Costimulation through CD28 Suppresses T Cell Receptor-dependent Activation of the Ras-like Small GTPase Rap1 in Human T Lymphocytes

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Members of the Ras superfamily of small molecular weight GTPases play diverse and critical roles in mediating cellular responses to extracellular stimuli, including mitogenesis, cytoskeletal maintenance and rearrangement, and integrin activation. In T lymphocytes, biochemical and genetic evidence demonstrate that Ras plays an essential role in coupling T cell receptor ligation to signaling cascades required for T cell proliferation and development. Recent observations that C3G, a guanine nucleotide exchange factor specific for the Ras-related GTPase Rap1, is recruited into tyrosine-phosphorylated protein signaling complexes in activated T cells have suggested that Rap1 may also play a role in T cell activation. Utilizing a recently developed technique for detection of endogenous, GTP-bound Rap1, we have found that Rap1, but not Rap2, is transiently activated following T cell receptor stimulation of normal human T lymphocytes. Increases in intracellular calcium is both necessary and sufficient to induce Rap1 activation. Remarkably, costimulation of T cells with mitogenic anti-CD28 antibody completely abolished T cell receptor-dependent activation of Rap1. This report demonstrates a potential role for Rap1 in T cell receptor signaling and suggests inactivation of Rap1 as a candidate target of CD28-dependent costimulatory signals required for T cell antigen responsiveness.

Biochemical, mutational, and genetic studies have demonstrated that physical and functional coupling of the T cell receptor (TCR) to associated Src (Fyn and Lck) and Syk (Syk, ZAP-70) family protein-tyrosine kinases (PTKs) plays a critical role in both T cell activation and development (reviewed in Refs. 1 and 2). The generation of lipid second messengers, mobilization of calcium stores, and gene transcription critical for T cell proliferation and cytokine secretion is entirely dependent on TCR-associated PTK activity (1, 3). In normal human T lymphocytes, ligation of the TCR alone is insufficient to induce proliferation and cytokine secretion, and instead in-ducees anergy, a long term unresponsiveness to antigen stimulation. Costimulation of T cells by soluble interleukins (ILs) (e.g., IL-2, -4, -7, or -15) or by ligation of the T cell accessory protein CD28 by antibodies or B7-1 and B7-2 ligands on antigen presenting cells avoids induction of anergy and leads to a full proliferative T cell response (4, 5). The mechanism(s) by which CD28 stimulation prevents anergy and cooperates with TCR-derived signals to induce T cell proliferation are poorly understood. CD28 can activate both Src and Tek family PTKs (reviewed in Ref. 6). CD28-dependent PTK activity phosphorylates a number of T cell signaling proteins previously shown to be tyrosine-phosphorylated in response to TCR stimulation, including phospholipase C-γ1, SLP-76, p36/36, and the proto-oncogene products Vav and Cbl (1, 2, 6–8). Similarly, CD28 stimulation also leads to activation of phosphatidylinositol 3-kinase and calcium mobilization in T lymphocytes (reviewed in Ref. 6). However, CD28 stimulation also generates second messengers distinct from TCR signaling, including ceramide (9) and reactive oxygen intermediates (10), which may play a role in the ability of CD28 stimulation to synergistically activate Jun kinases and enhance IL-2 mRNA transcription and stability in CD3-stimulated T cells (4, 6).

In T cells, stimulation by either anti-CD3 or anti-CD28 antibodies results in recruitment of the Ras nucleotide exchange factor Sos to tyrosine-phosphorylated Shc and p36/36 proteins via the adaptor protein Grb2 (7, 8, 11, 12). Subsequent conversion of Ras to its active, GTP-bound form (13) allows membrane localization and activation of Raf, which initiates a cascade of serine/threonine kinase activation, culminating in activation of transcription factors, gene transcription, and mitogenesis (14). Studies in transgenic mice and fetal thymic organ cultures have confirmed that an intact Ras signaling pathway is required for proper T cell development and function (15–19), and blocks in Ras signaling pathways are found in anergized T cells (20, 21).

Recent studies in B and T lymphocytes have suggested a novel signaling pathway in lymphocytes involving the Ras-related GTPase Rap1. Antigen receptor-dependent tyrosine phosphorylation of the proto-oncogene product Cbl induces Cbl association with SH2 domain-containing adaptor proteins, including Crk family proteins (22–24) and the p85 subunit of phosphatidylinositol 3-kinase (25–28). In these studies, it was observed that CrkI was constitutively associated via its SH3 domain to the guanine nucleotide exchange factor C3G, which has guanine nucleotide exchange activity specific for Rap1 (29, 30). As C3G could be detected in complex with tyrosine-phosphorylated Cbl (22), it was suggested that Rap1 may be activated as a consequence of TCR stimulation. Rap1 was originally identified by its ability to revert viral Kras oncogenic transformation in fibroblasts, yet potential roles and functions for Rap1 in physiological signaling pathways remain poorly understood.

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understood (31, 32). Rap1 has an effector domain nearly identical to Ras and binds to many of the same effector proteins as Ras, including Raf-1, the catalytic subunit p110 of phosphatidylinositol 3-kinase and the Ral guanine nucleotide exchange factors in vitro, suggesting that Rap1 might antagonize Ras signaling by sequestering potential Ras effectors in inactive complexes (33–36). Recent observations that Rap1 can mediate cAMP-dependent B-Raf and MAP kinase activation in PC12 cells (37) and can enhance mitogenic signaling pathways in Swiss 3T3 responses to insulin (38) suggest that in certain cells, Rap1 could contribute positive signals to mitogenic responses.

In this study, we utilize a recently developed technique for detecting GTP-bound Rap proteins (39) and demonstrate that Rap1 is transiently activated in TCR-stimulated normal human T cell PHA blasts and the cytotoxic T cell clone D11 but not in the leukemic T cell line Jurkat. Although TCR-mediated Rap1 activation is calcium-dependent, either phorbol ester or calcium ionophore alone are sufficient to induce Rap1 activation. Remarkably, activation of Rap1 by cross-linking TCR/CD3 could be completely inhibited by costimulation through CD28, suggesting that Rap1 may play a role in discerning the presence of costimulation during TCR-dependent activation.

MATERIALS AND METHODS

Cells—The human T cell leukemia cell line Jurkat was maintained as described previously (25). Human T cell PHA blasts were derived from fresh citrated human blood obtained from healthy volunteers (Minidonor Blood System, Academic Hospital, Utrecht and the Red Cross Blood Bank, Utrecht). Enriched peripheral blood lymphocytes were obtained by centrifugation over Ficoll-Paque (Pharmacia Biotech, The Netherlands) and two successive rounds of monocyte depletion by plastic adhesion. Resulting cells were then stimulated for 48 h with PHA (1 μg/ml; Sigma) and recombinant IL-2 (20 units/ml; ICN Biomedicals, The Netherlands) in RPMI 1640 media (containing 10% fetal bovine serum [Integro B.V., Netherlands], 0.03% glutamine, 100 units/ml penicillin and streptomycin) (all media components from Gibco, Grand Island, NY). Jurkat cells were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum (Integro B.V., Netherlands), 0.03% glutamine, 100 units/ml penicillin and streptomycin) (all media components from Gibco, Grand Island, NY) unless otherwise noted, washed, and expanded for an additional 4-8 days in media containing 20 units/ml IL-2 alone. PHA blasts were removed from IL-2 24 h before experiments to obtain quiescent cells. Maintenance, culture, and stimulation of the D11 allogeneic cytotoxic CD8+ human T cell clone was as described previously (40).

Lymphocyte Activation—Washed cells were resuspended in RPMI 1640 media and incubated for 30 min on ice in the absence or presence of activating monoclonal antibodies against CD3 (Spv T3b, a generous gift of Dr. Hergen Spiks, Netherlands Cancer Institute, Amsterdam) and/or CD28 (3851, 1 μg/ml) (a gift kindly provided by Dr. René van Lier, Red Cross Central Blood Bank, Amsterdam) (41). After centrifugation at 4 °C and two washes in cold RPMI 1640, cells were activated by resuspension in 37 °C RPMI 1640 containing 10 μg/ml cross-linking goat anti-mouse antibodies (Cappel). Cold lysis buffer containing 0.5% Triton X-100, 50 mM Tris, pH 7.6, 150 mM sodium chloride, and phosphatase and protease inhibitors (1 μM phenylmethanesulfonfyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μM sodium orthovanadate, and 10 mM sodium fluoride) was added after 2 min, unless indicated otherwise in figure legends, and were cells lysed for 45 min at 4 °C.

In some experiments, cells were stimulated by resuspension in pre-warmed media containing 100 ng/ml TPA (Sigma) or 1 μM ionomycin (calcium salt; Calbiochem). For experiments using pharmacological inhibitors, cells were incubated with BAPTA-AM (30 min), staurosporine (30 min), or herbimycin A (2 h) (all from Sigma) at 37 °C on the concentrations indicated in figure legends before washing and incubation with antibodies as described above.

Antibodies and Fusion Proteins—Monoclonal antibodies used were anti-Rap1 (Transduction Laboratories) and anti-phosphotyrosine 4G10 (Upstate Biotechnology). Anti-Rap1 antibodies were generated in our laboratory by immunization of rabbits with synthetic Rap1A carboxyl-terminal peptide (amino acids 100–114, CDLVQRQIKRT-PVERK, cysteine added for coupling) (Eurogentec, Belgium) coupled to keyhole limpet hemocyanin (Pierce). Anti-MAP kinase ERK2 polyclonal antibodies have been previously described (42).

Plasmid-encoding glutathione S-transferase (GST) fused to amino acids of the Rap binding domain (RBD) of Rap-GDS and purification of GST-RBD fusion protein have been previously described (36).

Immunoprecipitations, Binding Reactions, SDS-PAGE, and Immunoblotting—Lysates were clarified for 10 min at 4 °C by centrifugation at 14,000 rpm. For GST-RBD binding reactions, lysate corresponding to 5–7.5 × 106 cell equivalents were incubated for 45 min at 4 °C with 5 μg of GST-RBD precoupled to glutathione-agarose beads (Pharmacia). Resolution of bound proteins by SDS-PAGE and detection by immunoblotting and ECL using horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin antibodies (New England BioLabs) were as described previously (39). For all Rap binding experiments, equivalent portions of cell lysates used for binding reactions were loaded on separate SDS-PAGE gels and analyzed for Rap1 content to confirm that equivalent amounts of Rap1 were available for binding (data not shown).

RESULTS AND DISCUSSION

TCR Stimulation Transiently Activates Rap1 in T Lymphocytes—Recent reports have suggested that Rap1 may be involved in B and T cell antigen receptor signaling in lymphocytes, as these studies reported recruitment of the Rap1-specific guanine nucleotide exchange factor C3G to tyrosine-phosphorylated Cbl, an early and prominent substrate of antigen receptor-associated PTKs, via the CrkL adaptor protein (22). To assess if TCR stimulation activated Rap1 in T lymphocytes, we utilized a recently developed novel technique using the RBD of Rap-GDS to precipitate GTP-bound Rap1 from cell lysates. GST-RBD fusion proteins bind with high affinity to GTP-bound Rap1 and Rap2 (Kd 10 nM) although binding negligibly to GDP-bound Rap proteins (Kd 10 μM) (36) and can be utilized as sensitive probes for detecting activated GTP-bound Rap1 proteins in cell lysates. Our laboratory has previously used this technique to report the thrombin-dependent activation of Rap1 in human platelets, where treatment with thrombin led to rapid, calcium-dependent activation of Rap1 (39).

We first compared levels of GTP-bound active Rap1 in normal human T cell PHA blasts before or after TCR/CD3 stimulation. T cells were incubated in the absence or presence of anti-CD3 antibody, followed by washing and activation by cross-linking antibody for 2 min. After lysis, endogenous GTP-bound Rap1 was detected by anti-Rap1 immunoblotting (Fig. 1). Small amounts of activated Rap1 were detected in unstimulated T cells, and after TCR stimulation, a distinct increase in GTP-bound Rap1 was observed. Anti-Rap1 immunoblotting of whole cell lysates confirmed that equivalent amounts of Rap1 were available for binding in all samples (data not shown). The basal levels of GTP-bound Rap1 varied between experiments, but in each of four independent experiments, an estimated 2–5-fold increase in Rap1 GTP content was observed.

To further characterize the activation of Rap1, we examined the kinetics of Rap1 activation in T cells after TCR cross-linking (Fig. 2, upper panel). Anti-Rap1 immunoblotting of
GST-RBD binding reactions revealed that Rap1 activation was strongly induced within 1 min of TCR cross-linking, reaching a maximal level of activation 2 min after stimulation. Levels of active Rap1 then decreased slowly and were still above basal levels 10 min after stimulation. In other experiments, Rap1 activation was found to return to basal activation levels by 30 min post-stimulation (data not shown). Thus, Rap1 is quickly activated and transiently converted to its active GTP-bound form after TCR stimulation. Transient Rap1 activation was also observed in TCR-stimulated D11 cells, where Rap1 activation was maximal at 2 min and decreased quickly thereafter (Fig. 2, lower panel).

In contrast, we have been unable to detect CD3-dependent activation of Rap1 in three independently maintained Jurkat cell lines, although tyrosine phosphorylation of Cbl was observed (Fig. 2, middle panel and data not shown). Thus, it is possible that mutations affecting either C3G catalytic activity or Rap1 itself occurred in Jurkat, or, alternatively, recruitment of C3G into signaling complexes may not be sufficient for TCR-dependent Rap1 activation, and an as yet unidentified novel exchange factor or cofactor present in normal T cells may be lacking in Jurkat.

Activation of Rap1 by TCR Stimulation is Calcium-dependent—Previous studies in human platelets demonstrated that treatment of platelets with phorbol ester or calcium ionophore was sufficient to induce Rap1 activation, and activation of Rap1 by thrombin was completely dependent on calcium (39). To assess if CD3-dependent activation of Rap1 in T cells was similarly regulated, we examined the ability of phorbol ester and calcium ionophore, alone or in combination, to activate Rap1 in T cell clones. T cells were either unstimulated or stimulated with the calcium ionophore ionomycin, alone or in combination, to activate Rap1 in T cells (Fig. 3, lower panel).

We also assessed whether TCR stimulation of PHA blasts could also induce activation of the closely related Rap2 GTPase. However, probing of RBD-GST binding reactions with anti-Rap2A antibody failed to detect CD3-stimulated Rap2 activation in PHA blasts. GST-RBD binding reactions from lysates of unstimulated or anti-CD3-stimulated PHA blasts were performed as in Fig. 1. C3G stimulation of Jurkat T cells failed to activate Rap2, and no change in GTP content was observed after TCR stimulation of PHA blasts (Fig. 3, lower panel). We also assessed whether TCR stimulation of PHA blasts could also induce activation of the closely related Rap2 GTPase. However, probing of RBD-GST binding reactions with anti-Rap2A antibody failed to detect CD3-stimulated Rap2 activation in PHA blasts. GST-RBD binding reactions from lysates of unstimulated or anti-CD3-stimulated PHA blasts were performed as in Fig. 1. C3G stimulation of Jurkat T cells failed to activate Rap2, and no change in GTP content was observed after TCR stimulation of PHA blasts (Fig. 3, lower panel).
observed with 10 mM BAPTA-AM. Partial inhibition of Rap1 activation was observed with inhibitor BAPTA-AM and PHA blasts were pretreated with staurosporine in the absence of inhibitors (lane 2) served as controls. In a separate experiment, PHA blasts were stimulated with anti-CD3 antibodies and staurosporine had no effect on CD3-dependent Rap1 activation (Fig. 4). Consistent with results obtained in thrombin-treated platelets, CD3-dependent Rap1 activation (Fig. 4). As these results demonstrated that both phorbol esters and calcium ionophores could induce Rap2 activation. Stripping of blots shown in Fig. 3A and reprobed with anti-Rap2 antibodies surprised a weak activation of Rap2 by TPA (Fig. 3C, lower panel). Ionomycin also induced barely detectable activation of Rap2 (data not shown). Overlay of anti-Rap1 and anti-Rap2 blots shown here clearly demonstrated that Rap2 migrated with a faster mobility than Rap1 and that Rap2 activation did not represent residual signals from anti-Rap1 immunoblotting. GTP-bound Rap2 recovered by GST-RBD represents more than 5% of total Rap2 available cellular lysate (whole cell lysate lanes represent 5% of cellular protein used in binding reactions). It is uncertain whether this reflects weak activation of a Rap2-specific exchange factor by calcium and TPA or whether the calcium/TPA-responsive exchange factor activating Rap1 has weak activity against Rap2. C3G does not display exchange activity for Rap2 (43), and exchange factors for Rap2 have yet to be identified. Together, these data indicate that Rap1 is selectively activated following TCR stimulation.

As these results demonstrated that both phorbol esters and increased intracellular calcium concentrations were individually sufficient to induce Rap1 activation in T cells, we next examined if either protein kinase C or calcium was required for CD3-dependent Rap1 activation (Fig. 4). Consistent with results obtained in thrombin-treated platelets, CD3-dependent Rap1 activation was inhibited in a dose-dependent manner by pretreatment of PHA blasts with the intracellular calcium chelator BAPTA-AM. Partial inhibition of Rap1 activation was observed with 10 mM BAPTA-AM (lane 3), whereas treatment with 50 mM BAPTA-AM completely inhibited CD3-dependent Rap1 activation (compare lanes 2 and 6). In contrast, pretreatment of T cells with the broad specificity protein kinase C inhibitor staurosporine had no effect on CD3-dependent Rap1 activation (lane 3) except at high concentrations (1 mM, lane 4), where staurosporine inhibited protein kinases with little specificity (44). Given that staurosporine could inhibit Rap1 activation at high concentrations, we examined in a separate experiment the effect of pretreatment with the tyrosine kinase inhibitor herbimycin A. Pretreatment with herbimycin A completely abolished Rap1 activation after TCR cross-linking (compare lanes 9 and 10), consistent with the absolute requirement for tyrosine kinases in transmitting signals from the TCR and the proposed regulation of Rap1 activation by recruitment of C3G to tyrosine-phosphorylated Cbl (22). However, activation of Rap1 by both thrombin and TCR stimulation in platelets and T cells, respectively, displayed similar calcium dependences. Analysis of the primary structure of C3G indicates that C3G lacks consensus calcium binding motifs as well as conserved amino acid motifs found in the calmodulin binding IQ domains of the Ras calcium-responsive exchange factors Ras-guanine nucleotide releasing factor (Ras-GRF) and Ras-GRF2 (45, 46). Although, additionally, although the majority of Rap1 is rapidly activated in thrombin-stimulated platelets, protein levels of C3G in human platelets are nearly undetectable. Perhaps a thus far unidentified calcium-dependent exchange factor or cofactor may be mediating Rap1 activation in hematopoietic cells.

Costimulation through CD28 Prevents CD3-dependent Rap1 Activation—It has recently been reported that ligation of the T cell accessory protein CD28, like TCR stimulation, also leads to tyrosine phosphorylation of the Cbl proto-oncogene (8), and we therefore anticipated that CD28 ligation might also induce Rap1 activation in T lymphocytes. Quiescent T cells were incubated with media alone or media containing anti-CD3, anti-CD28, or a combination of anti-CD3 and anti-CD28 antibodies. Stimulation by CD3 Alone increased the amount of GTP-bound Rap1 as compared with unstimulated T cells (Fig. 5A, left panel). In contrast, stimulation of CD28 failed to activate Rap1. Strikingly, costimulatory anti-CD28 antibodies, when combined with anti-CD3 antibodies, completely blocked CD3-dependent Rap1 activation. Indeed, levels of GTP-bound Rap1 were reduced below levels observed in unstimulated T cells. Identical results were observed in three independent experiments, excluding donor-specific effects. Stimulation through CD28, either alone or in combination with anti-CD3 antibodies, failed to modulate Rap2 activation (Fig. 5A, right panel).
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come primarily from experimental systems that measure the

sights into potential roles for Rap1 in signal transduction have

B). Treatment of T cells with anti-CD28 antibodies alone resulted in a slow decrease in GTP-bound Rap1, as compared with levels in unstimulated cells over a period of 10 min, to nearly undetectable levels (Fig. 5B, left panel). Costimulation through TCR and CD28 led to a more rapid decrease in levels of GTP-bound Rap1 (right panel). Under these conditions, GTP-bound Rap1 could no longer be detected 2 min after stimulation. Neither CD28 or TCR plus CD28 stimulation resulted in activation of Rap2 (data not shown). Thus, stimulation through CD28 initiates signals that result in a decrease in GTP-bound Rap1 and completely blocks CD3-dependent conversion of Rap1 to its active state.

To confirm that costimulation through CD28 was not generally antagonizing signaling through CD3, we assessed the ability of anti-CD3 and anti-CD28 antibodies to stimulate MAP kinase activation in PHA blasts. As seen in Fig. 5C, stimulation with either anti-CD3 or anti-CD28 antibodies both induced gel mobility shifts (correlating with activation) of the MAP kinase ERK2, maximal 2–5 min post-stimulation. Importantly, costimulation through CD28 did not antagonize CD3-dependent MAP kinase activation and slightly enhanced ERK2 activation. These results demonstrate that antagonism of CD3-dependent Rap1 activation is a specific consequence of CD28 costimulation.

The mechanism by which CD28 costimulation blocks CD3-dependent activation is currently under investigation and could include regulation of one or more of several potential checkpoints. CD28, unlike CD3, activates the Tek family kinase Itk (6), which could result in differential phosphorylation of Cbl (preventing association with CrkL) or phosphorylation of CrkL (potentially disrupting ligand binding to both the SH2 and SH3 domains of CrkL) (47, 48). Alternatively, generated second messengers unique to CD28 signaling, including ceramide and reactive oxygen intermediates, might directly regulate Rap1 or its exchange factor(s) or activate Rap-specific GTPase-activating proteins, such as tuberin or Rap-GTPase-activating protein (49–51). CD3 and CD28 stimulation both induce calcium influx in T cells (1, 6), so it is therefore likely that CD28-mediated antagonism of Rap1 occurs downstream of the calcium-dependent regulatory component.

Rap1 was originally identified in screens for suppressors of viral Kras-mediated fibroblast transformation. Consequent insights into potential roles for Rap1 in signal transduction have come primarily from experimental systems that measure the effect of exogenously introduced wild-type and mutant Rap1 on receptor signaling pathways. Consistent with the ability of Rap1 to suppress transformation by viral Kras, overexpression of active Rap1 antagonizes Ras-dependent MAP kinase activation after lysophosphatidic acid and epidermal growth factor stimulation of fibroblasts (52), and a dominant gain-of-function mutation in the Drosophila homolog of Rap1, Roughened, interferes with Ras-dependent photoreceptor development (53). In contrast, overexpression of Rap1 can also potentiate insulin-dependent mitogenesis in Swiss 3T3 fibroblasts, and recent work reports regulation of CAM-dependent B-Raf/MAP kinase signaling by Rap1 in PC12 cells (37), indicating that in some cell types, Rap1 can positively regulate mitogenic signaling pathways as well. Rap1 is closely related to Ras, and the core GTPase effector domain is completely identical between Rap1 and Ras. This has led to the suggestion that the effects of Rap1 on Ras-dependent signaling result from competition by Rap1 for binding to the same effector proteins utilized by Ras (35).

Indeed, Rap1 binds with high affinity in vitro to Raf and Ral-GDS family members. Association of activated Rap1 with a Ras effector protein could then either stimulate pathways similar to Ras, or sequester the effector in an inactive complex, inhibiting Ras-dependent signaling events.

TCR stimulation in the absence of costimulation results in long-term antigen responsiveness of normal T lymphocytes, while costimulatory signals allow elevated production of IL-2 and proliferation. Recent studies demonstrate that an intact Ras signaling pathway is critical for proliferative T cell responses, and that blocks in this pathway induce an anergic state in T cells (15–19). Moreover, in anergized T cells, there is a block in Ras-dependent MAP kinase activation (20, 21). While this manuscript was in preparation, Boussiotis et al. reported that levels of GTP-bound Rap1 were constitutively elevated in an anergized T cell clone, compared with control cells (52). Elevated Rap1-GTP levels were found to correlate with a block in CD3-dependent c-Raf activation and association with Ras. Therefore, in addition to our proposal that Rap1 could play a role in discerning between productive and nonproductive antigenic stimulation, Rap1 may also play a role in maintaining T cell anergy, once established.

As we have observed that Rap1 is activated in normal T cells following stimulation by CD3 alone, but not when costimulatory signaling through CD28 is provided, it will be of interest to examine if Rap1 plays a role in allowing T cells to discern between anergizing and proliferative stimuli. Given the known capacity of Rap1 to antagonize Ras signaling pathways, it is tempting to speculate that CD3-dependent Rap1 activation, in the absence of costimulation, might serve to attenuate Ras signaling by sequestering away one or more potential Ras effector proteins (Raf, phosphatidylinositol 3-kinase, Ral-GDS). This in turn might hold Ras-dependent signaling cascades below a certain threshold required for mitogenesis and possibly contribute to T cell anergy. Although stimulation with anti-CD3 and anti-CD28 antibodies, but not CD28 ligands B7–1 and B7–2 (8), can both induce Ras and MAP kinase activation, detailed analyses of the extent and duration of CD3-dependent Ras effector activation in the absence and presence of CD28 costimulation have not been reported.

Alternatively, Rap might induce selective transcriptional activation distinct from Ras. In this regard, it is interesting that overexpression of C3G can enhance c-Jun NH2 kinase activation, and dominant negative C3G can block serum- and Crk-dependent c-Jun NH2 kinase activation (53). Lastly, Rap1 may also regulate signaling pathways unrelated to mitogenesis. Mutant Rap1 has been demonstrated to interfere with NADPH oxidase assembly in myeloid leukemia cells (54, 55), whereas mutations in the Rap1 homolog Bud1/Rsr1 1 protein disrupt polarized budding in Saccharomyces cerevisiae (56). By analogy, Rap1 might also play a role in the targeted secretion of cytotoxic granules in T lymphocytes. Ongoing studies in our laboratory are now addressing the mechanisms by which CD3 and CD28 differentially regulate Rap1 binding to GTP and examining the role of Rap1 in T cell activation.

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