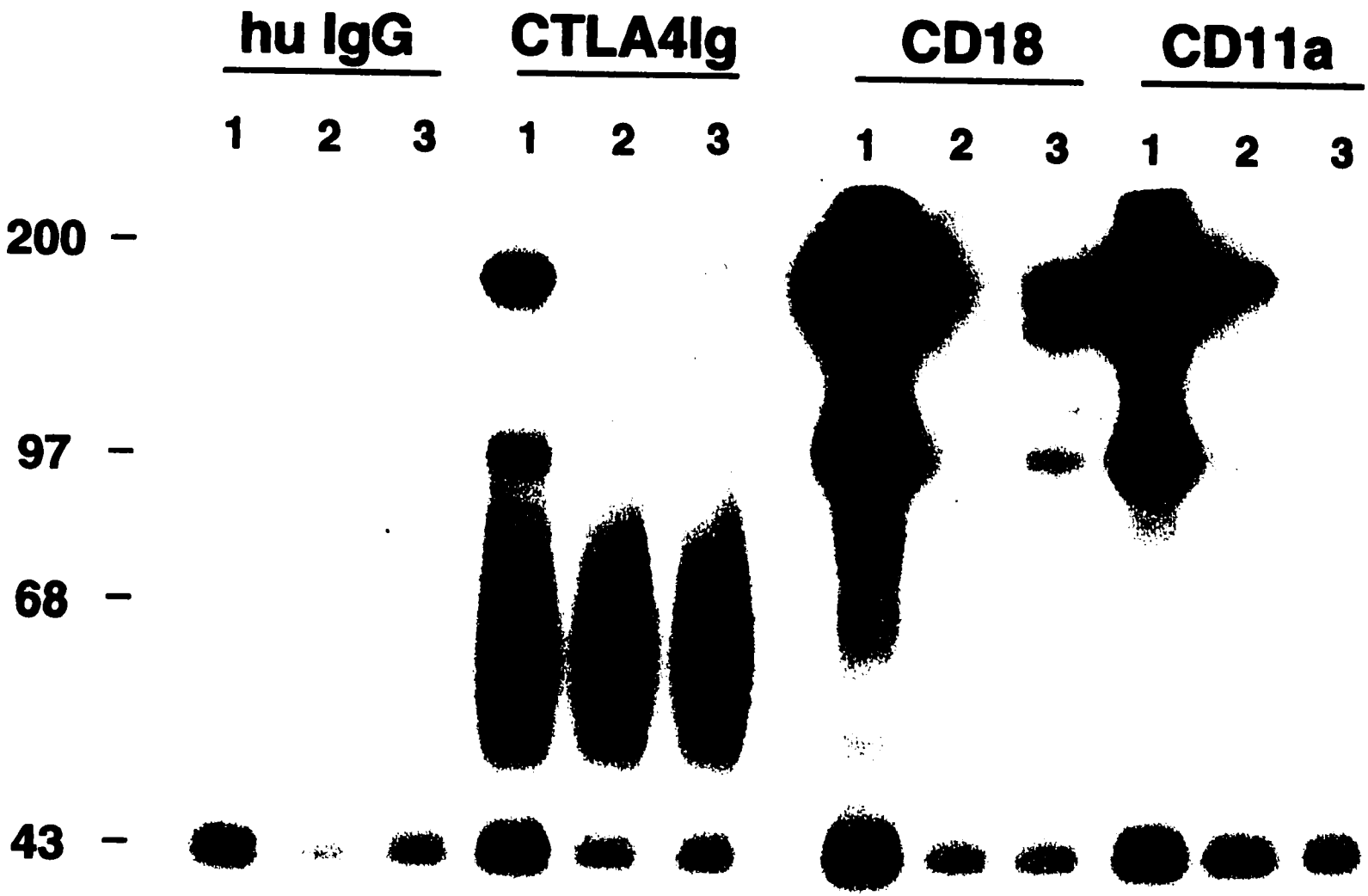
CTLA4Ig can bind to a p180/100 complex on CD80⁻ cells.

CTLA4Ig bound to Hut 78 cells, a CD80⁻ cell line, suggesting that binding to the p180/100 complex is independent of CD80 binding. To test this possibility we precipitated proteins from Hut 78 with CTLA4Ig (Fig. 4.1B). CTLA4Ig precipitated the p180/100 protein complex from Hut 78 in the absence of any detectable CD80. The p180/100 protein complex has molecular weights similar to those of the CD11a/18 (LFA-1) heterodimer, 180 kD and 95 kD, and, comigrate with anti-CD18 precipitates in both normal B cells and in Hut 78 (Fig. 4.1B).

The p180/100 complex is CD11a/18.

To test whether the p180/100 complex was in fact CD11a/18, we precleared CESS lysates with anti-CD18, anti-CD11a, or a control mAb then performed a sequential immunoprecipitation with CTLA4Ig (Fig. 4.2). When

Figure 4.2. CTLA4Ig binds to CD11a/CD18 in addition to CD80.
NP-40 lysates of ^{125}I-labeled CESS cells were prepared as in Figure 4.1 and extensively precleared with either the isotype control G10-1 mAb, anti-CD18, or anti-CD11a, lanes 1 to 3 respectively. The precleared lysates were then precipitated with either human IgG, CTLA4Ig, anti-CD18, or anti-CD11a as indicated in the figure. Molecular weight standards are indicated in kDa.


hu IgG
CTLA4Ig
CD18
CD11a
1 2 3 1 2 3 1 2 3 1 2 3
200 –
97 –
68 –
43 –

CESS lysates were precleared with mAbs to either chain of CD11a/18, the two p180/100 bands present in CTLA4Ig precipitates were eliminated, demonstrating that CTLA4Ig was precipitating CD11a/18 (Fig. 4.2). The possibility that CD11a/18 is interacting non-specifically with CTLA4Ig is unlikely because neither control CD28Ig, which contains the same Ig fusion construct as CTLA4Ig, nor the CD80 mAb precipitated CD11a/18 under the same conditions (Fig. 4.1A). As is evident in Fig. 4.2, CTLA4Ig precipitated only a fraction of the CD11a/18 that could be precipitated with either anti-CD18 or anti-CD11a. To test for the presence of possible contaminating mAb to CD11a or CD18 in our CTLA4Ig preps, we absorbed CTLA4Ig preps with CD80 CHO cells; absorbed CTLA4Ig lost all binding to both Hut 78 and to CESS (data not shown), *i.e.*, cells expressing only CD80 absorbed out all reactivity of CTLA4Ig with CD11a/18, again demonstrating that the binding to CD11a/18 was mediated by CTLA4Ig.

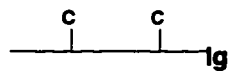
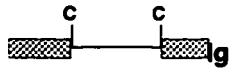
CTLA-4 binds to CD11a/18 through its Ig-like extracellular domain.

To determine which region of CTLA-4 mediates binding to CD11a/18, we compared the binding of CTLA4Ig and CD28Ig with the binding of DS2, a CTLA-4/CD28 chimeric fusion protein. The DS2 fusion protein contains the central Ig-like domain of CD28, while the N-terminal and C-terminal regions are the same as CTLA4Ig (Fig. 4.3A). At saturating doses, the DS2 and CTLA4Ig fusion proteins bound CD80 CHO at approximately equivalent levels, indicating that DS2 binds to CD80; however, it did not bind to the $CD80^-$ Hut

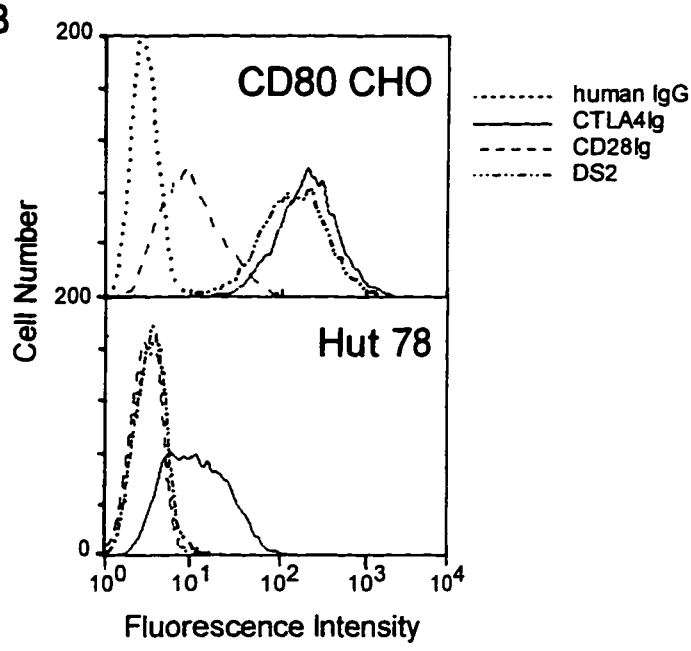
Figure 4.3. Identification of the CD11a/18 binding region of CTLA-4.

(A) Schematic representation of CD28Ig, CTLA4Ig, and the domain swap chimera, DS2, and a summary representation of their binding activity towards the CESS, CD80 CHO, and Hut 78 cells as determined by flow cytometry and/or immunoprecipitation analysis. (B) Flow cytometric analysis of fusion proteins binding to cell lines. The cells indicated were incubated with the following biotinylated mAbs: human IgG control (· · · · · ·), CTLA4Ig (———), CD28Ig (– – –), or DS2 (– · · –) followed by goat anti-human IgG-FITC. The axis of the histograms are cell number vs. log fluorescence intensity. (C) Immunoprecipitation analysis of CESS with the domain swap chimera DS2. NP-40 lysates of ^{125}I-labeled CESS cells were prepared as in Figure 4.1, then precipitated with either human IgG, CTLA4Ig, CD28Ig, DS2, or anti-CD18 as indicated in the figure.

A

	Construct	CESS CD80+, CD18+	CD80 CHO CD80+, CD18−	Hut 78 CD80−, CD18+
CD28Ig		+	+	−
CTLA4Ig		+	+	+
DS2		+	+	−

B



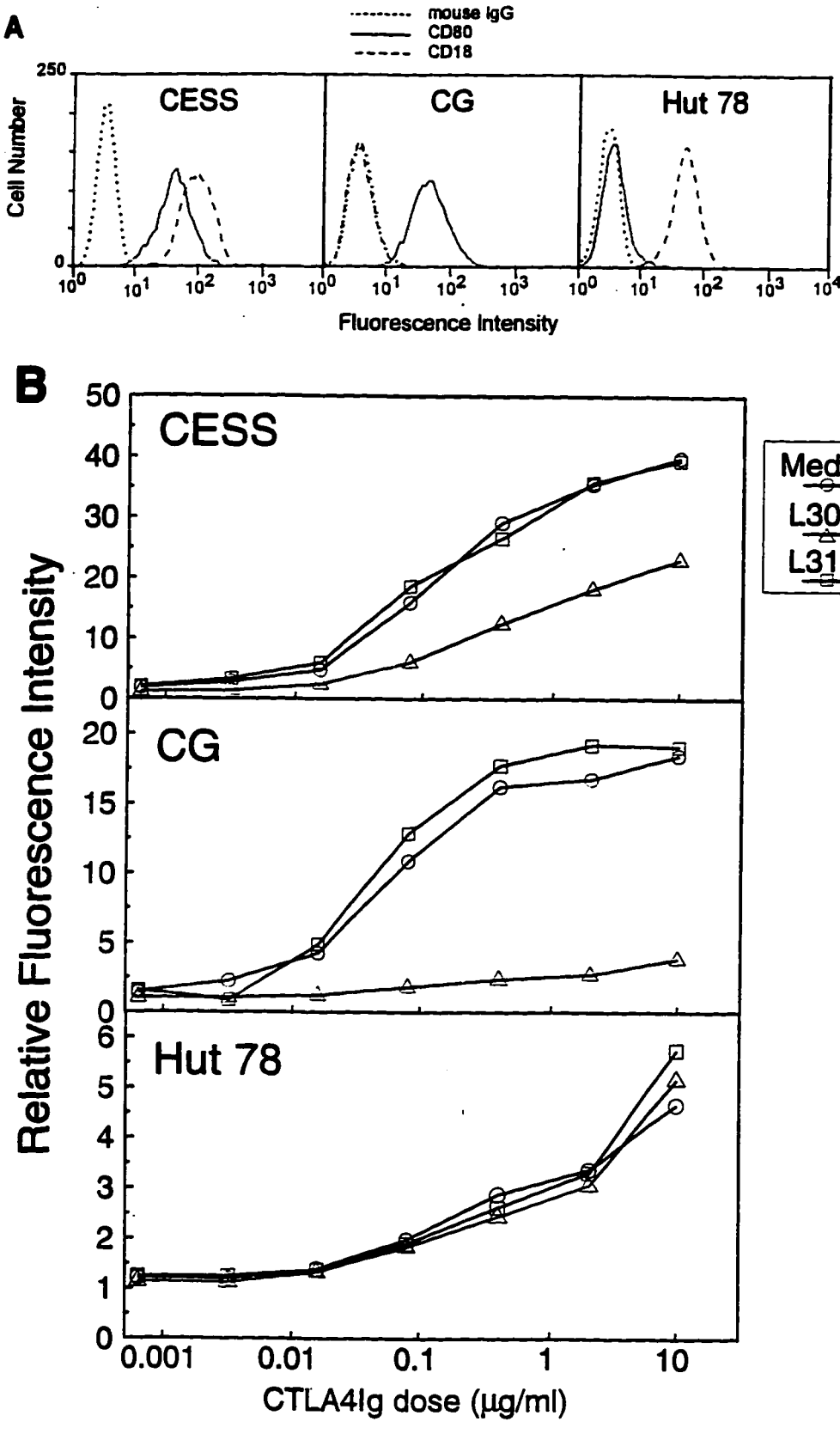
C

78 cells (Fig. 4.3B). The results summarized in Fig. 4.3A demonstrate the binding reactivity of CD28Ig, CTLA4Ig, and DS2 to cells expressing CD80 and/or CD11a/18. These results suggest that the central Ig-like domain of CTLA-4 is required for binding to CD11a/18. To test this further we compared the ability of the three fusion proteins to precipitate CD80 and CD11a/18 from CESS cells (Fig. 4.3C); all three fusion proteins precipitated CD80 from CESS, but only CTLA4Ig was able to precipitate CD11a/18, demonstrating that the extracellular Ig-like domain of CTLA-4 is required for CTLA4Ig to bind to and precipitate CD11a/18.

CTLA-4 binds independently to CD80 and CD11a/18.

To test whether the presence of CD80 affected the ability of CTLA4Ig to bind to CD11a/18, we compared the binding curves of CTLA4Ig on three lymphoid lines expressing different combinations of CD11a/18 and CD80 in the presence of saturating amounts of CD80 mAbs which either block (L307) or do not block (L310) CTLA4Ig binding to CD80. CESS is $CD80^+$, $CD11a/18^+$; CG is $CD80^+$, $CD11a/18^-$; and Hut 78 is $CD80^-$, $CD11a/18^+$ (Fig. 4.4A). CTLA4Ig binding to Hut 78 was detectable by flow cytometry at 16 ng/ml with a FI of 1.4, and increased to a FI of 5 at 10 μg/ml (Fig. 4.4B). The CTLA4Ig binding to Hut 78 did not reach saturating levels at any of the doses tested, including 50 μg/ml (data not shown), which suggests that the binding affinity of CTLA4Ig for the CD11a/18 complex is low. Neither L307, a CD80 mAb that blocks CTLA4Ig binding to CD80, nor L310, a CD80 mAb that does not block CTLA4Ig binding

Figure 4.4. Role of CD11a/18 in CTLA4Ig binding to target cells. CESS ($CD80^+$, $CD11a/18^+$), CG ($CD80^+$, $CD11a/18^-$), or Hut 78 ($CD80^-$, $CD11a/18^+$) cells were stained by indirect immunofluorescence. (A) Cells were incubated with 10 μg/ml control murine IgG (· · · · ·), anti-CD80 (———), or anti-CD18 (– – –) for 40 min, washed, and incubated with goat anti-mouse IgG-FITC for 40 min, followed by flow cytometry. The data are presented as cell number vs. log fluorescence intensity. (B) Cells were incubated for 15 min with media (○) or either of the CD80 mAbs, L310 (□) or L307 (△), at a final concentration of 20 μg/ml, followed by biotin-CTLA4Ig at the doses indicated for 40 min. The cells were washed and the biotin-CTLA4Ig was detected by strepavidin-PE. FI was calculated by dividing the log fluorescence intensity of the sample mean by that of the control sample (biotinylated human IgG) so a FI of 1.0 was background. CESS and CG express approximately equivalent levels of CD80 as detected with mAb L307, FI of 12.7 and 11.8, respectively.


A
mouse IgG
CD80
CD18
250
0
Cell Number
CESS
CG
Hut 78
10^0 10^1 10^2 10^3 10^4
Fluorescence Intensity
B
CESS
CG
Hut 78
Media
L307
L310
Relative Fluorescence Intensity
0.001
0.01
0.1
1
10
CTLA4Ig dose (µg/ml)

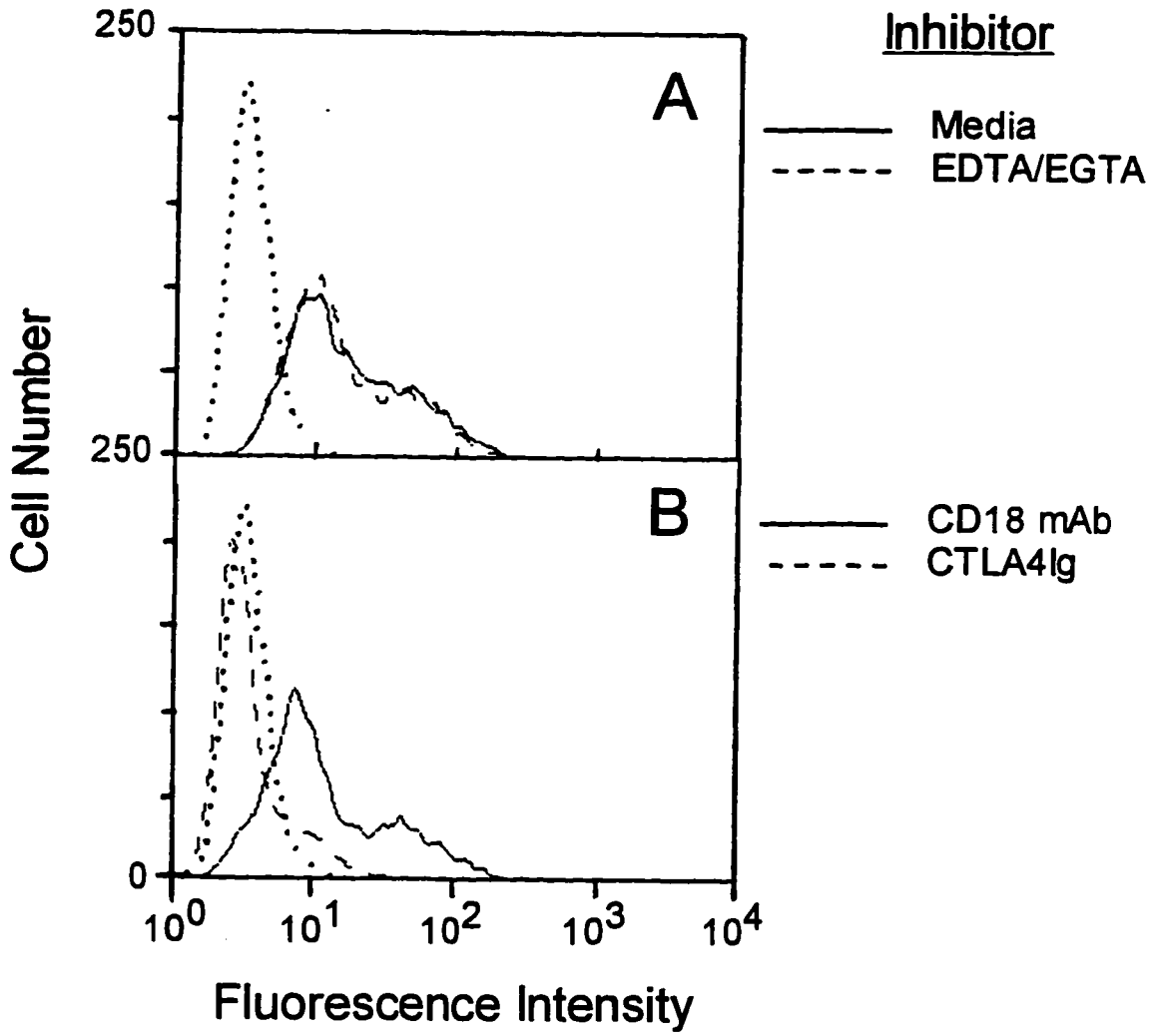
had any effect on CTLA4Ig binding to Hut 78 (Fig. 4.4B). This was expected since Hut 78 expresses CD11a/18 and not any detectable CD80. CTLA4Ig binding to CG was detectable at about 100 pg/ml, FI = 1.3 (data not shown), and reached a maximum FI of 17 at 400 ng/ml. L307 blocked over 90% of CTLA4Ig binding to CG at all doses of CTLA4Ig, even when CTLA4Ig was present at 10 μg/ml, demonstrating how efficiently L307 can block CTLA4Ig binding to CD80 (Fig. 4.4B). CTLA4Ig binding to CESS was also detectable at about 100 pg/ml, but unlike with CG cells the binding did not reach saturating levels at any dose tested, which is similar to the results on Hut 78 cells. L307 blocked most of the binding of CTLA4Ig to $CD80^+$, $CD11a/18^+$ CESS cells when low doses of the fusion protein were used (over 80% blocking at CTLA4Ig doses < 5 ng/ml), but it could only block about 50% of the binding when higher doses of CTLA4Ig were used. Thus, anti-CD80 blocked binding of CTLA4Ig to its high affinity ligand, CD80 but not to its low affinity ligand, CD11a/18, as expected. The binding curve of CTLA4Ig to CESS appears to be the sum of CTLA4Ig binding independently to both low and high affinity ligands. While we have found high levels of CD86 expressed on CESS, CD80 mAbs were able to block 80-90% of CTLA4Ig binding to high affinity ligands on both CESS and CG (Fig. 4.4), thus CD80 appears to be the major high affinity ligand on these cells.

CTLA4Ig binding to CD11a/18 is divalent cation independent and can block CD18-dependant adhesion.

The binding of CD11a/18 to its ligand CD54 is dependent on the presence of divalent cations, and the addition of chelating agents or CD18 mAbs can inhibit PMA- or mAb-induced aggregation of B lineage cells (Barrett *et al.*, 1991; Dransfield *et al.*, 1992; Patarroyo and Makgoba, 1989). Thus, we tested whether the binding of CTLA4Ig to CD11a/18 was dependent on divalent cations (Fig. 4.5), and whether CTLA4Ig inhibits CD18-dependent aggregation of B lineage cells (Fig. 4.6 and Table VI). The combination of EDTA and EGTA did not inhibit the binding of CTLA4Ig to Hut 78 cells as detected by flow cytometry, but was partially inhibited by the CD18 mAb 60.3 (Fig. 4.5). This suggests that CTLA4Ig can bind to CD11a/18 in a manner which is fundamentally different than other ligands.

PMA-induced aggregation of B lineage cells depends on CD18 and can be inhibited by either CD18 mAbs or EDTA/EGTA (Barrett *et al.*, 1991; Patarroyo and Makgoba, 1989). We have previously used the Daudi B cell line ($CD80^+$, $CD11a/18^+$) to examine CD18-dependent aggregation, so we tested whether CTLA4Ig was able to inhibit PMA-induced aggregation of these cells. Based on dose-response analysis (*e.g.*, Fig. 4.4B) we used CTLA4Ig at a dose of 10 μg/ml, and we assayed for aggregation after 24 hr (Barrett *et al.*, 1991). CTLA4Ig significantly inhibited the PMA-induced aggregation of the Daudi cells, but not

Figure 4.5. CTLA-4 binding to CD11a/18 does not depend on divalent cations.
Hut 78 T cells were incubated with (A) media (———) or 5 mM each EDTA and EGTA (– – –), or (B) 20 μg/ml anti-CD18, (———) or CTLA4Ig (– – –) for 30 min before a two-step immunofluorescence assay was performed with 10 μg/ml of biotin-CTLA4Ig as described in Figure 4.4. Each panel contains a biotinylated human IgG control (· · · · ·). The histograms are presented as cell number vs. log fluorescence intensity.


250
250
0
A
B
Cell Number
10^0
10^1
10^2
10^3
10^4
Fluorescence Intensity
Inhibitor
Media
EDTA/EGTA
CD18 mAb
CTLA4Ig

as well as 60.3 or EDTA/EGTA (Fig. 4.6, Table VI). Since it was possible that CTLA4Ig reduced aggregation of the B lineage cells by interacting with CD80, we compared the ability of the CD80 mAb, L307, and of DS2 to inhibit aggregation. Neither L307 nor the DS2 fusion protein had any effect on PMA-induced B cell aggregation (Table VI) again suggesting that CTLA4Ig inhibits B cell aggregation by binding to CD11a/18.

Discussion

CTLA-4, previously identified as a ligand for CD80, and CD86, shares functionally significant regions with CD28, including the regions required for binding to CD80 and CD86 (Harper *et al.*, 1991; Linsley *et al.*, 1991a; Linsley *et al.*, 1991b; Azuma *et al.*, 1993a; Hathcock *et al.*, 1993). However, CTLA-4 differs from CD28 not only in 70% of its amino acid sequence but also in that it can bind to CD11a/18. Although CTLA4Ig and DS2, which contains the central Ig-like domain of CD28, both bind to CD80, only CTLA4Ig can bind to CD11a/18 and inhibit CD18-dependent aggregation of lymphocytes. Thus, the site within the extracellular Ig-like domain of CTLA-4 that is required for binding to CD11a/18 is distinct from the site required for binding to CD80.

The CTLA-4–CD11a/18 interaction was not detected previously most likely because the PM line used in previous studies expresses much less CD11a/18, and higher levels of CD80 than CESS cells; after long exposures of PM cell precipitates it was difficult to identify the additional protein bands on films. Alternatively, the preparations of CTLA4Ig used in the previous study

Figure 4.6. CTLA4Ig inhibits CD18-dependent B cell aggregation. Daudi B cells were treated for 24 hr with media alone or with 3.2 ng/ml PMA to induce CD18- dependent aggregation. 5 mM EDTA/EGTA, or 10 μg/ml of anti-CD18, human IgG, or CTLA4Ig were added to inhibit the PMA-induced aggregation. The cells were resuspended and allowed to settle at least 30 min before being scored and photographed. The samples were scored as in Table VI: Medium, 1; PMA alone, 4; or with human IgG, 4; CTLA4Ig, 2; mouse IgG, 4; anti-CD18, 0. Magnification 100x.

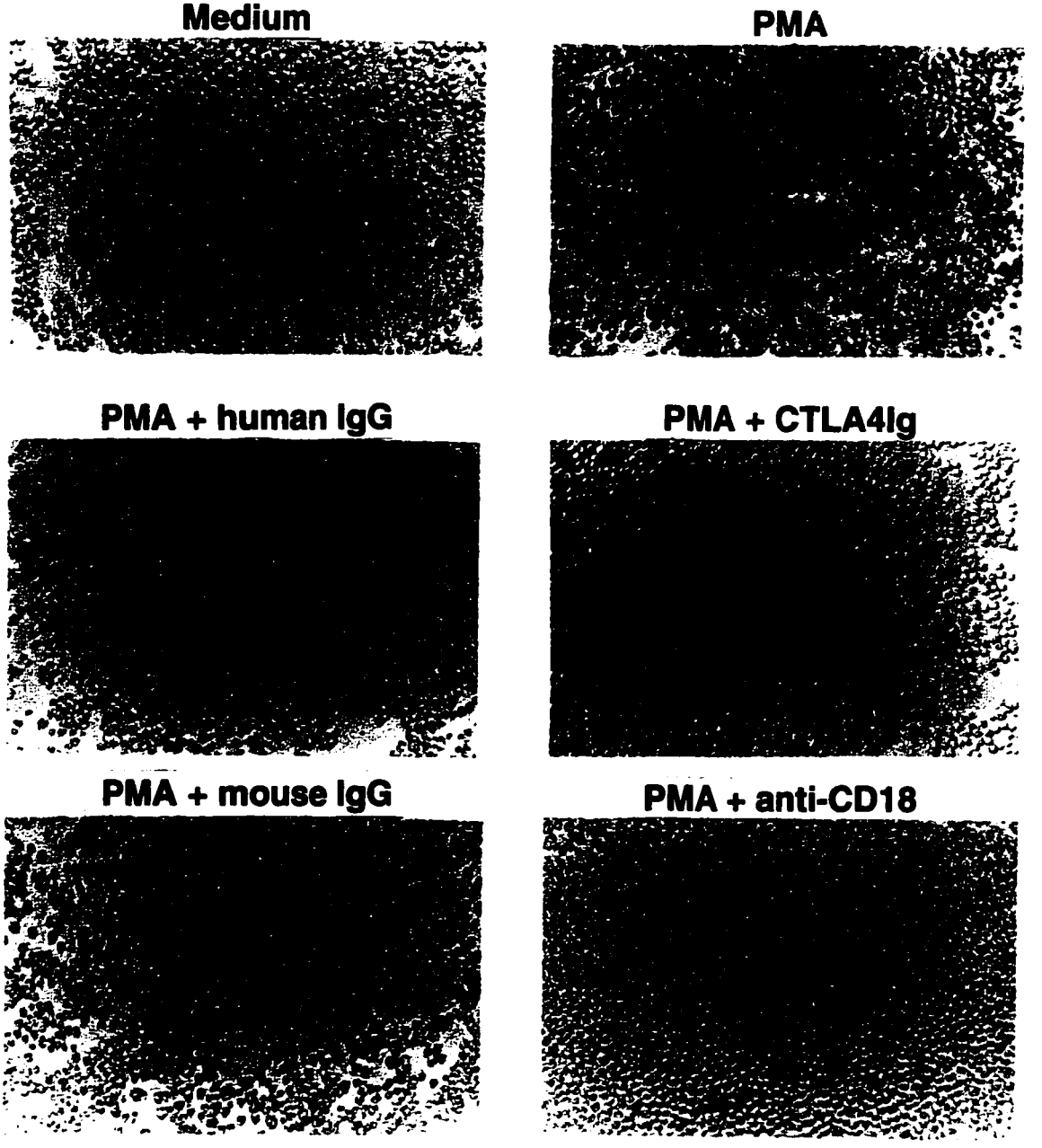

Medium
PMA
PMA + human IgG
PMA + CTLA4Ig
PMA + mouse IgG
PMA + anti-CD18

Table VI. CTLA4Ig Inhibits CD18-dependent B Cell Aggregation.

Treatment[a]		Aggregation Score[b]				
PMA	inhibitor	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5
–	None	1	0	1	1	1
+	None	4	4	4	4	4
+	EDTA/EGTA	0	0	0	0	0
+	mouse IgG	4	4	4	4	4
+	anti-CD18	2	1	1	1	0
+	anti-CD80	4	4	ND	ND	ND
+	human IgG	3	4	4	4	4
+	CTLA4Ig	2	1	2	2	2
+	DS2	ND	ND	ND	4	4

[a]Daudi cells were cultured at 0.15 x 10^6 cells/well in 200 µl of RPMI-10 with 3.2 ng/ml PMA, 5 mM each EDTA and EGTA, or 10 µg/ml of anti-CD18, anti-CD80, mouse IgG, human IgG, or the indicated fusion protein. After 24 hr the cells were resuspended by being pipetted three times through a 200 µl tip, 1 cycle/s, and allowed to settle for at least 30 min before being scored.

[b]The aggregation was scored on a scale of 0 to 4 as follows: 0, no cell aggregation; 1, 1-10% of the cells were in clumps; 2, 11-30% in clumps; 3, 30-80% in clumps; 4, greater than 80% in clumps. ND indicates not tested.

may not have had any binding activity to CD11a/18, as is the case with several lots of CTLA4Ig. The possibility that the CD11a/18 precipitated by CTLA4Ig was due to a non-specific interaction is highly unlikely since the L307 mAb or CD28Ig did not precipitate CD11a/18 (Fig. 4.1A), and binding to CD11a/18 requires the extracellular Ig-like domain of CTLA-4 (Fig. 4.3C). Furthermore, the ability of unlabeled CTLA4Ig to compete for the binding sites of biotin-CTLA4Ig on Hut 78 cells indicates that it binds specifically (Fig. 4.5B).

The possibility that CTLA-4 does not interact directly with CD11a/18 but precipitates it because of its proximity to CD80 is also unlikely. First, we used 0.5% NP-40 in our lysis buffer and wash buffer, under the same conditions we have used previously to disassociate non-covalent but closely associated molecules (Leprince *et al.*, 1993). Second, CTLA4Ig precipitated CD11a/18 from and bound to CD11a/18 on cells that did not express any detectable CD80 (Fig. 4.1B and Fig. 4.3). Finally, the DS2 chimeric fusion protein did not bind to surface CD11a/18 or precipitate CD11a/18 from cells under conditions that CTLA4Ig did (Fig. 4.3C).

CTLA4Ig precipitates only a fraction of the total CD11a/18 present in the lysates (Fig. 4.2), which could be caused by several mechanisms. If the binding of CTLA4Ig to CD80 prevented CTLA4Ig from binding to CD11a/18, then we would expect CTLA4Ig to precipitate a higher fraction of CD11a/18 from lines that are $CD80^-$. This is clearly not the case: CTLA4Ig only precipitates a small fraction of CD11a/18 from Hut 78 cells (Fig. 4.1B). It is possible that CTLA4Ig

binds to a particular subset of CD11a/18, such as an "activated" form, but we have been unable to alter CTLA4Ig binding to Hut 78 and normal T cells with PMA, Mg^{++}, or Ca^{++} (data not shown), all of which have been reported to increase ligand binding or mAb binding to CD11a/18 (Barrett *et al.*, 1991; Dransfield *et al.*, 1992; Rothlein and Springer, 1986). The most likely cause for the small fraction of total CD11a/18 precipitated with CTLA4Ig is a low avidity for CD11a/18 relative to the mAbs used. This is further supported by the incomplete inhibition of CD18-dependent aggregation with CTLA4Ig as compared with anti-CD18 (Fig. 4.6, Table VI). Even though we did not directly measure the affinity of CTLA4Ig for CD11a/18, the CTLA4Ig binding titration to Hut 78 in Figure 4.4 suggests that the CTLA4Ig–CD11a/18 interaction is a relatively low-affinity interaction.

CD54 binding to CD11a/18 is dependent on divalent cations (Dransfield *et al.*, 1992; Rothlein and Springer, 1986), whereas, CTLA4Ig binding to CD11a/18 is not (Fig. 4.5), suggesting that CTLA-4 binds to a unique epitope of CD11a/18. CTLA-4 and the three other CD11a/18 ligands, CD54, CD102, or CD50 (ICAM-1, -2, -3, respectively), each have immunoglobulin superfamily domains involved in binding to CD11a/18. However, CTLA-4 has no obvious regions of sequence identity with these other ligands suggesting that CTLA-4 may interact with CD11a/18 through a novel epitope. In this study we examined only the interaction of CTLA-4 with CD11a/18, and did not address the precise role of the CD11a or the CD18 chains. Thus it remains to be

determined whether CTLA-4 can interact with the other CD18 integrin pairs, CD11b/18 and CD11c/18.

The initial activation of T cells by APC leads to engagement of the antigen receptor on T cells and MHC class II on the APC, which in turn induces expression of CD40 ligand (Spriggs *et al.*, 1992) and CD80 (Koulova *et al.*, 1991), respectively. Engagement of CD40 on the B cell by CD40 ligand (Barrett *et al.*, 1991; Ranheim and Kipps, 1993) and of CD28 on the T cell by CD80 (Linsley and Ledbetter, 1993; Linsley *et al.*, 1991a) then can further augment B and T cell proliferation. This reciprocal dialogue (Clark and Ledbetter, 1994) may be regulated, *e.g.*, by other cell-cell interactions or by cytokines. The fact that CTLA-4 is expressed after T cell activation (Linsley *et al.*, 1992a) and can bind to CD11a/18 in addition to CD80 and CD86 suggests that it functions differently than does CD28 to regulate the T cell-APC dialogue. Moy and Brian (1992) demonstrated that CD11a/18 stimulation enhanced the APC function of B cells; CTLA-4 may be able to provide this stimulus to the APC by directly stimulating CD11a/18.

I have subsequently tested 7 lots of CTLA4Ig, including an independently cloned construct, in addition to the three used in these studies to characterize CTLA-4 binding to CD11a/18. I found that the binding of CTLA4Ig to CD11a/18 was not reproducible with these additional lots of fusion proteins. While this prevents subsequent studies necessary to characterize this unique interaction, we cannot rule out the possibility that CTLA-4 and CD11a/18 are ligands. I can

rule out contamination as a possible explanation for several reasons. First, CD80 CHO cells (CD11a/18$^-$) were able to absorb all CD11a/18 binding reactivity to Hut 78 cells from samples of CTLA4Ig, ruling out a contaminating anti-CD11a/18 mAb. Using goat anti-human IgG-FITC, I could detect CTLA4Ig binding to Hut 78, but I could not detect any binding of mouse mAbs to CD18 with the goat anti-human IgG-FITC. Furthermore, ICAMIg fusion proteins do not display any detectable binding to CD11a/18 molecules expressed on cells (J.A. Ledbetter, personal communication). These results suggest that CD80 and CD11a/18 binding were both mediated by the same protein and there were no detectable mAbs or other fusion proteins contaminating the CTLA4Ig preparations used.

Since CTLA4Ig binding to CD11a/18 is dependent upon the individual preparation of fusion protein, it is unlikely that the binding is strictly dependent upon the protein sequence, but may be altered by the presence of particular carbohydrate moieties on either molecule. To date, the best explanation for CTLA4Ig binding to CD11a/18 is that there was a specific pattern of glycosylation present on the three lots of CTLA4Ig which bound CD11a/18, that is absent on subsequent lots. The difference between the estimated molecular weight of CD11a/18, 129/85 kDa compared to the actual molecular weight of 180/95 reveal approximately 60 kDa of carbohydrate reside on CD11a/18. Thus the binding of CTLA4Ig to CD11a/18 in a cation-independent manner may be attributed to specific carbohydrate residues on CTLA4Ig binding those on

CD11a/18. While this raises concerns about using recombinant proteins to define protein-protein interactions, we cannot rule out the possibility that the binding is relevant *in vivo*.

Chapter 5: Concluding Remarks

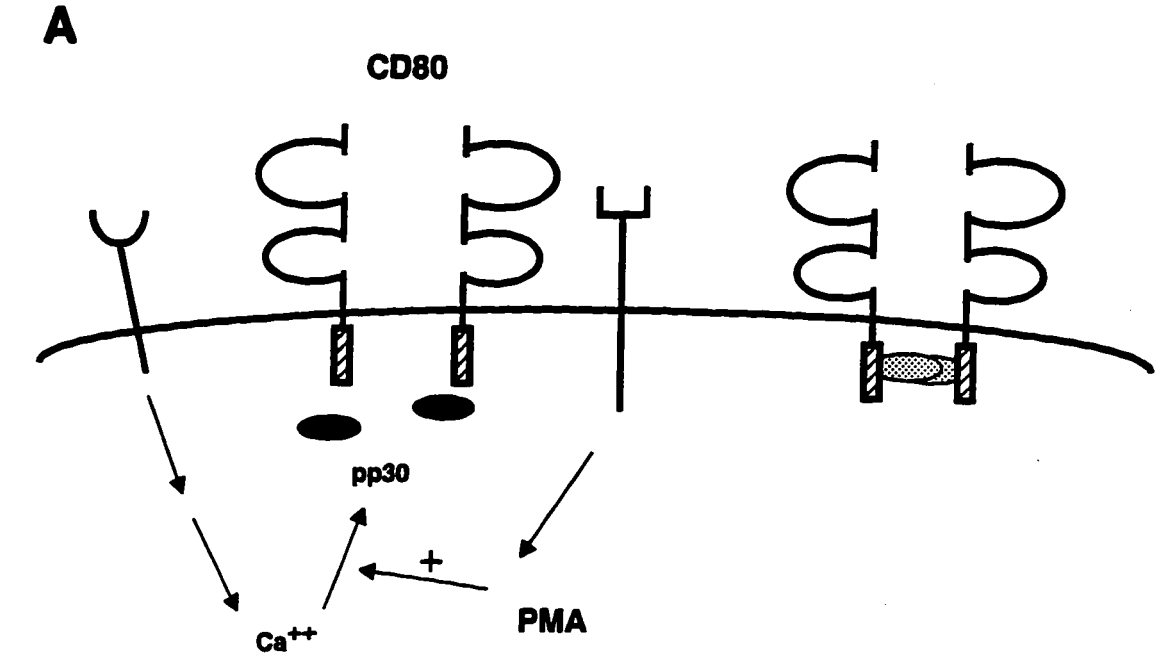
As I have shown in the preceding chapters, CD80 binding to CD28 is clearly insufficient to initiate CD28 costimulation. CD80 must be redistributed, thereby aggregating CD28 and inducing costimulation. While it is clear that CD80 must be redistributed into caps or patches, the specifics of CD80 redistribution are still unknown. Transfectants expressing mutant CD80 molecules (R80/4A and R80/SA) do not costimulate T cells, although they do cap and patch CD80 similar to cells expressing wildtype CD80, but at a significantly slower rate. This suggests there is a threshold rate of CD80 capping required to maintain engagement of CD28 and induce costimulatory signals. The avidities of CTLA-4 and CD28 for CD80 and CD86 are dramatically altered by oligomerization of CD80 or CD86 (Greene *et al.*, 1996). Slow or inadequate patching and capping of CD80 may directly result in insufficient avidity between CD28 and CD80; thus, CD28 aggregation does not reach a threshold required to induce signaling.

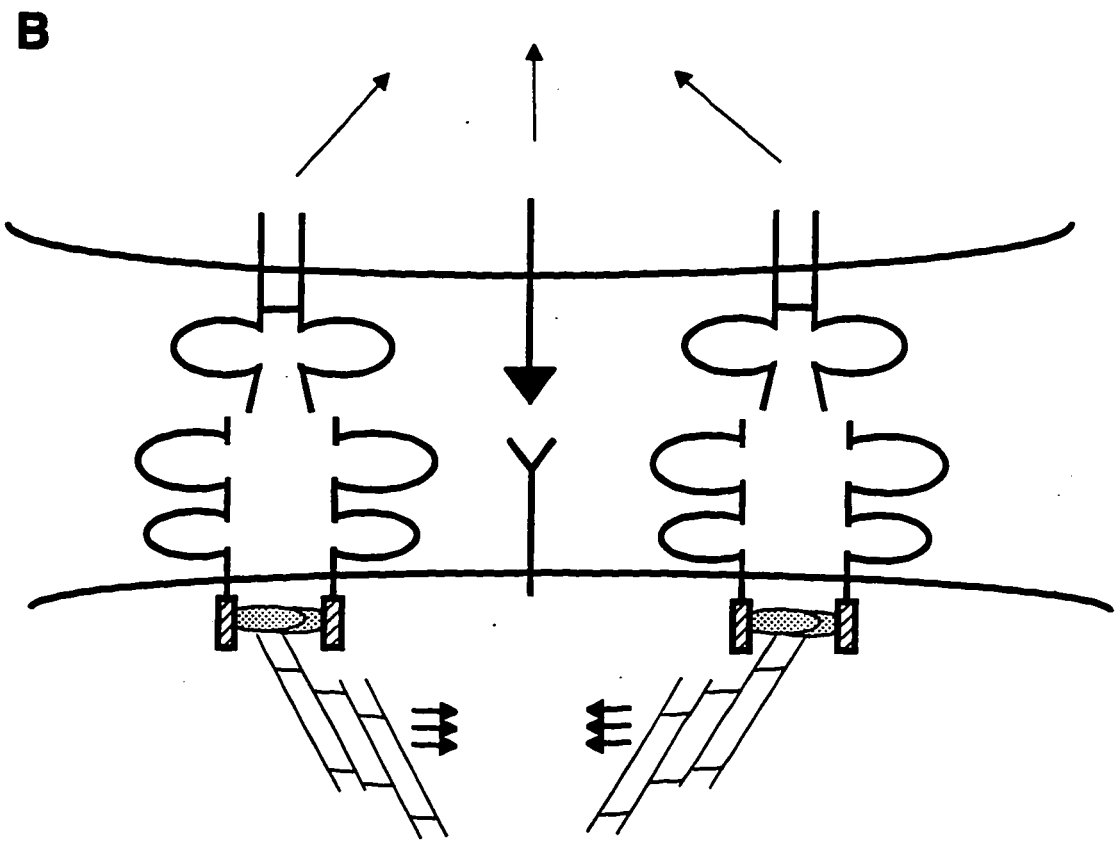
While the foci of CD80 may have sufficient numbers of molecules to maintain high avidity binding to CD28, CD80 is monomeric, thus there is no inherent ability for it to form rigid oligomers required to aggregate CD28. Thus, another possibility is that proteins which interact with the cytoplasmic tail of CD80, such as pp30, maintain or induce large patches of CD80 such that it can aggregate CD28 and induce signaling. Individual foci of CD80 are incapable of inducing CD28 aggregation and signaling. The interaction of the cytoplasmic

tail of CD80 with cytosolic proteins, and indirectly with components of the actin cytoskeleton, is required to form the rigid oligomers of CD80 necessary to aggregate CD28.

It is most likely a combination of these two mechanisms which actually occurs. A CD28 homodimer binds to CD80 monomers present in foci of CD80 with high avidity. Antigen receptor initiated (calcium dependent) signals will induce CD80 to interact with pp30 (Fig. 5.1A). Independent signals, mimicked by PMA, will enhance the CD80-pp30 association. The association of CD80 with pp30 will dramatically alter the nature of the CD80 foci from a loosely associated group of CD80 molecules to a tightly associated CD80 oligomer. This rigid oligomer of CD80 binds to CD28 with high avidity and interacts with the actin cytoskeleton. Once CD80 has formed a stable interaction with pp30 and the cytoskeleton, it redistributes towards the site of B cell-T cell contact, which is the site of the cognate MHC-TCR interaction (Fig. 5.1B). Once CD80 has sufficiently aggregated CD28, it will initiate signal transduction. Thus, if CD80 does not redistribute fast enough to initiate CD28-dependent signals before the T cell and APC disengage, there will be no costimulation. If CD28 signaling occurs in close proximity to the TCR/CD3 complex, it may directly modify CD3-mediated proximal signal events in addition to generating CD3-independent signals. If this model is correct, then it may be possible to enhance antigen presentation by crosslinking APC cell surface molecules which increase intracellular calcium levels. Thus, crosslinking IgG on the CESS cell line, or

Figure 5.1. Regulation of CD80 mediated costimulation.
(A) CD80 molecules are loosely associated in foci (left). If the APC has encountered a calcium inducing signal such as a signal through surface Ig or MHC Class II, then pp30 will tightly associate with CD80 (right). Accessory molecule signals, such as CD40, mediated through phosphorylation, mimicked by PMA, will increase the pp30-CD80 association. (B) pp30 associated CD80 forms rigid oligomers with high avidity for ligand and associate with the actin-dependent cytoskeleton. The actin cytoskeleton then redistributes CD80 towards the site of MHC–TCR interaction, aggregating CD28 proximal to the CD3 signaling complex, where CD28 initiates signal transduction.


A
CD80
pp30
+
PMA
Ca++


B

CD19 on the Reh CD80 transfectants may improve their function as APC by increasing cytoplasmic calcium levels and subsequent CD80 association with the actin cytoskeleton.

We do not know yet if the same will hold true for the other CD28 ligand, CD86. I would speculate that it is expressed in foci in a manner similar to CD80, and that it also needs to be relocalized to 'competent' structures to induce costimulation. However, since the cytoplasmic tails of CD80 and CD86 are quite different, it would be likely that CD86 associates with different proteins than does CD80. The regulation of proteins associating with the cytoplasmic tails of CD80 and CD86 may be distinct since CD80 and CD86 are uniquely regulated *in vivo*.

While the roles of CD28 and CTLA-4 signals in regulating T cell development are fairly well understood, it is unclear whether CD80 (or CD86) has direct effects on B cell development. We have been unable to detect traditional signals such as inducible phosphorylation, associated kinases, or changes in intracellular calcium levels after crosslinking CD80. We found CD80 undergoes antibody-mediated actin-dependent redistribution in response to crosslinking (Doty and Clark, 1996; Chapter 2), suggesting that CD80 does induce some signals in B cells. In support of this, Hirokawa *et al.* (1996) recently found that crosslinking CD80 on the B lymphoma line Raji induced tyrosine phosphorylation of several proteins, inhibited cell growth, and induced cell flattening and spreading. This is the first evidence of direct signal

transduction to the APC by CD80; however, none of the effects was unique to crosslinking CD80, as very similar results were obtained by crosslinking CD29.

This study provides new insights into old experiments, making it possible to reevaluate some experiments which now have alternative interpretations. Sethna *et al.* (1994) found that B7-1 (CD80) transgenic mice have markedly reduced humoral responses to T dependent antigens. The T cells in these mice are primed normally *in vivo* to TNP-KLH, and the B cells are potent *in vitro* stimulators of allogeneic MLR. The authors' interpretation of these results is that chronic costimulation may either inhibit T cell help, or induce CTL responses against the B cells which chronically over-express CD80. It is unlikely that T cells are being inhibited since the T cells respond normally *ex vivo*. They dismiss the possibility that CD28 ligation of CD80 may directly inhibit B cell responses, which is a possible interpretation in light of the findings by Hirokawa *et al.* (1996).

The presence of CD80 but not CD86 on centroblasts in the dark zone of the germinal center (Vyth-Dreese *et al.,* 1995) suggests CD80 plays a role in B cell development independent of its role as a costimulatory molecule for T cell growth. There are relatively few T cells present in germinal centers and because of this, centroblasts probably are not involved in presenting antigen to T cells to induce T cell growth. If CD80 crosslinking does indeed inhibit B cell proliferation (Hirokawa *et al.,* 1996), it is possible to speculate that $CD80^+$

centroblasts encounter CD28-expressing cells and drop out of cell cycle. The cells then migrate to the marginal light zone where as non-cycling centrocytes, they stop expressing CD80 and begin to express CD86. T cells, which are present in the germinal center in very few numbers, or plasma cells, which express CD28 (Kozbor *et al.*, 1987), may be the CD28-bearing cells present in the germinal center which bind to $CD80^+$ centroblasts. The defect in humoral responses in CD80 transgenic mice (Sethna *et al.*, 1994) may be a direct effect of CD80-mediated signals inhibiting B cell expansion. If this is the case and CD80 is constitutively expressed at high levels on B cells during the initial activation, CD28 ligation of CD80 may inhibit the cells from undergoing clonal expansion, somatic hypermutation, and class switching, all of which occur in the dark zone of the germinal center (MacLennan, 1994). This is consistent with the phenotype of the CD80 transgenic mice (Sethna *et al.*, 1994).

Recently Borriello *et al.* (1997) found that serum IgM, IgG_1, and IgG_{2a} levels to T-dependent antigens were relatively normal in CD80 deficient mice, suggesting that CD80 does not play a direct role in class switching. However, they only examined serum Ig titers from mice 7 days or more after immunization. Thus, it is possible that there are differences in the kinetics of the antibody response in CD80 deficient mice, present at earlier times. While this study doesn't directly support a role for CD80 in centroblast development, it doesn't exclude the possibility. Analysis of CD80 and CD86 deficient mice revealed CD80 and CD86 have similar roles in humoral immunity *in vivo*. The

one major difference was that CD86 deficient mice were severely compromised in class switching in response to intravenous antigen without adjuvant (Borriello *et al.*, 1997). Immunization with adjuvant, or subcutaneously without adjuvant revealed no significant differences in serum Ig levels among wildtype, $CD80^{-/-}$ or $CD86^{-/-}$ mice. This is greatly different than what was observed in mice deficient in both CD80 and CD86. In these mice, there were no significant IgG_1 or IgG_{2a} responses to T-dependent antigen. Furthermore, spleens from these mice had primary follicles but no germinal centers. This phenotype is remarkably similar to the phenotype of mice deficient in CD28 expression, and to mice with defects in the CD40/CD40L pathway (Ferguson *et al.*, 1996; Klaus *et al.*, 1994). These results strongly support the reciprocal dialog model of T cell-B cell collaboration (Clark and Ledbetter, 1994). Interfering with any number of receptor-ligand interactions will prevent or compromise all subsequent events in normal immune responses, and signals to both the T cell and the B cell are crucial for normal cell activation and induction of the immune response.

The fact that CD80/CD86 deficient mice have a phenotype very similar to that of CD28 deficient mice lends no support to the possibility of additional ligands for CD28 (Borriello *et al.*, 1997). While it is still formally possible that additional CD28 ligands exist, it is unlikely that these ligands, if they exist, have a significant impact on CD28-mediated costimulation in peripheral immunity. Because of the complexity of the CD80/86–CD28/CTLA-4 pathways, it is very difficult to examine the role of potential CD80 signals in B cell

development. By deleting CD80 and CD86, T cell helper functions such as cytokine secretion are dramatically reduced, directly affecting B cell growth. It is possible to make mice which express chimeric CD80 molecules by replacing exons encoding the transmembrane and cytoplasmic tail of CD80 with those of other molecules such as CD54 or CD2. The chimeric molecules would be expressed just as wildtype CD80 and they are still able to induce CD28-mediated costimulation (Doty and Clark, 1996). However, they would not be able to induce any CD80 specific signals back into the APC. Studies of mice expressing these chimeric molecules may provide clues to what the role of the cytoplasmic tail of CD80 is in B cell development.

References

Aversa, G., Chang, C.C.J., Carballido, J.M., Cocks, B.G., and de Vries, J.E. (1997). Engagement of the signaling lymphocytic activation molecule (SLAM) on activated T cells results in IL-2-independent, cyclosporin A-sensitive T cell proliferation and IFN-γ production. J. Immunol. *158*, 4036-4044.

Azuma, M., Cayabyab, M., Buck, D., Phillips, J.H., and Lanier, L.L. (1992). Involvement of CD28 in MHC-unrestricted cytotoxicity mediated by a human natural killer leukemia cell line. J. Immunol. *149*, 1115-1123.

Azuma, M., Ito, D., Yagita, H., Okumura, K., Phillips, J.H., Lanier, L.L., and Somoza, C. (1993a). B70 antigen is a second ligand for CTLA-4 and CD28. Nature *366*, 76-79.

Azuma, M., Yssel, H., Phillips, J.H., Spits, H., and Lanier, L.L. (1993b). Functional expression of B7/BB1 on activated T lymphocytes. J. Exp. Med. *177*, 845-850.

Bajorath, J., Peach, R.J., and Linsley, P.S. (1994). Immunoglobulin fold characteristics of B7-1 (CD80) and B7-2 (CD86). Protein Sci. *3*, 2148-2150.

Banchereau, J., dePaoli, P., Vallé, A., Garcia, E., and Rousset, F. (1991). Long-term human B cell lines dependent on interleukin-4 and antibody to CD40. Science *251*, 70-72.

Barnstable, C.J., Bodmer, W.F., Brown, G., Galfre, G., Milstein, C., Williams, A.F., and Ziegler, A. (1978). Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens--new tools for genetic analysis. Cell *14*, 9-20.

Barrett, T.B., Shu, G., and Clark, E.A. (1991). CD40 signaling activates CD11a/CD18 (LFA-1) mediated adhesion in B cells. J. Immunol. *146*, 1722-1729.

Beatty, P.G., Ledbetter, J.A., Martin, P.J., Price, T.H., and Hansen, J.A. (1983). Definition of a common leukocyte cell-surface antigen (Lp95-150) associated with diverse cell-mediated immune functions. J. Immunol. *131*, 2913-2918.

Beatty, P.G., Ochs, H.D., Harlan, J.M., Price, T.H., Rosen, H., Taylor, R.F., Hansen, J.A., and Klebanoff, S. (1984). Absence of monoclonal-antibody-defined protein complex in boy with abnormal leukocyte function. Lancet *1*, 535-537.

Berberich, I., Shu, G., and Clark, E.A. (1994). Crosslinking CD40 rapidly induces NF-κB in B cells. J. Immunol. *153*, 4357-4366.

Borriello, F., Freeman, G.J., Edelhoff, S., Disteche, C.M., Nadler, L.M., and Sharpe, A.H. (1994). Characterization of the murine B7-1 genomic locus reveals an additional exon encoding an alternative cytoplasmic domain and a chromosomal location of chromosome 16, band B5. J. Immunol. *153*, 5038-5048.

Borriello, F., Sethna, M.P., Boyd, S.D., Schweitzer, A.N., Tivol, E.A., Jacoby, D., Strom, T.B., Simpson, E.M., Freeman, G.J., and Sharpe, A.H. (1997). B7-1 and B7-2 have overlapping, critical roles in immunoglobulin class switching and germinal center formation. Immunity *6*, 303-313.

Boussiotis, V.A., Freeman, G.J., Gribben, J.G., Daley, J., Gray, G., and Nadler, L.M. (1993). Activated human B lymphocytes express three CTLA-4 counterreceptors that costimulate T-cell activation. Proc. Natl. Acad. Sci. USA *90*, 11059-11063.

Boussiotis, V.A., Freeman, G.J., Griffin, J.D., Gray, G.S., Gribben, J.G., and Nadler, L.M. (1994). CD2 is involved in maintenance and reversal of human alloantigen-specific clonal anergy. J. Exp. Med. *180*, 1665-1673.

Bretscher, P.A. and Cohn, M. (1970). A theory of self-nonself discrimination. Science *169*, 1042-1049.

Brunet, J.-F., Denizot, F., Luciani, M., Roux-Dosseto, M., Suzan, M., Mattei, M.-G., and Golstein, P. (1987). A new member of the immunoglobulin superfamily-CTLA-4. Nature *328*, 267-270.

Brunschwig, E.B., Levine, E., Trefzer, U., and Tykocinski, M.L. (1995). Glycosylphosphatidylinositol-modified murine B7-1 and B7-2 retain costimulator function. J. Immunol. *155*, 5498-5505.

Buelens, C., Willems, F., Delvaux, A., Pierard, G., Delville, J.-P., Velu, T., and Goldman, M. (1995). Interleukin-10 differentially regulates B7-1 (CD80) and B7-2 (CD86) expression on human peripheral blood dendritic cells. Eur. J. Immunol. *25*, 2668-2672.

Cambier, J.C., Pleiman, C.M., and Clark, M.R. (1994). Signal transduction by the B cell antigen receptor and its coreceptors. Ann. Rev. Immunol. *12*, 457-486.

Carpén, O., Pallai, P., Staunton, D.E., and Springer, T.A. (1992). Association of intracellular adhesion molecule-1 (ICAM-1) with actin-containing cytoskeleton and α-actinin. J. Cell Biol. *118*, 1223-1234.

Cassell, D.J. and Schwartz, R.H. (1994). A quantitative analysis of antigen-presenting cell function: activated B cells stimulate naive CD4 T cells but are inferior to dendritic cells in providing costimulation. J. Exp. Med. *180*, 1829-1840.

Chapin, S.J., Enrich, C., Aroeti, B., Havel, R.J., and Mostov, K.E. (1996). Calmodulin binds to the basolateral targeting signal of the polymeric immunoglobulin receptor. J. Biol. Chem. *271*, 1336-1342.

Chen, L., Ashe, S., Brady, W.A., Hellström, I., Hellström, K.E., Ledbetter, J.A., McGowan, P., and Linsley, P.S. (1992). Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA-4. Cell *71*, 1093-1102.

Chen, L., McGowan, P., Ashe, S., Johnson, J., Li, Y., Hellstrom, I., and Hellstrom, K.E. (1994). Tumor immunogenicity determines the effect of B7 costimulation on T cell-mediated tumor immunity. J. Exp. Med. *179*, 523-532.

Clark, E.A., Ledbetter, J.A., Holly, R.C., Dinndorf, P.A., and Shu, G. (1986). Polypeptides on human B cells associated with cell activation. Hum. Immunol. *16*, 100-113.

Clark, E.A. and Ledbetter, J.A. (1986). Activation of human B cells mediated through two distinct cell surface differentiation antigens, Bp35 and Bp50. Proc. Natl. Acad. Sci. USA *83*, 4494-4498.

Clark, E.A. and Ledbetter, J.A. (1994). How B and T cells talk to each other. Nature *367*, 425-428.

Cocks, B.G., Chang, C.C., Carballido, J.M., Yssel, H., de Vries, J.E., and Aversa, G. (1995). A novel receptor involved in T-cell activation. Nature *376*, 260-263.

Coligan, J.E., Kruisbeek, A.M., Marguelies, D.H., Shevach, E.M., and Strober, W. (1991). Current Protocols in Immunology (New York: John Wiley & Sons).

Croft, M., Duncan, D.D., and Swain, S.L. (1992). Response of naive antigen-specific $CD4^+$ T cells *in vitro*: characteristics and antigen-presenting cell requirements. J. Exp. Med. *176*, 1431-1437.

Damle, N.K., Doyle, L.V., Grosmaire, L.S., and Ledbetter, J.A. (1988). Differential regulatory signals delivered by antibody binding to the CD28 (Tp44) molecule during the activation of human T lymphocytes. J. Immunol. *140*, 1753-1761.

Damle, N.K., Linsley, P.S., and Ledbetter, J.A. (1991). Direct helper T cell-induced B cell differentiation involves interaction between T cell antigen CD28 and B cell activation antigen B7. Eur. J. Immunol. *21*, 1277-1282.

Damle, N.K., Klussman, K., and Aruffo, A. (1992a). Intercellular adhesion molecule-2, a second counter-receptor for CD11a/CD18 (leukocyte function-associated antigen-1), provides a costimulatory signal for T-cell receptor-initiated activation of human T cells. J. Immunol. *148*, 665-671.

Damle, N.K., Klussman, K., Linsley, P.S., and Aruffo, A. (1992b). Differential costimulatory effects of adhesion molecules B7, ICAM-1, LFA-3, and VCAM-1 on resting and antigen-primed $CD4^+$ T lymphocytes. J. Immunol. *148*, 1985-1992.

Damle, N.K. and Doyle, L.V. (1989). Stimulation via the CD2 and CD28 molecules induces responsiveness to IL-4 in $CD4^+CD29^+CD45R^-$ memory T lymphocytes. J. Immunol. *143*, 1761-1767.

Dariavach, P., Mattei, M.G., Golstein, P., and Lefranc, M.P. (1988). Human Ig superfamily CTLA-4 gene: chromosomal localization and identity of protein sequence between murine and human CTLA-4 cytoplasmic domains. Eur. J. Immunol. *18*, 1901-1905.

DeLisser, H.M., Chilkotowsky, J., Yan, H.-C., Daise, M.L., Buck, C.A., and Albelda, S.M. (1994). Deletions in the cytoplasmic domain of Platelet-Endothelial Cell Adhesion Molecule-1 (PECAM-1, CD31) result in changes in ligand binding properties. J. Cell Biol. *124*, 195-203.

Doty, R.T. and Clark, E.A. (1996). Subcellular Localization of CD80 Receptors Is Dependent on an Intact Cytoplasmic Tail and Is Required for CD28-Dependent T Cell Costimulation. J. Immunol. *157*, 3270-3279.

Dransfield, I., Cabañas, C., Craig, A., and Hogg, N. (1992). Divalent cation regulation of the function of the leukocyte integrin LFA-1. J. Cell Biol. *116*, 219-226.

Ehlin-Henriksson, B., Clark, E.A., Jonsson, V., and Klein, G. (1983). Studies on the B lymphoblast antigen No. 1 (BB-1) on a series of Burkitt lymphoma lines

differing in the expression of the EBV/C3 receptor complex. J. Immunol. *130*, 2448-2452.

Fargeas, C.A., Truneh, A., Reddy, M., Hurle, M., Sweet, R., and Sékaly, R.-P. (1995). Identification of residues in the V domain of CD80 (B7-1) implicated in functional interactions with CD28 and CTLA4. J. Exp. Med. *182*, 667-675.

Feramisco, J.R., Glass, D.B., and Krebs, E.G. (1980). Optimal spatial requirements for the location of basic residues in peptide substrates for the cyclic AMP-dependent protein kinase. J. Biol. Chem. *255*, 4240-4245.

Ferguson, S.E., Han, S., Kelsoe, G., and Thompson, C.B. (1996). CD28 is required for germinal center formation. J. Immunol. *156*, 4576-4581.

Fraser, J.D., Irving, B.A., Crabtree, G.R., and Weiss, A. (1991). Regulation of interleukin-2 gene enhancer activity by the T cell accessory molecule CD28. Science *251*, 313-316.

Freedman, A.S., Freeman, G.J., Horowitz, J.C., Daley, J., and Nadler, L.M. (1987). B7, a B-cell-restricted antigen that identifies preactivated B cells. J. Immunol. *139*, 3260-3267.

Freedman, A.S., Freeman, G.J., Rhynhart, K., and Nadler, L.M. (1991). Selective induction of B7/BB-1 on interferon-gamma stimulated monocytes: a potential mechanism for amplification of T cell activation through the CD28 pathway. Cell. Immunol. *137*, 429-437.

Freeman, G.J., Freedman, A.S., Segil, J.M., Lee, G., Whitman, J.F., and Nadler, L.M. (1989). B7, a new member of the Ig superfamily with unique expression on activated and neoplastic B cells. J. Immunol. *143*, 2714-2722.

Freeman, G.J., Gray, G.S., Gimmi, C.D., Lombard, D.B., Zhou, L.J., White, M., Fingeroth, J.D., Gribben, J.G., and Nadler, L.M. (1991). Structure, expression, and T cell costimulatory activity of the murine homologue of the human B lymphocyte activation antigen B7. J. Exp. Med. *174*, 625-631.

Freeman, G.J., Borriello, F., Hodes, R.J., Reiser, H., Gribben, J.G., Ng, J.W., Kim, J., Goldberg, J.M., Hathcock, K., Laszlo, G., Lombard, L.A., Wang, S., Gray, G.S., Nadler, L.M., and Sharpe, A.H. (1993a). Murine B7-2, an alternative CTLA4 counter-receptor that costimulates T cell proliferation and interleukin 2 production. J. Exp. Med. *178*, 2185-2192.

Freeman, G.J., Borriello, F., Hodes, R.J., Reiser, H., Hathcock, K.S., Laszlo, G., McKnight, A.J., Kim, J., Du, L., Lombard, D.B., Gray, G.S., Nadler, L.M., and Sharpe, A.H. (1993b). Uncovering of functional alternative CTLA-4 counter-receptor in B7-deficient mice. Science *262*, 907-909.

Freeman, G.J., Gribben, J.G., Boussiotis, V.A., Ng, J.W., Restivo, V.A.J., Lombard, L.A., Gray, G.S., and Nadler, L.M. (1993c). Cloning of B7-2: a CTLA-4 counter-receptor that costimulates human T cell proliferation. Science *262*, 909-911.

Freeman, G.J., Boussiotis, V.A., Anumanthan, A., Bernstein, G.M., Ke, X.Y., Rennert, P.D., Gray, G.S., Gribben, J.G., and Nadler, L.M. (1995). B7-1 and B7-2 do not deliver identical costimulatory signals, since B7-2 but not B7-1 preferentially costimulates the initial production of IL-4. Immunity *2*, 523-532.

Gillis, S., Ferm, M.M., Ou, W., and Smith, K.A. (1978). T cell growth factor: parameters of production and a quantitative microassay for activity. J. Immunol. *120*, 2027-2032.

Gimmi, C.D., Freeman, G.J., Gribben, J.G., Gray, G., and Nadler, L.M. (1993). Human T-cell clonal anergy is induced by antigen presentation in the absence of B7 costimulation. Proc. Natl. Acad. Sci. USA *90*, 6586-6590.

Glass, D.B., El-Maghrabi, M.R., and Pilkis, S.J. (1986). Synthetic peptides corresponding to the site phosphorylated in 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase as substrates of cyclic nucleotide-dependent protein kinases. J. Biol. Chem. *261*, 2987-2993.

Glass, D.B. and Smith, S.B. (1983). Phosphorylation by cyclic GMP-dependent protein kinase of a synthetic peptide corresponding to the autophosphorylation site in the enzyme. J. Biol. Chem. *258*, 14797-14803.

Greene, J.L., Leytze, G.M., Emswiler, J., Peach, R., Bajorath, J., Cosand, W., and Linsley, P.S. (1996). Covalent dimerization of CD28/CTLA-4 and oligomerization of CD80/CD86 regulate T cell costimulatory interactions. J. Biol. Chem. *271*, 26762-26771.

Greenfield, E.A., Howard, E., Paradis, T., Nguyen, K., Benazzo, F., McLean, P., Hollsberg, P., Davis, G., Hafler, D.A., Sharpe, A.H., Freeman, G.J., and Kuchroo, V.K. (1997). B7.2 expressed by T cells does not induce CD28-mediated costimulatory activity but retains CTLA4 binding. J. Immunol. *158*, 2025-2034.

Gribben, J.G., Freeman, G.J., Boussiotis, V.A., Rennert, P., Jellis, C.L., Greenfield, E., Barber, M., Restivo, Jr., Ke, X., Gray, G.S., and Nadler, L.M. (1995). CTLA4 mediates antigen-specific apoptosis of human T cells. Proc. Natl. Acad. Sci. USA *92*, 811-815.

Griffith, T.S., Brunner, T., Fletcher, S.M., Green, D.A., and Ferguson, T.A. (1995). Fas ligand-induced apoptosis as a mechanism of immune privilege. Science *270*, 1189-1192.

Guerder, S., Meyerhoff, J., and Flavell, R. (1994). The role of the T cell costimulator B7-1 in autoimmunity and the induction and maintenance of tolerance to peripheral antigen. Immunity *1*, 155-166.

Hansen, J.A., Martin, P.J., and Nowinski, R.C. (1980). Monoclonal antibodies identifying a novel T cell antigen and Ia antigens of human lymphocytes. Immunogenetics *10*, 247-260.

Hansen, J.A., Martin, P.J., Beatty, P.G., Clark, E.A., and Ledbetter, J.A. (1984). Human T lymphocyte cell surface molecules defined by the workshop monoclonal antibodies ("T Cell Protocol"). In Leukocyte Typing: Human Leukocyte Differentiation Antigens Detected by Monoclonal Antibodies. A. Bernard, A. Boumodl, J. Danoset, C. Milsteon, and S.F. Schlossman, eds. (New York: Springer Verlag), pp. 195

Harper, K., Balzano, C., Rouvier, E., Mattéi, M.G., Luciani, M.F., and Golstein, P. (1991). CTLA-4 and CD28 activated lymphocyte molecules are closely related in both mouse and human as to sequence, message expression, gene structure, and chromosomal location. J. Immunol. *147*, 1037-1044.

Hathcock, K.S., Laszlo, G., Dickler, H.B., Bradshaw, J., Linsley, P.S., and Hodes, R.J. (1993). Identification of an alternative CTLA-4 ligand costimulatory for T cell activation. Science *262*, 905-907.

Hathcock, K.S., Laszlo, G., Pucillo, C., Linsley, P., and Hodes, R.J. (1994). Comparative analysis of B7-1 and B7-2 costimulatory ligands: expression and function. J. Exp. Med. *180*, 631-640.

Hirokawa, M., Kuroki, J., Kitabayashi, A., and Miura, A.B. (1996). Transmembrane signaling through CD80 (B7-1) induces growth arrest and cell spreading of human B lymphocytes accompanied by protein tyrosine phosphorylation. Immunology Letters *50*, 95-98.

Hood, L., Kronenberg, M., and Hunkapiller, T. (1985). T cell antigen receptors and the immunoglobulin supergene family. Cell *40*, 225-229.

Hurtado, J.C., Kim, Y.-J., and Kwon, B.S. (1997). Signals through 4-1BB are costimulatory to previously activated splenic T cells and inhibit activation-induced cell death. J. Immunol. *158*, 2600-2609.

Inobe, M., Linsley, P.S., Ledbetter, J.A., Nagai, Y., Tamakoshi, M., and Uede, T. (1994). Identification of an alternatively spliced form of the murine homologue of B7. Biochem. Biophys. Res. Commun. *200*, 443-449.

Inobe, M., Aoki, N., Linsley, P.S., Ledbetter, J.A., Abe, R., Murakami, M., and Uede, T. (1996). The role of the B7-1a molecule, an alternatively spliced form of B7-1 (CD80), on T cell activation. J. Immunol. *157*, 582-588.

Isono, T. and Seto, A. (1995). Cloning and sequencing of the rabbit gene encoding T-cell costimulatory molecules. Immunogenetics *42*, 217-220.

Jack, L.J. and Mather, I.H. (1990). Cloning and analysis of cDNA encoding bovine butyrophilin, an apical glycoprotein expressed in mammary tissue and secreted in association with the milk-fat globule membrane during lactation. J. Biol. Chem. *265*, 14481-14486.

Judge, T.A., Liu, M., Christensen, P.J., Fak, J.J., and Turka, L.A. (1995). Cloning the rat homolog of the CD28/CTLA-4-ligand B7-1: structural and functional analysis. Int. Immunol. *7*, 171-178.

Kaufman, J. and Salomonsen, J. (1992). B-G: we know what it is, but what does it do? Immunol. Today *13*, 1-3.

Kawamura, T. and Furue, M. (1995). Comparative analysis of B7-1 and B7-2 expression in Langerhans cells: differential regulation by T helper type 1 and T helper type 2 cytokines. Eur. J. Immunol. *25*, 1913-1917.

Kishimoto, A., Nishiyama, K., Nakanishi, H., Uratsuji, Y., Nomura, H., Takeyama, Y., and Nishizuka, Y. (1985). Studies on the phosphorylation of myelin basic protein by protein kinase c and adenosine 3':5'-monophosphate-dependent protein kinase. J. Biol. Chem. *260*, 12492-12499.

Klaus, S.J., Berberich, I., Shu, G., and Clark, E.A. (1994). CD40 and its ligand in the regulation of humoral immunity. Seminars in Immunol. *6*, 279-286.

Koulova, L., Clark, E.A., Shu, G., and Dupont, B. (1991). The CD28 ligand B7/BB1 provides costimulatory signal for alloactivation of CD4+ T cells. J. Exp. Med. *173*, 759-762.

Koyasu, S., Lawton, T., Novick, D., Recny, M.A., Siliciano, R.F., Wallner, B.P., and Reinherz, E.L. (1990). Role on interaction of CD2 molecules with lymphocyte function-associated antigen 3 in T-cell recognition of nominal antigen. Proc. Natl. Acad. Sci. USA *87*, 2603-2607.

Kozbor, D., Moretta, A., Messner, H.A., Moretta, L., and Croce, C.M. (1987). Tp44 molecules involved in antigen-independent T cell activation are expressed on plasma cells. J. Immunol. *138*, 4128-4132.

Krummel, M.F. and Allison, J.P. (1995). CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. J. Exp. Med. *182*, 459-465.

Kuchroo, V.K., Das, M.P., Brown, J.A., Ranger, A.M., Zamvil, S.S., Sobel, R.A., Weiner, H.L., Nabavi, N., and Glimcher, L.H. (1995). B7-1 and B7-2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways: application to autoimmune disease therapy. Cell *80*, 707-718.

Kuiper, H.M., Brouwer, M., Linsley, P.S., and Van Lier, R.A.W. (1995). Activated T cells can induce high levels of CTLA-4 expression on B cells. J. Immunol. *155*, 1776-1783.

Kupfer, A. and Singer, S.J. (1989). The specific interaction of helper T cells and antigen-presenting B cells. J. Exp. Med. *170*, 1697-1713.

Lane, P.J., McConnell, F.M., Schieven, G.L., Clark, E.A., and Ledbetter, J.A. (1990). The role of class II molecules in human B-cell activation: association with phoshatidyl inositol turnover, protein tyrosine phosphorylation and proliferation. J. Immunol. *144*, 3684-3692.

Lanier, L.L., O'Fallon, S., Somoza, C., Phillips, J.H., Linsley, P.S., Okumura, K., Ito, D., and Azuma, M. (1995). CD80 (B7) and CD86 (B70) provide similar costimulatory signals for T cell proliferation, cytokine production, and generation of CTL. J. Immunol. *154*, 97-105.

Ledbetter, J.A., Tsu, T.T., Draves, K.E., and Clark, E.A. (1985). Differential expression of pan T cell antigens on human T cell subsets. In Perspectives in Immunogenetics and Histocompatibility. E. Heise, ed. (New York: American Society of Histocompatibility and Immunogenetics), pp. 325-340.

Ledbetter, J.A., June, C.H., Grosmaire, L.S., and Rabinovitch, P.S. (1987). Crosslinking of surface antigens causes mobilization of intracellular ionized calcium in T lymphocytes. Proc. Natl. Acad. Sci. USA *84*, 1384-1388.

Ledbetter, J.A., Imboden, J.B., Schieven, G.L., Grosmaire, L.S., Rabinovitch, P.S., Lindsten, T., Thompson, C.B., and June, C.H. (1990). CD28 ligation in T-cell activation: evidence for two signal transduction pathways. Blood *75*, 1531-1539.

Lenschow, D.J., Zeng, Y., Thistlethwaite, J.R., Montag, A., Brady, W., Gibson, M.G., Linsley, P.S., and Bluestone, J.A. (1992). Long-term survival of zenogeneic pancreatic islet grafts induced by CTLA4Ig. Science *257*, 789-792.

Lenschow, D.J., Sperling, A.I., Cooke, M.P., Freeman, G.J., Rhee, L., Decker, D.C., Gray, G., Nadler, L.M., Goodnow, C.C., and Bluestone, J.A. (1994). Differential up-regulation of the B7-1 and B7-2 costimulatory molecules after Ig receptor engagement by antigen. J. Immunol. *153*, 1990-1997.

Lenschow, D.J., Ho, S.C., Sattar, H., Rhee, L., Gray, G., Nabavi, N., Herold, K.C., and Bluestone, J.A. (1995). Differential effects of anti-B7-1 and anti-B7-2 monoclonal antibody treatment on the development of diabetes in the nonobese diabetic mouse. J. Exp. Med. *181*, 1145-1155.

Leprince, C., Draves, K.E., Geahlen, R.L., Ledbetter, J.A., and Clark, E.A. (1993). CD22 associates with the human surface IgM-B-cell antigen receptor complex. Proc. Natl. Acad. Sci. USA *90*, 3236-3240.

Levine, B.L., Ueda, Y., Craighead, N., Huang, M.L., and June, C.H. (1995). CD28 ligands CD80 (B7-1) and CD86 (B7-2) induce long-term autocrine growth of $CD4^+$ T cells and induce similar patterns of cytokine secretion *in vitro*. Int. Immunol. *7*, 891-904.

Lindsten, T., June, C.H., Ledbetter, J.A., Stella, G., and Thompson, C.B. (1989). Regulation of lymphokine messenger RNA stability by a surface-mediated T cell activation pathway. Science *244*, 339-343.

Linsley, P.S., Clark, E.A., and Ledbetter, J.A. (1990). T-cell antigen CD28 mediates adhesion with B cells by interacting with activation antigen B7/BB-1. Proc. Natl. Acad. Sci. USA *87*, 5031-5035.

Linsley, P.S., Brady, W., Grosmaire, L., Aruffo, A., Damle, N.K., and Ledbetter, J.A. (1991a). Binding of the B cell activation antigen B7 to CD28 costimulates

T cell proliferation and interleukin 2 mRNA accumulation. J. Exp. Med. *173*, 721-730.

Linsley, P.S., Brady, W., Urnes, M., Grosmaire, L.S., Damle, N.K., and Ledbetter, J.A. (1991b). CTLA-4 is a second receptor for the B cell activation antigen B7. J. Exp. Med. *174*, 561-569.

Linsley, P.S., Greene, J.L., Tan, P., Bradshaw, J., Ledbetter, J.A., Anasetti, C., and Damle, N.K. (1992a). Coexpression and functional cooperation of CTLA-4 and CD28 on activated T lymphocytes. J. Exp. Med. *176*, 1595-1604.

Linsley, P.S., Wallace, P.M., Johnson, J., Gibson, M.G., Greene, J.L., Ledbetter, J.A., Singh, C., and Tepper, M.A. (1992b). Immunosuppression in vivo by a soluble form of the CTLA-4 T cell activation molecule. Science *257*, 792-795.

Linsley, P.S., Greene, J.L., Brady, W., Bajorath, J., Ledbetter, J.A., and Peach, R. (1994a). Human B7-1 (CD80) and B7-2 (CD86) bind with similar avidities but distinct kinetics to CD28 and CTLA-4 receptors. Immunity *1*, 793-801.

Linsley, P.S., Peach, R., Gladstone, P., and Bajorath, J. (1994b). Extending the B7 (CD80) gene family. Protein Sci. *3*, 1341-1343.

Linsley, P.S. (1995). Distinct roles for CD28 and cytotoxic T lymphocyte-associated molecule-4 receptors during T cell activation? J. Exp. Med. *182*, 289-292.

Linsley, P.S., Ledbetter, J.A., Peach, R., and Bajorath, J. (1995a). CD28/CTLA-4 receptor structure, binding stoichiometry and aggregation during T-cell activation. Res. Immunol. *146*, 130-140.

Linsley, P.S., Nadler, S.G., Bajorath, J., Peach, R., Leung, H.T., and Rogers, J. (1995b). Binding stoichiometry of the cytotoxic T lymphocyte-associated molecule-4. J. Biol. Chem. *270*, 15417-15424.

Linsley, P.S. and Ledbetter, J.A. (1993). The role of the CD28 receptor during T cell responses to antigen. Ann. Rev. Immunol. *11*, 191-212.

Liu, Y., Jones, B., Aruffo, A., Sullivan, K.M., Linsley, P.S., and Janeway Jr., C.A. (1992a). Heat-stable antigen is a costimulatory molecule for CD4 T cell growth. J. Exp. Med. *175*, 437-445.

Liu, Y., Jones, B., Brady, W., Janeway Jr., C.A., and Linsley, P.S. (1992b). Co-stimulation of murine CD4 T cell growth: cooperation between B7 and heat-stable antigen. Eur. J. Immunol. *22*, 2855-2859.

MacLean-Fletcher, S. and Pollard, T.D. (1980). Mechanism of action of cytochalasin B on actin. Cell *20*, 329-341.

MacLennan, I.C.M. (1994). Germinal centers. Ann. Rev. Immunol. *12*, 117-139.

Martin, R., McFarland, H.F., and McFarlin, D.E. (1992). Immunological aspects of demyelinating diseases. Ann. Rev. Immunol. *10*, 153-187.

Masten, B.J., Yates, J.L., Pollard Koga, A.M., and Lipscomb, M.F. (1997). Characterization of accessory molecules in murine lung dendritic cell functions: roles for CD80, CD86, CD54, and CD40L. Am. J. Respir. Cell Mol. Biol. *16*, 335-342.

McHugh, R.S., Ahmed, S.N., Wang, Y.-C., Sell, K.W., and Selvaraj, P. (1995). Construction, purification, and functional incorporation on tumor cells of glycolipid-anchored human B7-1 (CD80). Proc. Natl. Acad. Sci. USA *92*, 8059-9063.

Moingeon, P.E., Lucich, J.L., Stebbins, C.C., Recny, M.A., Wallner, B.P., Koyasu, S., and Reinherz, E.L. (1991). Complementary roles for CD2 and LFA-1 adhesion pathways during T cell activation. Eur. J. Immunol. *21*, 605-610.

Moy, V.T. and Brian, A.A. (1992). Signaling by lymphocyte function-associated antigen 1 (LFA-1) in B cells. J. Exp. Med. *175*, 1-7.

Muraguchi, A., Kishimoto, T., Miki, Y., Kuritani, T., Kaieda, T., Yoshizaki, K., and Yamamura, Y. (1981). T cell-replacing factor- (TRF) induced IgG secretion in a human B blastoid cell line and demonstration of acceptors for TRF. J. Immunol. *127*, 412-416.

Nabavi, N., Freeman, G.J., Gault, A., Godfrey, D., Nadler, L.M., and Glimcher, L.H. (1992). Signalling through the MHC class II cytoplasmic domain is required for antigen presentation and induces B7 expression. Nature *360*, 266-268.

Nagata, S. and Golstein, P. (1995). The Fas death factor. Science *267*, 1449-1456.

Nickoloff, B.J., Mitra, R.S., Lee, K., Turka, L.A., Green, J., Thompson, C., and Shimizu, Y. (1993). Discordant expression of CD28 ligands, BB-1, and B7 on keratinocytes *in vitro* and psoriatic cells *in vivo*. Am. J. Pathol. *142*, 1029-1040.

Norton, S.D., Zuckerman, L., Urdahl, K.B., Shefner, R., Miller, J., and Jenkins, M.K. (1992). The CD28 ligand, B7, enhances IL-2 production by providing a costimulatory signal to T cells. J. Immunol. *149*, 1556-1561.

Offringa, R. and Bierer, B.E. (1993). Association of CD2 with Tubulin: evidence for a role of the cytoskeleton in T cell activation. J. Biol. Chem. *268*, 4979-4988.

Palacios, R. (1985). Mechanism by which accessory cells contribute in growth of resting T lymphocytes initiated by OKT3 antibody. Eur. J. Immunol. *15*, 645-651.

Patarroyo, M. and Makgoba, M.W. (1989). Leucocyte adhesion to cells. Molecular basis, physiological relevance, and abnormalities. Scand. J. Immunol. *30*, 129-164.

Peach, R.J., Bajorath, J., Brady, W., Leytze, G., Greene, J., Naemura, J., and Linsley, P.S. (1994). Complementarity determining region 1 (CDR1)- and CDR3-analogous regions in CTLA-4 and CD28 determine the binding to B7-1. J. Exp. Med. *180*, 2049-2058.

Peach, R.J., Bajorath, J., Naemura, J., Leytze, G., Greene, J., Aruffo, A., and Linsley, P.S. (1995). Both extracellular immunoglobin-like domains of CD80 contain residues critical for binding T cell surface receptors CTLA-4 and CD28. J. Biol. Chem. *270*, 21181-21187.

Pearson, T.C., Alexander, D.Z., Winn, K.J., Linsley, P.S., Lowry, R.P., and Larsen, C.P. (1994). Transplantation tolerance induced by CTLA4-Ig. Transplantation *57*, 1701-1706.

Peltz, G.A., Trounstine, M.L., and Moore, K.W. (1988). Cloned and expressed human Fc receptor for IgG mediates anti-CD3-dependent lymphoproliferation. J. Immunol. *141*, 1891-1896.

Perrin, P.J., Scott, D., June, C.H., and Racke, M.K. (1995). B7-mediated costimulation can either provoke or prevent clinical manifestations of experimental allergic encephalomyelitis. Immunol. Res. *14*, 189-199.

Pilon, M., Gullberg, M., and Lundgren, E. (1991). Transient expression of the CD2 cell surface antigen as a sortable marker to monitor high frequency transfection of human primary B cells. J. Immunol. *146*, 1047-1051.

Prabhu Das, M.R., Zamvil, S.S., Borriello, F., Weiner, H.L., Sharpe, A.H., and Kuchroo, V.K. (1995). Reciprocal expression of co-stimulatory molecules, B7-1 and B7-2, on murine T cells following activation. Eur. J. Immunol. *25*, 207-211.

Ranheim, E.A. and Kipps, T.J. (1993). Activated T cells induce expression of B7/BB1 on normal or leukemic B cells through a CD40-dependent signal. J. Exp. Med. *177*, 925-935.

Rattis, F.-M., Peguet-Navarro, J., Staquet, M.-J., Dezutter-Dambuyant, C., Courtellemont, P., Redziniak, G., and Schmitt, D. (1996). Expression and function of B7-1 (CD80) and B7-2 (CD86) on human epidermal Langerhans cells. Eur. J. Immunol. *26*, 449-453.

Rosenman, S.J., Ganji, A.A., Tedder, T.F., and Gallatin, W.M. (1993). *Syn*-capping of human T lymphocyte adhesion/activation molecules and their redistribution during interaction with endothelial cells. J. Leukoc. Biol. *53*, 1-10.

Rothlein, R. and Springer, T.A. (1986). The requirement for lymphocyte function-associated antigen 1 in homotypic leukocyte adhesion stimulated by phorbol ester. J. Exp. Med. *163*, 1132-1149.

Rousset, F., Garcia, E., and Banchereau, J. (1991). Cytokine-induced proliferation of human B lymphocytes triggered through their CD40 antigen. J. Exp. Med. *173*, 705-710.

Sanchez-Madrid, F., Krensky, A.M., Ware, C.F., Robbins, E., Strominger, J.L., Burakoff, S.J., and Springer, T.A. (1982). Three distinct antigens associated with human T-lymphocyte mediated cytolysis: LFA-1, LFA-2, and LFA-3. Proc. Natl. Acad. Sci. USA *79*, 7489-7493.

Sayegh, M.H., Akalin, E., Hancock, W.W., Russell, M.E., Carpenter, C.B., Linsley, P.S., and Turka, L.A. (1995). CD28-B7 blockade after alloantigenic challenge *in vivo* inhibits Th1 cytokines but spares Th2. J. Exp. Med. *181*, 1869-1874.

Schweitzer, A.N., Borriello, F., Wong, R.C.K., Abbas, A.K., and Sharpe, A.H. (1997). Role of costimulators in T cell differentiation: studies using

antigen-presenting cells lacking expression of CD80 or CD86. J. Immunol. *158*, 2713-2722.

Schwinzer, R., Franklin, R.A., Domenico, J., Renz, H., and Gelfand, E.W. (1992). Monoclonal Antibodies directed to different epitopes in the CD3-TCR complex induce different states of competence in resting human T cells. J. Immunol. *148*, 1322-1328.

Seed, B. and Aruffo, A. (1987). Molecular cloning of the CD2 antigen, the T-cell erythrocyte receptor, by a rapid immunoselection procedure. Proc. Natl. Acad. Sci. USA *84*, 3365-3369.

Selvakumar, A., Mohanraj, B.K., Eddy, R.L., Shows, T.B., White, P.C., and Dupont, B. (1992). Genomic organization and chromosomal location of the human gene encoding the B-lymphocyte activation antigen B7. Immunogenetics *36*, 175-181.

Selvakumar, A., White, P.C., and Dupont, B. (1993). Genomic organization of the mouse B-lymphocyte activation antigen B7. Immunogenetics *38*, 292-295.

Sethna, M.P., Parijs, L.V., Sharpe, A.H., Abbas, A.K., and Freeman, G.J. (1994). A negative regulatory function of B7 revealed in B7-1 transgenic mice. Immunity *1*, 415-421.

Shahinian, A., Pfeffer, K., Lee, K.P., Kündig, T.M., Kishihara, K., Wakeham, A., Kawai, K., Ohashi, P.S., Thompson, C.B., and Mak, T.W. (1993). Differential T cell costimulatory requirements in CD28-deficient mice. Science *261*, 609-612.

Sidorenko, S.P. and Clark, E.A. (1993). Characterization of a cell surface glycoprotein IPO-3, expressed on activated human B and T lymphocytes. J. Immunol. *151*, 4614-4624.

Simmons, D., Makgoba, M.W., and Seed, B. (1988). ICAM, an adhesion ligand of LFA-1, is homologous to the neural cell adhesion molecule NCAM. Nature *331*, 624-627.

Spriggs, M.K., Armitage, R.J., Strockbine, L., Cliford, K.N., Macdiff, B.M., Sato, T.A., Maliszewski, C.R., and Fanslow, W.C. (1992). Recombinate human CD40 ligand stimulates B cell proliferation and immunoglobulin E secretion. J. Exp. Med. *176*, 1543-1550.

Steinman, L. (1993). Connections between the immune system and the nervous system. Proc. Natl. Acad. Sci. USA *90*, 7912-7914.

Steinman, R.M. (1991). The dendritic cell system and its role in immunogenicity. Ann. Rev. Immunol. *9*, 271-296.

Symington, F.W., Brady, W., and Linsley, P.S. (1993). Expression and function of B7 on human epidermal Langerhans cells. J. Immunol. *150*, 1286-1295.

Tan, P., Anasetti, C., Hansen, J.A., Melrose, J., Brunvand, M., Bradshaw, J., Ledbetter, J.A., and Linsley, P.S. (1993). Induction of alloantigen-specific hyporesponsiveness in human T lymphocytes by blocking interaction of CD28 with its natural ligand B7/BB1. J. Exp. Med. *177*, 165-173.

Tivol, E.A., Borriello, F., Schweitzer, A.N., Lynch, W.P., Bluestone, J.A., and Sharpe, A.H. (1995). Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity *3*, 541-547.

Townsend, S.E. and Allison, J.P. (1993). Tumor rejection after direct costimulation of $CD8^+$ T cells by B7-transfected melanoma cells. Science *259*, 368-370.

Tsoukas, C.D., Landgraf, B., Bentin, J., Valentine, M., Lotz, M., Vaughan, J.H., and Carson, D.A. (1985). Activation of resting T lymphocytes by anti-CD3 (T3) antibodies in the absence of monocytes. J. Immunol. *135*, 1719-1723.

Vallé, A., Garrone, P., Yssel, H., Bonnefoy, J.-Y., Freedman, A.S., Freeman, G., Nadler, L.M., and Banchereau, J. (1990). MAb 104, a new monoclonal antibody, recognizes the B7 antigen that is expressed on activated B cells and HTLV-1-transformed T cells. Immunology *69*, 531-535.

van der Merwe, P.A., Bodian, D.L., Daenke, S., Linsley, P.S., and Davis, S.J. (1997). CD80 (B7-1) binds both CD28 and CTLA-4 with a low affinity and very fast kinetics. J. Exp. Med. *185*, 393-403.

Van Gool, S.W., de Boer, M., and Ceuppens, J.L. (1994). The combination of anti-B7 monoclonal antibody and cyclosporin A induces alloantigen-specific anergy during primary mixed lymphocyte reaction. J. Exp. Med. *179*, 715-720.

Van Seventer, G.A., Shimizu, Y., Horgan, K.J., and Shaw, S. (1990). The LFA-1 ligand ICAM-1 provides an important costimulatory signal for T cell receptor-mediated activation of resting T cells. J. Immunol. *144*, 4579-4586.

Vandenberghe, P., Delabie, J., de Boer, M., De Wolf-Peeters, C., and Ceuppens, J.L. (1993). *In situ* expression of B7/BB1 on antigen-presenting cells and activated B cells: an immunohistochemical study. Int. Immunol. *5*, 317-321.

Vyth-Dreese, F.A., Dellemijm, T.A.M., Majoor, D., and de Jong, D. (1995). Localization *in situ* of the co-stimulatory molecules B7.1, B7.2, CD40 and their ligands in normal human lymphoid tissue. Eur. J. Immunol. *25*, 3023-3029.

Wakasugi, H., Bertoglio, J., Tursz, T., and Fradelizi, D. (1985). IL-2 receptor induction on human T lymphocytes: role for IL-2 and monocytes. J. Immunol. *135*, 321-327.

Walunas, T.L., Lenschow, D.J., Bakker, C.Y., Linsley, P.S., Freeman, G.J., Green, J.M., Thompson, C.B., and Bluestone, J.A. (1994). CTLA-4 can function as a negative regulator of T cell activation. Immunity *1*, 405-413.

Waterhouse, P., Penninger, J.M., Timms, E., Wakeham, A., Shahinian, A., Lee, K.P., Thompson, C.B., Griesser, H., and Mak, T.W. (1995). Lymphoproliferative disorders with early lethality in mice deficient in *Ctla-4*. Science *270*, 985-988.

Watts, T.H., Alaverdi, N., Wade, W.F., and Linsley, P.S. (1993). Induction of costimulatory molecule B7 in M12 B lymphomas by cAMP or MHC-restricted T cell interaction. J. Immunol. *150*, 2192-2202.

White, M.W., McConnell, F., Shu, G.L., Morris, D.R., and Clark, E.A. (1991). Activation of dense human tonsilar B cells. Induction of c-myc gene expression via two distinct signal transduction pathways. J. Immunol. *146*, 846-853.

Woodgett, J.R., Gould, K.L., and Hunter, T. (1986). Substrate specificity of protein kinase C. Eur. J. Biochem. *161*, 177-184.

Wu, Y., Guo, Y., and Liu, Y. (1993). A major costimulatory molecule on antigen-presenting cells, CTLA4 ligand A, is distinct from B7. J. Exp. Med. *178*, 1789-1793.

Xu, J., Foy, T.M., Laman, J.D., Elliott, E.A., Dunn, J.J., Waldschmidt, T.J., Elsemore, J., Noelle, R.J., and Flavell, R.A. (1994). Mice deficient for the CD40 ligand. Immunity *1*, 423-431.

Yokochi, T., Holly, R.D., and Clark, E.A. (1982). B lymphoblast antigen (BB-1) expressed on Epstein-Barr virus-activated B cell blasts, B lymphoblastoid cell lines, and Burkitt's lymphomas. J. Immunol. *128*, 823-827.

Young, J.W., Koulova, L., Soergel, S.A., Clark, E.A., Steinman, R.M., and Dupont, B. (1992). The B7/BB1 antigen provides one of several costimulatory signals for the activation of $CD4^+$ T lymphocytes by human blood dendritic cells *in vitro*. J. Clin. Invest. *90*, 229-239.

Zurzolo, C., Lisanti, M.P., Caras, I.W., Nitsch, L., and Rodriguez-Boulan, E. (1993). Glycosylphosphatidylinositol-anchored proteins are preferentially targeted to the basolateral surface in Fischer rat thyroid epithelial cells. J. Cell Biol. *121*, 1031-1039.

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Law, C.-L., A. Aruffo, K.A. Chandran, **R.T. Doty**, and E.A. Clark. Ig domains 1 and 2 of murine CD22 constitute the ligand-binding domain and bind multiple sialylated ligands expressed on B and T cells. J. Immunol. 155: 3368-3376, 1995.

Doty, R.T. and E.A. Clark. Subcellular localization of CD80 receptors is dependent upon an intact cytoplasmic tail and is required for CD28-dependent T cell costimulation. J. Immunol. 157:3270-3279, 1996.

Doty, R.T. and E.A. Clark. Multiple sites in the CD80 cytoplasmic tail regulate CD80 redistribution and T cell costimulation. (Submitted)

Pinchuk, L.M., D.M. Magaletti, G. Grouard, **R.T. Doty**, J.W. Young, R.G. Andrews, and E.A. Clark. Macaque dendritic cells generated from bone marrow progenitors can be infected with HIV-2. (Submitted).

Abstracts:

Doty, R.T., W.A. Brady, P.S. Linsley, and E.A. Clark. CTLA-4 is a ligand of the leukocyte integrin CD11a/18 (LFA-1). J. Cell. Biochem. (Abstract supplement 18D): p. 297, Abstract V135. 1994.

Doty, R.T., and E.A. Clark. The cytoplasmic tail of CD80 is required for CD28-dependent T cell Costimulation. The 9th International Congress of Immunology. Abstract 340, 1995.