GRAIL: An E3 Ubiquitin Ligase that Inhibits Cytokine Gene Transcription Is Expressed in Anergic CD4+ T Cells

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Summary

T cell anergy may serve to limit autoreactive T cell responses. We examined early changes in gene expression after antigen-TCR signaling in the presence (activation) or absence (anergy) of B7 costimulation. Induced expression of GRAIL (gene related to anergy in lymphocytes) was observed in anergic CD4+ T cells. GRAIL is a type I transmembrane protein that localizes to the endocytic pathway and bears homology to RING zinc-finger proteins. Ubiquitination studies in vitro support GRAIL function as an E3 ubiquitin ligase. Expression of GRAIL in retrovirally transduced T cell hybridomas dramatically limits activation-induced IL-2 and IL-4 production. Additional studies suggest that GRAIL E3 ubiquitin ligase activity and intact endocytic trafficking are critical for cytokine transcriptional regulation. Expression of GRAIL after an anergizing stimulus may result in ubiquitin-mediated regulation of proteins essential for mitogenic cytokine expression, thus positioning GRAIL as a key player in the induction of the anergic phenotype.

Introduction

CD4+ T lymphocytes are able to either mount an active immune response or become tolerized depending upon the context in which they encounter antigen displayed on the surface of antigen-presenting cells (APCs). T cell anergy is one form of peripheral CD4+ T cell tolerance that results in nonresponsiveness to antigen recall from insufficient initial T cell costimulation. The induction of T cell anergy and other mechanisms of peripheral tolerance limit the expansion of self-reactive T cells in vivo. Loss of self-tolerance contributes to the pathophysiologycl of human autoimmune diseases including multiple sclerosis, rheumatoid arthritis, and type I insulin-dependent diabetes (Ridgway et al., 1994). Conversely, T cell unresponsiveness to tumor antigens presents a significant obstacle in the immune-mediated eradication of tumors (Antonia et al., 1998; Staveley-O'Carroll et al., 1998).

Productive CD4+ T cell activation leading to clonal expansion occurs after the efficient engagement of T cell receptor (TCR) by peptide ligand (signal one) along with costimulatory signals provided by APCs predominantly via the interaction of B7 with CD28 (signal two). In contrast to T cell activation, CD4+ T cell clones are rendered anergic when engaged by peptide bound to MHC class II molecules on a planar membrane (signal one) in the absence of costimulatory signals (signal 2) (Quill and Schwartz, 1987). The delivery of costimulatory signals from accessory molecules prevents the induction of T cell anergy (Mueller et al., 1989). Additional forms of anergy have subsequently been demonstrated in T cells using stimulation with plate-bound anti-CD3 monoclonal antibodies (mAb) (Jenkins et al., 1990), antigen presented by APCs lacking B7 expression, stimulation with the calcium ionophore ionomycin (Jenkins et al., 1987), and by blocking costimulation on conventional APCs with anti-CD28 monoclonal antibody (mAb) (Harding et al., 1992). B and T cell anergy can also result from low avidity antigen-TCR interactions in the presence of potential costimulation as demonstrated in double-transgenic mouse models and in experiments using altered peptide ligands (Cooke et al., 1994; Girgis et al., 1999; Sloan-Lancaster et al., 1993).

Anergized T cells differ markedly from activated T cells, which mount an effective proliferative response, undergo clonal expansion, and produce robust amounts of the T cell mitogenic cytokine interleukin-2 (IL-2). In contrast, anergic T cells are characterized by a failure to proliferate in response to subsequent delivery of activation and costimulatory signals as well as by a greatly diminished production of IL-2. Anergic T cell clones can be restored to a responsive state by the addition of recombinant IL-2 to cultures during antigen rechallenge (Bev- erly et al., 1992), and anergy induction can be prevented by signaling through the IL-2 receptor (Boussiotis et al., 1994). Events correlated with anergic signaling include blockade of the cell cycle in the G1 phase (Boussi- otiis et al., 1994, 2000; Greenwald et al., 2001), diminished ras GTP binding (Fields et al., 1996), and altered composition of the immunologic synapse (Eisenbraun et al., 2000). The observations that CD4+ T cell anergy induction could be inhibited by the addition of cyclohexamide or cyclosporin A suggest that anergy induction is an active process, dependent upon the expression of newly synthesized proteins as well as intact Ca2+ flux and calcineurin activity (Quill and Schwartz, 1987). Fusion of anergic murine T cell clones to a human Jurkat T cell leukemia line resulted in a dramatic block in signal transduction to the human IL-2 gene in response to TCR stimulation or to PMA-ionomycin, rather than resulting in complementation of a signaling defect. These early experiments strongly supported the existence of a domin-ant acting repressor (Telander et al., 1999).

Using differential display to examine differences in transcripts expressed early in the induction of T cell anergy in antigen-specific murine CD4+ T cell clones,
we have identified a transcript encoding a zinc binding RING finger protein, GRAIL (gene related to anergy in lymphocytes). Human and mouse GRAIL share homology to other murine RING finger proteins as well as to those from other species including A. italiana, D. melanogaster, and C. elegans, whose functions are poorly understood. Here we present data characterizing GRAIL function as an E3 ubiquitin ligase whose expression results in diminished gene transcription of the cytokine IL-2. Our studies demonstrate that GRAIL localizes to the transferrin recycling pathway and suggest a requirement for intact endocytic processes in order to inhibit cytokine gene transcription.

Results

T Cell Anergy Is Rapidly Induced In Vitro

To identify early gene products specific for the inductive events in anergy, we utilized a T cell- and APC-based system in vitro to induce phenotypic anergy, inhibition of proliferation, and diminished IL-2 production following recall antigen challenge (Ruberti et al., 1992). To function as surrogate APCs, RT 7.7 fibroblasts were transfected to express the appropriate MHC in the presence or absence of B7 to deliver either activating or anergy-inducing signals, respectively. These surrogate APCs were cultured with the sperm whale myoglobin (SWM) reactive 3A T cell clone in the presence of B7 and SWM peptide (activating), the presence of peptide but absence of B7 (anergic), or in the presence of B7 but absence of peptide (resting). T cell clones cocultured under anergizing conditions for 4 or more hours, separated, rested, and rechallenged failed to proliferate (Figure 1A) and to produce IL-2 (data not shown). The addition of IL-2 to the reactivation cultures restored proliferation (Figure 1B). These data suggest that absence of proliferation in cells activated for 4–6 hr with signal one in the absence of costimulation is a result of functional anergy and not diminished viability. We also observed that addition of anti-IL-2 monoclonal antibody to T cell clones cultured under activating conditions blocked proliferation in response to antigen rechallenge (data not shown). At the time of antigen rechallenge, activated, resting, and anergized T cell clones had equivalent TCR levels (data not shown). These data suggested that the delivery of signals required to induce anergy was achieved within 4 hr of coculture and that at this 4 hr time point transcriptional changes could be observed in anergic T cells.

GRAIL Is Expressed Early in the Induction of T Cell Anergy In Vitro

In order to identify gene products specific to early events in the induction of CD4⁺ T cell anergy, we used the in vitro model system described above and differential display (ddPCR) to compare mRNA transcripts from T cell clones following 4 hr anergizing, resting, and activating conditions. Of the five differentially displayed gene products confirmed by quantitative PCR (qPCR) analysis to be expressed at higher levels in anergic T cell clones, only one transcript encoded an unknown protein with low to undetectable constitutive transcript levels in resting T cells (Figure 1C). A ³²P-labeled gene fragment was used to screen a mouse anergic T cell library. A 2145 bp cDNA clone encoding a 1287 bp open reading frame was identified and called GRAIL. Following in vitro T cell, peptide, and surrogate APC coculture under anergy-inducing conditions, GRAIL was rapidly transcribed. Northern blot analysis confirmed ddPCR data; GRAIL mRNA level increased as early as 4 hr by 5- to 7-fold in anergized cultures as compared to resting or activated cells (Figure 1D).

GRAIL Induction in Ionomycin-Treated Cells Is Calcineurin Dependent

The calcium ionophore ionomycin has been previously used to mimic early events in the induction of T cell
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Figure 2. Kinetics of Ionomycin-Induced Tolerance and GRAIL Expression
(A) SWM T cell clone 2B was treated with 1.5 μM ionomycin for increasing amounts of time (0, 6, and 18 hr) and rechallenged with conventional APCs and peptide antigen in a standard proliferation assay.
(B) Proliferation after the addition of 5 U/ml recombinant murine IL-2 added to T cell clone 2B during 10 μM antigen recall.
(C) Effect of cyclosporin A addition on mean stimulation index at 18 hr after ionomycin treatment.
(D) Quantitative PCR analysis of GRAIL expression in untreated 2B T cells, in the presence of 1.5 μM ionomycin (0, 6, and 18 hr), 1.5 μM ionomycin plus 1 μM cyclosporin A (6 and 18 hr), 1.5 μM PMA + 1.5 μM ionomycin (6 hr).
(E) Western analysis of GRAIL expression in control untreated cells (lane 3) after 6 hr 1.5 μM ionomycin treatment (lane 2) or in the presence of 1.5 μM ionomycin and 1 μM cyclosporin A (lane 1). Anti-Jab loading control (below).

Anergy (Macian et al., 2002). We additionally examined GRAIL expression in T cells made unresponsive through ionomycin treatment. In cells treated with 1.5 μM ionomycin for 6–18 hr, proliferation in response to antigen rechallenge was markedly diminished at both time points (Figure 2A). Addition of IL-2 to these cultures at the time of antigen rechallenge restored proliferation to levels comparable to those of untreated cells (Figure 2B). After 6 hr of ionomycin treatment, GRAIL transcript was increased 3.3-fold compared to resting and activated cells by qPCR analysis and by 18 hr was rapidly restored to resting levels (Figure 2D). While ionomycin treatment induced anergy and GRAIL expression in vitro, addition of 1 μM cyclosporin A to ionomycin-treated cells markedly reduced anergy induction (Figure 2C). We noted in parallel cyclosporin A addition blocked ionomycin-induced expression of GRAIL transcripts at 6 hr. Moreover, T cells activated with PMA and ionomycin also had minimal GRAIL transcript levels (Figure 2D).

Using a polyclonal antibody raised against the C-terminal GRAIL protein, we examined the kinetics of GRAIL induction at the protein level. We observed markedly increased protein expression after 6 hr of ionomycin treatment. GRAIL protein expression was undetectable in untreated T cells or in T cells treated with ionomycin in the presence of cyclosporin A (Figure 2E).

GRAIL Contains a Highly Conserved Zinc Binding RING Finger
Sequence analysis of the GRAIL cDNA revealed identity to a mouse brain cDNA clone (GenBank accession AB041548) that differed by a single nucleotide. The predicted translation product of GRAIL (Figure 3A) is a 428 amino acid protein that exhibits homology to several zinc RING finger-containing proteins from mammalian species as well as from a variety of invertebrate and plant species including C. elegans, A. thaliana, and the D. melanogaster Goliath (G1) protein. Homology is clus-
Figure 3. Sequence Analysis of the GRAIL Protein and Multitissue Northern Expression

(A) The predicted amino acid sequence of GRAIL based on the longest open reading frame (428 aa).

(B) The predicted GRAIL protein sequence was multiply aligned (Clustal W) with other proteins containing Zinc RING finger domains including: D. melanogaster Goliath, a mouse RING finger protein, a C. elegans homolog of Drosophila Goliath, a human hypothetical protein with similarity to Goliath, and a mouse cDNA with similarity to Goliath called g1rp (g1-related protein). Only the region of highest homology containing the Zinc RING domain is shown. Gray shading indicates identity, whereas black shading indicates similarity.

(C) A hypothetical GRAIL primary structure with putative protein motifs is shown.

(D) Northern blot analysis of GRAIL mRNA expression in multiple murine tissues using an antisense full-length GRAIL probe.

tered in a stretch of approximately 75 residues in the RING finger domain (Figure 3B), and this conservation across diverse species suggested functional significance. Additionally, we cloned a human GRAIL homolog hGRAIL (AF394689) with 97% amino acid identity to murine GRAIL. Using various secondary structure predictive algorithms, we identified other putative structural motifs in addition to the zinc RING finger (C3H2C3 type)
domain, including transmembrane, coiled-coil, and protease-associated (PA) domains (Figure 3C). Analysis of GRAIL expression in multiple tissues by Northern blotting revealed that, in addition to its expression in anergic T cell clones, GRAIL message was found in tissues from brain, kidney, heart, liver, ovary, testes, and thymus (Figure 3D). In vitro translated GRAIL, endogenous GRAIL protein from hepatocytes, and GRAIL protein induced in anergic T cells was found to migrate between 62–66 kDa. Glycosylation of GRAIL was demonstrated by treatment of cells with tunicamycin reducing GRAIL protein to a single band migrating at 46 kDa (data not shown).

**GRAIL Demonstrates E3 Ligase Activity In Vitro**

A number of RING finger-containing proteins have well-characterized E3 ligase activity (cbl-b, anaphase promoting complex-cyclosome, Skp-1-cullin 1-F box). To test the functional significance of the C terminus and RING domain, we performed a yeast two-hybrid screen of a murine liver library because of high constitutive GRAIL expression in liver (Figure 3D). Four murine homologs of human E2 ubiquitin transferase proteins were isolated by this procedure and identified by sequence analysis (data not shown). Therefore, we asked whether GRAIL could function in vitro as an E3 ubiquitin ligase. During a ubiquitination reaction, ubiquitin is transferred from the E1 ubiquitin-activating enzyme (which forms the initial thiol-ester bond to ubiquitin) by an E2 ubiquitin transferase enzyme to an E3 ubiquitin ligase/conjugase via direct binding of the E2 with the E3 ligase. Substrate specificity is achieved via E3 ligase substrate binding, which results in the mono- or polyubiquitination of the bound substrate. Therefore, one criterion with which to evaluate GRAIL ligase activity is the ability of the candidate E3 to directly bind an E2 ubiquitin transferase, while a second criterion is ATP-dependent catalytic activity of ubiquitinating enzymes resulting in autoubiquitination (Hershko and Ciechanover, 1998; Gluckman and Ciechanover, 2002).

To test the ability of GRAIL to directly bind E2 ubiquitin-conjugating enzymes, GRAIL was expressed in E. coli as a recombinant human GRAIL-GST fusion protein lacking the signal sequence and the transmembrane region. GRAIL-GST was bound to a column, and several recombinant E2s were run over the column in pull-down assays. Recombinant human GST-GRAIL bound several recombinant E2s including E2-H5a (Figure 4A and data not shown). Given that recombinant GRAIL binds E2 ubiquitin-transferring enzymes, we addressed the ability of GRAIL to act as an E3 ligase when coexpressed with these E2s in vitro by autocatalyzing the formation of polyubiquitin chains. Autoubiquitinating activity of purified C-terminal GRAIL was examined in vitro through expression of recombinant human His-tagged E1, various recombinant human E2s, ATP, and biotinylated ubiquitin, followed by Western blotting with avidin-peroxidase. When hGRAIL-GST was assayed in the presence of E2-H5a or E2-H6 (and to a lesser extent with E2-H2), we observed a characteristic laddering pattern that results from progressive polyubiquitination and formation of higher molecular weight ubiquitin conjugates (Figure 4B) in an E1- and ATP-dependent manner (Figure 4C). Mutation analysis of the RING finger domain was used to examine its function in ligase activity. Substitution of two basic histidine residues with two basic asparagine residues that reside in the RING finger domain and coordinate zinc binding (H2N2 GRAIL) entirely abolished autoubiquitination activity in vitro (Figure 4C). These data suggested that E3 ligase activity required an intact RING finger domain. From these studies, we observed that GRAIL fulfills two criteria for E3 ligase activity: the ability to bind E2s and the ability to autocatalyze formation of higher molecular weight ubiquitin species in vitro.

A role for ubiquitination in the regulation of immune responses recently has become more evident. Cbl-b, an E3 ligase, was demonstrated to be a negative regulator of lymphocyte activation inhibiting TCR signaling through PI3 kinase and blocking cytoskeletal rearrangements downstream of CD28 signaling (Bachmeier et al., 2000; Chiang et al., 2002; Krawczyk et al., 2000; Fang et al., 2001). Cbl-b was one of the five gene products whose expression was increased in anergic cells (C. Seroogy et al., submitted) in the in vitro anergy assay and differential display described above. We now demonstrate that GRAIL is an E3 ubiquitin ligase induced under anergic conditions.

**GRAIL Inhibits Expression of IL-2 in T Cells**

We identified GRAIL transcripts in anergic CD4+ T cells characterized by deficient IL-2 cytokine production. To determine whether GRAIL expression was sufficient to confer this phenotype, IL-2 production was assayed following TCR engagement in MBP reactive T cell hybridomas (ANTC) retrovirally transduced to express a CDNA of GRAIL bearing a C-terminal epitope tag. Transduced T cell hybridomas were generated that expressed either wild-type or H2N2 RING finger mutant v5 tagged GRAIL in the first cistron and GFP in the second cistron. The viral IRES enabled efficient transcription of both the first and second cistrons resulting in comparable bicistronic transcription of GFP and GRAIL. T cell hybridomas transduced with vector, wild-type GRAIL, or H2N2 GRAIL were sorted by flow cytometry for equivalent GFP and clonotypic TCR Vβ expression. In T cell hybridomas expressing wild-type GRAIL, we observed greatly reduced IL-2 production in response to anti-CD3 and anti-CD28 antibody activation when compared to IL-2 production in the same T cell hybridomas transduced with the vector control (Figure 5A). This inhibitory activity was lost in cells that expressed H2N2 mutant GRAIL containing the two amino acid substitutions in the RING finger domain. Following stimulation, H2N2 mutant GRAIL exhibited IL-2 production comparable to vector-transduced controls. Wild-type but not H2N2 mutant GRAIL was also found to inhibit IL-4 production following stimulation to less than 20% of levels seen in activated cells expressing vector alone (Figure 5B). RNase protection assay demonstrated that this deficiency was due to diminished IL-2 and IL-4 transcript levels (Figure 5C). To determine whether TCR proximal signaling events were required to inhibit cytokine production in the presence of full-length GRAIL, transduced T cell hybridomas were stimulated with PMA and ionomycin to bypass cell surface activation. Inhibition of both IL-2 (0.399 U/ml vector versus 0.146 U/ml wild-type GRAIL) and IL-4 (0.073 ng/ml vector versus
**Figure 4. In Vitro Ubiquitination Activity of Wild-Type but Not RING Finger Mutant GRAIL**

(A) Wild-type recombinant human GST-tagged GRAIL lacking the signal sequence and transmembrane domain (lane 1), H2N2 RING finger mutant recombinant C-terminal GST-tagged GRAIL lacking the signal sequence and transmembrane domain (lane 2), or GST alone (lane 3) was incubated with S35-labeled in vitro translated E2-H5a. Pull-down assays were performed using beads and SDS-PAGE separation.

(B) Ubiquitination assays were performed using recombinant wild-type GST-tagged GRAIL lacking the signal sequence and transmembrane domain along with an E1, biotinylated ubiquitin and ATP, and various recombinant human E2 enzymes.

(C) Recombinant wild-type (lane 1) and H2N2 RING finger mutant (lane 2) GST-tagged GRAIL lacking the signal sequence and transmembrane domain, or GST alone (lane 3) was expressed in *E. coli*, purified and coincubated with an E1, E2 UbcH5a, biotinylated ubiquitin, and ATP (lane 1) or in the absence of ATP (lanes 4–6). Wild-type GST GRAIL was also assayed in the absence of either E2 (lane 7) or E1 (lane 8). Western blots were probed with avidin-peroxidase.

0.001 ng/ml wild-type GRAIL) protein expression was observed (Figure 5D). Quantitative PCR analysis confirmed inhibition of IL-2 and IL-4 transcripts (data not shown). Similar inhibition of IL-2 and IL-4 protein expression was seen in wild-type but not H2N2 mutant-expressing Jurkat T cells, EL-4 T cells, and a collagen-reactive hybridoma by cytokine ELISA (data not shown). These data suggest that GRAIL functions as an inhibitor of cytokine gene transcription and that this activity is likely to be mediated by E3-ligase activity.

**GRAIL Is Localized to the Transferrin-Recycling Endocytic Pathway**

From sequence analysis, GRAIL is predicted to have a putative signal peptide and single transmembrane-spanning domain suggesting that GRAIL is a membrane-bound protein. Based on functional assays, inhibition of cytokine transcription in GRAIL-expressing cells still occurred when cell surface-proximal membrane signaling events were bypassed with PMA-ionomycin activation, suggesting that GRAIL activity was likely to occur distal to the cell surface. The subcellular localization of GRAIL protein was analyzed by generating stable retrovirally transduced NIH 3T3 cell lines in which v5 epitope-tagged GRAIL was expressed. Immunohistochemistry revealed that GRAIL localized in an asymmetric perinuclear punctate manner that partially colocalized with the ER resident Bip/GRP 78, with Golgi resident syntaxin 5, and with the late endosomal GTPase Rab7 (Figures 6A–6I). GRAIL localization closely associated with the recycling endosomal compartments defined by transferrin receptor recycling. In cells preincubated for 1 hr at 4°C to slow endosomal traffic, loaded with Texas Red-labeled transferrin at 4°C for an additional hour, and then chased with 37°C media to allow transferrin internalization for 30 min at 37°C, GRAIL was found to colocalize with transferrin (Figures 6J–6L). Using an anti-GRAIL rabbit-polyclonal antibody, it was possible to compare localization in NIH 3T3 cells to endogenous GRAIL expression in H1 hepatocytes as well as in the GRAIL-transduced T cell hybridoma lines used in the cytokine assays. All cells exhibited a similar
Figure 5. Wild-type but not RING finger mutant GRAIL inhibits IL-2 and IL-4 production
(A) T cell hybridomas were transduced to express GFP alone (empty vector) (right), GFP and wt GRAIL (left), or GFP and H2N2 RING finger mutant GRAIL (middle) and sorted for equivalent TCR and GFP expression. Sorted T cells were assayed for IL-2 by ELISA 18–24 hr after stimulation with plate-bound anti-CD3 and anti-CD28 antibodies (5 μg/ml of each). Data show the mean of six independent experiments in which triplicate ELISA wells were assayed per experiment and show relative ratio to vector.
(B) IL-4 ELISA; one representative experiment of three with triplicate values assayed per well.
(C) RNase protection assay using extracted mRNA from T cell hybridomas 4 hr after stimulation with plate-bound anti-CD3 and anti-CD28 antibody (lanes 4–6: WT, H2N2, vector) or unstimulated controls (lanes 1–3: WT, H2N2, vector). L32 and GAPDH loading controls are shown below.
(D) IL-2 and IL-4 ELISA for cells treated with 1 μg/ml PMA + 1 μg/ml ionomycin for 6 hr.

GRAIL staining pattern (see supplemental data at http://www.immunity.com/cgi/content/full/18/4/535/DC1).

GRAIL inhibition of cytokine gene transcription is dependent upon intact endocytic traffic
In contrast to antibody or PMA-ionomycin activation, we observed that activation of T cell hybridomas with 2.5 μg/ml concanavalin A (Con A) in the presence of freshly irradiated APCs resulted in comparable IL-2 production between GRAIL, H2N2 GRAIL, and vector-expressing cells (Figure 7A). Furthermore, addition of ConA to hybridomas stimulated on plate-bound anti-CD3 and anti-CD28 minimized the observed differences in IL-2 production (Figure 7A). In light of GRAIL localization and because in addition to mitogenic properties Con A efficiently inhibits clathrin-mediated endocytosis and G protein-coupled receptor internalization (Hansen et al., 1993, Gicquiaux et al., 2002; Budd et al., 1999; DeGraff et al., 1999), we hypothesized that GRAIL activity might require intact endosomal traffic and that the IL-2 production observed might be due to ConA inhibition of endocytosis. In T cell hybridomas treated with ConA, endocytosis of transferrin was efficiently blocked (see supplemental data at http://www.immunity.com/cgi/content/full/18/4/535/DC1). We stimulated cells with anti-CD3 and anti-CD28 in the presence or absence of latrunculin B, a drug demonstrated to increase coated pit mobility at low doses and to disrupt endocytic sorting of transferrin through actin-monomer sequestration (Gadairov et al., 1999; Sheff et al., 2002). Latrunculin B addition entirely abolished GRAIL-mediated IL-2 inhibition (Figure 7C), further suggesting a requirement for actin-based endocytic sorting.

Discussion
We have identified and characterized GRAIL, an E3 ubiquitin ligase whose expression is rapidly induced in anergic T cells. While a requirement for the synthesis of new proteins and intact calcineurin activity in the induction of T cell anergy has been reported, no proteins have been identified in anergic CD4+ T cells that are not expressed at significant constitutive levels in resting T cells and that are also induced in a calcineurin-dependent fashion. We observed that GRAIL expression alone recapitulates certain hallmarks of the anergic phenotype, dramatically limiting the transcription of both the IL-2 and IL-4 genes. These cytokine genes (whose pro-
GRAIL Localizes to the Internal Pool of the Transferrin Recycling Endosomal Pathway

(A) NIH 3T3 retrovirally transduced lines expressing wild-type GRAIL with a C-terminal v5 epitope tag were used for immunofluorescence analysis of subcellular GRAIL (100× oil). Confocal sections of .5 μM were taken at 100× magnification of NIH 3T3 cells costained (C, F, and I) for GRAIL-v5 (B, E, and H) and anti-GRP78 (ER) (A), anti-syntaxin 5 (Golgi) (D), or anti-Rab7 (late endosomes) (G). Recycling endosomal localization in NIH 3T3 cells was assayed after transferrin uptake for 30 min. Shown are .5 μM confocal sections for GRAIL-V5 (K) and Texas Red-transferrin (J), with overlay (L).
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Figure 7. GRAIL Does Not Inhibit IL-2 Production in T Cells When Endocytosis Is Inhibited

(A) IL-2 cytokine ELISA of wild-type GRAIL, H2N2 GRAIL, and vector-expressing T cells stimulated with ConA and APCs. Shown is the mean relative ratio to vector from three independent experiments.

(B and C) Anti-CD3 and anti-CD28 in the presence (gray bars) or absence (black bars) of 2.5 μg/ml ConA (B) or .1 μg/ml Latrunculin B. (C) One representative experiment of three is shown with triplicate wells assayed.

Motors synergistically integrate activity of NFAT and AP-1 downstream of independent signals) were previously demonstrated to be induced downstream of TCR and CD28 signaling (DeSilva et al., 1996; Seder et al., 1994; June et al., 1994). Consistent with a functional association of GRAIL expression and T cell anergy in vitro, our lab has recently described GRAIL transcript upregulation in a CD25+ suppressor in vitro model system (Ermann et al., 2001) as well as in two classic models of T cell anergy induction in vivo (C. Seroogy et al., submitted).

Mechanisms suggested for anergy induction and maintenance included the transcriptional regulation of cytokines, inhibition of cell cycle progression, and blockade of intracellular signaling. More recently, a role for ubiquitin-related mechanisms has emerged in T cells, highlighted by the regulation of phosphatidyl-inositol 3 kinase in lymphocytes by the E3 ubiquitin ligase Cbl-b (Fang et al., 2001). Cbl-b-deficient mice develop spontaneous autoimmunity as demonstrated by autoantibody production, organ infiltration by T and B cells, and T cell hyperproliferation of IL-2 independent of CD28-mediated signaling (Bachmeier et al., 2000). In addition to more traditional roles in targeting substrates for proteosome-mediated degradation via ubiquitination, E3 ubiquitin ligases have recently been shown to alter ligand-mediated signaling in T cells by targeting cell surface receptors, including TCR and IL-2Rβ chain, into recycling pathways via monoubiquitination (Cenciarelli et al., 1992; Rocca et al., 2001). The induction of E3 ligase expression and regulation of T cell anergy induction via the ubiquitin proteosome system is one posttranslational mechanism of protein regulation that may help explain the earlier findings that fusion of anergic cells to transformed Jurkat T cells did not rescue signaling defects but rather transferred IL-2 transcriptional repression.

In view of the ability of wild-type but not H2N2 mutant GRAIL to function as an E3 ligase and to inhibit cytokine gene transcription, GRAIL inhibition of IL-2 expression is likely to occur through GRAIL-mediated substrate ubiquitination. Specificity in ubiquitin-mediated processes is achieved by E3 binding of target substrates. Identification of the target(s) of GRAIL ubiquitination will likely uncover novel mechanisms of cytokine transcriptional regulation, such as those seen in T cell anergy. We have demonstrated an association between intact actin-driven endocytic transport and GRAIL-mediated inhibition of cytokine gene transcription, suggesting that GRAIL function is intimately tied to this compartment. GRAIL mutants, including H2N2, which no longer impact cytokine gene transcription, demonstrate markedly altered subcellular localization (see supplemental data at http://www.immunity.com/cgi/content/full/18/4/535/DC1) lending further support to localization-dependent function.
Ubiquitin-mediated regulation of endocytic sorting and internalization has been reported in \textit{S. cerevisiae} and is likely to serve a homologous function in mammalian species (Hicke, 2001). While the internalization of a large number of mammalian cell surface receptors is driven by ubiquitination, very few mammalian E3 ligases have actually been demonstrated to localize to endosomal compartments. Endosomal turnover provides a highly efficient means of rapidly reorganizing signaling complexes (Di Fiore and De Camilli, 2001). Kinetic studies have demonstrated a half-life of 7–8 min for transferrin recycling (Edwards et al., 1996). We observe GRAIL localization in the transferring-recycling pathway, through which surface expression of a number of T cell signaling molecules are regulated, including the TCR (Salmeron et al., 1995; Alcover and Alarcon, 2000), CTLA-4 (Bradshaw et al., 1997; Chuang et al., 1997), and Ick (Richie-Ehrlich et al., 2002). That GRAIL has an N-terminal protease-associated domain, a motif originally defined by transferrin receptor sorting and involved in plant vacuolar sorting pathways, further supports a possible sorting function (Luo and Hofmann, 2001; Cao et al., 2000).

A potential mechanism by which GRAIL might inhibit the delivery of mitogenic signals could be through activity analogous to that demonstrated for Cbl: selective targeting of tyrosine phosphorylated proteins into the endocytic pathway via ubiquitination. In this model, GRAIL-mediated ubiquitination may promote substrate sequestration toward or away from a subcellular compartment as a means of terminating signaling.

A second possible mechanism for GRAIL regulation of mitogenic signal delivery could be through GRAIL-mediated ubiquitination of substrate, resulting in substrate degradation via the proteosome. GRAIL is a type I transmembrane protein, suggesting that its N-terminal protease-associated domain would be internal to an endocytic vesicle and that the C terminal C3H2C3 RING finger domain would project into the cytosolic lumen. This positioning affords the possibility of its interaction with numerous cytosolic proteins that may be substrates for ubiquitination and degradation.

The coordinated regulation of mitogenic signaling on endosomes has been established for several signaling pathways, including components of those pathways downstream of CD28 such as JNK and WASP (Cavalli et al., 2001; Di Fiore and De Camille, 2001). CD28 costimulation mediates actin reorganization resulting in productive synapse formation through activation of the WASP-Vav-CDC42 pathway and is intimately tied to endocytic traffic via Rho family GEF proteins (Costello et al., 1999), adaptor molecules such as intersectin-2 (McGavin et al., 2001), and the actin cytoskeleton (Silvin et al., 2001; Zhang et al., 1999; Snapper et al., 1998). Thus, a third possible model would have GRAIL regulate events linking endocytosis and actin reorganization that act upstream of cytokine transcription, as has been shown for Vav/Slp 76 activity in which IL-2 transcription independent of MAPK activity may occur (Raab et al., 2001). Consistent with such a model, we did not observe any differences in the activation status of \(p38\), JNK, or ERK1/2 in GRAIL-expressing cells (L. Su and C.G.F., unpublished data). Furthermore, we have observed that the expression of GRAIL zinc finger deletion mutants in NIH 3T3 fibroblasts results in filopodia formation, membrane ruffling, and other actin-related phenotypes (see supplemental data at http://www.immunity.com/cgi/content/full/18/4/535/DC1). We have also identified in preliminary studies binding of GRAIL and Rit, a ras-related membrane-associated GTPase (Lee et al., 1996) whose constitutive activity results in actin-based phenotypes such as neurite outgrowth (Spencer et al., 2002). In this model, GRAIL-mediated ubiquitination of substrate might occur within a macromolecular, endosomal complex which may serve to link or unlink adaptor molecules to the cytoskeleton or endosomes.

Given the conserved domain homology of GRAIL to RING finger proteins across mammalian, plant, and invertebrate species, it is likely that these other RING finger-containing proteins may also function as E3 ligases in signaling pathways. The function of Goliath and Goliath-related homologs of GRAIL is unclear; however, they appear to be critical in numerous developmental processes. Restricted Goliath expression has been observed in the developing mesodermal tissues of \textit{Drosophila} (Bouchard and Cote, 1993), and increased expression of \(g1\)rp \((g1\)-related protein\) has been observed during IL-3 growth-factor withdrawal-induced apoptosis of myeloid precursor cells (Baker and Reddy, 2000). A role for GRAIL E3 ligase activity in developmental processes is supported by the findings of Borchers et al. (2002) who observe that GRAIL expression in \textit{Xenopus} can sensitize ectoderm to neutralizing signals resulting in conversion of ectoderm to an anterior fate. That GRAIL is induced in a highly regulated manner in peripheral CD4\(^+\) T cells while constitutive GRAIL expression is observed in the CNS, kidney, and liver suggests tissue-specific substrate interactions, regulation of GRAIL itself at a posttranslational level, or, most intriguingly, that the program of T cell anergy is similar to regulatory pathways in these organs. Understanding GRAIL function in concert with other anergy-specific genes will expose the functional mechanisms that underlie anergy induction in T cells. In light of GRAIL E3 ligase activity, endocytic residence, and domain homology, understanding GRAIL function and substrate association is likely to reveal highly conserved mechanisms for rapidly altering cell fate in diverse cellular contexts.

**Experimental Procedures**

**Cell Lines and Culture**

T cell clones were carried in RPMI-C media and restimulated with freshly irradiated DBA/2 splenocytes and 10 \(\mu\)M SWM peptide every 10–14 days. T cell clone 11.3.7 has been described (Ruberti et al., 1992). The RT 7.7 fibroblast cell line transfected with the mixed isotype (Ea’A’a’) MHC class II gene and the B7-1 cDNA construct pL444-mB7 used to generate a B7 (+) RT 7.7 line by stable transfection were kindly provided by Dr. Germain (NIH, Bethesda, MD).

**Fibroblast Anergy Assays**

B7 (+) RT 7.7 fibroblasts (anergic) or costimulatory competent, B7 (+) RT7.7 APCs (activated, resting) were irradiated at 3000 rads prior to use in anergy assays and mixed at a 1:1 ratio with SWM T cell clones 11.3.7 or 3A in RPMI-C media in the presence (anergic, activated) or absence (resting) of 10 \(\mu\)M SWM 110-121 peptide. At selected time intervals, aliquots of cultures were mixed with anti-CD4 microbeads (Miltenyl Biotech) to separate CD4\(^+\) T cells from fibroblast APCs; T cells were rested 24 hr. To test for anergy, 5 \(\times\) 10\(^5\) T cells were cultured with 5 \(\times\) 10\(^5\) irradiated DBA/2 splenocytes.
and 0–10 μM peptide in triplicate wells of a 96-well plate. In selected cultures murine rIL-2 (Genzyme) was added at 5 units/ml during restimulation to test viability. After 54 hr, the cells were pulsed for 18 hr with [3H]-thymidine and counted in a β-counter.

Ionomycin Anergy

SWM T cell clone 2B was treated for 0, 1, 6, or 18 hr with 1.5 μM ionomycin (Sigma) at 37°C, with 1.5 μM PMA (Sigma) and 1.5 μM ionomycin, or with 1.5 μM ionomycin and 1 μM cyclosporin A for 6 hr and 18 hr. Cells were washed and cell pellets were prepared for quantitative PCR or Western blot analysis. To test for anergy, 5 × 10^5 T cell clones were tested by proliferation as described above.

Differential Display

RNA was isolated from 11.3.7 T cells treated under resting, activating, or anergic conditions (Qiagen) and reverse transcribed for differential display PCR (Genomyx). Templates were amplified for 40 cycles in the presence of [35S]-dATP. cDNA fragments present at higher levels in anergic lanes were gel excised, reamplified for TA cloning (Invitrogen), and sequenced.

Real-Time Quantitative PCR

Standard RNA isolation, reverse transcription, and PCR protocols were used (ABI 5700, Applied Biosystems). GRAIL primers and fluorogenic probes were designed to span intron-exon-regions. Probes and primer sets specific for normalizing gene 18S rRNA were used (Applied Biosystems). PCR was performed in triplicate on test samples and murine liver cDNA standards allowing changes in GRAIL RNA to be quantitated and normalized to RNA levels. GRAIL arbitrary units (aU) is GRAIL quantity expressed as the mean of triplicate normalized values ± standard deviation.

Cloning of the GRAIL cDNA

Poly A+ mRNA isolated from anergized 11.3.7 T cells was sent to Stratagene for cDNA library construction. Phage plaques were screened with a 32P-labeled ddPCR fragment, and positive clones were sequenced (Stanford PAN facility). The longest positive clone was 2145 bp and contained an ORF of 1284 bp (clone 25-3_pbCMV). Sequence comparisons between GRAIL and related proteins were done using EXPASY and NCBI-Blast. Orosphila Goliath (G1) protein (AA285882) (35% identity over 191 amino acids (aa), mouse g1-related protein (g1rp) (AAF05310) (41%, 315 aa), the uncharacterized PMA-ionomycin stimulation, 1 (AAA28582) (35% identity over 191 amino acids (aa), mouse g1- Pharmingen). ELISA supernatants were harvested at 18–24 hr. For done using EXPASY and NCBI-Blast.

Western Blot Analysis of GRAIL Expression

Recombinant C-terminal GRAIL protein was expressed in bacteria as a GST fusion (pET41 vector, Novagen); purified protein was used to produce rabbit polyclonal antiseraum (Zymed). Affinity-purified rabbit polyclonal antibody against GRAIL was screened against hepatocyte and GRAIL-expressing T cell hybridomas by Western blot and immunofluorescence analysis. Brij 96 lysis buffer was used for cell lysis.

Northern Blot Analysis of GRAIL Expression

Northern analysis was performed on total RNA from 11.3.7 T cells treated for 4 hr under resting, activating, or anergizing conditions. Membranes were hybridized with a 1.8 kb random-primed 32P-labeled GRAIL cDNA fragment according to standard protocols, stripped, and reprobed with the DECAprobe-GAPDH-Mouse cDNA fragment (Ambion) to assess loading. For multissue analysis, GRAIL ORF cDNA was cloned into pCR (Invitrogen) and linearized, and [32P]-UTP labeled antisense transcripts were generated using T3 polymerase (StriAble RNA probe synthesis, Ambion). Full-length antisense mouse GRAIL probe was hybridized at 10^6 cpm/ml to Ambion's FirstChoice Mouse Blot 1 (Ambion). The blot was stripped and reprobed with antisense probe generated from pTRI-ji-actin-mouse control template (Ambion).

Ubiquitination Assays

Ubiquitination assays were performed as described (Huang et al., 2000), with some modifications. Ten microliter reactions contained 100 nM human His-tagged E1 (Calbiochem), 1 μM E2 enzyme (Boston Biochemicals), 5 μM biotinylated-ubiquitin, 2 mM ATP, and 1 μg of either GST-GRAIL, GST-GRAIL H2N2, or GST alone in buffer (50 mM Tris-HCl [pH 7.8], 2 mM MgCl2, and 1 mM DTT). Reactions were incubated at 25°C for 60 min and terminated by the addition of reducing Laemmli sample buffer. Samples were resolved on a 4%–12% gradient polyacrylamide gel (NuPage).

Pull-Down Assays

Bacterial expressed GST-GRAIL isoforms (2 μg) were incubated with 1 × 10^6 cpm of 32P-labeled in vitro translated E2-Hs5 (TNT-T7) (Promega). Reactions were rotated for 2 hr at 4°C. Glutathioneagarose beads were added for an additional 2 hr. Beads were collected by centrifugation, washed three times in PBS-0.1% NP-40, and boiled for 3 min in Laemmli sample buffer. Samples were run on a 12% SDS-PAGE, and the gel was fixed. Detection of radioactive E2-Hs5 was captured using a Phosphor Imager (Bio-Rad).

Retroviral Transduction

The ANTC T cell hybridoma was previously described (Nakajima et al., 2001). P3 constructs were transfected into 293 phoenix packaging cells using standard calcium phosphate transfection to generate virus containing either the P3 vector alone, the FL-P3 vector containing the 1.3 kb GRAIL open reading frame and v5 epitope tag (Invitrogen), or with the mH2N2-P3 vector identical to FL-P3 but with histidine 297 and 300 substituted with arginine. In PMA-ionomycin experiments, ANTC lines were used expressing GRAIL in the pGC-ires vector. Cells were retrovirally transduced by standard protocols and sorted every 4 weeks for equivalent TCR and GFP expression between vector, wt GRAIL, and H2N2 mutant-expressing lines. Wild-type GRAIL-P3, H2N2-P3, and empty P3 backbone vectors were also used for transduction of NIH 3T3 cell lines.

ELISA

Cells from ANTC lines P3, FL, and mH2N2 were seeded at 8 × 10^4 cells/ml 48 hr prior to use. After 48 hr, 4 × 10^5 cells/ml were seeded for antibody stimulation into 1 ml RPMI-C onto a 6-well precoated with 5 μg/ml anti-CD2 and anti-CD28 antibodies (BD Pharmingen). ELISA supernatants were harvested at 18–24 hr. For PMA-ionomycin stimulation, 1 × 10^5 cells/ml were seeded in RPMI-C with 1 μg/ml PMA plus 1 μg/ml ionomycin; supernatants were harvested at 6 hr. Europium Sandwich ELISA (Pharmingen and Wallac) was performed on triplicate wells in 96-well plates (Nunc). Based on standard curves, the lower limits of detection were 0.078 units/ml for IL-2 and .01 ng/ml for IL-4. Actual experimental values for IL-2 ranged between .15 and 2.5 U/ml activity and for IL-4 between .0001 (below detectable limits) and .1 ng/ml under anti-CD3 anti-CD28 stimulation conditions.

RPA

The Riboquant Multiprobe RNase Protection System was used to generate mouse cytokine probe mCK-1, and standard Riboquant procedures (Pharmingen) were used to detect products in 5 μg total RNA.

Immunofluorescence


Concanavalin A and Latrunculin B Treatment

1 × 10^6 ANTC cells were seeded with 6–8 × 10^5 B10.PL female (Jackson) irradiated splenocytes in the presence of 2.5 μg/ml concanavalin A (Sigma). For anti-CD3, anti-CD28 activation cells were cultured with anti-CD3 and anti-CD28 as described above but with addition of either 2.5 μg/ml concanavalin A or .1 μg/ml Latrunculin B.

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