Lymphocyte Adhesion Is Mediated by C3G and Rap1

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T-lymphocyte adhesion plays a critical role in both inflammatory and autoimmune responses. The small GTPase Rap1 is the key coordinator mediating T-cell adhesion to endothelial cells, antigen-presenting cells, and virus-infected cells. We describe a signaling pathway, downstream of the cytotoxic T-lymphocyte antigen 4 (CTLA-4) receptor, leading to Rap1-mediated adhesion. We identified a role for the Rap1 guanine nucleotide exchange factor C3G in the regulation of T-cell adhesion and showed that this factor is required for both T-cell receptor (TCR)-mediated and CTLA-4-mediated T-cell adhesion. Our data indicated that C3G translocates to the plasma membrane downstream of TCR signaling, where it regulates activation of Rap1. We also showed that CTLA-4 receptor signaling mediates tyrosine phosphorylation in the C3G protein, and that this is required for augmented activation of Rap1 and increased adhesion mediated by leukocyte function-associated antigen type 1 (LFA-1). Zap70 is required for C3G translocation to the plasma membrane, whereas the Src family member Hck facilitates C3G phosphorylation. These findings point to C3G and Hck as promising potential therapeutic targets for the treatment of T-cell-dependent autoimmune disorders.

L Leukocyte function-associated antigen type 1 (LFA-1) is an integrin that is a critical mediator of T-cell adhesion. Integrins are heterodimeric transmembrane proteins that function not only as adhesive structures but also as receptors. In the mode of a receptor, LFA-1 is capable of transmitting outside information into the cell (“outside-in” signaling) as well as transmitting the activation status of the cell to the extracellular matrix (“inside-out” signaling) (1). The affinity of LFA-1 for its ligand, intercellular adhesion molecule 1 (ICAM-1), is variable and is controlled by several inside-out signaling events, including activation of Rap1, a small GTPase that cycles between active GTP-bound and inactive GDP-bound states (2). Like all small GTPases, activation of Rap1 is mediated by guanine nucleotide exchange factors (GEFs) that induce release of GDP and thereby facilitate GTP binding. Among the GEFs that activate Rap1 is C3G, which is known to be expressed in lymphocytes (3). Our understanding of LFA-1 regulation has been greatly advanced by structural studies that reveal the existence of at least three affinity states in which LFA-1 is bent, extended, or maximally open. The ability of Rap1 to control LFA-1 affinity for its ligand and LFA-1-mediated adhesion of lymphocytes is well established. T cells from Rap1-deficient mice have diminished adhesive capacity (2). In humans, the physiological relevance of Rap1 is evident in patients suffering from a congenital defect in the kindlin-3 protein that is required for proper Rap1 signaling (4). Such patients manifest leukocyte adhesion deficiency type III syndrome, whose characteristics include an immunocompromised state.

T cells require at least two signals in order to become fully activated (5). An initial signal, which is antigen specific, is delivered by the T-cell receptor (TCR) through its interaction with antigenic peptides in complex with major histocompatibility complex molecules on the antigen-presenting cell (APC) membrane. A second signal, the costimulatory signal, is not antigen specific and is provided by the interaction between costimulatory molecules expressed on the APC membrane and receptors on the T cell. Costimulation of T cells is necessary for proliferation, differentiation, and survival. Activation of T cells without costimulation can lead to T-cell anergy, T-cell deletion, or development of immune tolerance. One of the best-characterized costimulatory molecules expressed by T cells is CD28, which interacts with CD80 and CD86 on the APC membrane (6). Another coreceptor expressed on T cells is cytotoxic T-lymphocyte antigen 4 (CTLA-4), Zap70 is required for C3G translocation to the plasma membrane, whereas the Src family member Hck facilitates C3G phosphorylation. These findings point to C3G and Hck as promising potential therapeutic targets for the treatment of T-cell-dependent autoimmune disorders.

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CTLA-4 is associated with autoimmune diseases. Polymorphisms in CTLA-4 have been linked to diabetes mellitus, Hashimoto's thyroiditis, celiac disease, and primary biliary cirrhosis (13). It has been suggested that in the presence of these polymorphisms the inhibitory effect of CTLA-4 is impaired (12). In systemic lupus erythematosus, an aberrant splice variant of CTLA-4 is produced and can be detected in the serum of patients with active disease (14). Agents that modulate signaling by CTLA-4 (ipilimumab) were recently approved for the treatment of patients with melanoma, but the signaling pathway(s) downstream of this receptor remains poorly understood (15).

We report a new signaling pathway downstream of CTLA-4 that mediates Rap1 activation and cellular adhesion. We discovered that CTLA-4 signals through the Src kinase family member Hck in order to phosphorylate the Rap1 GEF C3G at the plasma membrane (PM). This event was found to be associated with enhanced exchange activity, higher levels of GTP-Rap1, and increased adhesion to ICAM-1. These findings may reveal novel ways of interfering with lymphocyte adhesion and autoimmunity by inhibiting the activation of Rap1.

MATERIALS AND METHODS

General reagents. RPMI medium, 5-carboxyfluorescein (CFSE), SNARF-1 carboxylic acid, and Opti-MEM-I were purchased from Invitrogen/Molecular Probes. Doxycycline, hygromycin, phorbol 12-myristate 13-acetate (PMA), ionomycin, staurosporine, PP2, and LY294 were obtained from Sigma-Aldrich. Ficol-Paque was purchased from GE Healthcare. ACK lysing buffer was obtained from Lonza. Protein concentrations were measured with a bichinonic acid (BCA) assay (Pierce). ICAM-1 was a gift from Mike Dustin (Skirball Institute, NYU).

Cell culture, transfection, and stimulation. Jurkat T cells were obtained from the American Type Culture Collection. Cells were maintained in 5% CO₂ at 37°C in RPMI 1640 medium supplemented with 10% fetal bovine serum and 100 U/ml penicillin G and streptomycin. CTLA-4 expressing Jurkat T cells induced with doxycycline overnight were a gift from Joaquin Madrenas (University of Western Ontario). Transfection of Jurkat T cells was performed either with DNRIE-C reagent (Invitrogen) or by nucleofection (Lonza). Introduction of DNA constructs into primary T cells was accomplished by nucleofection (Lonza). Both primary and Jurkat T cells were stimulated by application of magnetic beads (Dynabeads antibody coupling kit) coated with immobilized antibodies. For low-dose anti-CD3 and for anti-CD28 stimulation, 1 μg of antibodies was coupled with 1 mg of beads. For high-dose anti-CD3 stimulation, 5 μg of antibodies was coupled with 1 mg of beads. For anti-CTLA-4 and CD80-Ig stimulation, 10 μg of proteins was coupled with 1 mg of beads. For costimulation, beads were coupled with a mixture of antibodies at the same concentrations. Beads coupled with isotype control antibodies served as a control. Cells were serum starved for 2 h before stimulation with beads at a bead-to-cell ratio of 1:1. Primary T cells were treated with 1 ng/ml of PMA and 100 ng/ml of ionomycin for 48 h to induce expression of CTLA-4. Jurkat T cells were cultured overnight with 1 μg/ml of doxycycline to induce expression of CTLA-4.

Antibodies. Mouse anti-CD3 and anti-CD28 (ANC28.1) antibodies were purchased from Ancell Corporation. Other antibodies used were anti-Rap1 (610195), anti-RhoGDI (clone 16), anti-CTLA-4 (555853) (all from BD Biosciences), anti-C3G (C-19) (Bethyl Laboratories), monoclonal anti-Ras antibody (Ras10) (Calbiochem), antiphosphotyrosine (4G10) (Millipore), anti-green fluorescent protein (anti-GFP) antibody (Roche USA), antiaxin (A1978) (Sigma), and anti-phospho-mitogen-activated protein kinase (anti-phospho-MAPK) (9106) (Cell Signaling). Anti-Hck (N-30), anti-CD28 (PV-1), anti-Epac (N-16), anti-Fgr (M-60), anti-Erk1 (K-23), anti-Lck (3A5), anti-Zap70 (4H386), and anti-CTLA-4 (9094) were obtained from Santa Cruz Biotechnology. Recombinant mouse B7-1/CD80Fc chimera was purchased from R&D Systems.

ELISA. A mouse interleukin 2 enzyme-linked immunosorbent assay (ELISA) kit was purchased from Aviva System Biology.

DNA constructs. pGFP (Clontech), Rap1GAP, Cherry-Rap1, GFP-Rap1, and the GFP-RalGDS-RBD construct were previously described (15). pCAGGS-C3G was a gift from Michiyuki Matsuda (Kyoto University). GFP-C3G was kindly provided by Philip Stork (Vollum Institute). GFP-C3G-Y504V was synthesized using a site-directed mutagenesis kit (Agilent Technologies). GFP-C3G-AC was subcloned from pCAGGS-C3G by the use of nested primers. Cherry-K-Ras tail (a fusion construct that delivers proteins to the PM) was fabricated by PCR amplification of the last 19 codons of the K-Ras gene, which were then subcloned into empty Cherry vector (Invitrogen). CTLA-4-GFP was a gift from Mike Dustin (Skirball Institute). All primer sequences are available upon request. Plasmids were treated with an UltraClean endotoxin removal kit (MoBio Laboratories) and verified by bidirectional sequencing.

Short inhibitory RNA. Short inhibitory oligonucleotides for human C3G, Hck, Fgr, Lck, and Zap70 and mouse C3G (SMARTpool) were designed and obtained from Dharmaco (Thermo Scientific). Oligonucleotides were introduced by nucleofection, and cells were analyzed 10 h later.

Adhesion assay. Jurkat and primary T-cell adhesion to ICAM-1-coated plates was performed as previously described (16). Cells were either transfected with GFP-tagged constructs or labeled with 5-carboxyfluorescein or SNARF-1. Cells were plated for 20 min, and nonadherent cells were then removed by serial washes. The number of adherent cells, expressed as a percentage of the total number of labeled cells, was determined with a plate reader at 525 nm (Synergy HT; BioTek Instruments).

Ras and Rap1 activation assay, Western blotting. Activated Ras and Rap1 were detected by the gluthathione S-transferase (GST) pulldown assay, as described previously (17, 18). Cell lysates were separated by Tris-glycine or Bis-Tris polyacrylamide gel electrophoresis and transferred to nitrocellulose filters. Blots were blocked, incubated with the primary antibodies at 4°C, washed, and incubated for 45 min at room temperature with conjugated secondary antibodies (Li-COR Biosciences). Immunoreactive bands were visualized using the Odyssey imaging system (Li-COR Biosciences).

Microscopy. Live cells were plated in 35-mm dishes, each containing a glass coverslip bottom (MatTek) or coverslip-like bottom (µ-Dish; ibidi GmbH). Cells were maintained at 37°C using a stage incubator (PeCon GmbH). Individual cells were imaged continuously, before and after stimulation, for periods of up to 30 min. Images were acquired with an inverted Zeiss 700 laser scanning confocal microscope (Carl Zeiss Microimaging). Recruitment of C3G to the PM was measured by colocalization analysis with Pearson’s correlation coefficient (Rp) and overlap coefficient calculated according to Manders’ M1 (R). The PM was labeled by its expression of Cherry-K-Ras tail. GFP-C3G was expressed concomitantly, and the degree of colocalization was calculated by means of Zen 2012 software (Carl Zeiss).

Mice. Wild-type C57BL/6 mice were bred under pathogen-free conditions. Primary T lymphocytes were isolated from 6- to 8-week-old female mice using density gradient centrifugation followed by a Pan T-cell isolation kit II (Miltenyi Biotec) and were kept in supplemented RPMI 1640 medium. Prior to a 24-h washout, the cells were stimulated with PMA and ionomycin to induce expression of CTLA-4.

Membrane fractionation. T cells were washed 3 times in ice-cold phosphate-buffered saline. They were resuspended in relaxation buffer (10 mM HEPES, 100 mM KCl, 3 mM NaCl, 3.5 mM MgCl₂, 0.1 mM phenylmethylsulfonyl fluoride, and protease inhibitors) and equilibrated with N₂ at 350 lb/in² for 20 min at 4°C in a nitrogen bomb (Parr Instrument Company). Dropwise release from the bomb resulted in disruption of the cells by cavitation. The cavitated sample was centrifuged at 500 × g for 10 min to remove unbroken cells and nuclei. For C3G translocation assays, cytosolic (S175) and membrane (P175) fractions were generated from postnuclear supernatants of nitrogen cavitation samples by centrifugation at 175,000 × g for 45 min at 4°C.
Differential activation of Ras and Rap1 by the coreceptors CD28 and CTLA-4. Jurkat T cells expressing CTLA-4 were stimulated with magnetic beads coated with antibodies (as described in Materials and Methods) for 2 min, as indicated, and subjected to GST pulldown assays for GTP-loaded Ras (A) and Rap1 (B). The histograms present the results (means ± SEM) of at least 3 experiments. *, P < 0.05; **, P < 0.001.

**RESULTS**

Differential activation of Ras and Rap1 by the coreceptors CD28 and CTLA-4. Activation of Ras downstream of TCR signaling is well established (11, 16, 19). As expected, we observed a dose response of Ras activation when Jurkat T cells were stimulated with increasing concentrations of anti-CD3 antibodies (Fig. 1A). Whereas cross-linking the TCR stimulated GTP loading on Ras, cross-linking of neither CD28 nor CTLA-4, in the absence of TCR stimulation, had a significant effect on Ras activation. Costimulation of CD28 along with engagement of the TCR with low-dose anti-CD3 augmented Ras activation. In contrast, costimulation of CTLA-4 inhibited TCR-stimulated Ras activation. When both CD28 and CTLA-4 were engaged simultaneously Ras activation was diminished, consistent with a dominant effect of CTLA-4. These data demonstrate that signaling through the TCR for Ras activation is differentially regulated by the coreceptors CD28 and CTLA-4.

We next examined whether the same coreceptors would modulate Rap1 activation in T cells, and if so, how. Stimulation by cross-linking of the TCR with low-dose anti-CD3 antibodies resulted in an increase in GTP-Rap1 (Fig. 1B). Similar to Ras activation, treatment with high-dose anti-CD3 antibodies resulted in further activation of Rap1 (11, 20). Cross-linking of neither CD28 nor CTLA-4 in the absence of TCR stimulation had an effect on GTP loading of Rap1, also consistent with the lack of effect on Ras. As was the case for Ras, engagement of each of the two coreceptors modulated the effect of TCR stimulation in opposite directions. Strikingly, however, the roles of the two coreceptors were reversed; CD28 decreased TCR-stimulated Rap1 activation, whereas CTLA-4 augmented TCR-stimulated GTP loading of Rap1. Stimulation with anti-CTLA-4 antibodies overcame the inhibition induced by anti-CD28 antibodies, suggesting that, as was the case for Ras, the effects of CTLA-4 on Rap1 are dominant over that of CD28. Similar results were obtained in primary T cells (PTC) stimulated through the TCR and the same coreceptors (see Fig. S1A in the supplemental material). To validate that stimulation of CTLA-4 by cross-linking antibodies is similar to stimulation via its natural ligands, we substituted anti-CTLA-4 antibodies for CD80-Ig. Since CD80 is the ligand for both CD28 and CTLA-4, we pretreated the cells with anti-CD28 blocking antibodies (PV-1). As shown in Fig. S1B in the supplemental material, Rap1 was activated to the same extent when PTC were stimulated with either CTLA-4 cross-linking antibodies or CD80-Ig.

Thus, although Ras and Rap1 are small GTPases that are so closely related that they have identical effector binding domains (19), they are differentially regulated by CD28 and CTLA-4. Importantly, whereas CTLA-4 inhibits TCR-stimulated activation of Ras, consistent with its characterization as a negative regulator of T cells, this same transmembrane receptor synergizes with the TCR to stimulate Rap1 activation.

**CTLA-4 signaling activates Rap1 at the plasma membrane of T lymphocytes.** The biochemical assays employed to establish the differential effects of CD28 and CTLA-4 on Rap1 activation described above are uninformative with regard to the subcellular location of Rap1 signaling. To characterize the spatiotemporal aspects of Rap1 activation, we used the probe GFP-RalGDS-RBD, which binds with significantly higher affinity to activated Rap1 than to Ras, making it possible for us to detect on which membrane compartments endogenous Rap1 is activated (16). In Jurkat T cells, both in the resting condition and upon stimulation, the localization of Cherry-Rap1 was restricted to the PM and a perinuclear vesicular compartment (Fig. 2A, upper). These vesicles included the Golgi apparatus (see Fig. S2 in the supplemental material). However, the activated pool of Rap1 was distributed differently. Under resting conditions, the probe was homogenously distributed throughout the cytosol and nucleoplasm such that organelles, including nucleoli, were negatively imaged (Fig. 2A, lower left). Upon stimulation of the cells with anti-CD3 antibodies, the probe translocated to the PM and to the vesicular com-
partment, indicating activation of endogenous Rap1 at these locations. Stimulation of neither CD28 nor CTLA-4 in the absence of TCR stimulation caused relocalization of the probe (Fig. 2A). Interestingly, when cells expressing the probe for activated Rap1 were concomitantly stimulated with anti-CD3 and anti-CD28 antibodies, activated Rap1 was observed predominantly on the vesicular compartment and was entirely excluded from the PM in ∼50% of the cells observed (while in the other half the probe was recruited in both locations) (Fig. 2A). Remarkably, when the cells were concomitantly stimulated with anti-CD3 and anti-CTLA-4 antibodies, the probe was recruited mainly to the PM (in ∼70% of the cells). These data suggested that costimulation with CD28 and CTLA-4 not only alters the amounts of activated Rap1 (Fig. 1B) but also changes its localization within the cells (Fig. 2A).

We next addressed the question of whether the pattern of Rap1 modulation by coreceptors is associated with different cellular be-
haviors. The principal function of Rap1 is to regulate integrin-mediated cellular adhesion (19). Stimulation of PTC by cross-linking the TCR resulted in increased adhesion of these cells to ICAM-1 relative to that of resting cells (Fig. 2B). Costimulation of PTC with anti-CD3 antibodies resulted in a decrease in the percentage of adherent cells, while costimulation with anti-CTLA-4 antibodies resulted in significantly enhanced adhesion, supporting the idea that CTLA-4-enhanced Rap1 activation at the PM induces increased adhesiveness (Fig. 2B).

Rap1 is a negative regulator of interleukin-2 (IL-2) release, and interference with its signaling results in increased release (21). While concurrent stimulation of PTC with anti-CD3 and anti-CD28 antibodies resulted in substantial release of IL-2, this release was significantly inhibited by concurrent stimulation with anti-CD3 and anti-CTLA-4 antibodies (Fig. 2C). It has been reported that stimulation of T cells with anti-CD3 antibodies in the absence of costimulation produces anergy that is associated with elevated Rap1 activation (21, 22). Importantly, stimulation with both anti-CD3 and anti-CTLA-4 antibodies resulted not just in increased Rap1 activation and decreased IL-2 release but also in decreased p-Erk levels (Fig. 2D). These data are consistent with the idea that CTLA-4-mediated Rap1 activation at the PM coincides with anergic phenotype.

Because CTLA-4 augmented TCR activation of Rap1, we wondered whether these proteins colocalize on the same subcellular compartments. In resting cells, CTLA-4-GFP was localized on vesicles that were also decorated with Cherry-Rap1 (Fig. 2E). In Jurkat T cells stimulated through the TCR, CTLA-4-GFP was observed on both perinuclear vesicles and the PM. Thus, Rap1 and CTLA-4 colocalize on membrane compartments both in resting and stimulated cells, but only in stimulated cells do the two molecules reside on the PM.

**C3G activates Rap1 at the plasma membrane downstream of CTLA-4.** Because CTLA-4 activates Rap1, we hypothesized that it must be mediated by the GEF C3G (the most common Rap1 GEF found in T cells) (22). To examine the contribution of C3G to T-cell adhesion, we overexpressed wild-type C3G or C3G lacking its catalytic domain (C3G-ΔC) in PTC. Relative to their behavior in the resting condition, both groups of cells demonstrated increased adhesion to ICAM-1 after stimulation induced by cross-linking the TCR (Fig. 3A). However, the enhanced adhesion was significantly greater in cells expressing wild-type C3G than in cells expressing C3G-ΔC, indicating that functional C3G is required for Rap1-mediated adhesion to ICAM-1.

To further verify that C3G participates in lymphocyte adhesion, we knocked down this protein using short inhibitory RNAs (siRNAs) (Fig. 3B). Adhesion of PTC to ICAM-1 was significantly inhibited when C3G was knocked down, indicating that this GEF is required for T-cell adhesion downstream of both TCR and CTLA-4 (Fig. 3C). This effect was mediated by Rap1 activation, since in cells depleted of C3G (Fig. 3D), the amount of GTP-Rap1 downstream of the receptors was significantly decreased.

To further characterize this finding, we searched for the subcellular localization of activated Rap1 in cells lacking C3G. When cells expressing GFP-RBD-RalGDS were stimulated with anti-CD3 antibodies, in 90% of the cells the probe translocated to the PM and the vesicular compartment (Fig. 3E). When C3G was depleted, however, this pattern was altered: while Rap1 activation at the PM was demonstrated in only 45% of the cells (inset), it was absent from the majority of the cells (55%). Thus, it seems that C3G is required for Rap1 activation and that this takes place mainly at the PM.

We have previously shown biochemically that C3G translocates to the membrane fraction of T cells after stimulation with anti-CD3 antibodies (16). In the present study, we were unable to image the translocation of C3G to the PM in cells overexpressing GFP-C3G (see Fig. S3 in the supplemental material). This inability was probably attributable to the fact that around 10% of C3G translocates from the cytosol to the PM under these conditions, and our methods were not sensitive enough for the purpose. We then tried a different approach, in which we overexpressed both Cherry-K-Ras tail (to decorate the PM) and GFP-C3G (Fig. 3F). In cells that were stimulated with anti-CD3 antibodies, both Manders’ and Pearson’s colocalization coefficients were higher than those of resting cells, suggesting that TCR signaling triggers trafficking of C3G to the PM. Interestingly, concomitant stimulation with anti-CD3 and anti-CTLA-4 antibodies failed to induce higher coefficients (Fig. 3F), suggesting that CTLA-4-induced Rap1 activation is not mediated by shuttling of more C3G to the PM.

**CTLA-4 signaling leads to phosphorylation of C3G tyrosine 504.** To find out whether CTLA-4 and C3G interact physically, we subjected whole T-cell lysates to immunoprecipitation assay. Interaction was not detected under resting conditions, but when the cells were stimulated with anti-CD3 antibodies we were able to detect a strong signal, suggesting that under these conditions C3G and CTLA-4 interact or at least are found in the same signaling complex (Fig. 4A). These results are in accord with our previous finding that C3G, upon stimulation, translocates to the PM, where CTLA-4 is also found (16).

Rap1 is activated downstream of the TCR as a result of C3G translocation to the PM, but it is not clear how its costimulation with CTLA-4, while utilizing the same GEF leads to a further increase in its activation. We were unable to show biochemically that there is more C3G at the membrane fraction than was observed after stimulation with CD3 alone (discussed below) (Fig. 4E). In view of reports that C3G activity in the cells is regulated by phosphorylation, we investigated whether their stimulation via CTLA-4 would result in C3G phosphorylation, and if so, whether this phosphorylation was associated with gain of function. Treatment of the cells with pervanadate, a phosphatase inhibitor, resulted in C3G phosphorylation (Fig. 4B). Most interestingly, it was only when we stimulated the cells with both anti-CD3 and CTLA-4 antibodies that C3G was phosphorylated (Fig. 4B).

One of the phosphorylated tyrosines of C3G is reportedly located at position 504 (23). To determine whether this is the location at which C3G is phosphorylated downstream of CTLA-4, we generated a mutant version of GFP-C3G, GFP-C3G-Y504V (in which the tyrosine at that position was replaced by valine), that cannot be phosphorylated. Stimulation of the Jurkat T cells with anti-CTLA-4 antibodies did not phosphorylate C3G in the absence of tyrosine 504, suggesting that the tyrosine at position 504 is the one that is phosphorylated downstream of CTLA-4 (Fig. 4C).

In an attempt to gain insight into the biological significance of C3G phosphorylation in this setting, we assessed adhesion to ICAM-1 in PTC overexpressing the wild-type C3G or C3G-ΔC or C3G-Y504V mutant. Overexpression of wild-type C3G but not of C3G-ΔC (Fig. 4D) resulted in increased cellular adhesion compared to that of empty vector (GFP)-transfected cells. Impor-
tantly, adhesion to ICAM-1 by C3G-Y504V-expressing cells stimulated with anti-CD3 antibodies was similar to that of cells expressing wild-type C3G, suggesting that this tyrosine is not required for TCR-induced adhesion of T cells (Fig. 4D). However, when the same cells were concomitantly stimulated with anti-CTLA-4 antibodies, their adhesion was not amplified, suggesting that this tyrosine contributes exclusively to signaling downstream of CTLA-4 (Fig. 4D).

We next examined where in the cell C3G is phosphorylated. As we reported previously (16), when the cells were stimulated with anti-CD3 antibodies, a small proportion of C3G protein (~10%) was detected in the membrane fraction (Fig. 4E). Concomitant stimulation of the cells with anti-CD3 and anti-CTLA-4 antibodies yielded a similar proportion of C3G protein in the membrane fraction. Notably, phosphorylated C3G was detected only in the membrane fraction of cells stimulated with both antibodies (Fig. 4E). These data suggested either that the phosphorylation of C3G occurs at the PM or that C3G, once phosphorylated, cannot return to the cytosol.

At an earlier stage of the present study, we were not able to directly detect translocation of GFP-C3G to the PM in stimulated cells (see Fig. S3 in the supplemental material). Indirect evidence

FIG 3 C3G activates Rap1 at the plasma membrane downstream of CTLA-4. (A) PTC expressing GFP, GFP-C3G, or GFP-C3G lacking its catalytic domain (C3G-ΔC) were stimulated as indicated and subjected to adhesion assay as described in the text. (B) PTC treated with either scrambled or C3G siRNA for 48 h, followed by immunoblotting with anti-C3G and anti-Rap1 antibodies. (C) PTC treated with either scrambled or C3G siRNA were subject to stimulation, as indicated, and adhesion to ICAM-1-coated wells was recorded. (D) PTC treated with either scrambled or C3G siRNA and stimulated as indicated. The level of GTP-Rap1 was studied by GST pulldown assay, as described in the text. (E) Jurkat T cells expressing GFP-RBD-RalGDS were treated with either scrambled or C3G siRNA, stimulated with anti-CD3 antibodies, and imaged alive. The percentage of cells sharing the same phenotype is marked (inset). (F) Jurkat T cells overexpressing Cherry-K-Ras tail and GFP-C3G were stimulated with anti-CD3 and anti-CTLA-4 antibodies, and images were analyzed for colocalization coefficients at the plasma membrane. The histograms present the results (means ± SEM) from at least 3 experiments. The bar represents 10 μm. *, P < 0.05; **, P < 0.001.
for such translocation came from colocalization studies (Fig. 3F) and from biochemical analyses of cellular fractions (Fig. 4E). To substantiate the finding that phosphorylated C3G is localized to the PM, we imaged the localization of C3G in pervanadate-treated cells. Remarkably, GFP-C3G was found to translocate to the PM within 1 min of treatment (Fig. 4F). When we treated cells overexpressing C3G-Y504V with pervanadate, translocation to the PM did not occur. These striking findings further supported the no-
tion that phosphorylated C3G is “locked” at the PM, where it can activate Rap1, an event that leads to enhanced LFA-1-mediated adhesion.

The Src family member Hck phosphorylates C3G downstream of CTLA-4. The finding that C3G is phosphorylated downstream of CTLA-4, together with the fact that the cytosolic tail of CTLA-4 does not possess kinase properties, suggested that an additional kinase must be present in order to mediate these events. Of the 518 kinases identified so far, only 90 target tyrosine residues. Since the CTLA-4 receptor does not possess kinase properties, we narrowed the search to the 32 known nonreceptor tyrosine kinases. When we treated the cells with PP2, an inhibitor of the Src family, C3G phosphorylation downstream of CTLA-4 was blocked (see Fig. S4 in the supplemental material), suggesting that our kinase is a member of this family. The Src family contains 9 known members, not all of which are constitutively expressed in T cells (24, 25). Based on that, we decided to apply siRNA oligonucleotides specific for the kinases Hck, Fgr, Lck, and Zap70 (non-Src family control) (Fig. 5A). Depletion of Hck or Fgr resulted in repressed TCR-stimulated adhesion, but CTLA-4-augmented lymphocyte adhesion was significantly inhibited (Fig. 5B). In contrast, depletion of Lck or Zap70 resulted in a decrease of both TCR-stimulated and CTLA-4-augmented lymphocyte adhesion (Fig. 5B). For the strongest and the most exclusive association with CTLA-4-mediated adhesion, the leading candidate among our kinases was Hck. This was not surprising, as previous studies (as well as our data) have shown physical interaction between C3G and Hck (see Fig. S5 in the supplemental material) (26).

Whether or not Hck is expressed in T cells is still a matter of controversy (24, 26, 27, 28). Interestingly, the basal levels of Hck were very low in resting T cells but were significantly upregulated after stimulation (Fig. 5C) or in cells obtained from patients with active inflammatory diseases (unpublished study). Importantly, depletion of Hck resulted not only in decreased adhesion but also in decreased GTP-Rap1 after concurrent stimulation of the cells with anti-CD3 and anti-CTLA-4 antibodies (Fig. 5D). In contrast, GTP-Rap1 levels in T cells deficient in Fgr were not lower than those in the control (Fig. 5D). We next showed that phosphorylated C3G was decreased after Hck (but not Fgr) knockdown in cells stimulated with anti-CD3 and anti-CTLA-4 antibodies, providing direct evidence that C3G is the target of Hck downstream of CTLA-4 (Fig. 5E).

Since both Lck and Zap70 are involved in TCR signal transduction, a plausible explanation for the influence of knockdown of these kinases upon adhesion is blocked TCR-mediated recruitment of C3G to the PM. To address this, we knocked down these kinases and measured C3G translocation to the membranous fraction (Fig. 5F). Depletion of Zap70 resulted in less membranous C3G after TCR stimulation (Fig. 5F); subsequently, less phosphorylated C3G was recorded (Fig. 5E). Surprisingly, partial removal of Lck failed to block C3G trafficking (Fig. 5F) and phosphorylation (Fig. 5E). Since this kinase was required, at least in part, for Rap1 activation (Fig. 5D), we suggest that its contribution to T-cell adhesion is mediated by an unidentified pathway.

**DISCUSSION**

The overall aim of this work was to gain a better understanding of the biology of Rap1 and how it is related to CTLA-4 signaling in T cells. We discovered, downstream of CTLA-4, a new signaling pathway that mediates Rap1 activation and cellular adhesion. Signaling via TCR and Zap70 resulted in recruitment of C3G to the PM. Concomitant stimulation of C3G and CTLA-4 led to phosphorylation of C3G by the Src family member Hck at position 504. C3G phosphorylation was accompanied by higher exchange activity toward Rap1, ultimately leading to an increase in GTP-bound Rap1. Activated Rap1 at the PM increased the affinity state of C3G for ICAM-1, an event directly related to increased T-cell adhesion (Fig. 6). The role of C3G in Rap1-dependent CTLA-4-mediated T-cell adhesion is novel but not surprising. Nolz et al. presented data in which Abl affected C3G phosphorylation downstream of the TCR (29). Our findings are complementary, as we identified that Hck, and not Abl, phosphorylated C3G downstream of CTLA-4. Interestingly, Ohashi et al. reported that TCR signaling resulted in Cas-L but not C3G phosphorylation (30). Whether Cas-L or Crk is a target for Hck phosphorylation is a subject for future studies.

The primary mechanism underlying the biological function of CTLA-4 suggested that it competes with CD28 for binding with CD80 and CD86 on APC (31). The affinity of CTLA-4 for these molecules is greater than that of CD28 and can explain why CTLA-4 prevents CD28 ligation and downstream signaling (5, 31, 32). Another suggested mechanism derives from the ability of CTLA-4 signaling to reverse the stop signal induced by the TCR (33, 34). The fact that CTLA-4-augmented LFA-1-mediated adhesion and inhibited IL-2 release provided further support for additional CTLA-4-related mechanisms. In the present research, we discovered another novel and specific mechanism downstream of CTLA-4. Further work will be needed to determine how CTLA-4 interacts with Hck and whether additional proteins participate in this process.

Apparent paradoxes regarding Rap1 signaling demand resolution. Our previous reports suggested that Rap1 inhibits the Ras/Erk pathway while positively regulating T-cell adhesion, which in turn is accompanied by Ras/Erk pathway activation (35). Likewise, it is unclear how Rap1 can increase T-cell adhesion while simultaneously decrease the threshold for TCR activation and inhibit IL-2 secretion. In this work, we showed that some of these contradictions could be reconciled by the compartmentalization of Rap1 signaling (Fig. 2), which permits a single molecule to have multiple and even opposite effects based on geographic segregation. Thus, activation of Rap1 at the PM resulted in increased adhesion, whereas activation of the same Rap1 in the vesicles failed to inhibit IL-2 secretion.

Rap1 was initially identified as a Ras antagonist (36). Subsequent data showed that this is not the case and that, in some situations, Rap1 and Ras function as allies (19, 37). Here, we showed that these small GTPases can be differently modified by CD28 and CTLA-4, and that their effects on Rap1 and Ras are dissimilar, supporting opposite relationships, at least in this setting (Fig. 1). Our model to explain the effects of CD28 on Rap1 activation is distinctive from a previously published work (12). In that important paper, the investigators studied the mechanism by which CD28 regulated Rap1 activation. They overexpressed Rap1, recorded loss of effect but did not exclude the possibility that CD28 signaling to Rap1 is secondary to inhibition of a GEF. In addition, the authors did not study the subcellular localization of activated Rap1. In our work (Fig. 2), we showed that Rap1 was inhibited at the PM; therefore, any GAP that would be recruited to the PM is a potential candidate to fill the gap between the CD28 receptor signals and Rap1 activity. The ability of CD28...
FIG 5 Src family member Hck phosphorylates C3G downstream of CTLA-4. (A) Activated Jurkat T cells were treated with scrambled or C3G siRNA for 48 h, and cell lysate was immunoblotted with anti-Hck, Fgr, Lck, Zap70, and actin antibodies. (B) Hck, Fgr, Lck, and Zap70 siRNAs were introduced into Jurkat T cell by nucleofection, stimulated as indicated, and subjected to adhesion assay with ICAM-1-coated wells. (C) Resting and treated (1 ng/ml of PMA and 100 ng/ml of ionomycin for 48 h) primary T cells (PTC) and polymorphonuclear cells (Non T cells) were lysed and assayed for Hck expression. (D) Jurkat T cells were treated with scramble, Hck, Fgr, Lck, and Zap70 siRNA for 48 h, stimulated as indicated, and subjected to GST pull-down assay to detect GTP-Rap1. (E) Jurkat T cells were treated with siRNAs as indicated, followed by stimulation with anti-CD3 and anti-CTLA-4 antibodies, lysate collection, immunoprecipitation with anti-C3G antibodies, and immunoblotting with anti-C3G and antiphosphotyrosine antibodies. (F) Jurkat T cells where treated with siRNAs as indicated and stimulated with anti-CD3 antibodies, and the membrane fraction was prepared by nitorgen cavitation (as described in Materials and Methods). Samples were immunoblotted for C3G and RhoGDI. The histograms present the results (means ± SEM) from at least 3 experiments. #, no statistical difference. *, P < 0.05.
to remove active Rap1 from the PM raises the question of whether this is actual removal of GTP-Rap1 or hydrolysis of GTP. Based on published data, there is no evidence for non-membrane-bound activated Rap1 (e.g., Rap1N17 is excluded from all membranes) (2). Therefore, it is more likely that hydrolysis of the GTP and not removal of activated Rap1 occurs. To support that, we overexpressed Rap1 (Fig. 2A, upper) and showed that the levels of Rap1 were the same before and after CD28 stimulation, favoring hydrolysis and not actual removal.

Adhesion molecules are promising targets for anti-inflammatory therapies, and the development of antagonists is among the most actively pursued areas in autoimmune pharmaceutical research (38). Proof of principle for therapeutic antiadhesive T-cell strategies in autoimmunity comes in the form of biologics that block T-cell adhesion and were initially approved for clinical use (39). Unfortunately, these agents cause increased risk for infection, leading to their market withdrawal (40). Thus, although targeting the adhesion process has major clinical immunosuppressive benefits, an alternative, more “tunable” approach is needed. Kinases are attractive targets for intervention (41). More than 23 FDA-approved kinase inhibitors are currently available either as small molecules or as soluble antibodies. We suggest that Hck also could be a suitable target, and that blocking of its activity would inhibit T-cell adhesion and migration to the injured organs. Because of the known redundancy in the functions of kinases, the contribution of additional members (such as Abl and PKCθ) should be investigated as well (24). It will be extremely interesting to study the effects of dasatinib, BCR/Abl, and Src family tyrosine kinase inhibitors on cellular adhesion. Finally, although in this study Hck levels in resting PTC were low, they were upregulated after prolonged activation. We intend to study the factors involved in this regulation and if Hck expression is more pronounced in certain T-cell subsets.

The possible contribution of other kinases is appreciated. The finding that Fgr knockdown does not affect TCR plus CTLA-4-induced Rap1 activation seems to rule out this kinase as a mediator, but at the same time the effect of Fgr knockdown upon adhesion was significant. One way to reconcile this finding is the understanding that Rap1 is important, but not exclusively required, for T-cell adhesion. It is possible that Fgr regulates signaling events distal to Rap1 and modulates adhesion without direct interference with Rap1 activity. Both Lck and Zap70 are involved in TCR signal transduction. A plausible explanation for the influence of knockdown of Zap70 upon adhesion is blocked TCR-mediated recruitment of C3G to the PM (Fig. 5F). Partial removal of Lck inhibited adhesion and Rap1 activation but failed to modulate C3G translocation/phosphorylation (Fig. 5). This is intriguing, since Lck has been shown to physically interact with CTLA-4 (42), suggesting that other mechanisms contribute to Rap1 activation by this kinase.

The most intriguing goal remains to identify the specific human diseases in which this new signaling pathway may be a significant player. Through ongoing studies in our laboratory, we attempt to answer this question and hopefully translate these signaling events to human diseases.

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FIG 6 CTLA-4 signaling mediates Rap1 activation and cellular adhesion (1). TCR/Zap70 stimulation recruits the GEF C3G to the plasma membrane (2). Concomitant CTLA-4 stimulation activates the kinase Hck to phosphorylate C3G. Phosphorylated C3G facilitates Rap1 activation and subsequent LFA-mediated adhesion (3).