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Antigen receptor and co-stimulatory signals differentially regulate RapGAP family protein expression in human T lymphocytes

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Manuscript in preparation

Abstract

The quality of T cell immune responses is exquisitely regulated by the coordinated triggering of the T cell antigen receptor (TCR) and costimulatory proteins, such as CD28-like proteins and TNF family ligands. Costimulatory proteins are differentially expressed depending on the activation and differentiation status of the T cell. Recent independent observations have suggested that T cell costimulatory proteins may exert their effects via Rap1GAP-dependent regulation of Rap1 activation. However, little is known regarding the regulation of distinct Rap1GAPs in human T cells during T cell activation and differentiation. Here, we provide evidence that each of the five Rap1GAPs is expressed in human T lymphocytes in both lineage- and activation- dependent manners, regulated at both transcriptional and post-translational levels. Our results indicate that control of Rap1 function is tightly orchestrated during both T cell differentiation and activation, and each of the Rap1GAPs plays a role in this process.

Introduction

Antigen-specific T cells are activated following T cell receptor (TCR) engagement by antigenic peptide complexed to major histocompatibility (MHC) proteins on antigen-presenting cells (APCs). Resultant effector T cell cytokine secretion, proliferation, differentiation into effector and memory T cells, and contraction of the T cell pool by apoptosis are tightly regulated processes[1,2]. Signaling by co-stimulatory cues, especially ligands of the CD28-like and tumor necrosis factor receptor family members, dictate the difference between antigenic unresponsiveness, successful immune responses, and chronic inflammatory responses. Specific T cell functional responses are coupled to these co-stimulatory cues via intracellular signaling pathways.

The intracellular signaling protein Rap1, a member of the Ras superfamily of GTPases, has emerged as playing a central role in interpreting TCR and co-stimulatory signals to coordinate T cell immune responses in vitro and in vivo. Like other small GTPases, Rap1 is activated by guanine nucleotide exchange factors (GEFs) which catalyze dissociation of the GTPase from GDP[3]. Subsequent binding of Rap1 to cytosolic GTP activates Rap1, allowing it to interact with downstream effector proteins[4]. A large number of potential Rap1 effectors have been identified, including adaptor proteins involved in integrin function, exchange factors for Rho family GEFs which mediate cytoskeletal rearrangements, and components of Ras signaling pathways such as Ras GEFs and Raf kinases[4]. Rap1 signaling is ultimately terminated by GTPase-activating proteins (GAPs) which enhance the intrinsic GTPase activity of Rap1, converting GTP to GDP and returning Rap1 to its inactive state[3]. Known GAPs for Rap1 include Rap1GAP1A, Rap1GAP1B, Rap1GAP2, Spa-1, and E6TP1 (Spa-L1/SPAR)[3;5;6].

Activation of Rap1 by chemokines drives polarization of T cells and activates integrins needed for chemotaxis and initial attachment to APCs[7]. TCR-dependent activation of Rap1 also drives cytoskeletal rearrangements and integrin activation required for establishment of a productive immunological synapse with the APC[8;9]. T cell co-stimulatory receptors, such as CD28 and CTLA-4, coordinate with the TCR to modulate Rap1 activity, which in turn influences TCR-dependent ERK activation [10-12] and reactive oxygen species production[13;14]. These latter functions of Rap1 are needed for optimal T cell cytokine transcription and proliferative responses, and two transcription factors sensitive to Rap1 signaling in T cells are NFAT and Elk[10;12]. CD28 and CTLA4 may modulate Rap1 activation by, respectively, stimulating and inactivating Rap1GAP1A[15-17].

Influences of Rap1 on T cell function in vitro have suggested that Rap1 might regu-

late the quality of T cell responses in vivo, and this has largely been confirmed in studies utilizing genetic manipulation of Rap1 signaling in mice[5]. Atlhough Rap1 participates in positive and negative selection of thymocytes, expression of constitutively active Rap1 in the T cell compartment has little overt effect on thymocyte development, but can enhance positive selection for low affinity antigens[11;18;19]. Transgenic expression of the active Rap1 mutant Rap1E63, but not the weaker RapV12 active mutant, decreases T helper cell function and increases the frequency and function of CD103-expressing regulatory T cells[11;19]. A similar phenotype is observed in mice lacking Spa-1, where thymocyte development appears normal[20]. However, if endogenous Rap1 is further activated by exogenous expression of a Rap1 GEF, Spa-1 –deficient mice display expanded DP thymocyte populations and develop T-cell leukemia[21]. Spa-1 –deficiency also results in an age-dependent acquisition of T cell hypo-responsiveness to mitogens and antigen recall challenges[20], and in human T cells, elevated Rap1 activation has been linked to T cell anergy[10;22]. Reciprocally, genetic ablation of Rap1A has no observable effect on T cell development, but does impair T cell polarization and integrin-dependent adhesion[23;24]. More severe phenotypes are observed in mice transgenically over-expressing Rap1GAPs in the T cell compartment. Spa-1 transgenic mice display a block in α/β thymocyte development at the DN stage[25]. Rap1GAP1A transgenic mice display normal thymocyte development and peripheral T cell populations, but accumulate activated, hyper-responsive lymph node T lymphocytes during aging[17]. A similar effect of T cell Rap1 inactivation with pathological consequences may occur in humans. In patients with rheumatoid arthritis, a block in Rap1 activation in observed in synovial fluid T cells[13;14], associated with enhanced TCR-dependent cytokine and proliferative responses[26].
Coupling of extracellular stimuli to Rap1 activation in T cells is mediated primarily by two Rap1 GEFs, C3G and CalDAG-GEFI. In general, GEF activity is modulated by inducible conformational changes in GEFs[3]. TCR stimulation recruits Crk-bound C3G to the TCR[27], which in turn stimulates GEF activity of C3G[28;29]. The conformation and activity of CalDAG-GEF I is regulated by the soluble second messengers calcium and diacyl glycerol (DAG), both of which are generated following phospholipase C γ1 activation[30]. CalDAG-GEF is required for TCR and chemokine -dependent Rap1 activation in human T cells[30;31]. In contrast to GEFs, Rap1GAPs are thought to be constitutively active[3], and their ability to inactivate Rap1 is instead regulated by changes in expression levels and recruitment to the site of activated Rap1[32]. Three Rap1GAPs have been reported in T lymphocytes, RapGAP1A, RapGAP1B, and Spa-1[33;34]. TCR-dependent Rap1 activation is inhibited by co-ligation of CD28[15], which is RapGAP1A-dependent[16;17]. RapGAP1B can bind to G-coupled receptors[34]. Spa-1 is

expressed in proliferating lymphoid cells[33;35] and its participation in TCR signaling is suggested by its recruitment to the immunological synapse during antigen-specific stimulation of T cells[36]. Variation in phenotypes of mice transgenically expressing different Rap1GAPs raises the possibility that each Rap1GAP family member may make distinct contributions to T cell activation. Here, we find that Rap1GAPs are differentially expressed in resting and TCR/CD28-stimulated human T lymphocytes, and that expression of Rap1GAPs is regulated by both transcriptional and post-translational mechanisms. Distinct Rap1GAPs may thus differentially couple external stimuli to Rap1 regulation, or inactivate Rap1 in distinct cellular subcompartments, dependent upon the activation status of the T cell.

Results

Differential expression of Rap1GAP family member mRNA in resting and activated human T lymphocytes

We initiated our studies by investigating mRNA expression of each of the Rap1GAP family members in freshly isolated resting and CD3/CD28-stimulated human peripheral blood T lymphocytes. In unstimulated T cells, Spa-1 mRNA was expressed at low to undectable levels, but increased following CD3/CD28 stimulation (Fig. 1). Rap1GAP1A mRNA was readily detected in resting T cells, and increased further following activation. A similar pattern of expression was observed for Rap1GAP1B. In contrast, Rap1GAP2 and E6TP1 mRNA, both expressed in unstimulated T cells,

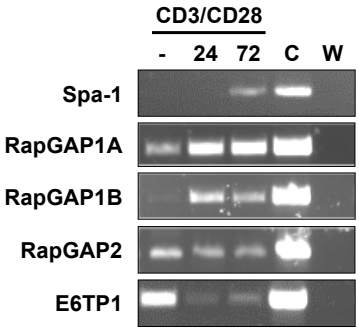

Figure 1. Rap1GAP family member mRNA expression is differentially regulated by CD3/CD28 stimulation. *Qualitative PCR analysis of Spa-1, Rap1GAP1A, Rap1GAP1B, Rap1GAP2 and E6TP1 mRNA expression in freshly isolated unstimulated (-) human T lymphocytes, and lymphocytes stimulated for 24 or 72 hours with anti-CD3/CD28 antibodies. Positive controls (C) consisted of mRNA from COS-7 cells transfected with 10 ng of expression plasmid for the appropriate Rap1GAP, while water (W) served as a negative control. Results are representative of three independent experiments.*

were down-regulated within 24 hours of CD3/CD28 ligation. Together, these results suggested that each of the Rap1GAP family members could be expressed in T lymphocytes, and that regulation of specific Rap1GAPs was a dynamic process dependent upon T cell activation status.

Spa-1 protein expression is specifically up-regulated in activated CD4$^+$ T lymphocytes

We next examined if changes in Rap1GAP family member mRNA expression following T cell activation resulted in changes in protein expression. Purified CD3$^+$ T lymphocytes were cultured in medium alone or stimulated with activating anti-CD3/CD28 antibodies for up to 72 hours, and cellular lysates examined for Spa-1 protein expression by immunoblotting (Fig. 2). Consistent with mRNA expression, Spa-1 protein was also detected at low levels in freshly isolated T cells (Fig. 2A). Following CD3/CD28 stimulation, Spa-1 protein expression increased in a time-dependent manner throughout the time course of activation (Fig. 2A). We further investigated if Spa-1 expression was similarly regulated in CD4$^+$ T helper cells and CD8$^+$ cytotoxic T cells. Purified T cell subsets were isolated by negative selection,

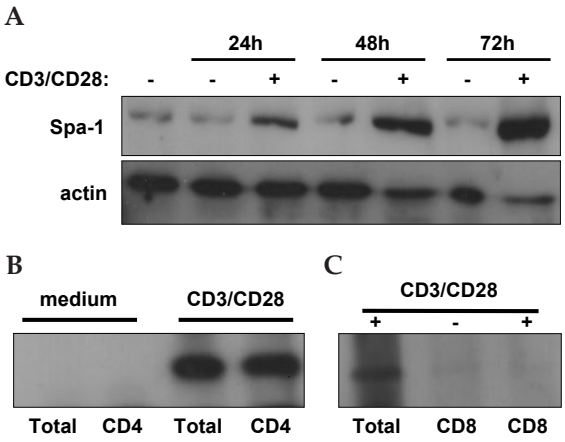

Figure 2. Spa-1 protein protein is selectively up-regulated in CD4$^+$ T lymphocytes following CD3/CD28 stimulation. *(A) Total T lymphocytes were left unstimulated (-) or stimulated (+) for the indicated number of hours with anti-CD3/CD28 antibodies and Spa-1 and actin expression determined by immunoblotting of cell lysates. (B) Immunoblotting of total and purified CD4$^+$ T lymphocyte lysates with Spa-1 antibodies after 72 hour incubation in medium alone or in the presence of anti-CD3/CD28 antibodies. (C) Immunoblotting of total and purified CD8$^+$ T lymphocyte lysates with Spa-1 antibodies after 72 hour incubation in the absence (-) or presence (+) of anti-CD3/CD28 antibodies. Results shown are representative of 3-6 independent experiments. Experiments shown are representative of three to five independent experiments.*

and cultured in the absence or presence of anti-CD3/CD28 antibodies for 72 hours. Remarkably, Spa-1 protein expression was up-regulated only in stimulated CD4+ T lymphocytes (Fig. 2B), but not CD8+ cytotoxic T cells (Fig. 2C). Spa-1 thus represents a lineage and activation status –specific regulator of Rap1 function in human T lymphocytes.

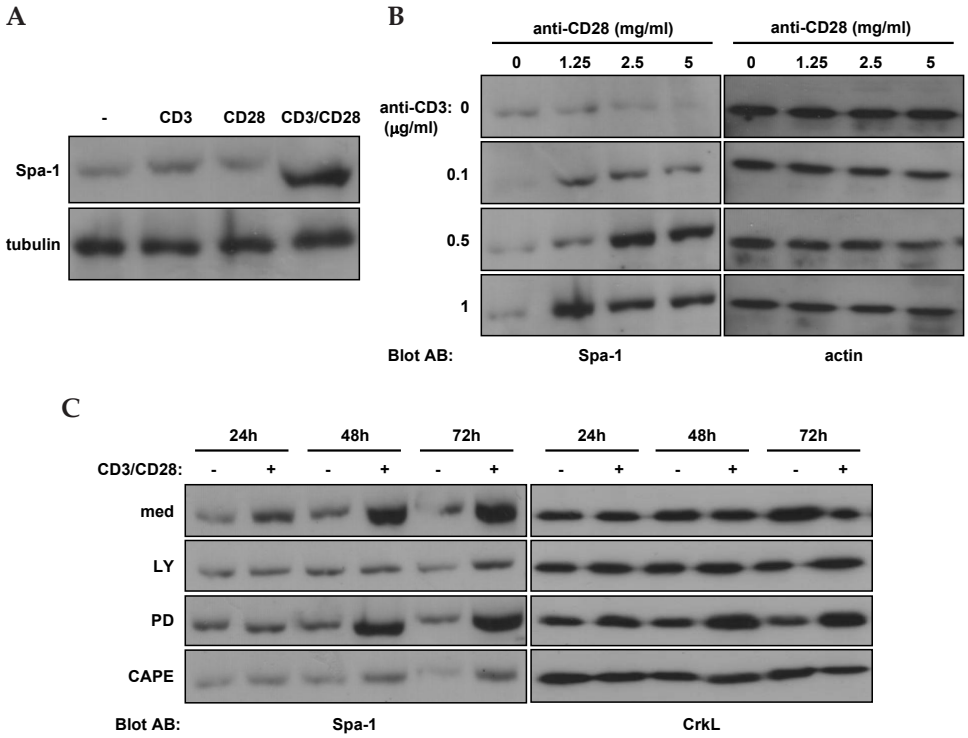

Figure 3. Spa-1 up-regulation requires CD28 costimulation and involvement of PI3-kinase and NF-kB signaling pathways. *(A) Immunblotting of lysates from total T lymphocytes with anti-Spa-1 and anti-tubulin antibodies following 72 hours culture in medium alone (-), or medium containing anti-CD3 and anti-CD28 antibodies, alone or in combination. (B) Immunoblotting of lysates from total T lymphocytes with anti-Spa-1 and anti-actin antibodies following 72 hours culture in the presence of increasing concentrations of anti-CD3 and anti-CD28 antibodies. (C) Immunoblotting of lysates from total T lymphocytes with anti-Spa-1 and anti-CrkL antibodies following incubation for the indicated number of hours in the absence (-) or presence (+) of anti-CD3/CD28 antibodies and vehicle control (med), LY294002 (LY, 20 µM), PD98059 (PD, 50 µM), or CAPE (40 µM). Experiments shown are representative of three to five independent experiments.*

T lymphocyte Spa-1 expression requires CD28 costimulatory activation of PI3-kinase and NF-κB signaling pathways

To further dissect the signaling requirements needed for induction of Spa-1 expression in T cells, we examined the relative contributions of CD3 and CD28 stimulation. Purified T cells were cultured for 72 hours in medium alone, or anti-CD3 and anti-CD28 antibodies, alone or in combination (Fig. 3A). Stimulation via CD3, or CD28 ligation in the absence of CD3 stimulation, failed to induce Spa-1 expression. Dose-dependency experiments, titrating in increasing amounts of anti-CD3 and anti-CD28 antibodies, demonstrated that induction of Spa-1 expression was sensitive to the strengthes of both CD3 and CD28 signals (Fig. 3B). To gain insight into which CD3 and CD28-dependent intracellular signaling events might contribute to Spa-1 induction, isolated T lymphocytes were pre-incubated with pharmacological inhibitors of PI3-kinase catalytic subunits (LY294002), the MEK/ERK pathway (PD98059), and NF-κB activation (CAPE) (Fig. 3C). Inhibition of either PI3-kinase or NF-κB signaling pathways almost completely abolished Spa-1 induction, while suppression of MEK/ERK signaling components had no effect. As both PI3-kinase and NF-κB signaling pathways are known important downstream mediators of CD28 signaling, this may explain the requirement for CD28 costimulation in the induction of Spa-1.

Mitogenic stimuli and homeostatic cytokines fail to induce Spa-1 expression

Initial descriptions of Spa-1 characterized it as a protein expressed specifically in proliferating lymphoid cells[33;35]. To examine if there was a strict relationship between T cell proliferation and Spa-1 expression, we compared Spa-1 protein expression in

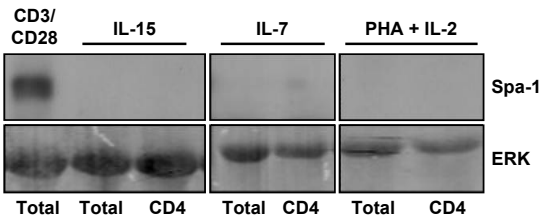

Figure 4. Homeostatic cytokines and mitogenic stimuli fail to induce Spa-1 expression. *Total and CD4⁺ T lymphocytes were stimulated for 72 hours with anti-CD3/CD28 antibodies, or 7 days with IL-15 (10 ng/ml), IL-7 (10 ng/ml), or PHA (1 ng/ml) + IL-2 (50 U/ml). Cellular lysates were prepared and Spa-1 expression detected by immunoblotting. Experiments shown are representative of three independent experiments.*

CD3/CD28-stimulated T cells with T cells exposed to homeostatic cytokines and mitogens. Total purified T cells and $CD4^+$ T cells were treated for 72 hours with anti-CD3/CD28 antibodies, or for seven days with IL-7, IL-15, or PHA+IL-2 (Figure 4). Of these stimuli, only CD3/CD28 antibodies induced Spa-1 expression. IL-7, IL-15, and PHA+IL-2 also failed to upregulate Spa-1 expression in $CD8^+$ T cells, and Spa-1 induction was not evident at shorter time points tested, including 48 and 96 hours (data not shown). Thus, in freshly isolated T lymphocytes, Spa-1 induction appears to be exquisitely dependent upon CD28 costimulatory signals, rather than associated with T cell proliferation.

CD3/CD28 stimulation promotes degradation of Rap1GAP1 and Rap1GAP2 proteins

Finally, we turned our attention to potential protein expression of other Rap1GAP family members in T cells. Total purified T cells were left unstimulated or stimulated for 24 and 72 hours in the presence of anti-CD3/CD28 antibodies, and cellular lysates prepared for immunoblotting. Although E6TP1 mRNA expression was detected in resting T cells (Figure 1), and the E6TP1 antibodies used for immunoblotting could detect exogenous E6TP1 in transfected COS cells, we could not detect any endogenous E6TP1 protein expression in T lymphocytes (data not shown). mRNA analysis indicated that both Rap1GAP1A and Rap1GAP1B expression was induced following CD3/CD28 stimulation (Figure 1). Surprisingly, using an antibody recognizing both Rap1GAP1A and Rap1GAP1B, we observed that expression of Rap1GAP1 proteins was suppressed in a time-dependent manner following CD3/CD28 stimulation (Figure 5A). This was accompanied by the appearance of apparent degradation products of Rap1GAP1 proteins recognized by the antibody. Similar results were also observed in purified $CD4^+$ and $CD8^+$ T cell subsets following stimulation. Rap1GAP2 protein expression, like Rap1GAP2 mRNA, also decreased following CD3/CD28 stimulation (Figure 5B). At 24 hours post-stimulation, decreases in full-length Rap1GAP2 expression were already observed, accompanied by an upward shift in gel motility of the remaining proteins. This may indicate activation-dependent phosphorylation and/or ubiquitination of Rap1GAP2. Additionally, disappearance of full-length Rap1GAP2, like Rap1GAP1s, was also associated with the appearance of degradation products. Activation-dependent mobility shifts and degradation of Rap1GAP2 occurred in both $CD4^+$ and $CD8^+$ T lymphocytes. Together, our results indicate that post-translational modifications play an important role in the suppres-

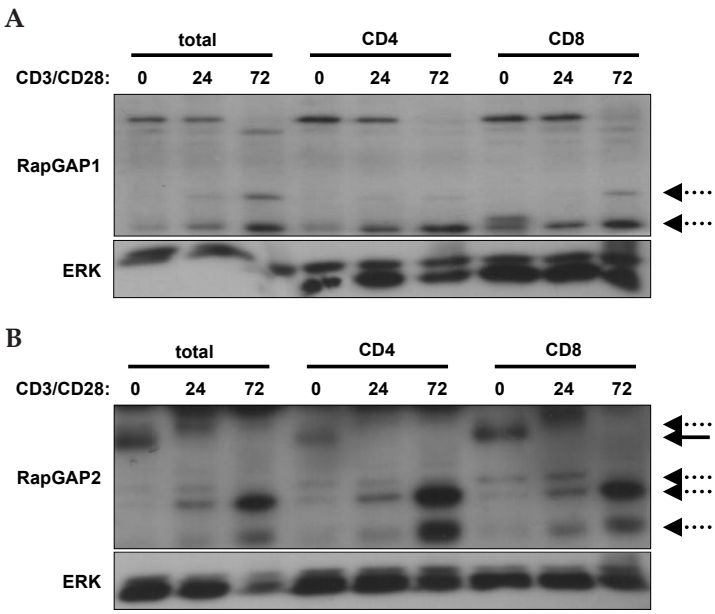

Figure 5. CD3/CD28 stimulation induces degradation of Rap1GAP1 and Rap1GAP2 proteins in T lymphocytes. *Total, CD4⁺ and CD8⁺ T lymphocytes were immediately lysed or stimulated for 24 or 72 hours with anti-CD3/CD28 antibodies. Lysates were prepared and assessed by immunoblotting with anti-Rap1GAP1, anti-Rap1GAP2, and control anti-ERK antibodies. (A) Rap1GAP1 expression. (B) Rap1GAP2 expression. Full-length Rap1GAP expression is noted by solid arrows to the right of immunoblots. Altered protein mobility and/or degradation products are noted by dotted arrows. Experiments shown are representative of three independent experiments.*

sion of Rap1GAP1 and Rap1GAP2 expression in activated T lymphocytes.

Discussion

Our results demonstrate that expression of Rap1GAP family members is dynamically regulated by both transcriptional and post-translational mechanisms in human T lymphocytes. Previous studies have independently but collectively provided evidence for the expression or potential expression of Spa-1, Rap1GAP1A, Rap1GAP1B, Rap1GAP2 and E6TP1 in murine or human T lymphocytes[6;20;33;34;37]. However, comparative analysis of Rap1GAP expression within the T cell lineage has been confined to analysis of Rap1GAP1A and Spa-1 expression in murine thymocytes and splenic T cells20. Here, both GAPs were expressed in thymocytes, while Spa-1 was relatively enriched in peripheral T lymphocytes. In human T lymphocytes, we find that expression of each Rap1GAP is dependent upon T cell lineage and activation status.

Spa-1 was originally identified in proliferating murine lymphoid cells and T cell hybridomas[33;35]. Spa-1 expression in lymphoid cells was suppressed following IL-2 withdrawal, associated with cell cycle arrest. In T cell hybridomas, stimulation with anti-CD3 antibodies also suppressed Spa-1 expression, again accompanied by cell cycle arrest. Conversely, mitogenic stimulation of splenic cells with concanavalin A stimulated Spa-1 expression[35]. However, it is unclear as to how far these observations can be extended to normal murine and human T lymphocytes, as subsequent studies from the same group found that Spa-1 could be readily detected in freshly isolated peripheral murine T lymphocytes, only a small percentage of which would be expected to be actively cycling[20]. Here, we find that in human T lymphocytes, Spa-1 is expressed at low to undetectable levels in resting cells, and upregulated only in $CD4^+$ T lymphocytes following CD3/CD28 stimulation. Spa-1 expression requires specific input from both CD3 and CD28, signals which can not be recapitulated by homeostatic cytokines and mitogens used in our studies. Two signaling pathways which are cooperatively activated by CD3 and CD28, PI3-kinase and NF-κB, appear to be important in the induction of Spa-1 expression. The requirement for CD28 co-stimulation in the induction of Spa-1 expression may be relevant for reports of a role for elevated Rap1 activity in anergic T cells. One common model for inducing anergy or tolerance in mature T cells is to stimulate naive cells with antigen in the absence of appropriate co-stimulatory input, such as that provided by CD28. Induction of anergy in human T cells in vitro is associated with constitutive activation of Rap1[10]. Similarly, $CD4^+$ T lymphocytes isolated from mice following in vivo antigen tolerization also display elevated levels of Rap1[22]. As T cells from mice lacking Spa-1 develop age-dependent hyporesponsiveness to mitogens and recall antigen[20], it may be the case that up-regulation of Spa-1 expression, and subsequent dampening of Rap1 activity, may be a necessary element of appropriate T cell costimulation.
Although we could detect increased mRNA expression of Rap1GAP1A and Rap-1GAP1B in T lymphocytes following CD3/CD28 stimulation, this did not correlate with changes in protein expression. Instead, Rap1GAP1 protein expression was suppressed in activated T cells, accompanied by protein degradation. Post-translational regulation of protein stability has appeared as an emerging theme in Rap-1GAP expression. Thyroid-stimulating hormone (TSH) stabilizes Rap1GAP1A protein in thyroid cells by inactivating glycogen synthase kinase 3β, which otherwise phosphorylates Rap1GAP1A and promotes its proteasomal degradation[38]. $G\alpha_o$ and $G\alpha_i$ –coupled receptors can also promote Rap1GAP1B proteasomal degradation[39]. Finally, E6TP1 is targeted by the papilloma virus E6 oncogene for degradation[37;40]. Here, we extend these studies to show that both Rap1GAP1 and Rap1GAP2 proteins

are degraded following CD3/CD28 stimulation in T cells. The mechanism(s) leading to degradation of these Rap1GAPs in T cells remains to be determined. In platelets, cAMP and cGMP can promote phosphorylation of Rap1GAP2[6;41]. However, these events do not appear to affect Rap1GAP2 stability, but rather inhibits suppression of Rap1GAP2 by 14-3-3 proteins[42]. The c-Cbl and Cbl-b proto-oncogene products have previously been indentified as E3 ubiquitin ligases regulating Rap1 function in T cells. However, these proteins suppress Rap1 activation by promoting degradation of the Crk-bound Rap1 GEF C3G[43;44].

It has been generally thought that diversity in Rap1GAP expression represented tissue-specific enrichment of enzymes performing redundant functions in the inactivation of Rap1. Indeed, in over-expression systems Rap1GAP1A, Rap1GAP1B, Spa-1[45] and E6TP1 (our unpublished observation) can each effectively suppress integrin-dependent adhesion in T cells. However, mice transgenically expressing RapGAP1A and Spa-1 have distinct phenotypes[17;25]. Inappropriate expression of Rap1GAP1A, driven by the β-actin promoter, has no obvious effect on thymocyte development, but leads to accumulation of T cells with an activated phenotype in peripheral lymph nodes46. In contrast, transgenic expression of Spa-1 under control of the lck promoter, expressed early in thymocyte development, blocks thymocyte maturation at an early $CD4^{-}CD8^{-}$ stage. Delaying Spa-1 over-expression until later in development, using a CD4 promoter, failed to modulate further thymocyte maturation[25]. These observations, along with our findings, suggest that each Rap1GAP may make distinct contributions during T cell development and activation. One possibility is that each Rap1GAP may couple to distinct cell surface signaling proteins. In vitro studies have indicated that Rap1GAPs inactivate Rap1 primarily at the cell membrane[32]. This may be due to specific recruitment of GAPs to cell-surface proteins. Initial evidence supporting this model has already been provided. For example, Rap1GAP1A is regulated directly or indirectly by CD28 and CTLA-4 ligation in T cells[16;17]. Rap1GAP1B, which compared to Rap1GAP1A, contains an N-terminal amino acid extension encoding a GoLoCo motif, can bind to Gi and Ga subunits of G-coupled receptors[34]. Indirect evidence that Spa-1 interacts with cell-surface proteins in T cells is seen in observations that Spa-1 is recruited to the T cell immunological synapse following antigen stimulation[36]. A second possibility is that in addition to inactivating Rap1, each Rap1GAP may also serve independent functions. In the case of Spa-1, protein-protein interactions with Brd4[47] and aquaporin-2[48] have been observed, although the relevance of these interactions to T cell function remains to be investigated. The wealth and variation of co-stimulatory proteins and other signaling receptors expressed on distinct T cell subsets during activation

and differentiation, in conjunction with the differential expression of Rap1GAPs we observe in T cells, raises the possibility that Rap1 function can be therapeutically modulated in distinct T cell populations to clinically enhance or suppress T cell-dependent immune responses.

Materials and Methods

Reagents

Recombinant human cytokines used in these studies included IL-2 (Dreiech, Germany), IL-7 (Strathmann Biotech GMBH, Germany), and IL-15 (R&D, Abingdon, UK). PHA was purchased from Sigma, LY294002 (used at 20 μM), and PD 98059 (50 μM) from Calbiochem, and CAPE (40 μM) from Biomol.

T cell isolation and culture

Peripheral blood mononuclear cells (PBMC) were isolated from healthy volunteers by Ficoll-Isopaque density gradient centrifugation (Nycomed, Pharma, Oslo, Norway). $CD3^+$ T lymphocytes were purified from PBMC using a negative isolation procedure (T Cell Negative Isolation Kit, Dynal Biotech, Oslo, Norway) according to the manufacturer's instructions. In some experiments, purified $CD4^+$ and $CD8^+$ T lymphocytes were also obtained using negative isolation procedures (MACSisolation kits, Miltenyi). T cells and T cell subset purity was greater than 95% as assessed by FACS analysis (described below). T cells were cultured at 37°C under 5% CO_2 at a density of $1x10^6$/ml in IMDM medium supplemented with 10% FCS, 200 μM L-glutamine, 25 mM HEPES, streptomycin (100 ng/ml) and penicillin (10 U/ml) (all from Invitrogen, Carlsbad, CA). T cells were cultured in medium alone or stimulated for up to 72 hours with activating anti-CD3 antibodies 1XE (Sanquin, Amsterdam, The Netherlands) or 16E9 (provided by Dr. R. van Lier, our institute), and/or anti-CD28 antibody (clone 15E8, Sanquin) antibodies. Alternatively, T cells were stimulated for up to 7 days with IL-7 (10 ng/ml), IL-15 (10 ng/ml), or PHA (1 ng/ml) + IL-2 (50 U/ml).

Flow Cytometric Analysis

Purity of T cell populations was assessed by staining with anti-CD3 antibody. Effects of antibodies and cytokines on T cell proliferation was assessed by CFSE dilution analysis. T cells were resuspended at 5-10x10^6 cells in PBS and labeled with 2.5 μM CFSE (Molecular Probes Europe BV, Leiden, The Netherlands) for 10 minutes at 37°C. Cells were then washed, resuspended at 1x10^6 cells/ml in complete culture medium, and left untreated, or stimulated with anti-CD3 and anti-CD28 antibodies, alone or in combination, for up to 72 hours, or PHA+IL-2, IL-7 or IL-15 for 7 days. Proliferation was measured using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) and CellQuest Pro software (BD Biosciences).

RNA extraction and PCR analysis

T lymphocytes were left untreated or stimulated for 24 or 72 hours with anti-CD3/CD28 antibodies. T lymphocytes were then harvested, washed with PBS and total RNA isolated using a GenElute RNA isolation kit (Sigma-Aldrich). RNA was reverse-transcribed using SuperScript™ II Reverse Transcriptase (Invitrogen) and cDNA amplified by polymerase chain reaction (PCR) using primers specific for GADPH (Eurogentec, Philadelphia, PA), Rap1GAP1a, Rap1GAP1b, Rap1GAP2, Spa-1 and E6TP1α (all synthesized by Invitrogen). RapGAP primer sequences and PCR conditions were as previously described6. Positive controls used were total RNA isolated from COS-7 cells transfected with 10 ng pCMV-myc-E6TP1 (kindly provided by Dr. V. Band, Northwestern University, Chicago, IL), pMT2HA-Rap1GAP1A, pMT2HA-Rap1GAP1B, pSR-His-Spa-1, and pCDNA3-Rap1GAP2 expression vectors[37;45] using Lipofectamine 2000 transfection reagent (Invitrogen). PCR products were separated by electrophoresis and visualized using a Gene Flash imaging system (Westburg, Leusden, The Netherlands).

Western blot analysis

Cells were counted, equivalent numbers of T lymphocytes were lysed in 1x Laemmli's buffer, and clarified protein lysates were resolved by electrophoresis on 3-8% gradient Bis-Tris SDS NuPAGE® gels (Invitrogen). Proteins were then transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA)

using a semi-dry transfer apparatus (Invitrogen). Membranes were washed in Tris-buffered saline (pH 8.0) containing 0.05% Tween-20 (Bio-Rad) (TBS/T), blocked in 2% milk (Bio-Rad)/TBS/T, and incubated overnight at 4°C in primary antibody diluted in TBS/T. Primary antibodies used included antibodies specific for Rap1GAP1a, Rap1GAP1b, E6TP1, actin, CrkL (all from Santa Cruz Biotechnology, Santa Cruz, CA), Spa-1, ERK 1/2 (Cell Signaling, Beverly, MA), and tubulin (Sigma-Aldrich). Rap1GAP2 rabbit anti-serum and purified rabbit anti-Rap1GAP2 antibodies have been previously described[6;42]. Following incubation with primary antibodies, blots were washed in TBS/T and then incubated in TBS/T containing IRDye 680 or IRDye 800 -conjugated anti-rabbit or anti-mouse immunoglobulin antibodies (LI-COR, Bad Homburg, Germany) and staining detected using an Odyssey Imager (LI-COR) and Odyssey 3.0 software.

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