

Molecular Basis of T Cell Inactivation by CTLA-4

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CTLA-4, a negative regulator of T cell function, was found to associate with the T cell receptor (TCR) complex ζ chain in primary T cells. The association of TCR ζ with CTLA-4, reconstituted in 293 transfectants, was enhanced by p56^{lck}-induced tyrosine phosphorylation. Coexpression of the CTLA-4-associated tyrosine phosphatase, SHP-2, resulted in dephosphorylation of TCR ζ bound to CTLA-4 and abolished the p56^{lck}-inducible TCR ζ -CTLA-4 interaction. Thus, CTLA-4 inhibits TCR signal transduction by binding to TCR ζ and inhibiting tyrosine phosphorylation after T cell activation. These findings have broad implications for the negative regulation of T cell function and T cell tolerance.

CTLA-4 is a T cell activation molecule essential for normal homeostasis of T cell reactivity. Engagement and cross-linking of CTLA-4 blocks production of interleukin-2, cell cycle progression, and cell differentiation, whereas in vivo blockade of CTLA-4-B7 interaction enhances autoreactive and tumor-specific T cell activity (1). Although it has been proposed that CTLA-4 affects signals downstream of initial T cell signaling events, several lines of evidence suggest that the negative signaling may occur at the T cell "activation cap" (2). Therefore, we investigated whether engagement of CTLA-4 directly affects proximal events of TCR-induced signaling pathways.

Primary T cells were activated for 2 days with monoclonal antibodies (mAbs) to CD3 and CD28 for optimal CTLA-4 expression and then rested to maximize anti-CD3-mediated signaling events. CTLA-4 cross-linking during restimulation with anti-CD3 mAbs resulted in decreased tyrosine phosphorylation of multiple intracellular proteins migrating between 18 and 40 kD (Fig. 1). Immunoblot analyses demonstrated that the affected proteins migrating at 18 to 23 kD represented the TCR ζ chains, whereas the protein migrating

at 36 kD was LAT (linker for activation of T cells) (3), an adaptor molecule critical for TCR signaling. In addition, tyrosine phosphorylation of mitogen-activated protein kinases stimulated by the anti-CD3 mAbs was reduced after CTLA-4 cross-linking (4). These data suggest that CTLA-4 can inhibit early TCR signaling events within the TCR complex.

To define the molecular mechanism by which CTLA-4 affected TCR signaling events, we analyzed anti-CTLA-4 immunoprecipitates, prepared from metabolically labeled and activated T cells, by two-dimensional SDS-polyacrylamide gel electrophoresis (PAGE) to identify interacting proteins (Fig. 2A). The bands migrating at 32 kD (nonreduced) and 16 kD (reduced) were demonstrated to be TCR ζ based on immunoblot analyses with antibodies to TCR ζ (Fig. 2B) (5), which suggests that CTLA-4 was associated with TCR ζ in these cells. The specificity of CTLA-4-TCR ζ binding was confirmed as the 16-kD band was absent in anti-CTLA-4 immunoprecipitates prepared from CTLA-4-deficient T cells (Fig. 2C) and could be specifically blocked by the addition of CTLA-4 immunoglobulin during immunoprecipitation (4, 6).

The interaction of CTLA-4 with TCR ζ in activated T cells is likely to be complex and may require additional T cell-specific proteins. Therefore, we examined the CTLA-4-TCR ζ association in a non-T cell transfection system. Human embryonic kidney epithelial (293) cells were transiently transfected with a plasmid containing murine TCR ζ and one encoding murine CTLA-4. The 16-kD TCR ζ chain was coprecipitated with CTLA-4 (Fig. 3A). The TCR ζ association was specific as mAb to CTLA-4 did not precipitate the 16-kD protein from cells transfected with vector alone or with a truncated form of CTLA-4 lacking the cytoplasmic tail. The importance of the CTLA-4 tail in the TCR ζ interaction was confirmed by coexpressing a construct

challenged mice were categorized according to the abundance of PAS⁺ goblet cells and assigned numerical scores (0: <5% goblet cells; 1: 5 to 25%; 2: 25 to 50%; 3: 50 to 75%; 4: >75%). The sum of the airway scores from each lung was divided by the number of airways examined for the histologic goblet cell score (expressed as arbitrary units; U). BAL samples were analyzed as described (7). Statistical significance was calculated using Student's *t* test (PC₂₀₀) or Wilcoxon test (goblet cell score, BAL cytology).

10. PC₂₀₀ of saline-treated controls was 0.55 ± 0.06 mg/kg. PC₂₀₀ of OVA-challenged wild-type, IL-4-deficient, and IL-4R α -deficient mice were 0.24 ± 0.025, 0.36 ± 0.026, and 0.45 ± 0.058 mg/kg, respectively.
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12. After subcutaneous priming with OVA on days -14 and -7, BALB/c wild-type mice were administered intranasally 55 μ l of IL-13R-Fc (13) or immunoglobulin control protein (Ig control) in phosphate-buffered saline (PBS). We administered 110 μ g of IL-13R-Fc on days 0, 1, 2, 3, 3.5, 4, and 4.5, and 110 μ g of Ig control was given on days 0, 1, 2, and 4. Both proteins were administered with 750 μ g of OVA on days 1, 2, and 4. Data were collected on day 5 (9).
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14. Wild-type, IL-4R α ^{-/-}, and RAG1^{-/-} mice were administered 3 to 5 μ g of recombinant IL-4 or IL-13, or control protein (bovine serum albumin or irrelevant rat antibody) intranasally on days 1, 3, and 5, and data were collected 12 to 15 hours later (9). For clarity, only data from BALB/c wild-type mice are shown.
15. CD4⁺ T cell lines were prepared using splenocytes from D011.10 T cell receptor (TCR) transgenic mice that express an OVA-specific TCR transgene (25). Equal numbers of T cells and antigen presenting cells (mitomycin c-treated and T cell-depleted BALB/c splenocytes) were incubated with 1 μ M OVA peptide, IL-4 (300 IU/ml), and antibody to IFN- γ (R46A2; 100 μ g/ml) for 5 days. Wild-type or IL-4R α ^{-/-} mice were reconstituted with 1.2 × 10⁷ washed cells intravenously. The mice were challenged intranasally with OVA for 6 consecutive days. Data were collected on day 7 (9).
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encoding a concatamer of three cytoplasmic tails of CTLA-4 fused to a glutathione *S*-transferase [GST-(CTLA-4)₃] with TCR ζ (Fig. 3B). To ensure that the interaction of TCR ζ with CTLA-4 occurred at the cell membrane, we examined CTLA-4–TCR ζ association in 293 cells transfected with a cell surface-expressed chimeric Tac- ζ construct (extracellular and transmembrane domains of Tac fused to the cytoplasmic domain of TCR ζ) (7). A significant amount of Tac- ζ , migrating between 43 and 60 kD, was coprecipitated with CTLA-4 in these transfectants (Fig. 3C). Thus, the association of CTLA-4 with TCR ζ can occur at the membrane and does not depend on other T cell-specific proteins.

Previous studies have shown that p56^{lck} can

regulate phosphorylation of both CTLA-4 and TCR ζ (8). Therefore, we examined the effect of p56^{lck} on CTLA-4–TCR ζ association. Cotransfection of p56^{lck} resulted in increased CTLA-4–TCR ζ association, especially with the higher, phosphorylated form (p18) of TCR ζ (Fig. 3A). p56^{lck} also enhanced TCR ζ binding to the GST-(CTLA-4)₃ fusion protein (Fig. 3B). A kinase-defective mutant p56^{lck} (K273A, KA) was not able to enhance CTLA-4–TCR ζ binding and failed to recruit p18 TCR ζ to CTLA-4 (Fig. 3D). These results suggest that the tyrosine kinase activity of p56^{lck} was required for enhanced association of CTLA-4 with TCR ζ . Two tyrosines (Y201 and Y218) located in the cytoplasmic domain of CTLA-4 are substrates for src family tyrosine kinases (8). Thus, the role of these tyrosines in mediating TCR ζ as-

sociation was examined in cells expressing a tyrosine-deficient [Tyr²⁰¹ to Phe/Tyr²¹⁸ to Phe (Y201F/Y218F)] CTLA-4 double mutant. The mutant CTLA-4 bound to TCR ζ to a similar extent as wild-type CTLA-4 (Fig. 3A), which suggests that CTLA-4 tyrosine phosphorylation was not required for TCR ζ binding. Therefore, the enhancement of TCR ζ –CTLA-4 association by p56^{lck} likely depends on tyrosine phosphorylation of TCR ζ , although p56^{lck}-mediated phosphorylation of other unidentified molecules may be involved.

CTLA-4 can also bind to a tyrosine phosphatase, SHP-2 (9). Thus, we explored the possibility that CTLA-4 formed complexes with SHP-2 and TCR ζ in activated T cells, which accounts for the lack of p18 TCR ζ in CTLA-4 immunoprecipitates (Fig. 4A). SHP-2 coprecipitated with antibodies to both TCR ζ and CTLA-4. Reciprocal immunoprecipitation of SHP-2 and subsequent immunoblotting with mAbs to TCR ζ or CTLA-4 as well as transfection studies (Fig. 4B) confirmed the existence of a multimolecular complex of CTLA-4–SHP-2–TCR ζ . Anti-CTLA-4 immunoprecipitates prepared from cells transfected with CTLA-4 and TCR ζ contained small but detectable amounts of endogenous SHP-2 present in 293 cells that were significantly enhanced by cotransfecting p56^{lck} (Fig. 4B). Overexpression of SHP-2 increased the amount of SHP-2 coprecipitated with CTLA-4. However, SHP-2 overexpression eliminated the binding of p18 TCR ζ to CTLA-4. The failure to observe p18 TCR ζ binding to CTLA-4 in SHP-2-overexpressing cells was not due to alterations in protein expression, as similar amounts of CTLA-4 (4) and TCR ζ were detected. Likewise, SHP-2 overexpression did not alter the overall amounts of the p18 form of TCR ζ within the cell, as equal amounts of p18 TCR ζ were present in SHP-2-transfected and control cells. Similar results were obtained with the cells expressing the Tac- ζ chimera (4).

Similar to wild-type CTLA-4, the tyrosine mutant CTLA-4 could interact with SHP-2

Fig. 1. Anti-CD3-induced LAT and TCR ζ tyrosine phosphorylation is inhibited by anti-CTLA-4 engagement. Activated T cells (5×10^6) (19) were admixed with 293 cells (2.5×10^6) transiently expressing a membrane-bound single-chain mAb to CD3 in the presence or absence of a membrane-bound single-chain mAb to CTLA-4 (20). The cells were incubated at 37°C for the time indicated and then subjected to lysis in buffer (LB). Immunoprecipitates (IPs) were prepared with mAb FB2 to phosphotyrosine (pTyr), rabbit antiserum to LAT (3), or mAb H146-968 to TCR ζ (21, 22). IPs separated on a reducing SDS-12% polyacrylamide gel were transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA) and subsequently immunoblotted (IB) with mAb 4G10 to pTyr (Upstate Biotechnology Inc., Lake Placid, NY). Bound proteins were detected by chemiluminescence (Pierce, Rockford, IL). CTLA-4 cross-linking resulted in a consistent but incomplete reduction of tyrosine phosphorylated p23TCR ζ and LAT. Results are representative of three independent experiments.

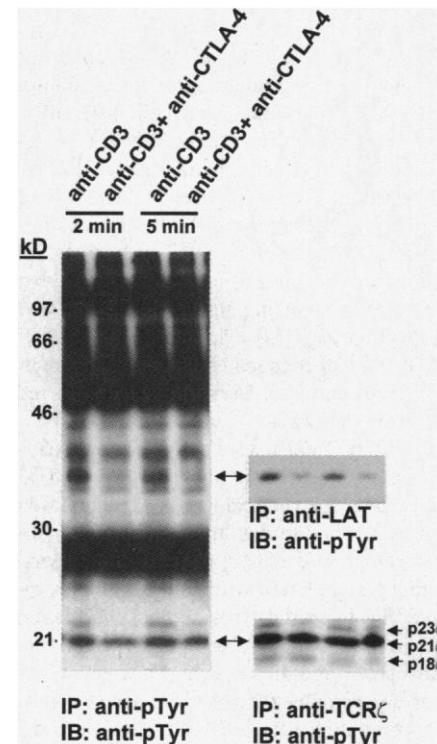
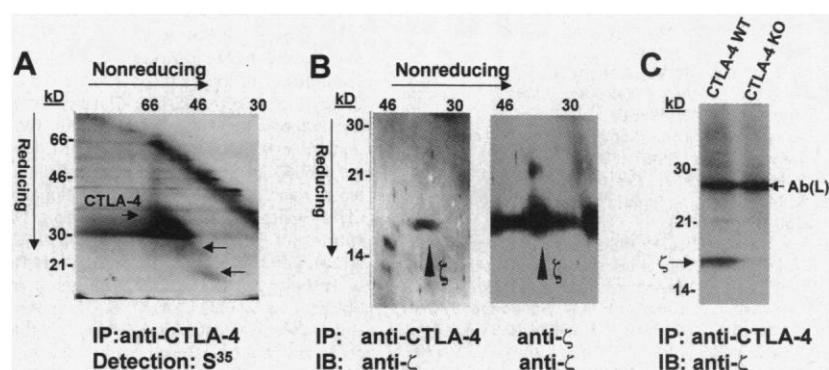
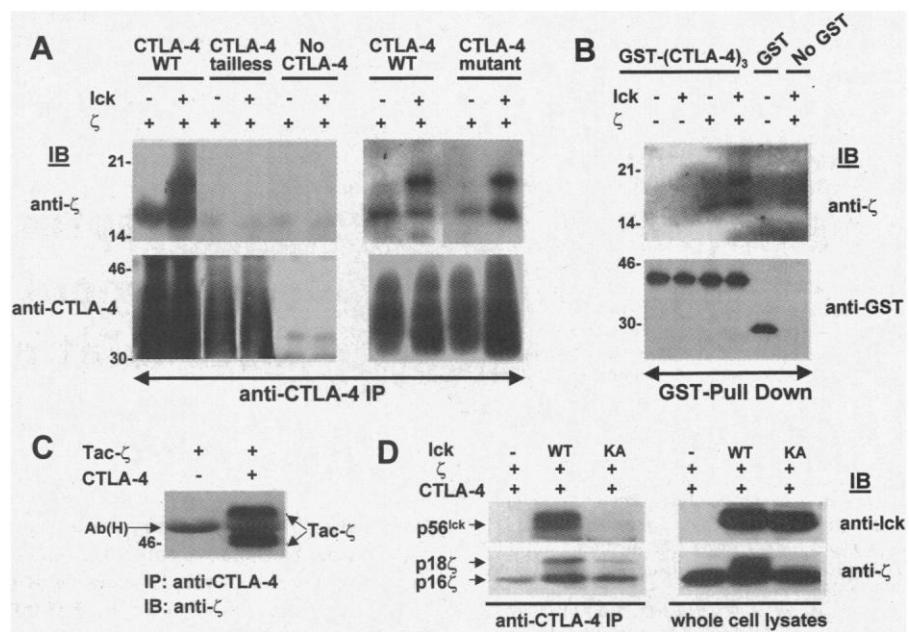


Fig. 2. (A) CTLA-4 coprecipitation of metabolically labeled proteins. Activated T cells (20×10^6 cells) (23) were labeled with ³⁵S-methionine and ³⁵S-cysteine (Amersham, Arlington Heights, IL). CTLA-4 was immunoprecipitated from cell lysates with mAb UC10-4F10 to CTLA-4 (15). Immune complexes were subjected to two-dimensional SDS-PAGE analysis with 10% acrylamide gels in both the first (nonreduced) and second (reduced) dimensions (5). Arrows indicate proteins coprecipitated with UC10-4F10. (B and C) Identification of p16 TCR ζ by immunoblotting. Activated T cells (100×10^6 cells per condition) (23) were lysed and subjected to immunoprecipitation with mAb UC10-4F10 to CTLA-4 or mAb H146-968 to TCR ζ . (B) IPs run on two-dimensional SDS-polyacrylamide gel (10% nonreduced, 12% reduced) were subjected to immunoblotting with a rabbit antiserum to TCR ζ (Ab 387) (24). The bound proteins were visualized by chemiluminescence. Upper bands in the anti- ζ IP (right) represented higher phosphorylated forms of TCR ζ and TCR η . (C) Anti-CTLA-4 IPs prepared from CTLA-4 wild-type (WT) or knockout (KO) mice were separated by one-dimensional reducing SDS-PAGE and immunoblotted with rabbit antiserum to ζ (Ab 387). Ab(L) represents antibody light chain. Independent experiments with similar results were performed five times.



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Fig. 3. Analysis of CTLA-4-TCR ζ association in 293 cell transfectants. **(A)** Coimmunoprecipitation of CTLA-4 with TCR ζ . 293 cells were transiently transfected with cDNA constructs encoding murine CTLA-4 WT, cytoplasmic domain-deficient (tailless) (12) CTLA-4, or CTLA-4 tyrosine double mutant (Y201F/Y218F) (25) in the absence or presence of WT p56^{lck} and murine TCR ζ (25). Cells were incubated for 40 hours and lysates were prepared. CTLA-4 was precipitated from an equal amount of protein [700 μ g per IP (left); 300 μ g per IP (right)]. IPs were analyzed by reducing SDS-PAGE and immunoblotted with the antibodies listed. The expression vectors used were pCDNA3 (CTLA-4 and TCR ζ , 2.5 μ g each) and pEF (p56^{lck}, 1.25 μ g). **(B)** GST-(CTLA-4)₃ fusion protein binds TCR ζ . 293 cells were transiently transfected with cDNA encoding GST-(CTLA-4)₃ (12) in the presence or absence of p56^{lck} and TCR ζ . GST proteins were precipitated from the lysates (200 μ g per sample) with glutathione Sepharose beads (12.5 μ l, Pharmacia) and analyzed by electrophoresis and immunoblotting with either rabbit antiserum to TCR ζ (Ab 387), or GST mAb (Santa Cruz, Santa Cruz, CA). GST and GST-(CTLA-4)₃ proteins migrated at 25 and 40 kD, respectively. **(C)** CTLA-4 coprecipitates Tac- ζ in 293 transfectants. 293 cells were transiently transfected with cDNAs for Tac- ζ chimera (7) in the absence or presence of WT CTLA-4. CTLA-4 IPs were subjected to SDS-PAGE and immunoblotted with Ab 387. Chimeric Tac- ζ , migrated as a doublet when detected with Ab 387. Ab(H) represents antibody heavy chain. **(D)** Kinase-defective lck (KA) failed to enhance TCR ζ association. 293 cells were transiently transfected with cDNAs for WT CTLA-4, murine TCR ζ , and either WT or kinase-defective p56^{lck} (26) and analyzed as described above.



(Fig. 4B) and abolished p18 TCR ζ binding to CTLA-4. These results are in contrast to the reported dependence of the *in vitro* interaction between CTLA-4 and SHP-2 on phosphotyrosines and SH2 domains (9). The difference may be due to the *in vivo* rather than *in vitro* analysis and suggests that there may be a phosphotyrosine-dependent as well as -independent association of CTLA-4 and SHP-2. As CTLA-4 does not possess SH2

domains, it is possible that TCR ζ binding to CTLA-4 is indirect and may depend on SHP-2 or the phosphotyrosine-dependent non-SH2 domain binding in CTLA-4.

A model can be envisioned to explain the process by which CTLA-4 regulates T cell responses. T cell activation, initiated by TCR ligation and CD28 costimulation, results in the recruitment of p56^{lck} to the TCR cap and phosphorylation of multiple substrates in-

cluding TCR ζ , ZAP-70, and LAT. CTLA-4, newly expressed (1) or preexisting in resting cells (10), is exported to the cell surface and binds to B7 molecules present in antigen presenting cells (11). CTLA-4 membrane localization may be facilitated by colocalization to the site of TCR engagement through direct interaction with TCR ζ and p56^{lck}-induced tyrosine phosphorylation of CTLA-4 (12). The interaction of TCR ζ and CTLA-4 brings the phosphatase, SHP-2, into the complex where it promotes TCR ζ dephosphorylation either directly by acting on TCR ζ or indirectly by regulating p56^{lck} kinase activity. In this regard, several studies have demonstrated a relationship between src family kinase activity and SHP-2 function (13). Currently, the molecular nature of TCR ζ -CTLA-4 binding is not known. Given the fact that p56^{lck} enhanced TCR ζ binding to the Y201F/Y218F CTLA-4 mutant, CTLA-4-TCR ζ interaction most likely depends on tyrosine phosphorylation of TCR ζ or other molecules that might be critical for their association. Further studies will help to elucidate the molecular basis of CTLA-4-TCR ζ binding. Finally, previous studies have shown that the ordered phosphorylation of TCR ζ establishes the threshold for T cell activation (14). The ability of CTLA-4 to bind to and dephosphorylate the p23 form of phosphorylated TCR ζ may decrease the extent and duration of TCR ζ phosphorylation and thereby antagonize TCR signal transduction. Further studies are needed to elucidate the functional importance of CTLA-4 in tolerance and antagonist peptide activity. However, the cur-

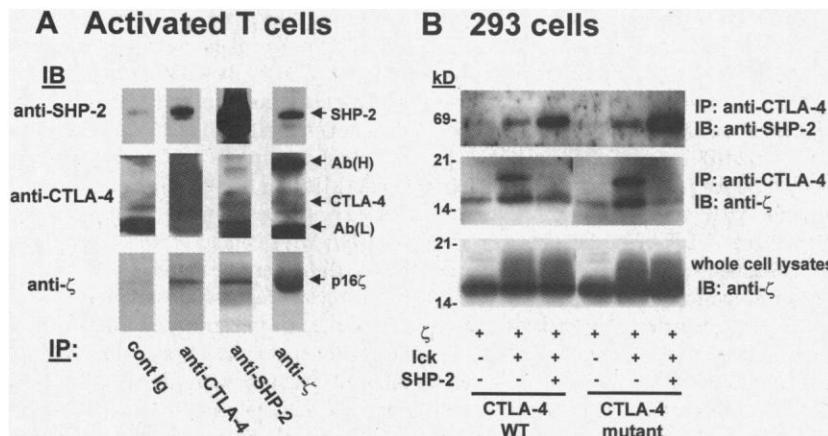


Fig. 4. SHP-2 associates with CTLA-4-TCR ζ complexes and regulates binding of TCR ζ to CTLA-4. **(A)** Equal numbers (100×10^6) of activated T cells (23) were lysed and subjected to IP with mAbs to CTLA-4 (UC10-4F10), rabbit antiserum against SHP-2 (27), and mAb to TCR ζ (H146). IPs were analyzed by SDS-PAGE and immunoblotted with a rabbit antiserum to SHP-2 (27), goat polyclonal antibodies to CTLA-4 (Q20, Santa Cruz, Santa Cruz, CA), or rabbit antiserum to TCR ζ (Ab 387). **(B)** 293 cells were transiently transfected with either WT CTLA-4 or mutated CTLA-4 (Y201F/Y218F) and TCR ζ in the presence or absence of p56^{lck} or SHP-2. CTLA-4 IPs prepared from lysates (400 μ g per sample) or whole cell lysates (35 μ g per lane) were electrophoresed and immunoblotted with rabbit antibody to SHP-2 (27) or antiserum to TCR ζ (Ab 387). Results are representative of three separate experiments.

rent studies provide a conceptual framework for developing approaches to regulate T cell function through CTLA-4.

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19. T cells from BALB/c lymph nodes were purified (15) and cultured for 60 hours in a dish coated with mAb to CD3 (anti-CD3) (145-2C11; 2 µg/ml) (16) and mAb to CD28 (anti-CD28) (PV-1; 2 µg/ml) (17). Cells were then washed three times to remove any residual antibodies present on the cell surface and rested at 37°C for additional 4 hours.
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21. Immunoprecipitations were performed with lysates prepared in LB [1% Nonidet P-40, 50 mM tris-HCl (pH 7.4), 150 mM NaCl, 20 mM EDTA (pH 8.0), 1 mM sodium vanadate, leupeptin (10 µg/ml), 10 µM aprotinin, 1 mM phenylsulfonysulfoxide]. Lysates were precleared twice with protein A beads and once with protein A beads coated with a mAb from a control hamster (UC3-10A6) (18) before precipitating antibodies were added. Immunoprecipitation was performed overnight at 4°C. Immune complexes were washed five times with LB and subjected to SDS-PAGE.
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25. The CTLA-4 tyrosine mutant (Y201F/Y218F) was generated by site-directed mutagenesis (Chameleon site-directed mutagenesis kit, Stratagene) and the presence of mutations was verified by DNA sequencing. A cDNA sequence incorporating the coding region of murine TCR ζ was generated by reverse transcription and polymerase chain reaction from RNA extracted from activated murine T cells and cloned into the mammalian expression vector pCDNA3.1(+).
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A Receptor/Cytoskeletal Movement Triggered by Costimulation During T Cell Activation

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During T cell activation, the engagement of costimulatory molecules is often crucial to the development of an effective immune response, but the mechanism by which this is achieved is not known. Here, it is shown that beads attached to the surface of a T cell translocate toward the interface shortly after the start of T cell activation. This movement appears to depend on myosin motor proteins and requires the engagement of the major costimulatory receptor pairs, B7-CD28 and ICAM-1-LFA-1. This suggests that the engagement of costimulatory receptors triggers an active accumulation of molecules at the interface of the T cell and the antigen-presenting cell, which then increases the overall amplitude and duration of T cell signaling.

The central event in T cell activation is the interaction of the T cell receptor (TCR) with the antigenic peptide presented by the major histocompatibility complex (MHC) of the antigen-presenting cell (APC). However, because the number of agonist peptide-MHC complexes can be very low, in the range of 10 to 100 per APC (1), and because the TCR is continuously modulated from the T cell surface (2), sustained T cell activation is likely to require signal amplification (3, 4). An important component of this amplification is thought to be provided by costimulatory molecules on the T cell, although the mechanism by which they accomplish it is unclear (5). The most important of the costimulatory receptors on T cells and their ligand on APCs are CD28-B7 (6) and LFA-1-ICAM-1 (7). Many different receptor couples, including TCR-peptide-MHC and LFA-1-ICAM-1, accumulate at the T cell-APC interface (8, 9). This accumulation has been assumed to be a passive, diffusion-limited cocapping mechanism (10). Here, we describe an active, cytoskeletal mechanism that appears to drive receptor accumulation at the T cell-APC interface. This mechanism requires the APC to express B7 and ICAM-1 and is independent of TCR signaling. We suggest that this mechanism is a central part of costimulation, as it would effectively amplify any TCR-mediated signals.

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To study whether receptor accumulation at the T cell-APC interface could be actively driven by the T cell cytoskeleton, we monitored the general movement of the cortical actin cytoskeleton and linked receptors (11) using the classical technique of attaching large beads to the surfaces of antigen-specific T cells. We coated 4.5-µm beads with an antibody to the T cell surface antigen ICAM-1 (12). Cross-linking ICAM-1 by beads in this way is not expected to influence T cell function (13). As has been observed with fibroblasts (11, 14), we find that in migrating 5C.C7 transgenic T cells (9, 15) the beads translocate from the anterior to the posterior end of the cell (16). We then mixed bead-loaded 5C.C7 T cells with B cell lymphoma cells that express the appropriate MHC molecule (I-E^k) and have been pulsed with the moth cytochrome c peptide 88-103. After contact with the APC, the T cells rapidly become activated (9, 17) and the beads move from the posterior end of the T cell to the newly formed interface with the B cells (Fig. 1, movie 1) beginning 4 ± 1 min after the first rise in intracellular calcium (n = 13). This suggests that the T cell cortical actin cytoskeleton reorients toward the T cell-APC interface soon after the start of T cell activation. The ensuing cytoskeletal flow would allow receptors that are linked to the actin cytoskeleton to be transported to the newly formed T cell-APC interface.

To rule out ICAM-1-specific effects, we also attached beads to T cells in two other ways. First, we surface-biotinylated the 5C.C7 T cells with an amine-reactive form of biotin and used