Role of reactive oxygen species in rheumatoid arthritis synovial T lymphocytes

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CHAPTER 3

Rap1 signaling is required for suppression of Ras-generated reactive oxygen species and protection against oxidative stress in T lymphocytes.

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J Immunol. 2004. 15;173: 920-31.

Abstract

Transient production of reactive oxygen species (ROS) plays an important role in optimizing transcriptional and proliferative responses to TCR signaling in T lymphocytes. Conversely, chronic oxidative stress leads to decreased proliferative responses and enhanced transcription of inflammatory gene products, and is thought to underlie the altered pathogenic behavior of T lymphocytes in some human diseases, such as rheumatoid arthritis (RA). Although the signaling mechanisms regulating ROS production in T lymphocytes has not been identified, activation of the small GTPase Ras has been shown to couple agonist stimulation to ROS production in other cell types. We find that Ras signaling via Ral stimulates ROS production in human T lymphocytes, and is required for TCR and phorbol ester-induced ROS production. The related small GTPase Rap1 suppresses agonist, Ras and Ral–dependent ROS production through a PI3K–dependent pathway, identifying a novel mechanism by which Rap1 can distally antagonize Ras signaling pathways. In synovial fluid T lymphocytes from RA patients we observed a high rate of endogenous ROS production, correlating with constitutive Ras activation and inhibition of Rap1 activation. Introduction of dominant-negative Ras into synovial fluid T cells restored redox balance, providing evidence that deregulated Ras and Rap1 signaling underlies oxidative stress and consequent altered T cell function observed in RA.

Introduction

Two members of the Ras family of GTPases, Ras and Rap1, play critical roles in regulating T cell proliferative responses. Ras plays an essential role in transmitting signals from the TCR to activation of the Raf-1/ERK signaling cascade, which is required for T cell proliferation, IL-2 production, and thymic maturation (1) The closely related Rap1, which is also activated in T lymphocytes following TCR stimulation, was identified as a suppressor of Ras-dependent transformation (2), and was proposed to act by sequestering Ras effector targets (Raf kinases, PI3K, and Ral GTPase, guanine nucleotide exchange factors (GEF)) away from Ras in an inactive complex (3-6). In overexpression studies, constitutive activation or inactivation of Rap1 signaling can profoundly modulate TCR-induced, Ras-dependent transcriptional events (6-9), and constitutive activation of Rap1 in anergic T lymphocytes has been suggested to contribute to the hyporesponsiveness of these cells to antigenic rechallenge (6). Although these studies have suggested that Rap1 mediates these effects through proximal antagonism of Ras signaling to the Raf/ERK pathway in T cells (6-10), no direct data support that endogenous Rap1 (5,11), or ectopically expressed active Rap1 (7,8,10), can directly interfere with Ras-dependent activation of Raf in T lymphocytes. This conclusion is most strongly supported by overexpression studies in T cell lines and analysis of transgenic mice expressing activated Rap1 in T lymphocytes at levels comparable to endogenous Rap1. In this study, active Rap1 induces integrin activation and enhances integrin-dependent activation of T cells without inhibiting TCR-dependent positive selection, proliferation or ERK activation (12-14). Thus, Rap1 modulation of Ras-dependent transcriptional activity in T cells likely occurs via Ras-distal rather than Ras-proximal signaling pathways.

In addition to the Raf/ERK pathway, Ras also signals through Ral family GTPases and PI3K (3,15). Each of these defined pathways, including the Raf/ERK pathway, is required for Ras-induced cellular transformation (15). Ras-dependent production of reactive oxygen species (ROS) is also required to induce cellular transformation (16). Of these signaling pathways, only the Raf/ERK pathway has been extensively studied in T lymphocytes (1). ROS, such as hydrogen peroxide, are proposed to act as important second messengers in TCR and phorbol ester-induced T cell activation. Both of these stimuli result in the transient production of ROS (17). Scavenging of intracellular ROS with antioxidants (e.g., *N*-acetylcysteine (NAc)) suppresses TCR-induced NF-κB, AP-1, and IL-2 promoter transcription (18,19). Conversely, acute exposure of T cells to

ROS can stimulate transcription from these promoters, and enhance TCR-induced transcription (20). Chronic oxidative stress, however, can lead to T cell hyporesponsiveness to proliferative stimuli and constitutive activation of NF-κB-dependent inflammatory gene products. In a number of human diseases, including rheumatoid arthritis (RA) (21,22) and HIV infection (23), chronic oxidative stress is thought to underlie altered, pathogenic T cell behavior.

RA is a chronic systemic disease characterized by inflammation of synovial tissue and destruction of cartilage and bone, marked by infiltration of the synovial joint by B and T lymphocytes, neutrophils, and macrophages (24). T lymphocyte interactions with synovial macrophages and fibroblast-like synoviocytes are regarded to play key roles in the pathogenesis and perpetuation of the disease (25,26). T lymphocytes isolated from rheumatoid joints display a number of signaling and proliferative abnormalities. They exhibit severe hyporesponsiveness to proliferative stimuli compared with autologous peripheral blood (PB) T cells, and produce little IL-2, IFN-γ, IL-4, TGF-β, or TNF-α in vitro or in situ (27). Despite this, RA synovial fluid (SF) T cells express markers of recent activation, such as CD69 and CD40 ligand (CD154), proinflammatory receptors for TNF-α and IL-1, and secrete matrix metalloproteinases, correlating with constitutive activation of NF-κB, a key ROS-dependent transcriptional regulator of these genes (25,28,29). Expression of these proteins by synovial T lymphocytes leads to activation of synovial macrophages and fibroblast-like synoviocytes, perpetuating inflammation, synovial hyperplasia, and joint destruction (25,26).

The hyporesponsiveness of SF T cells correlates strongly with oxidative stress, and in vitro replenishment with NAc restores reduced glutathione (GSH) equilibrium concomitant with recovery of TCR signaling defects and partial rescue of both SF T lymphocyte proliferation and IL-2 production (22). Conversely, depletion of intracellular GSH in peripheral T cells, or chronic exposure to TNF-α, mimicks signaling defects observed in SF T cells (30-32). Because ROS production has been described as a critical component of Ras-dependent transformation in fibroblasts, and Rap1 was first described as a suppressor of Ras-dependent transformation, we examined the possibility that Ras and Rap1 signaling may regulate ROS production and oxidative stress in normal and RA T lymphocytes.

Materials and methods

cDNA and transient transfections

Empty pMT2-hemagglutinin (HA), and pMT2 plasmid encoding HA-tagged RasV12, RasN17, Rap1V12, Rap1N17, RapGTPase-activating protein (RapGAP), RalV23, RalN17, Rlf-CAAX, and exchange protein directly activated by cAMP (EPAC)-CAT have been previously described (12,33). Empty pSG5 and pSG5 encoding RasV12, RasV12/G37, RasV12/E38, and RasV12/C40 have been previously described (33). Wild-type and catalytically inactive phospholipase C (PLC)ε in pCDNA were generously provided by Dr. M. Schmidt (University Hospital Essen, Essen, Germany) (34). Rap1V12 effector mutants V12/G37, V12/E38, and V12/C40, and Rap1V12-ΔCAAX in pMT2HA were generated using the Stratagene Quick-Change site-directed mutagenesis kit (La Jolla, CA) and two oligonucleotide primers containing the desired mutations, and the sequence integrity of the constructs was established by sequencing. For transient expression of cDNA in Jurkat, 6 x 10^6 cells were transfected by electroporation (250 V, 950 μF) with 15–30 μg of matching empty vector or cDNA expression vectors as indicated in figures, plus pCMV-CD20 expression vector (provided by Dr. R. Medema, Netherlands Cancer Institute, Amsterdam, The Netherlands), 10 μg of pEF-*myc*ERK2 (a kind gift of D. Cantrell, University of Dundee, Dundee, U.K.), or 5 μg of *Renilla* luciferase expression construct regulated by the thymidine kinase promoter to differentiate between transfected and untransfected cells.

Cells and FACS-based ROS detection assay

Jurkat T cells were maintained as previously described (31). Human PB T lymphocytes from healthy donors and PB and SF T lymphocytes from human RA patients were purified and maintained as previously described (30,31). Patient material was obtained following informed consent from patients according to regulations at each of the participating institutes.

For detection of ROS production, Jurkat cells were isolated 48 h posttransfection cells and stained with CyChrome-conjugated anti-CD20 mAbs (BD PharMingen, San Diego, CA), resuspended at 5 x 10^6 cells/ml in phenol red-free DMEM medium and loaded with 28 μM 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate-di(acetoxymethyl ester) (DCF; Molecular Probes, Eugene, OR) for 20 min at 37°C before stimulation with anti-CD3 Ab (1XE; Central Laboratory of the Netherlands, Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) or 100 ng/ml PMA (Sigma-Aldrich, St. Louis, MO) plus 1 μg/ml ionomycin (PMA/I; Sigma-Aldrich) and ROS detection. CD20-positive cells were analyzed on a FACScan (BD Biosciences) for the mean fluorescence intensity (MFI) of oxidated DCF in the FL1 channel at different time points after stimulation. Because DCF loading continues after the first 20 min, the data are presented as ΔROS generation, whereby the measured MFI at each time point is reduced by the measured MFI at corresponding time points in the unstimulated or unstimulated mock-transfected control group.

Detection of activated GTPases, immunoprecipitations and immunoblotting

PB T lymphocytes from healthy controls and RA patient PB and SF T cells were left unstimulated or stimulated for 5 min with anti-CD3 Ab (0.5 μg/ml), 100 ng/ml PMA/I or 500 μM H_2O_2 (Merck, West Point PA). In indicated experiments, cells were pretreated with LY294002 (1 μM, 24 h), NAc (5 mM, 48 h) or L-buthionine-(*S*,*R*)-sulfoximine (BSO, 200 μM, 72 h). Active GTP-bound Ral, Rap1, and Ras proteins from 5 x 10^6 T cells were precipitated with RLIP-Ras binding domain (RBD), Ral guanine nucleotide stimulator protein (GDS)-RBD, and Raf-RBD GST fusion proteins, respectively, resolved by SDS-PAGE, and detected by immunoblotting with anti-Ral, anti-Rap1, or anti-Ras Abs (BD Transduction Laboratories, Lexington, KY) as previously described (11,12). Briefly, cells were lysed in buffer containing 10% glycerol, 1% Nonidet P-40, 50 mM Tris (pH 7.6), 150 mM NaCl, 10 mM $MgCl_2$, 2 mM $NaVO_4$, 10 mM NaF, and 2 mM PMSF. Clarified lysates were incubated with ~5 μg RBD-GST fusion protein precoupled to glutathione-agarose and washed with lysis buffer before elution in Laemmli's sample buffer. Proteins were resolved on 13.5% SDS-PAGE gels, transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA), and proteins detected by immunoblotting and ECL. Total Rap1 and Ras was detected in lysates to confirm equal amounts of GTPases. For detection of ERK activation, transfected *myc*-tagged ERK2 was immunoprecipitated with anti-*myc* Abs (Santa Cruz Biotechnology, Santa Crus, CA), followed by resolution by SDS-PAGE, transfer to PVDF, and detection with anti-phospho-ERK1/2 Abs (New England Biolabs, Beverly, MA). Equivalent *myc*-ERK2 expression between independent transfection samples was confirmed by immunoblotting with anti-*myc* Abs. In all other transfection experiments, equivalent expression of constructs was confirmed by immunoblotting of cell lysates with anti-Ras, anti-FLAG (Sigma-Aldrich), or anti-HA Abs (Santa Cruz Biotechnology).

Adhesion assays

Adhesion assays were as previously described (12). Briefly, Jurkat cells were transfected with 10 μg of thymidine kinase-luciferase expression plasmid (to detect transfected cells) and 30 μg of empty plasmid or the indicated Rap1 construct. After 48 h, cells were washed and resuspended in

TSM medium (20 mM Tris, pH 8, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$). Costar Maxisorp 96-well plates were coated overnight at 4°C with goat anti-human IgG Abs (4 μg/ml; Jackson ImmunoResearch Laboratories, West Grove, PA) in TSM, washed with TSM, blocked for 30 min at 37°C in 0.5% BSA/TSM, and incubated with 50 ng/ml recombinant ICAM-1 (generously provided by C. Figdor, Nijmegen University, Nijmegen, The Netherlands) in BSA/TSM. Cells were allowed to adhere for 45 min at 37°C, nonadherent cells washed away with warm BSA/TSM, and adherent cells lysed and subjected to luciferase assays as previously described (12). Percentage of specific adhesion was calculated by dividing detected luciferase counts by counts obtained from lysates from total input cells.

Incubation of T lymphocytes with cell-permeable Tat fusion peptides

Tat-β-galactosidase (β-Gal) peptide was a generous gift of Dr. D. Hall (University of Wisconsin, Madison, WI) (35). Generation, purification, and characterization of Tat-RasN17 peptides have been previously described (36). Freshly isolated human PB and SF T lymphocytes were incubated at 4 x 10^6 cells/ml in culture medium alone, or medium containing 25 μg/ml Tat-β-Gal or Tat-RasN17 peptide for 16 h at 37°C. Following extensive washing in PBS, cells were left unstimulated, or stimulated for 5 min with medium, anti-CD3/CD28 Abs or PMA/I as previously described before lysis and detection of Ras, phospho-ERK and ERK protein by immunoblotting. Alternatively, ROS production was measured as previously described.

Results

Ras signaling via the GTPase Ral is both necessary and sufficient for ROS production in T lymphocytes

We first examined the role of Ras signaling in the regulation of ROS production in T cells. Treatment of Jurkat cells with either anti-CD3 Abs or PMA/I led to a rapid and transient generation of intracellular ROS that was maximal 2–5 min poststimulation (Fig. 1*A*). No further changes in rate of ROS production were observed up to 1 h poststimulation, the longest time point tested. Jurkat cells transiently expressing an active Ras mutant, RasV12, demonstrated a constitutively high basal level of intracellular free radical production that could not be further augmented by stimulation with anti-CD3 Abs or PMA/I. Conversely, expression of dominant-negative RasN17 completely blocked CD3 and PMA/I-dependent ROS production, demonstrating Ras activation is necessary and sufficient for ROS generation in T lymphocytes.

The signaling pathways coupling Ras activation to ROS production have not been identified, so we first used RasV12 effector domain mutants that selectively signal via members of the Ral (GEF) family and PLCε (RasV12/G37), Raf kinases (RasV12/E38), or PI3K (RasV12/C40) (15,37) (Fig. 1*A*). Of these three effector domain mutants, only RasV12/G37, but not RasV12/E38 or RasV12/C40, mimicked RasV12 in stimulating ROS generation in Jurkat cells. This finding suggested that Ras signaling to either the small GTPase Ral or PLCε was responsible for stimulating ROS production.

In initial experiments, we detected protein expression of two RalGEFs, RalGDS, and Rlf in T cell lysates by immunoblotting (data not shown), supporting the possibility that Ras could couple to Ral activation in T cells. Most importantly, CD3/CD28 stimulation of PB T lymphocytes led to activation of Ral, as determined using an activation-specific pulldown assay to detect GTP-bound Ral (Fig. 1*B*). To directly test whether Ral activation could stimulate ROS production in T cells, we transiently transfected Jurkat with a constitutively active mutant of the Ral-specific GEF Rlf, Rlf-CAAX (activating endogenous Ral), or an active Ral mutant, RalV23. Expression of either protein in Jurkat cells led to constitutive ROS production (Fig. 1*C*). Conversely,

expression of dominant-negative RalN blocked both PMA/I- and Ras-induced ROS production clearly indicating the relevance of the Ral signaling pathway in Ras-dependent ROS production. In the presence of dominant-negative Ras, active RalV23 still stimulated persistent generation of ROS, placing Ral downstream of Ras.

Figure 1

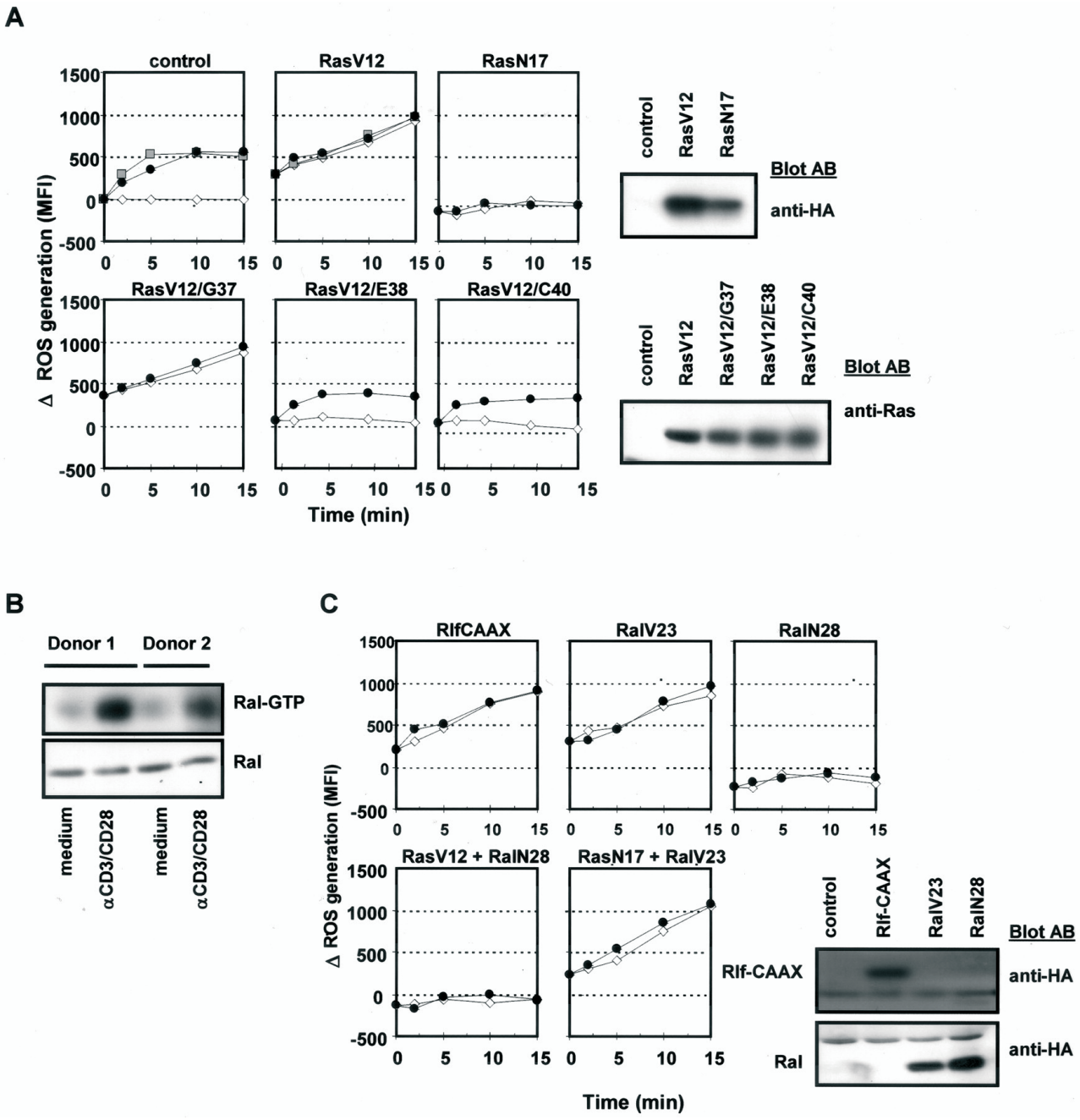


FIGURE 1. Ras signaling via a Ral-dependent pathway is sufficient and required for ROS generation in T cells. *A*, Ras is sufficient and required for ROS generation in T lymphocytes. A total of 6 x 10^6 Jurkat cells were transfected by electroporation (250 V, 950 μF) with pCMV-CD20, to differentiate between transfected and untransfected cells, and empty pMT2HA expression plasmid (control) or cDNA encoding Ras signaling constructs indicated in each panel. At 48 h posttransfection, cells were stained with CyChrome-conjugated anti-CD20 mAbs. Cells were resuspended at 5 x 10^6 cells/ml in phenol red-free DMEM medium and loaded with 28 μM DCF for 20 min at 37°C before stimulation and ROS detection. Cells were stimulated with anti-CD3 (▨) or PMA/I (•) or left unstimulated (◇) and analyzed on a FACScan for the mean fluorescence intensity (MFI) of oxidated 6-carboxy-DCF in the FL1-channel, as a measurement for the presence of intracellular ROS, of CD20-expressing cells at different time points after stimulation. The ΔROS generation is obtained by reducing the measured MFI with the measured MFI at corresponding time points in the unstimulated, control-transfected Jurkat cells. The data shown are the mean values of three or more independent experiments. Expression of all constructs was confirmed by immunoblotting of cell lysates with anti-HA (for HA-RasV12 and HA-RasN17) or anti-Ras Abs (for RasV12, RasV12/G37, RasV12/E38, and RasV12/C40). *B*, Ral participates in TCR signaling. A total of 5 x 10^6 PB T lymphocytes from two healthy donors were stimulated for 5 min with either medium or anti-CD3 and anti–CD28 Abs (▨ CD3/CD28). GTP-bound Ral was precipitated, resolved by SDS-PAGE and visualized by immunoblotting with anti-Ral Abs and ECL (*top*). Total Ral expression was assessed by immunoblotting of whole cell lysates (*bottom*). *C*, Ral is sufficient and required for ROS generation downstream of Ras. Jurkat T cells were transfected with cDNA encoding Rlf, Ral, and Ras signaling mutants and assessed for ROS production as in *A*. Expression of Rlf-CAAX, RalV23, and RalN28 was assessed by immunoblotting of whole cell lysates with anti-HA Abs. Constructs used in *A* and *B* were assessed in the same experiments, but are split into two sections for clarity of presentation.

Although our data clearly demonstrated that activation of endogenous Ral was required and sufficient for Ras-induced ROS generation, RasV12/G37 can also interact with PLCε (37), and overexpression of PLCε mimics RasV12/G37-induced cytokine transcription in T cells (38). We therefore examined whether PLCε might also mediate Ras-dependent ROS production. Jurkat cells were transfected with RasV12/G37 alone, or in combination with wild-type or previously characterized dominant-negative, catalytically inactive PLCε (34). If PLCε acted downstream of Ras, we would expect wild-type PLCε to enhance Ras-dependent ROS production, whereas inactive PLCε should be inhibitory. Instead, we found that cotransfection of either PLCε construct led to a small but reproducible decrease in Ras-induced ROS production (data not shown), suggesting that although PLCε might interact with Ras in this overexpression model, Ral, not PLCε, is the Ras effector responsible for ROS production in T lymphocytes. Modulation of ROS generation represents a novel function for Ral and is the first description of Ral participation in T cell signaling.

Rap1 is required for suppression of Ras-dependent ROS production

Because Rap1 can antagonize distal Ras-dependent proliferative and activation signals in T lymphocytes, we next examined whether Rap1 signaling could also influence ROS homeostasis in T lymphocytes. Transfection of Jurkat cells with an inactive mutant of Rap1, RapN17, led to a slight increase in basal ROS levels (Fig. 2*A*, *middle panel*). Strikingly, following PMA/I stimulation, persistent rather than transient ROS generation was observed. To verify that this was not a nonspecific effect of overexpressed RapN17, we used the Rap1-specific RapGAP to inactivate endogenous Rap1 (Fig. 2*A*, *right panel*). Inactivation of endogenous Rap1 by RapGAP also led to persistent ROS production in Jurkat T cells following PMA/I stimulation. Thus,

although inactivation of Rap1 signaling is not sufficient to generate ROS, endogenous Rap1 signaling is required for the down-regulation of agonist-induced ROS generation.

This interpretation was further strengthened by the finding that transient overexpression of active RapV12 completely blocked TCR-, PMA/I-, and RasV12-mediated ROS generation (Fig. 2*C*). Activation of endogenous Rap1 alone with constitutively active Rap1 GEF EPAC (Epac-CAT; Fig. 2*C*) and C3G (data not shown) also blocked ROS production by these stimuli. To determine whether Rap1 was antagonizing ROS production proximally to Ras, or further downstream, we cotransfected Jurkat cells with active RalV23 and RapV12. Active Rap1 suppressed Ral-induced ROS production (Fig. 2*C*), indicating Rap1 was interfering with Ras signaling indirectly.

Rap1 suppresses ROS production via a PI3K-dependent pathway

To gain more insight into how Rap1 might suppress ROS production in T cells, we generated RapV12 effector domain mutants analogous to those made for Ras (RapV12/G37, RapV12/E38, RapV12/C40), and examined their effect on agonist- and Ras-induced ROS generation. We also generated a RapV12 mutant lacking the carboxyl-terminal motif required for posttranslational modifications needed for membrane localization, RapV12-ΔCAAX. The analogous mutant of Ras, RasV12-ΔCAAX, acts as a dominant-negative (RasN17-like) mutant (39). Each of the mutants was transiently expressed at levels comparable to RapV12 in Jurkat cells (Fig. 3*A*). Of the three effector domain mutants only RapV12/C40, but not V12/G37 or V12/E38, abrogated ROS generation (Fig. 3*B*). If analogous to Ras, this mutant would be predicted to signal only via PI3K. Intriguingly, the RapV12-ΔCAAX mutant not only failed to suppress PMA/I–induced ROS production, but also prevented subsequent down-regulation of ROS production. This was the same phenotype observed in cells expressing RapN17 (Fig. 2*A*). These data indicate that membrane localization of Rap1 or its effector is required to suppress ROS production, and that the active Rap-ΔCAAX mutant may sequester the effector away from this cellular compartment.

To examine whether each of the Rap1V12 mutants retained biological activity, we examined their ability to induce integrin-dependent adhesion in Jurkat (Fig. 3*C*). In contrast to their differential effects on ROS production, each of the RapV12 effector mutants, as well as RapV12-ΔCAAX, supported LFA-1-dependent adhesion to immobilized ICAM as efficientlyas RapV12. Similarly, no differences were observed in the induction of VLA-4-dependent adhesion to fibronectin (data not shown), providing evidence that Rap regulates ROS production by a signaling pathway distinct from integrin regulation. If analogous to Ras, our observation that RapV12/C40 selectively blocked ROS production might suggest that a PI3K isoform was the downstream target of Rap1. However, in coimmunoprecipitation studies with transfected active Rap1, we have been unable to demonstrate a physical interaction between Rap1 and PI3K (data not shown). In separate studies we examined whether Rap-dependent suppression of ROS production was PI3K–dependent using the PI3K inhibitor LY294002. In control, mock-transfected cells LY294002 inhibited TCR-induced ROS production (Fig. 3*D*). This was likely due to inhibitory effects of the LY294002 compound on TCR-dependent Ras activation, as we observed that LY294002 inhibited Ras activation upon TCR activation (Fig. 3*E*). These findings were consistent with previous reports, which suggested the requirement of PI3K in coupling the TCR to ERK activation (40).

Use of RasV12 effector domain mutants in earlier experiments suggested that PI3K activity was not required for Ras-induced ROS generation (Fig. 1*A*). This was confirmed pharmacologically, as LY294002 had no effect on RasV12-induced ROS production (Fig. 3*D*). However, pretreatment of cells with LY294002 completely relieved the inhibition of Ras-dependent ROS generation by RapV12 (Fig. 3*F*). LY294002 has no effect on Rap-induced integrin adhesion

(Refs. 13,41 and data not shown), again indicating that Rap1 regulates integrin function and ROS production by distinct pathways. Together, these data indicate that a PI3K-dependent target acts downstream of or in parallel to Rap1 in the down-regulation of ROS production.

Figure 2

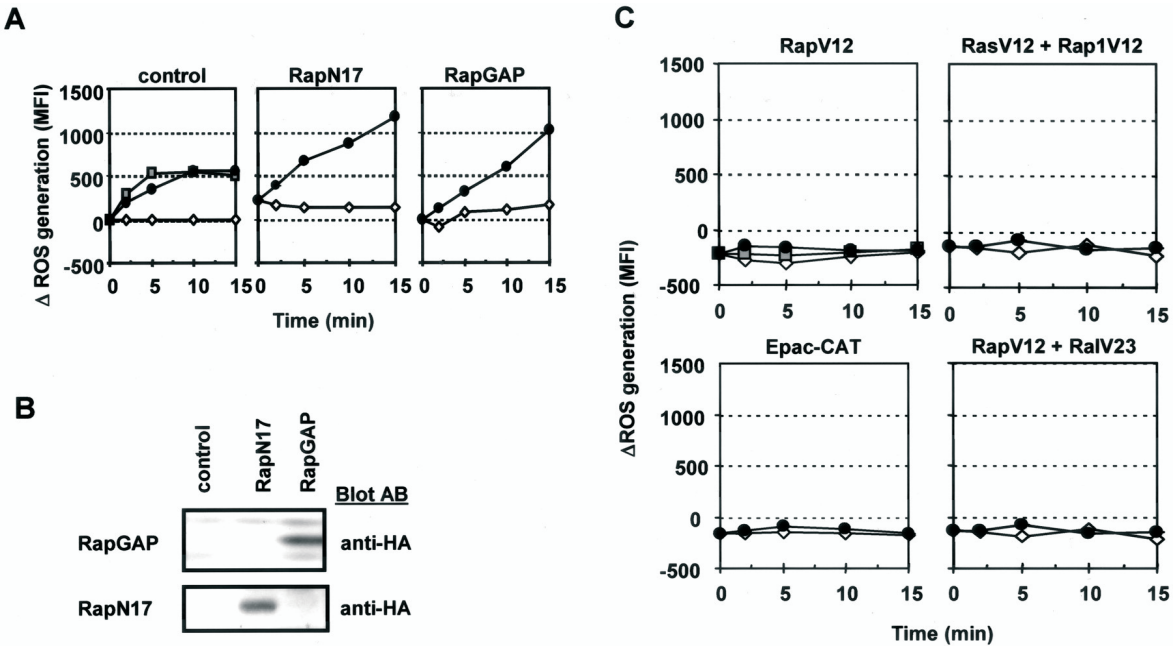
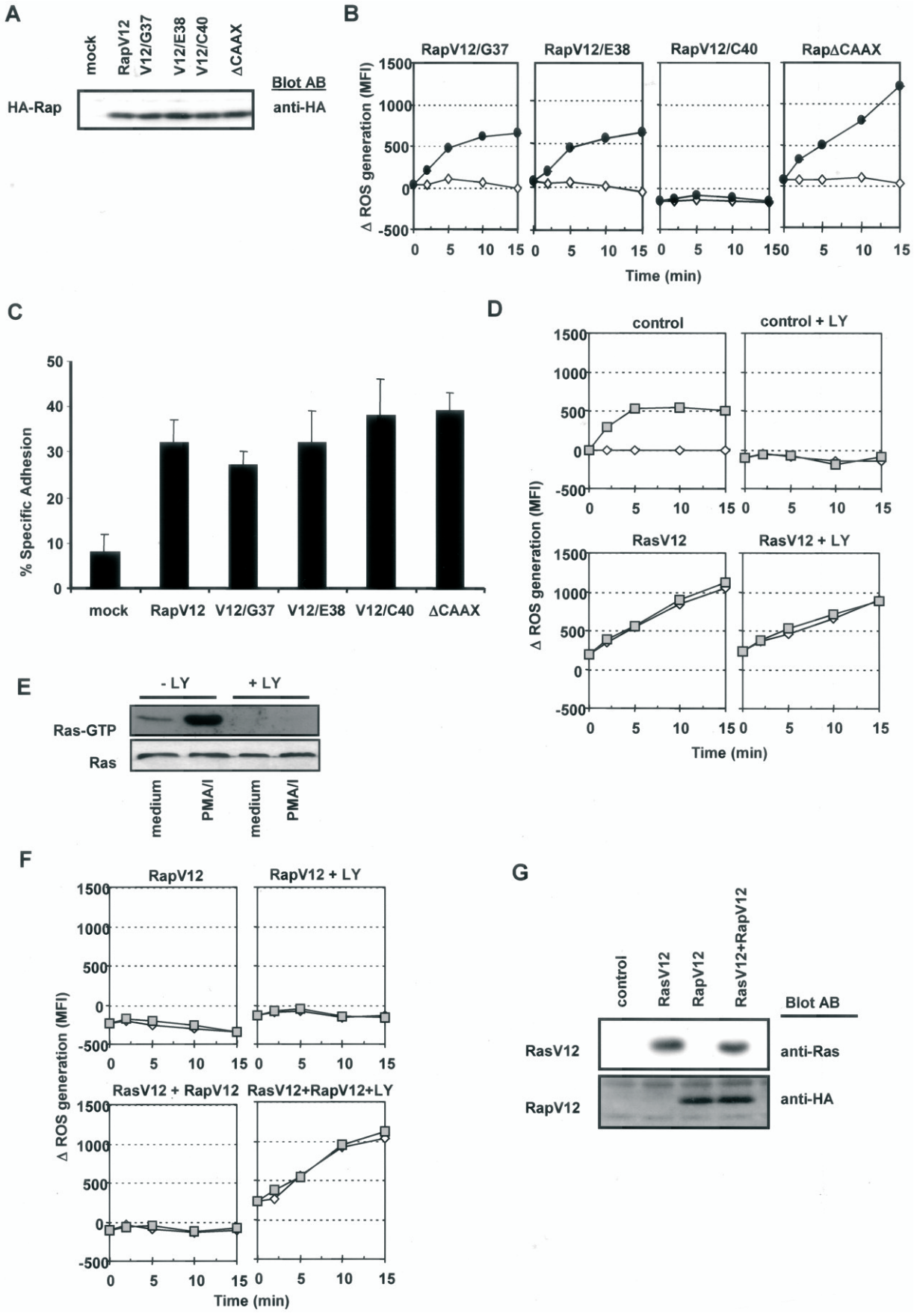


FIGURE 2. ROS generation in T cells is down-regulated through a Rap1-dependent pathway. *A*, Rap1 signaling is required for down-regulation of agonist-induced ROS production. Jurkat cells were transfected with CD20 cDNA alone (control) or in combination with RapGAP or inactive RapN17 cDNA in pMT2HA vector as indicated and processed for analysis of intracellular ROS as in Fig. 1. *B*, Expression of HA-tagged RapN17 and RapGAP in *A* were determined by immunoblotting of whole cell lysates with anti-HA Abs. *C*, Activation of Rap1 suppresses agonist-, Ras-, and Ral-induced ROS production. Jurkat cells were transfected with cDNA encoding CD20 and active RapV12 or the active catalytic domain of the Rap1 GEF EPAC (Epac-Cat), alone, or in combination with active Ras or Ral mutants in pMT2HA vector and analyzed for intracellular ROS production as in Fig. 1. In all sections, cells were left unstimulated (◇) or stimulated with anti-CD3 Abs (gray diamond) or PMA+I (•). Constructs used in *A* and *C* were analyzed in the same experiments, but are sectioned into two for clarity of presentation.

Figure 3


A
mock
RapV12
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HA-Rap
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RapV12/E38
RapV12/C40
RapΔCAAX
Δ ROS generation (MFI)
Time (min)
C
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mock
RapV12
V12/G37
V12/E38
V12/C40
ΔCAAX
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RasV12 + LY
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RasV12+RapV12
Blot AB
RasV12
anti-Ras
RapV12
anti-HA

FIGURE 3. Rap1 suppresses ROS production by a PI3K-dependent signaling pathway distinct from regulation of integrin function. *A*, Expression of RapV12 effector domain mutants. Jurkat cells were transfected with empty pMT2HA vector, or vector encoding HA-tagged RapV12, RapV12/G37,RapV12/E38, RapV12/C40, or RapV12/ΔCAAX. Whole cell lysates were resolved by SDS-PAGE, transferred to PVDF, and proteins detected by immunoblotting with anti-HA Abs and ECL. *B*, Selective suppression of ROS production by RapV12 effector domain mutants. Jurkat cells were transfected with CD20 cDNA and RapV12/G37, RapV12/E38, or RapV12/C40 effector domain mutants in pMT2HA as indicated and analyzed for intracellular ROS production as in Fig. 1. In all panels, cells were stimulated with either anti-CD3 (▨), PMA+I (•) or left unstimulated (◇). The data shown are the mean values of three or more independent experiments. *C*, Stimulation of integrin-dependent adhesion by RapV12 mutants. Jurkat T cells were transiently transfected with 10 μg of pG3-TK luciferase reporter plasmid and 30 μg of empty pMT2HA vector (mock) or pMT2HA vector encoding RapV12, RapV12/G37, RapV12/E38, RapV12/C40, or RapV12/Δ CAAX mutants as indicated. Cells were allowed to adhere for 45 min on immobilized ICAM, washed, lysed, and adherent cells quantitated by luciferase assay. Error bars represent the average mean binding + SE of the percentage of bound cells from four independent experiments performed in quadruplicate. Expression of constructs was confirmed by anti-HA immunoblotting of whole cell lysates of transfected cells (data not shown). *D*, CD3-induced ROS production requires PI3K signaling upstream of Ras. Jurkat cells were transfected with cDNA encoding CD20 and either empty vector (control) or RasV12 in pMT2HA. Where indicated, cells were pretreated with 1 μM LY294002 (LY) for 24 h before loading with 6-carboxy-DCF, and stimulated with anti-CD3 (▨) or left unstimulated (◇). Intracellular ROS production was measured as in Fig. 1. *E*, PI3K activity is required for TCR-dependent Ras activation. Healthy donor PBLs were incubated for 24 in the absence or presence of with 1 μM LY294002 (LY) before stimulation with medium or anti-CD3 Ab or PMA+I, as indicated. Ras-GTP was precipitated from cell lysates with GST-RafRBD fusion protein, resolved by SDS-PAGE, and detected by immunoblotting with anti-Ras Ab. F Suppression of Ras-induced ROS production by Rap1 requires PI3-kinase activity. Jurkat cells were transfected with CD20 and RapV12 cDNA, in the absence or presence of cotransfected RasV12 cDNA. Where indicated, cells were incubated with 1 μM LY294002 (LY) for 24 h before anti-CD3 stimulation and detection of intracellular ROS, as in Fig. 1. Constructs and conditions in *C* and *E* were assessed in the same experiment, but are shown as two sections for clarity of presentation. *G*, Expression of RasV12 and RapV12 constructs in *D* and *F* was detected by resolution of whole cell lysates by SDS-PAGE, transfer to PVDF, immunoblotting with anti-Ras and anti-HA Abs, respectively, and ECL.

Constitutive ROS production in RA SF T lymphocytes correlates with deregulated Ras and Rap1 activation

Because our previous experiments indicated that ROS production in Jurkat T cells was highly sensitive to Ras and Rap1 signaling pathways, we next examined whether chronic oxidative stress observed in RA SF T lymphocytes might reflect altered signaling by these GTPases. Several potential sources of ROS in the synovial joint have been proposed to contribute to disturbed redox homeostasis in SF T lymphocytes, including activated macrophages and neutrophils, ischemia/reperfusion compromised oxygen radical tension in the inflamed joint, and generation of hydroxyl radicals by $Fe2^{+}$ released from dying cells (42). However, because TCR or phorbol ester stimulation of T cells also induces ROS production, this raised the possibility that chronic oxidative stress in RA SF T cells results from endogenous ROS production. Therefore, we first compared intracellular ROS production in RA PB and SF T lymphocytes. PB and SF T cells were loaded with the ROS-reactive dye DCF, and left unstimulated or stimulated with anti-CD3 Abs or PMA/I, followed by FACS analysis of DCF reactivity with ROS. Resting PB T cells displayed a low basal rate of ROS production (Fig. 4, *left panel*), which transiently increased for 5–10 min following PMA/I (Fig. 4) or TCR (data not shown) stimulation. No further changes in the rate of ROS production were observed up to 1 h poststimulation. However, in contrast to PB T cells, RA SF T cells displayed a high basal rate of ROS production that could not be further elevated (Fig. 4, *right panel*). Basal and PMA/I (Fig. 4) or TCR-induced ROS levels were consistently lower in human PB T cells than in Jurkat T cells. It is unclear whether

this reflects differences in cell size or efficiency of ROS regulation between primary human T cells and the leukemic cell line. The same forward light-side light scatter gates were used in analyzing PB and SF T lymphocytes, eliminating the trivial possibility that observed differences in ROS production were due to differences in cell size. These results indicated that intracellular ROS production alone was sufficient to explain disturbed redox homeostasis in SF T cells.

Figure 4

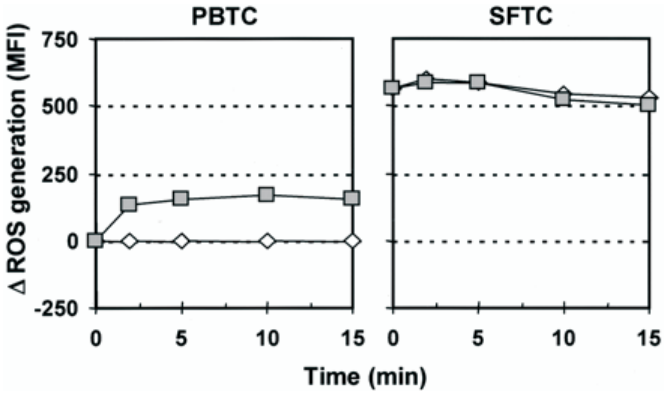

FIGURE 4. ROS is produced transiently in PB T lymphocytes and constitutively in RA SF T lymphocytes. Purified PB and SF T lymphocytes from RA patients were resuspended at 5 x 10^6 cells/ml in phenol red-free DMEM medium and loaded with DCF, stimulated with anti-CD3 (▣) or left unstimulated (◇) and analyzed for ROS production as in Fig. 1. The ΔROS generation is obtained by reducing the measured MFI with the measured MFI at corresponding time points in the unstimulated PB T lymphocyte group. The data shown are the mean values of three or more independent experiments.

We next examined the possibility that Ras and Rap1 signaling may be altered in oxidatively stressed RA SF T lymphocytes. SF and PB T lymphocytes were isolated from RA patients, left unstimulated or activated with anti-CD3 Abs or PMA/I. Active GTP-bound Ras and Rap1 proteins were precipitated with Raf1-RBD and RalGDS-RBD GST fusion proteins, respectively, and detected by immunoblotting. Both anti-CD3 and PMA/I stimulation potently activated Rap1 in PB T lymphocytes from RA patients (Fig. 5*A*), in accordance with previous observations in PB T lymphocytes from healthy controls (5). In contrast, basal levels of activated Rap1 were decreased in SF T lymphocytes and could not be increased through TCR or PMA/I stimulation (Fig. 5*A*). Minimal amounts of GTP-bound Rap1 were detected in SF T cells following long overexposure of the immunoblot, but again no increase was noted following cell stimulation (data not shown). Our failure to detect activation of Rap1 in SF T cells was not due to decreased expression of Rap1 in SF T cells, as no consistent differences in total Rap1 were observed between PB and SF T cells. TCR and PMA/I–dependent Ras activation was also observed in PB T cells from RA patients. However, unlike Rap1, Ras activation in SF T cells was constitutively high and could not be further enhanced through stimulation (Fig. 5*B*). Equivalent amounts of total Ras were observed in whole cell lysates of both PB and SF T cells. Despite constitutive Ras activation, TCR and PMA/I–dependent ERK activation, as detected by phospho-specific Abs, was blocked in SF T cells (Fig. 5*B*). No differences in total ERK expression were observed. Similar results were obtained for Ras and Rap1 in SF T cells from six different RA patients, whereas ERK activation was diminished in four of five patients examined (data not shown).

Unlike previously noted defects in RA SF T lymphocyte signaling, as observed for linker for activation of T cells phosphorylation and IL-2 production (30,31), we obtained no evidence that

deregulated Ras and Rap1 activation was due to chronic oxidative stress. Acute stimulation of PB T lymphocytes from healthy controls with hydrogen peroxide transiently activated Rap1, but not Ras (Fig. 5*C*). Pretreatment of PB T lymphocytes with BSO, which results in depletion of intracellular GSH and abrogated TCR-dependent linker for activation of T cells phosphorylation (data not shown) also failed to mimic the inhibition of Rap1 activity observed in SF T lymphocytes (Fig. 5*D*). Finally, restoration of GSH equilibrium in SF T lymphocytes with the antioxidant NAC, failed to rescue Rap1 activation (Fig. 5*E*). Together these data indicated that constitutive activation of Ras and a block in agonist-induced Rap1 activation observed in RA SF T lymphocytes was not resultant from chronic SF T lymphocyte exposure to ROS. Instead, these results raised the possibility that altered Ras and Rap1 signaling was responsible for chronic oxidative stress in these cells.

Figure 5

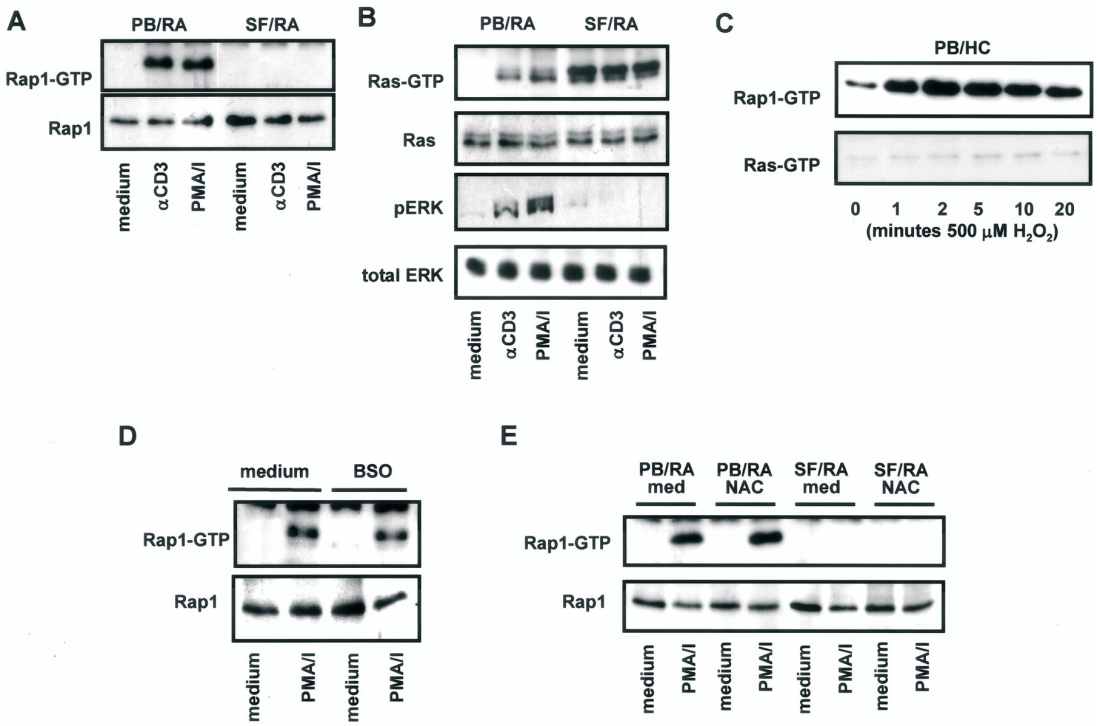

FIGURE 5. Ras and Rap1 signaling are deregulated in RA SF T lymphocytes. *A* and *B*, Blocked Rap1 activation and constitutive Ras activation in RA SF T lymphocytes. Peripheral blood (PB) and synovial fluid (SF) T lymphocytes of rheumatoid arthritis (RA) patients were isolated and stimulated for 5 min with medium, anti-CD3 Ab (0.5 μg/ml anti-CD3, αCD3) or 100 ng/ml PMA plus 1 μg/ml ionomycin (PMA/I). Active GTP-bound Rap1 (*A*) and Ras (*B*) proteins from 5 x 10^6 T cells were precipitated with RalGDS-RBD and Raf-RBD GST fusion proteins, respectively, resolved by SDS-PAGE, and detected by immunoblotting with anti-Rap1 or anti-Ras Abs. Total Rap1, Ras, phosphorylated ERK (pERK), and total ERK were detected in 35 μl whole cell lysate. *C*, Acute oxidative stress activates Rap1, but not Ras. PB T lymphocytes from healthy donors were left unstimulated or stimulated for the indicated times with 500 μM hydrogen peroxide (H_2O_2) before lysis and precipitation and detection of activated Rap1 and Ras as in *A* and *B*. *D*, Depletion of intracellular GSH levels does not affect Rap1 activation. Normal PB T lymphocytes were cultured in medium alone (medium) or medium containing 200 μM BSO for 72 h. Cells were then left unstimulated or stimulated for 5 min with PMA/I, and GTP-bound Rap1 (*upper*) and total Rap1 (*lower*) detected as in *A*. *E*, Supplementation of intracellular GSH levels does not restore Rap1 activation in SF or RA cells. PB and SF T lymphocytes from a representative RA patient were cultured in 5 mM NAc for 48 h before stimulation with medium or PMA/I and detection of GTP-bound Rap1 *A*.

Ras and Rap-1 dependent oxidative stress negatively regulates ERK activation

One important consequences of chronic oxidative stress in RA SF T lymphocytes is a decreased proliferative response to mitogenic stimuli. Therefore, we examined whether manipulation of Ras and Rap1 activation in Jurkat T cells could recapitulate altered ERK signaling observed in RA SF T lymphocytes. In initial experiments, we had noted that TCR and PMA/I–induced activation of ERK was diminished in RA SF T cells (Fig. 5*B*). Transfection of Jurkat cells with small quantities (1–3 μg) of RasV12 cDNA led to enhanced phosphorylation of cotransfected Myc epitope-tagged ERK2 (data not shown). However, higher concentrations of RasV12 cDNA used in ROS experiments failed to activate ERK2, and resulted in an ~50% inhibition of TCR-induced ERK2 phosphorylation (Fig. 6*A*). A similar reduction in TCR-stimulated ERK2 activation was observed in cells cotransfected with the active Ral exchange factor Rlf-CAAX (Fig. 6*B*). In both cases, maintenance of intracellular GSH levels by incubation in NAc restored ERK responsiveness to TCR stimulation, indicating that constitutive Ras and Ral activation inhibited ERK activation through ROS production. Cotransfection of active RapV12 with RasV12 (Fig. 6*C*) also rescued defective TCR-stimulated ERK activation. Thus, chronic oxidative stress resulting from constitutive activation of Ras and Ral diminishes TCR-proximal proliferative signals, whereas Rap1 activation protects against this inhibitory effect.

Activation of Ras is required to maintain ROS production in RA SF T lymphocytes

Finally, we directly examined whether altered Ras family GTPase signaling was responsible for oxidative stress in RA SF T lymphocytes. To test this, we incubated PB and SF T cells in medium alone or containing cell-permeable Tat peptides fused to control β-Gal (Tat-βGal) or dominant-negative RasN17 (Tat-RasN17). Introduced Ras protein was readily detected in T lymphocytes incubated with Tat-RasN17, but not control PB T lymphocyte lysates (Fig. 7*A*). As compared with cells treated with medium alone or Tat-βGal, TCR-dependent ERK activation was completely inhibited in PB T cells exposed to Tat-RasN17, demonstrating that the RasN17 peptide was internalized and functional (Fig. 7*B*). We observed no significant differences in the basal rate of ROS production between PB T cells incubated with Tat-βGal and Tat-RasN17 (Fig. 7*C*). In contrast, Tat-RasN17, but not control Tat-peptide, completely inhibited the enhanced rate of ROS production observed in SF T lymphocytes, providing direct evidence that altered GTPase signaling is responsible for oxidative stress in RA SF T lymphocytes.

Figure 6

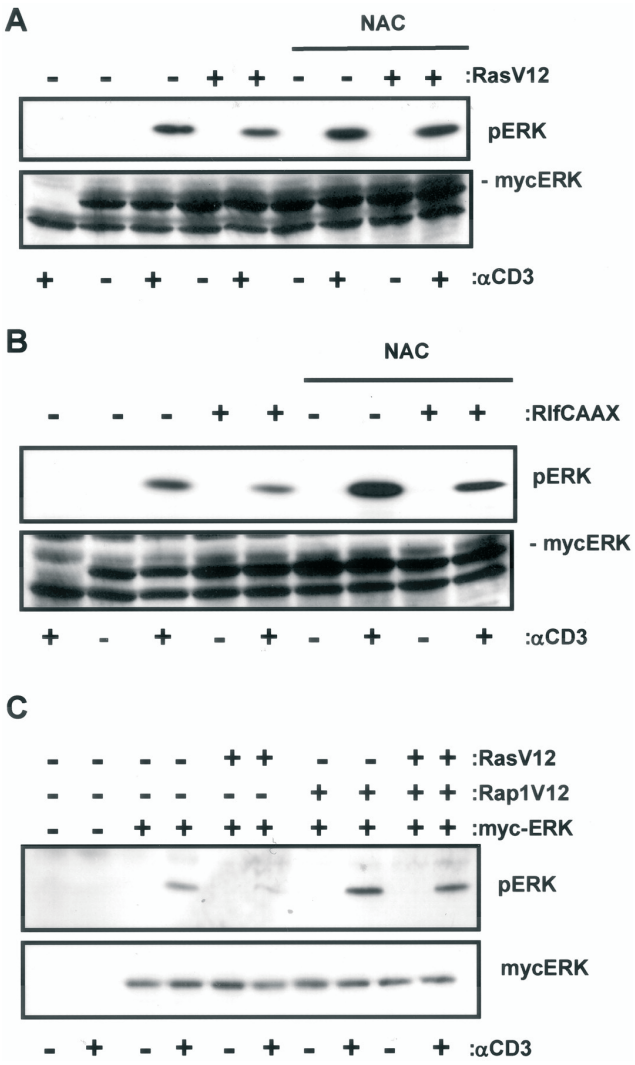

FIGURE 6. Chronic oxidative stress by constitutive Ras or Ral signaling diminishes TCR-dependent ERK phosphorylation that can be suppressed by Rap1. *A* and *B*, Constitutive Ras (*A*) or Ral (*B*) signaling inhibits TCR-dependent ERK activation via ROS production. Jurkat T cells were mock-transfected (*first lane, two columns*) or transfected with *myc*-tagged ERK, alone or in combination with Ras V12 (*A*) or Rlf-CAAX (*B*). Where indicated, cells were maintained in 5 mM NAc. At 48 h posttransfection, cells were harvested and left unstimulated, or stimulated for 5 min with anti-CD3 (α CD3) Ab. Cell lysates were immunoprecipitated with anti-*myc* Ab, and ERK activation detected by immunoblotting with phospho-specific anti-ERK1/2 Abs (pERK). *C*, Rap1 relieves inhibition of ERK activation by Ras. Jurkat T cells were mock transfected (*first lane*), or transfected with *myc*-tagged ERK alone or in combination with active RasV12 and/or Rap1V12 as indicated. Cells were activated, and ERK phosphorylation assessed as in *A*. Results are representative of two to three independent experiments. Equivalent levels of transfected *myc*-tagged ERK were detected within each experiment by resolution of whole cell lysates by SDS-PAGE, transfer to PVDF, immunoblotting

Figure 7

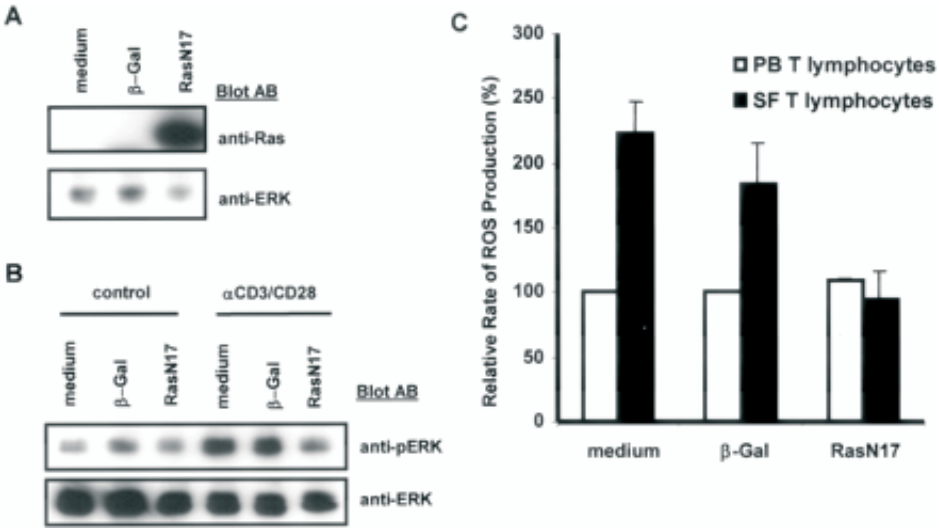

FIGURE 7. Inhibition of Ras signaling blocks oxidative stress in RA SF T lymphocytes. *A*, Expression of cell-permeable Tat-RasN17 peptide in T lymphocytes. PB T lymphocytes were cultured overnight in medium alone, or 25 μg/ml Tat-β-galactosidase (β-Gal) or Tat-RasN17 (RasN17) peptide. Cells were washed extensively, lysed, and Ras detected by SDS-PAGE resolution, immunoblotting with anti-Ras Ab and ECL (*top*). Immunoblotting with anti-ERK Ab demonstrated that equivalent amounts of protein were loaded from each sample (*bottom*). *B*, Tat-RasN17 peptide blocks TCR-dependent ERK activation in T lymphocytes. PB T cells were treated with medium, Tat-β-Gal or Tat-RasN17 peptides (*A*), and stimulated for 5 min with medium (control) or anti-CD3 and anti–CD28 Abs (αCD3/CD28), before lysis and analysis of ERK phosphorylation (pERK) and expression (ERK) by SDS-PAGE, immunoblotting and ECL. *C*, Inhibition of Ras signaling blocks ROS production in RA SF T lymphocytes. Purified RA PB (□) and SF T lymphocytes (■) were incubated with medium, Tat-β-Gal or Tat-RasN17 peptides (*A*), before measurement of basal ROS production as in Fig. 1. The data shown represent the mean and SD of three independent experiments. Within each experiment, the rate of ROS production was normalized to 100 for medium-treated PB T lymphocytes.

Discussion

Our results reveal a novel function for Ras, Ral and Rap1 GTPases: a critical role in the regulation of transient ROS production in T lymphocytes following agonist stimulation. In PB T cells, agonist-induced activation of Ras, signaling via Ral, leads to the transient production of ROS (Fig. 8, *left panel*). Concomitant activation of Rap1 serves to attenuate ROS production, distally from Ral. When both Ras and Rap1 are transientally activated, limited ROS production is used as a second messenger optimizing Ras-dependent activation of ERKs and transcription factors. Intriguingly, we find that acute stimulation of T cells with ROS activates Rap1, suggesting that Rap1 may serve as a sensor to protect against oxidative toxicity. The cellular machinery responsible for coupling Ral and Rap1 to ROS regulation in T cells remains to be identified. While this report was under review, Nagy and colleagues (43) reported a detailed pharmacological analysis of the relationship between TCR signaling, ROS production, and changes in mitochondrial integrity, an important step in T cell apoptosis. They found that TCR-dependent ROS production and calcium-release combined to increase NO production as a

requisite step in mitochondrial hyperpolarization, further ROS production, and apoptosis. Previous studies have shown that TCR stimulation results in ROS production via catalase-sensitive hydrogen peroxide production, requiring BAPTA-AM-sensitive calcium release, and diphenylene iodonium (DPI)-sensitive superoxide anion generation (44). Preliminary studies from our laboratory indicate that the primary source of ROS in SF T cells is hydrogen peroxide, but not superoxide anion. Additionally, we find that Ras-induced ROS, as well as enhanced ROS production in RA SF T cells, is also sensitive to catalase and BAPTA-AM, but not DPI. However, monitoring mitochondrial integrity with the potential-dependent J-aggregrate-forming dye 5,5',6,6'-tetrachloro-1,1'3,3'-tetraethylbenzimidazolocarbocyanine iodide (JC-1), we observed no differences in mitochondrial potential between PB and SF T cells, nor mock and RasV12-transfected Jurkat T cells. Rap1 has been shown to associate with NADPH complexes in neutrophil-differentiated human HL-60 cells (45), but many components of the NADPH oxidase are not expressed in T lymphocytes. A homologue of gp91^{phox} has been identified in T cells, but this enzyme is also DPI-sensitive (46).

Figure 8

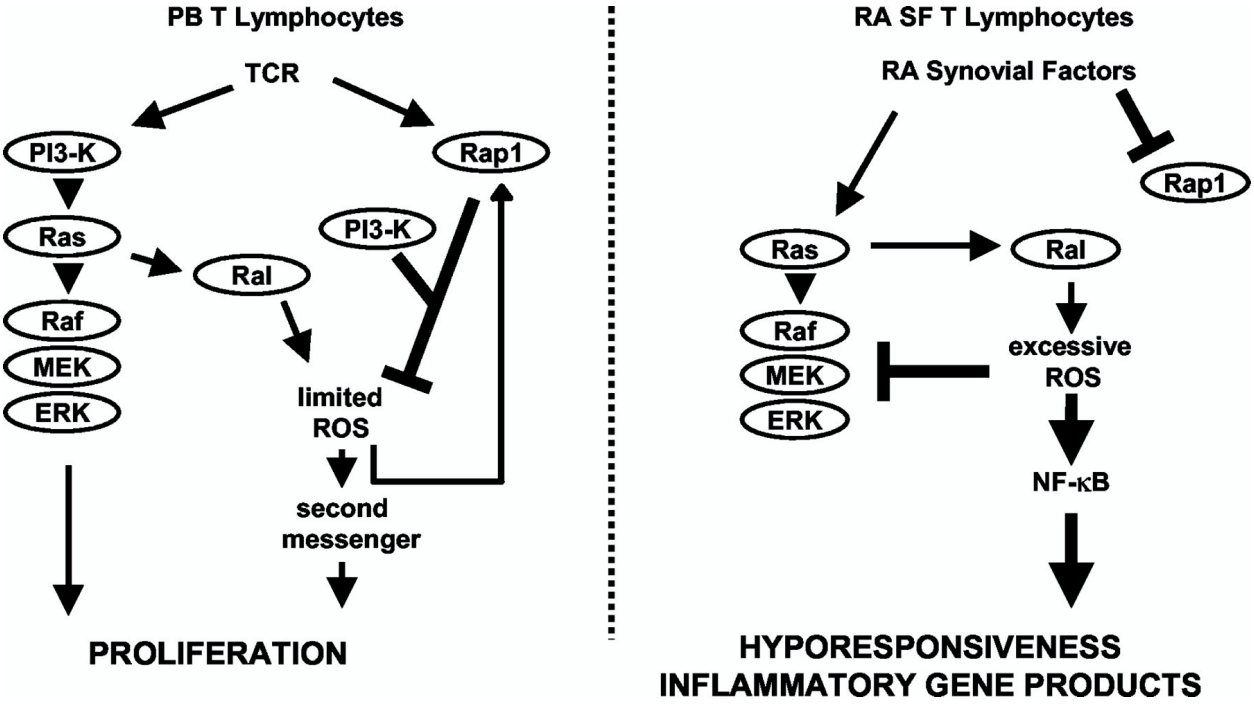

FIGURE 8. A hypothetical model for regulation of ROS production in PB and RA SF T lymphocytes by Ras family GTPases. *A*, In PB T lymphocytes, TCR stimulation leads to PI3K–dependent activation of Ras. Subsequent activation of Ral leads to production of ROS. Concomitantly, TCR stimulation activates Rap1. Rap1, signaling via a pathway dependent upon or acting in parallel to PI3K, down-regulates ROS production. Transient ROS production cooperates with the Raf/ERK pathway to optimize proliferative transcriptional events. *B*, In RA SF T lymphocytes, unidentified synovial microenvironment factors constitutively activate Ras and inhibit Rap1 signaling. In the absence of Rap1 signaling, ROS production by Ras and Ral is not down-regulated, leading to oxidative stress. Oxidative stress inhibits TCR signaling to proliferative pathways, such as Raf/ERK, causing antigenic hyporesponsiveness. Conversely, resultant oxidative stress may lead to enhanced NF-κB–dependent transcription of inflammatory gene products. Activating pathways are indicated by arrowheads and arrows, with proposed relative strength of signal correlating with arrow thickness. T bars indicate negative or suppressing signaling pathways.

Rap1 appears to regulate ROS production distally from Ras, rather than via competitive sequestration of Ras effectors by Rap1. We observe that Rap1-dependent inhibition of ROS is PI3K-dependent, whereas Ras-induced ROS generation is Ral-dependent. Moreover, RapV12 effectively inhibited Ral-induced ROS generation (Fig. 2), and Rap1 and Ral have dissimilar effector domains (3). Although our data show that Rap1 suppresses ROS production via a LY294002-sensitive pathway, we have been unable to demonstrate a direct association between Rap1 and PI3K in T cell lysates. Additionally, in a DO11 murine thymoma line stably expressing active RapV12, we detect no constitutive activation of PI3K, despite suppressed ROS production and enhanced integrin activation in these cells (data not shown). Together, this supports the idea that a PI3K-dependent effector of Rap, rather than PI3K itself, is responsible for ROS suppression in T lymphocytes.

It is clear from previous studies in T lymphocytes that constitutive Rap1 activation can profoundly influence Ras-dependent transcriptional activity, such as NFAT, the minimal IL-2 promoter, and Elk-1 (6-8). However, these are very distal readouts of Ras signaling, and little data support a model in which Rap1 activation in T lymphocytes antagonizes Ras-dependent Raf activation. In transgenic mice expressing activated Rap1 in the T cell compartment at levels equivalent to endogenous Rap1, no inhibition of TCR-dependent ERK activation is observed, despite constitutive integrin activation by Rap1 (14). Similarly, we and others (our study and Ref. 9) fail to observe inhibition of ERK activation in T cells overexpressing active Rap1. Although this strongly argues against a role for Rap1 in directly antagonizing Ras activation of the Raf/ERK pathway, it does not explain the previously observed effects on Ras-dependent transcriptional activity. Our finding that activation of Rap1 down-regulates Ras-induced ROS production in T cells provides a molecular mechanism by which Rap1 can antagonize Ras signaling pathways, distally, downstream of Ral. Reevaluation of Rap1 effects on TCR-dependent NFAT and Elk-1 activity within the context of ROS production will be important. Also, Rap1 antagonism of ROS production by Ras may represent a physiological mechanism by which Rap1 suppresses Ras-dependent oncogenic transformation. Our identification of Rap1 effector domain mutants that fail to regulate ROS production, yet support integrin-dependent adhesion, will serve useful in assessing these possibilities.

In RA SF T cells, as yet unidentified factors lead to constitutive activation of Ras and a block in Rap1 signaling (Fig. 8, *right panel*). In the presence of constitutive Ras-dependent ROS production, the resultant oxidative stress results in inhibition of TCR-proximal proliferative signals, via misfolding of linker for activation of T cells, and diminished ERK activation, possibly via redox sensitive interactions between Ras and Raf (47). Oxidative stress has also been proposed to induce constitutive NF-κB transcriptional activity in RA synovial T lymphocytes, contributing to expression of inflammatory markers such as TNF-α and IL-1 receptors that perpetuate inflammation in the synovial joint. Ral-dependent suppression of ERK signaling and activation of NF-κB have been previously reported in other cell types, but involvement of ROS was not addressed in these studies (48,49). Preliminary experiments indicate that Ras-dependent signaling via Ral also stimulates NF-κB transcriptional activity in Jurkat T cells, which can be blocked by Rap1 activation (P.H.J. Remans and K.A. Reedquist, unpublished observation). Further studies will be required to determine whether Ral and Rap1–dependent modulation of NF-κB activity is mediated by ROS.

The chronic oxidative stress observed in SF T cells can be mimicked by transient transfection of Jurkat cells with activating Ras and interfering Rap1 proteins, and inhibited in RA SF T cells with dominant-negative Ras. Therefore, we propose that deregulation of Rap and Ras are critical events leading to the disturbed intracellular redox balance underlying antigenic hyporesponsiveness and inflammatory gene transcription in RA synovial. It is likely that complex

interactions of T cells with other synovial cells and secreted stimuli contribute to deregulated Ras and Rap1 signaling in the synovial joint, and we are currently attempting to identify these factors. Many of the inflammatory stimuli encountered by T cells in the synovial joint (for example, TNF, IL-1, stromal-derived factor-1, and CD40) are known to activate Ras in T lymphocytes and other cell types. Similarly, ligation of CD28 on SF T lymphocytes and/or stimulation via $G_{\alpha i}$ subunits used by chemokine receptors might be responsible for inactivation of Rap1 (5,7,10).

The identification of Rap1 as a key regulatory protein involved in oxidative stress in SF T lymphocytes provides the first direct evidence for a role for Rap1 in human disease. In vitro, and in pharmacological and genetic studies in rodent models of arthritis, there are strong indications that ROS-dependent activation of NF-κB in T lymphocytes contributes to pathogenesis (21,50,51). Strategies aimed at restoring regulated Ras and Rap1 function in SF T lymphocytes may be therapeutic in RA. Recently, adenoviral-mediated expression of dominant-negative Ras in the joints of arthritic rats was shown to decrease inflammation and joint destruction, indicating the therapeutic potential in targeting these pathways (52). It will be of considerable interest to examine whether deregulated Ras and Rap1 signaling contributes to other human diseases in which chronic oxidative stress is thought to alter T cell function, such as systemic lupus erythematosus and HIV infection.

Acknowledgments

We thank Miranda van Triest (Utrecht, The Netherlands) for superb technical assistance, and Rene van Lier and Martijn Nolte (Academic Medical Center, Amsterdam, The Netherlands) for critical discussions of the manuscript.

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