A Novel E3 Ubiquitin Ligase Substrate Screen Identifies Rho Guanine Dissociation Inhibitor as a Substrate of Gene Related to Anergy in Lymphocytes

Leon Su, Neil Lineberry, Yul Huh, Luis Soares and C. Garrison Fathman

*J Immunol* 2006; 177:7559-7566; doi: 10.4049/jimmunol.177.11.7559

http://www.jimmunol.org/content/177/11/7559

---

**References**
This article cites 28 articles, 10 of which you can access for free at:
http://www.jimmunol.org/content/177/11/7559.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
A Novel E3 Ubiquitin Ligase Substrate Screen Identifies Rho Guanine Dissociation Inhibitor as a Substrate of Gene Related to Anergy in Lymphocytes

Leon Su, Neil Lineberry, Yul Huh, Luis Soares, and C. Garrison Fathman

Ubiquitination of eukaryotic proteins regulates a broad range of cellular processes, including regulation of T cell activation and tolerance. We have previously demonstrated that gene related to anergy in lymphocytes (GRAIL), a ring finger ubiquitin E3 ligase, is required for the induction of T cell anergy; however, the substrate(s) for GRAIL E3 ligase activity is/are unknown. In this study, we report a novel prokaryotic system developed to screen for substrates of E3 ligases. Using this screen, Rho guanine dissociation inhibitor (RhoGDI) was identified as a potential substrate of GRAIL. GRAIL was subsequently demonstrated to bind and ubiquitinate RhoGDI, although GRAIL-mediated ubiquitination of RhoGDI did not result in proteosomal degradation. Expression of GRAIL in T cells resulted in specific inhibition of RhoA GTPase activation; activation of Rac1, cdc42, and Ras GTPases were not affected. Interestingly, stable T cell lines expressing dominant-negative RhoA mimicked the GRAIL-mediated IL-2 inhibition phenotype, and T cells expressing constitutively active RhoA were able to overcome GRAIL-mediated inhibition of IL-2 expression. These findings validate our prokaryotic screen as a method of identifying substrates for ubiquitin E3 ligases and suggest a role for Rho effector molecules in T cell anergy. The Journal of Immunology, 2006, 177: 7559–7566.

The ability to recognize self from nonself is a basic tenet of the mammalian immune system. CD4+ T lymphocytes have the ability to mount an active immune response or become tolerized depending on the context in which they encounter Ag presented by MHC class II molecules on the surface of APCs. T cell anergy is one form of peripheral T cell tolerance that results in nonresponsiveness to Ag recall. The gene related to anergy in lymphocytes (GRAIL)3 was identified in a differential display screen as an upregulated gene product in anergized T cells (5). Subsequent studies have shown GRAIL to be a critical element in the induction of T cell anergy in murine CD4+ T cell clones in vitro and in OVA-tolerized DO.11 mice in vivo (6). Structure-function studies have characterized GRAIL as a zinc binding RING finger single subunit E3 ubiquitin ligase; however, the identity of GRAIL E3 substrate(s) has remained elusive. In this study, we report a novel and efficient method of screening for E3 ligase substrates based on a prokaryotic expression system. This new system resulted in the identification of Rho guanine dissociation inhibitor (RhoGDI) family members as substrates of the E3 ubiquitin ligase GRAIL.

Materials and Methods

Plasmids, Abs, and reagents

pACYC184, pCDF184, and pET21 vectors, T7-Tag Ab, and S-Tag HRP conjugate, along with BL21(DE3) competent cells and antibiotics were purchased from Novagen. pCMV-3xFlag vector and Flag M2 Ab were purchased from Sigma-Aldrich. Anti-RhoGDIα, anti-RhoGDIβ, anti-RhoA, and anti-His were obtained from Santa Cruz Biotechnology. pcdNA4/HisMax vector, anti-Xpress, and anti-His Abs were obtained from Invitrogen Life Technologies. Anti-phospho-c-Raf1, p44/42 ERK1/2, p38 MAPK, and JNK were purchased from Cell Signaling Technology. PMA, ionomycin, and MG-132 were purchased from Calbiochem.

Cloning of cDNA

Full-length UBE1, UBE2D1, and ubiquitin (UBB) were cloned by PCR from a human liver first-strand cDNA library. Full-length RhoGDIα,

Covalent attachment of ubiquitin or polyubiquitin chains to eukaryotic proteins regulates a broad range of cellular processes in addition to proteosomal degradation, including cell cycle progression, protein trafficking, inflammation, tumor suppression, and modulation of signaling pathways (1–3). The ubiquitination process occurs through a cascade of three discrete steps, involving different classes of modifying enzymes (4). The first step in this cascade involves an ATP-dependent attachment of ubiquitin to the ubiquitin-activating enzyme (E1) through the formation of a high-energy thiol-ester bond between the C terminus of ubiquitin and a cysteine within the E1. Next, the thiol ester-linked ubiquitin is transferred from the E1 enzyme to a cysteine residue in an ubiquitin-conjugating enzyme (E2). In the last step of the conjugation process, the E2 enzyme, together with ubiquitin-protein ligase (E3) transfers ubiquitin to target proteins, where a stable isopeptide bond is formed between the C terminus of ubiquitin and the ε-amino group of a lysine residue on the target protein. The ubiquitin monomer contains seven lysines, and ubiquitinated proteins can be further modified by the conjugation of additional ubiquitin molecules in a highly processive manner to form diverse polyubiquitin chains. Although the E3 is the central determinant of specificity in the substrate conjugation process, recognition of specific ubiquitination motifs or domains on target proteins have not been clearly defined. Consequently, the identification of specific ubiquitin E3 ligase substrates has been difficult.

Department of Medicine, Division of Immunology and Rheumatology, Stanford University, Stanford, CA 94305

Received for publication April 25, 2006. Accepted for publication September 19, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by National Institutes of Health Grant CA65237.

2 Address correspondence and reprint requests to Dr. C. Garrison Fathman, Stanford University School of Medicine, Center for Clinical Science and Research Building, Room 2240, 269 West Campus Drive, Stanford, CA 94305. E-mail address: cfathman@stanford.edu

Copyright © 2006 by The American Association of Immunologists, Inc. 0022-1767/06/$02.00
RhoGDIβ, and RhoA were cloned by PCR from a murine cDNA library. Cloning of GRAIL cDNA has been described previously (5). Dominant-negative RhoA T19N and constitutively active RhoA G14V were created by PCR-based site-directed mutagenesis (QuickChange; Stratagene).

E3 ligase substrate screen

Plasmids containing the ubiquitination components (5 ng each) along with 10–20 ng of pET21 cDNA library were cotransformed into BL21(DE3) bacterial expression competent cells by standard heat shock method and plated onto agar plates containing antibiotics appropriate for all vectors. Individual colonies were cultured in medium with appropriate antibiotics to log phase growth, then isopropyl β-D-thiogalactoside (IPTG) (1 mM final) was added for 3 h to induce protein expression. Bacterial cell lysates were prepared by sonication, and polyubiquitin laddering of potential substrate targets was revealed by standard SDS-PAGE and immunoblotting protocols. The clones displaying laddering were cultured in carbenicillin medium to selectively isolate the plasmids encoding potential substrate targets. These plasmids were sequenced (Stanford core facility) and compared with the National Center for Biotechnology Information database to identify the potential substrate targets.

Cell culture and stable cell lines

HEK293 cells and tetracycline (tet)-inducible Jurkat T cells (Invitrogen Life Technologies) were maintained in DMEM or RPMI 1640 supplemented with 10% FBS, respectively. Jurkat cell lines were electroporated (Multiporator; Brinkman Instruments) (240 V, 40 μsec), selected based on antibiotic resistance of transformed vector (blasticidin or Zeocin; Invitrogen Life Technologies), and established by limiting dilution. Retroviral transduction of T cells lines were conducted as described previously (5). In brief, retroviral constructs were transfected into 293 Phoenix-packaging cells using standard calcium phosphate transfection to generate virus containing either mock vector alone (P3) or with vector containing the constitutively active RhoA G14V substitution (CA RhoA G14V). Cells were transduced by standard protocol and sorted twice for bicistronic internal ribosome entry site-GFP expression.

Ubiquitination assay

HEK293 cells were transfected with GRAIL cDNA, RhoGDIα, or RhoGDIβ expression vector, and 3xFlag-Ubiquitin vector with Lipofectamine2000 (Invitrogen Life Technologies). Eighteen hours later, cells were incubated with 40 μM MG-132 for 6 h. Cells were lysed in a 1% Triton X-100/150 mM NaCl lysis buffer containing protease inhibitors and subjected to immunoprecipitation with indicated Ab. Immunocomplexes were analyzed by SDS-PAGE and blotted with anti-Flag Ab.

Activation of Rho, Rac, cdc42, and Ras GTPase

Rho activation was assayed with the EZ-Detect Rho Activation Kit (Pierce). The activation of Rac or cdc42 was assayed with the EZ-Detect Rac or cdc42 Activation kit (Pierce), respectively. Ras activity was assayed with the EZ-Detection Ras Activation Kit (Pierce). In brief, 1–2 × 10^6 cells were used for each treatment. Cells were cultured in the presence of tet (1 μg/ml) for 24 h to induce GRAIL expression. The next day, cells were cultured in low-serum medium (0.2% FCS) containing tet for 4–6 h before PMA (50 ng/ml)/ionomycin (1 μM) treatment. Cells were pelleted, washed with ice-cold TBS, and lysed in a 1% Nonidet P-40 lysis buffer containing protease inhibitors and subjected to immunoprecipitation with indicated Ab. Immunocomplexes were analyzed by SDS-PAGE and blotted with anti-Flag Ab.

RT-PCR

RT-PCR analysis of IL-2 production in Jurkat lines was performed using a MX4000 thermocycler (Stratagene). Primer sequences for human IL-2 were: forward, 5′-TGGAGGCTTACTGCTGGATT and reverse, 5′-TCAGTGTGAGGATGATGTTTG. Primer sequences for β-actin were as follows: forward, 5′-CAGCAATGCTGACAGGATCA and reverse, 5′-GGCCAGAGTGACCCACCCGATC.

Results

A prokaryotic-based E3 ligase screen

We have previously shown GRAIL to possess the ability to undergo ATP-dependent auto ubiquitination in vitro in the presence of recombinant E1 (UBE1) and E2 (UBE2D1) enzymes (5). However, determination of substrate-dependent E3 ligase activity remains a challenge due to a lack of a systematic method of identifying E3 ligase substrates. Attempts to identify potential substrates by mass spectrometric analysis of immunoprecipitated GRAIL complexes purified from proteasome-inhibited cells have proven unsuccessful. Furthermore, due to the promiscuous nature of the ubiquitin conjugation process in eukaryotic cells, nonspecific background ubiquitination has hampered the development of screening strategies in mammalian cells. We attempted to overcome this challenge by designing a substrate-dependent E3 ligase activity screen in a prokaryotic system that lacks endogenous ubiquitin machinery. The use of bacterial expression vectors with different but compatible replications, together with different antibiotic resistance markers, enabled the introduction of the components necessary for the ubiquitination process into Escherichia coli bacteria (Fig 1A).

The pET21 vector carries the ColE1 replicon, whereas the pCVDuet-1 and pACYC-Duet-1 vector contains the compatible CloDF13 and PI5A replications, respectively. Additionally, these Duet vectors were designed to contain two expression units, controlled by separate T7lac promoters, allowing expression of multiple target proteins. The ubiquitin-activating enzyme UBE1 and the ubiquitin-conjugating enzyme UBE2D1 were cloned into the multiple cloning sites (MCS) of pCVDuet-1 vector. UBE2D1, also known as UBC5, was chosen to catalyze the second step of the conjugation process because it was found to associate with GRAIL in pull-down assays and mediated in vitro autoubiquitination of GRAIL. cDNA encoding the RING domain, containing C-terminal GRAIL lacking the single pass transmembrane domain, was cloned into the first MCS of the pCVDuet-1 vector (His-ATM GRAIL). A Flag-tagged monomeric unit of ubiquitin was inserted into the second MCS of pCVDuet-1. A cDNA library constructed from primary murine tissues and cloned into the pET21 vector was used as the source for putative GRAIL E3 ligase substrates. The ubiquitination components and cDNA library were cloned in-frame with epitope tags to facilitate detection (shown in Fig. 1B). We hypothesized that coexpression of the cDNA library with the eukaryotic ubiquitination components would result in identification of proteins that could be specifically ubiquitylated by GRAIL. As expected, approximately one-third of the selected clones yielded translatable protein due to the directional cDNA library design (Fig. 1C, lanes 1–10). We reasoned that the observed “laddering” pattern of select cDNA clones was due to progressive polyubiquitination of the target protein (Fig. 1C, lanes 4, 5, and 8). Interestingly, not all expressed proteins resulted in this laddering pattern, indicative of a semiselective ubiquitination process in our prokaryotic system (Fig. 1C, lanes 2, 6, 7, and 10).

RhoGDI is a candidate GRAIL E3 substrate

Sequence analysis of ubiquitin-tagged cDNA clones revealed the identity of multiple GRAIL E3 ligase substrate candidates. Because previous studies have shown that ectopic expression of GRAIL resulted in structural morphological changes in the GRAIL-expressing transductants (5), cDNA clones encoding proteins involved in pathways regulating the actin cytoskeleton were of particular interest. Several of the ubiquitin-conjugated clones obtained from the E3 ligase screen were identified as members of the RhoGDI family (Fig. 1C, lane 4). To date, three members of the mammalian RhoGDI family have been identified. RhoGDIα is widely expressed in tissues, whereas RhoGDIβ (D4/LyGDI) is expressed predominantly in cells of hemopoietic origin, particularly T and B lymphocytes, and RhoGDIγ is preferentially expressed in brain, pancreas, lung, kidney, and testis (7). We designed primers corresponding to the 5′- and 3′-end sequences to clone full-length cDNA of RhoGDIα and RhoGDIβ. The cDNA clones were
inserted into a mammalian expression vector (pcDNA4) with a N-terminal polyhistidine/Xpress (HisXp) epitope tag and transiently expressed in HEK293 cells (Fig. 2A).

Having established that GRAIL and RhoGDI can interact in a bacterial expression system, we asked whether GRAIL could associate with RhoGDI in mammalian cells. Full-length GRAIL, containing the transmembrane domain, was used in all subsequent mammalian experiments to maintain proper GRAIL topology and subcellular localization. Expression of C-terminal 3xFlag-tagged GRAIL with HisXp- RhoGDI and RhoGDI in 293 cells, followed by anti-Flag immunoprecipitation and anti-His immunoblot analysis revealed protein:protein interactions between GRAIL and RhoGDI, and RhoGDIβ (Fig. 2B).

**GRAIL, but not enzymatically inactive H2N2 GRAIL, can polyubiquitinate RhoGDI**

To validate the GRAIL-mediated polyubiquitination activity observed in the prokaryotic system, we asked whether GRAIL could also ubiquitinate RhoGDI in mammalian cells. V5-tagged full-length GRAIL or enzymatically inactive GRAIL mutants containing 2-aa substitutions in the RING domain (H2N2 GRAIL) and HisXp-tagged RhoGDIα along with N-terminal triple Flag-tagged ubiquitin (3xFlag-Ub) were coexpressed in 293 cells. Eighteen hours posttransfection, the cells were treated with the proteosome inhibitor MG132 for 6 h, followed by immunoprecipitation with an anti-Xpress (Xp) Ab. SDS-PAGE and immunoblot analysis of 3xFlag-Ub-conjugated HisXp-RhoGDIα revealed a distinct polyubiquitinated laddering pattern of RhoGDIα in the presence of full-length GRAIL (Fig. 2C, lane 3). The histidine to asparagine substitutions in the RING domain (H2N2 GRAIL) abrogate the ability of GRAIL to form a cross-brace motif to bind zinc and has previously been shown to reduce the E3 ligase activity of GRAIL (5). Ubiquitin conjugation of RhoGDIα was markedly diminished in the presence of H2N2 GRAIL (Fig. 2C, lane 4).

To determine whether GRAIL E3 ligase activity could be detected on endogenous RhoGDI, V5-tagged full-length GRAIL or the H2N2 GRAIL mutant were expressed in 293 cells along with 3xFlag-Ub. Immunoprecipitation of endogenous RhoGDIα, followed by immunoblot analysis reveal that GRAIL ubiquitinated endogenous RhoGDI, and that this E3 ligase activity was dependent on the functional RING domain of GRAIL (Fig. 2D). These data validate the findings from the prokaryotic substrate-dependent E3 ligase activity screen and establish RhoGDI as a substrate for GRAIL E3 ligase activity.

**GRAIL uses nonlysine 48-ubiquitin linkage in polyubiquitinating RhoGDI**

Once a RING E3 ligase has bound and transferred ubiquitin to the target protein, the E3 ligase can continue to progressively form higher m.w. polyubiquitin conjugates via ubiquitin’s lysine residues. Formation of polyubiquitin chains through lysine 48 (K48) can result in degradation of the target protein by the 26S proteosome. However, polyubiquitin chains linked through other ubiquitin linkages are resistant to proteolysis, including endocytosis, vesicular sorting, membrane-directed protein trafficking, and DNA repair (8, 9). Because previous studies described a perinuclear, endosomal subcellular localization of GRAIL (5), we hypothesized that GRAIL E3 ligase activity might result in nonproteolytic functions for some substrate proteins and thus asked which lysine residue of ubiquitin was preferentially
FIGURE 2. GRAIL ubiquitinates RhoGDI. A, RhoGDIα (GDIα) and RhoGDIβ (GDIβ) were cloned from a first-strand murine cDNA library and inserted into a mammalian expression vector with N-terminal histidine/Xpress (His/Xp) epitope tag. HEK293 cells were transiently transfected with the indicated vectors (0.5 μg each), and lysates were subjected to SDS-PAGE and immunoblotted with RhoGDI-specific Ab. B, Interaction between RhoGDI and GRAIL. HEK293 cells were transiently transfected with a plasmid encoding C-terminal 3xFLAG GRAIL, along with either empty pcDNA vector, or vectors encoding His/Xp-tagged RhoGDIα or RhoGDIβ (1.0 μg each). Interaction was analyzed by anti-Flag immunoprecipitation, followed by immunoblotting with anti-His Ab (top). The membrane was stripped and reprobed with GRAIL antiserum to demonstrate equal GRAIL pull-down (bottom). C, GRAIL ubiquitinates RhoGDI. HEK293 cells were transiently transfected with the expression vector for His/Xp-tagged RhoGDIα (1.0 μg) (lane 1), plus 3xFLAG-tagged ubiquitin (0.2 μg) (lane 2) together with either V5-tagged full-length GRAIL (lane 3) or RING finger mutant H2N2 (0.8 μg each) (lane 4), and were treated with 40 μM MG132 for 6 h before lysis. Ubiquitination of transiently expressed RhoGDI was detected by immunoprecipitation with anti-Xpress Ab, followed by anti-Flag immunoblotting (top). The membrane was stripped and reprobed with anti-His Ab to show amount of immunoprecipitated RhoGDI (bottom). D, GRAIL ubiquitinates endogenous RhoGDIα. 3xFLAG-tagged ubiquitin (0.2 μg) plus GRAIL (lane 2) or H2N2 (0.8 μg each) (lane 3) were transfected into HEK293 cells, as described above, followed by immunoprecipitation with Ab specific for RhoGDIα (top). The membrane was stripped and reprobed with anti-RhoGDI Ab to show the amount of immunoprecipitated RhoGDIα (bottom). E, GRAIL promotes ubiquitin conjugation on RhoGDI primarily via lysine 63. HEK293 cells were transfected with His/Xp-RhoGDIα (1.0 μg) plus either a vector encoding
used in polyubiquitin chain formation on RhoGDI. Interestingly, an intense ladder pattern of polyubiquitinated RhoGDI mediated by GRAIL was observed in the presence of 3xFlag-Ub lacking lysine 48 (K48R), implicating non-K48 ubiquitin linkage formation. Conversely, weak ubiquitin conjugation to RhoGDI was detected when expressed together with 3xFlag-Ub containing a lysine to arginine substitution at residue 63 (K63R) (Fig. 2E). These data suggest that ubiquitin-ubiquitin polymers conjugated on RhoGDI are not formed via a K48 linkage, but rather predominantly via lysine 63 (K63) of ubiquitin. Because K63-linked ubiquitin chains have been reported to act as nonproteolytic signals in several intracellular pathways (10), we assessed the potential for GRAIL to affect steady-state RhoGDI expression levels. Eighteen hours after transfection of a fixed amount of RhoGDIα vector along with increasing amounts of cDNA encoding full-length GRAIL, H2N2 GRAIL, or RING deletion (dZF) GRAIL, the expression level of HisXp-RhoGDIα was analyzed. In accord with the nonlysine 48 polyubiquitination of RhoGDI by GRAIL, increased expression of GRAIL did not promote the degradation of RhoGDI. In fact, levels of RhoGDI increased directly with GRAIL expression levels, and this increase was dependent on an intact RING domain (Fig. 2F). These data demonstrate that ubiquitination of RhoGDI by GRAIL results in a nonproteolytic outcome.

GRAIL inhibits Rho GTPase activation

RhoGDIs have been described to regulate the activity of Rho small G-protein family members. The RhoGTPase family was initially described as a family of proteins that regulate changes in reorganization of the cytoskeletal framework in many cell types, including stress fiber, membrane ruffles, and filipodia formation (11). More recently, this family has been ascribed a broad role in cellular function, including regulation of cell morphology, vesicular trafficking, gene transcription, and cell cycle (12). Because of their crucial role in cell biology, the GTPase activity of this family of small G proteins is under the tight control of a large set of regulatory proteins (13). The activation of RhoGTPases through exchange of GDP for GTP is catalyzed by guanine nucleotide exchange factors. GTPase-activating proteins accelerate their intrinsic GTPase activity to inactivate the protein and terminate downstream signaling. In addition to regulation by this GTPase cycle, Rho activity is also controlled by cytosolic and membrane localization. This cytosol-membrane shuttling of Rho GTPase is regulated by the RhoGDI proteins, where RhoGDI has been described to bind to the C-terminal prenylated form of Rho. This binding extracts Rho from the membrane, blocks its accessibility to guanine nucleotide exchange factors and GTPase-activating proteins, thereby inhibiting nucleotide exchange and GTP-hydrolyzing activities (7).

Because GRAIL can interact and ubiquitinate RhoGDI, we asked whether GRAIL expression could affect Rho activation and downstream signaling events in T cells. Because GRAIL has been described as an anergy factor that exhibits potent antiproliferative effects, we decided to construct a tet-inducible Jurkat T cell system. Jurkat T cells with strong tet repressor expression were electroporated with either control vector or with a vector encoding GRAIL cDNA regulated with tandem tet binding/operator sites. Several stable lines were established by limiting dilution, and, as shown by data presented in Fig. 3A, addition of tet resulted in robust GRAIL expression. The observed basal GRAIL expression in the absence of tet is most likely due to tet contamination from bovine serum used in the growth culture medium. To detect Rho GTPase activity in Jurkat T cells, a GST-Rhoactive kinase RBD probe was used to specifically bind and precipitate GTP-loaded Rho. Although rapid and robust Rho activation was observed following PMA/ionomycin stimulation of vector control cells, no Rho activation was detected in the Jurkat cells expressing GRAIL (Fig. 3B, top). Probing whole cell lysates for RhoA demonstrates that the lack of Rho activation was not due to GRAIL-mediated degradative effects (Fig. 3B, bottom). The presence of GRAIL also resulted in an inhibition of RhoA activation when cells were stimulated via the TCR with cross-linking anti-CD3 and anti-CD28 Abs (data not shown).

Next, we investigated whether the activation of Rac1 and cdc42, the other defining members of the Rho GTPase family, were affected in GRAIL-expressing cells. Following PMA/ionomycin stimulation, active GTP-bound Rac1 and cdc42 were isolated by precipitation with a GST-PAK1 binding domain fusion protein and detected by blotting for Rac1 or cdc42, respectively. The inhibition in GTPase activity appears to be specific to Rho because GTP-bound Rac1 and GTP-bound cdc42 were detected even in the presence of GRAIL (Fig. 3C).

We next asked whether GRAIL could affect other signaling pathways that have been implicated in mechanism(s) of T cell clonal anergy. Previously, the selective inhibition of IL-2 production in anergic CD4+ T cells has been attributed to the inability to activate the Ras signaling pathway upon TCR stimulation (14). However, in our studies, PMA/ionomycin time-course treatment of control and GRAIL-expressing cells revealed similar kinetics of Ras activation (Fig. 3D, top). A GST fusion protein containing the Ras binding domain of Raf1 (GST-Raf1 RBD) was used to pull-down active GTP-bound Ras. We also observed similar rapid and robust serine phosphorylation of c-Raf, a downstream effector molecule in the Ras pathway, in stimulated whole cell lysates of control and GRAIL-expressing cells (Fig. 3D, bottom). T cell clones rendered anergic by T cell stimulation in the absence of costimulatory signals have also been reported to display defects in the three major mammalian MAPK signaling pathways: p44/42 MAPK, p38 MAPK, and JNK pathways (15–17). Furthermore, the JNK pathway has been reported to be regulated by the Ras signal transduction cascade (18). However, no difference in inducible threonine/tyrosine phosphorylation in the activation loop of JNK was observed in lysates of stimulated control and GRAIL-expressing Jurkat cells (Fig. 3E, top). Similarly, the presence of GRAIL did not affect the activation of MAPKs p44/42 MAPK and p38 MAPK (Fig. 3E, middle, bottom).

Rho is involved in IL-2 expression, and this activity is abrogated by GRAIL expression

GRAIL expression and RhoA activation have independently been linked to regulation of IL-2 expression in T cells. In this
study, we show that tet-induced GRAIL-expressing Jurkat T cells displayed diminished IL-2 gene transcription in response to PMA/ionomycin treatment compared with control vector-integrated cells (Fig. 4A). Repression expression and either integration of empty vector (Vector) or a tet-inducible vector encoding full-length GRAIL. Two representative inducible GRAIL-expressing Jurkat T cell clones are shown (GRAIL#1 and GRAIL#2). Cells were treated with 1 μg/ml tet for 24 h, followed by lystate preparation and immunoblot analysis with GRAIL antiserum. GRAIL inhibits Rho GTPase activation. Jurkat T cells were treated with 1 μg/ml tet for 24 h to induce GRAIL expression. The following day, the cells were cultured in medium containing low serum (0.2% FCS) for 4–6 h to decrease GTP-Rho background levels. Cells were stimulated with a combination of PMA (50 ng/ml) and ionomycin (1 μM) for times indicated, and activated GTP-loaded RhoA was isolated by GST-Rhoteckin RBD pull-down and detected by immunoblotting with RhoA-specific Ab (top). Whole cell extracts show abundant RhoA in Jurkat samples (bottom). GRAIL does not affect cdc42 or Rac1 GTPase activation. Jurkat cells were treated as in B and subjected to GST-PAK protein binding domain pull-down, followed by either immunoblotting for cdc42 (top) or Rac1 (bottom). Parental wild-type Jurkat lystate was either loaded with 0.1 mM GTPγS (+) or 1 mM GDP (−) to serve as positive and negative controls, respectively. D, GRAIL expression does not affect Ras activation. Cells were treated and prepared as in B and subjected to GST-Raf1 RBD pull-down, followed by Ras immunoblotting (top). tet-treated Jurkat T cells were stimulated with PMA (50 ng/ml) and ionomycin (1 μM) for times indicated, and the extent of c-Raf phosphorylation was evaluated by immunoblot analysis with phosphorylation-specific c-Raf (S338) Ab. E, GRAIL expression does not affect MAPK signaling pathways. Lysates of PMA/ionomycin-stimulated non-GRAIL and GRAIL-expressing cells were subjected to SDS-PAGE and subsequently immunoblotted with phosphorylation-specific JNK (T183/Y185), p44/42 MAPK (T185/Y187), and p38 MAPK (T180/Y182) Ab.
Data are presented as IL-2 mRNA levels, normalized to amounts of mRNA encoding internal actin, and samples were subjected to real-time quantitative-PCR analysis. GRAIL-expressing Jurkat T cells. cDNA was prepared by standard methods, and samples were subjected to real-time quantitative-PCR analysis. Data are presented as IL-2 mRNA levels, normalized to amounts of mRNA encoding internal β-actin. B, RhoA GTPase affects IL-2 expression in T cells. Stable Jurkat T cell lines expressing constitutively active (G14V) RhoA or dominant-negative (T19N) RhoA were stimulated with PMA (50 ng/ml) and ionomycin (1 μM) for 4 h. Levels of IL-2 expression were evaluated as in A. C, Constitutively active (G14V) RhoA can overcome the effects of GRAIL tet-inducible GRAIL-expressing cells (GRAIL#1) were retrovirally transduced with either mock vector (P3) or with a vector encoding constitutively active (G14V/P3) RhoA. Cells were sorted for bicistronic GFP expression and stable lines were selected. Cells were treated as in A and subjected to IL-2 real-time quantitative-PCR.

with the specific Rho kinase pharmacological inhibitor Y-27632, resulted in inhibition of IL-2 production in T cells (21, 22).

Next, we wished to determine whether the Rho regulation of IL-2 expression functions downstream of GRAIL activity, or whether regulation of IL-2 by GRAIL and Rho kinase is independently regulated. To achieve this, we retrovirally transduced inducible GRAIL-expressing Jurkat cells with either mock virus (P3) or constitutively active RhoA G14V-packaged virus (CA V14 RhoA/P3). Cells were sorted for bicistronic GFP expression and stable lines were established. After PMA/ionomycin treatment, GRAIL cells with RhoA G14V expressed greater levels of IL-2 than GRAIL transduced with mock virus (Fig. 4C). Similar to the cells stimulated in a TCR-independent fashion, TCR engagement of GRAIL cells with RhoA G14V expressed greater levels of IL-2 than mock virus-transduced GRAIL cells (data not shown). Because dominant active RhoA overcomes the GRAIL effect, these results demonstrate that the effect of RhoA on IL-2 transcription is downstream of GRAIL, and clearly establishes a critical contribution of the Rho signaling pathway in promoting IL-2 production in T cells.

**Discussion**

In this study, we report an efficient and systematic method to identify potential ubiquitin E3 ligase substrates by use of a bacterial system that allows expression of multiple interacting proteins. Because prokaryotes do not share mechanisms of ubiquitin posttranslational modification with eukaryotic cells, background nonspecific ubiquitin reactions are minimized. This system has limitations including the inability of this assay to isolate substrates that require posttranslational modifications of proteins that allows them to become targets for ubiquitination, such as protein phosphorylation. Nevertheless, because specific target ubiquitination domains on substrate proteins have not been clearly defined, this system allows quick identification of proteins that interact with the E3 ligase while minimizing background interference. Furthermore, this strategy can be used to screen for potential E3 ligases of target substrates. For instance, if a protein is known to be ubiquitin modified when the cells of interest are in a particular stage of development, this screen should be able to identify specific E3 ligase activity from a library constructed from these cells.

Using this screen, we found that RhoGDIα and RhoGDIβ are ubiquitin E3 substrates of GRAIL. This was verified in mammalian cells, and shown to be dependent on a functional RING domain, demonstrating the need for functional E3 ligase activity. We further demonstrated that polyubiquitination of RhoGDI by GRAIL proceeds preferentially via a nonlysine 48, predominantly K63 ubiquitin linkage pattern. How the E3 ligase dictates the linkage pattern on its specific target substrate is unclear, although there is increasing evidence that polyubiquitin chain formation can create nonproteolytic outcomes. For example, K63-linked polyubiquitination plays an important role in TNFR-associated factor 6-mediated activation of IκB kinase in the NF-κB pathway (10). In another example, the E3 ligase cbl-b exhibits nonproteolytic ubiquitination activity on the p85 subunit of PI3K, resulting in changes in the TCR signaling threshold (23). Our data suggest that ubiquitination of RhoGDI by GRAIL does not result in proteolytic degradation. In fact, GRAIL activity appeared to increase RhoGDI stability.

Identifying RhoGDI as a substrate for GRAIL E3 ligase activity provides a link between GRAIL function, diminished IL-2 production, and T cell anergy induction. The increased RhoGDI level in the presence of GRAIL can result in sequestration of Rho molecules in the cytosol, preventing Rho activation and initiation of the Rho signaling pathway. The Rho GTPase family members have been shown to play a pivotal role in regulating changes in actin cytoskeleton, which in turn affects many aspects of cellular function (12, 24, 25).

Previous studies have shown that specific inactivation of RhoA kinase activity in T cells resulted in defective TCR/CD3 complex polymerization (21, 22). Similarly, anergic T cells stimulated with anti-CD3-coated beads displayed impaired actin cup formation at the T cell/bead interface, due to defects in actin cytoskeleton reorganization (26). These studies suggest a model in which induction of T cell anergy results from incomplete cytoskeletal polarization or actin polymerization due to inactivation of the Rho signaling pathway.
Rho may also mediate T cell activation and proliferation through control of transcriptional activation. Activated Rho can regulate c-fos transcription by increased binding of serum response factor to the serum response element in the c-fos promoter (27). Fos can interact with c-Jun to form the AP-1 transcriptional activation complex. Furthermore, constitutively active RhoA (G14V) has been shown to potentiate AP-1 activity in Jurkat T cells (28). Interestingly, this enhanced AP-1 transcriptional activity is independent of the MEK-MAPK pathway, consistent with our findings that GRAIL did not affect MAPK pathways. Because the IL-2 promoter region proximal to the transcriptional start site includes binding sites for several transcriptional complexes, including AP-1, these results provide a mechanism of how the observed inactivation of RhoA in T cells resulted in diminished IL-2 production, whereas constitutive active RhoA was able to overcome the IL-2 inhibitory effect of GRAIL. Taken together, these data provide a molecular mechanism of GRAIL function and suggest a role for the Rho signaling pathway in establishing an anergy phenotype in T cells.

Acknowledgments
We thank R. Kizer and L. O’Brien for administrative support.

Disclosures
The authors have no financial conflict of interest.

References