ROG, Repressor of GATA, Regulates the Expression of Cytokine Genes

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Summary

GATA-3 is a T cell–specific transcription factor and is essential for the development of the T cell lineage. Recently, it was shown that the expression of GATA-3 is further induced in CD4^{+} helper T cells upon differentiation into type 2 but not type 1 effector cells. Here, we report the molecular cloning of a GATA-3 interacting protein, repressor of GATA (ROG). ROG is a lymphoid-specific gene and is rapidly induced in Th cells upon stimulation with anti-CD3. In in vitro assays, ROG represses the GATA-3-induced transactivation. Furthermore, overexpression of ROG in Th clones inhibits the production of Th cytokines. Taken together, our results suggest that ROG might play a critical role in regulating the differentiation and activation of Th cells.

Introduction

Upon encountering antigen, naive CD4^{+} precursor helper T (Thp) cells differentiate into mature effector Th cells that are capable of secreting high levels of cytokines. Th cells have been divided into two functional subsets based on the specific cytokines they secrete (Mosmann et al., 1986; Mosmann and Coffman, 1989; Seder and Paul, 1994). Type 1 helper (Th1) cells secrete IFN-γ, IL-2, and TNF-α. The Th1 immune response is responsible for eradication of intracellular organisms but is also responsible for mounting organ-specific autoreactive immune responses that occur in diseases such as Type I diabetes or multiple sclerosis (Correale et al., 1995; Katz et al., 1995; Wilson et al., 1998). Type 2 helper (Th2) cells produce IL-4, IL-5, IL-10, and IL-13. The Th2 immune response is critical for the production of noncomplement fixing antibody and eosinophil maturation. Overproduction of Th2 cells is characteristic of allergic diseases such as asthma and atopic skin disease (Kapsenberg et al., 1991, 1996; Ying et al., 1995).

The molecular mechanisms mediating the differentiation of Th cells and the cell lineage-specific expression of cytokine genes have begun to be elucidated in the last few years. Both signals generated from the antigen/ T cell receptor (TCR) and from cytokine receptors are critical in determining the fate of a Thp cell (Constant and Bottomly, 1997). These signaling events lead to the induction or activation of several cell lineage-specific

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or nonlineage-specific transcription factors (Szabo et al., 1997). For example, the differentiation of Th2 cells requires signaling via the IL-4 receptor (IL-4R) in addition to signaling via the TCR. Stimulation via the TCR upregulates the expression of a Th2 cell-specific transcription factor, c-maf, and induces nuclear translocation of nuclear factor of activated T cells (NF-AT) (Ho et al., 1996; Rao et al., 1997). On the other hand, activation via IL-4/ IL-4R induces the nuclear translocation of Stat6, which subsequently upregulates the expression of GATA-3, another Th2 cell-specific transcription factor (Zhang et al., 1997; Zheng and Flavell, 1997). Synergy between these factors results in high levels of IL-4 expression that drive a Thp cell into the Th2 pathway. In vivo or in vitro manipulation of these molecular events results in the alteration of Th cell differentiation and cytokine production. For example, mice that lack Stat6 do not mount Th2 responses in vitro or in vivo (Kaplan et al., 1996; Shimoda et al., 1996), and Th2 cells derived from c-maf-deficient mice, while capable of secreting IL-5 and IL-10, produce only very low levels of IL-4 (Kim et al., 1999). Furthermore, overexpression of GATA-3 promotes the differentiation of Th2 cells by an IL-4-independent mechanism (Ouyang et al., 1998).

GATA-3 is a zinc finger protein that belongs to the GATA family of transcription factors (Weiss and Orkin, 1995) and was initially cloned as a T cell–specific transcription factor that can bind to the TCR α and δ gene enhancers (Ho et al., 1991; Ko et al., 1991). Subsequently, GATA-3 was found to be critical in regulating the expression of several T cell–specific genes in addition to TCR and Th2 cytokine genes (Landry et al., 1993; Leiden, 1993; Merika and Orkin, 1993). Furthermore, GATA-3 is essential for normal embryonic development as well as for the generation of the T cell lineage (Pandolfi et al., 1995; Ting et al., 1996). Mice bearing a targeted disruption of the GATA-3 gene die in utero due to profound defects in hematopoiesis and in the development of the nervous system. Mice lacking GATA-3 only in the lymphoid lineage, as generated by RAG2 blastocyst complementation, have a complete absence of T cell development. Taken together, these observations clearly demonstrate that GATA-3 is a multifunctional factor that is essential for the development, differentiation, and activation of Th cells.

In addition to positive regulation by transcription factors such as GATA-3 and c-maf, the differentiation of Th2 cells can be negatively regulated by other nuclear proteins, such as Bcl-6. Bcl-6 belongs to the expanding family of POZ proteins (Bardwell and Treisman, 1994; Albagli et al., 1995). A majority of the POZ members contain a BTB (broad complex, tramtrack, and bric-a-brac) domain in the N terminus and a zinc finger domain in the C terminus. The BTB domain is a newly characterized protein–protein interaction domain found in a variety of proteins involved in a spectrum of biological functions ranging from repression of pair-rule segmentation in *Drosophila* to germinal center formation in mice (Albagli et al., 1995). While the functional role of the BTB domain in vivo remains unclear, most POZ proteins func-

A

Bait	Prey	Glu −UHW	Gal/Raf −UHWL	Glu −UHWL
GATA-3	vector			
GATA-3	ROG			
Max	ROG			
c-maf	ROG			

B

Transfection: GATA-3 GFP / GATA-3 GFP-ROG

IP: GATA-3 Control GATA-3

—130 KD
—90 KD

C

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  1  MPQTPTRLVS PYGSDRLVQL AARLRPALCD TLITVGGLEF PAHSLVLAGA
 51  SPRLGCRGRW ALVEDISPST FAQILTFVYG ESIELQPGEL GDLEEAAKAL
101  GVQALEEACQ RAQKGKDEDE LDPGLKRHQQ SEDFMRGSER GLGSPGEKQK
151  PEKDFRSNGR EQEMSHKHKA PGERPEMAGA TRMMSSEEVM RGIESHKGSE
201  ESLRGCPDPL SPPGSLLTSL IPRPWWAEVP RLGEGQALCW SILLWPSRYG
251  APFSHSTPIT AAWQVRPQDQ RIPLTLNHSK ALWSQNQLAS SSPTPGSFPQ
301  GTESLSPWQI ETSGQGFTGT LATCVSQERT LNCPSHQHPP LPSPARSRPY
351  SCSVCGKRFS LKHQMETHYR VHTGEKPFSC SLCPQRSRDF SAMTKHLRTH
401  GAAPYRCPLC RAGCPSLASM QAHMRGHSPS RLPPGWTIRS TFLYSSSRPT
451  RASSSPGSPT SSAAT
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D

ROG — BTB — Zn Finger [Zn][Zn][Zn]

36%   71%

PLZF — [Zn][Zn][Zn][Zn][Zn][Zn][Zn][Zn][Zn]

E

input DNA: — ROG

86 KD—
54 KD— ← ROG

Figure 1. Molecular Cloning and Protein Structure Analysis of ROG

(A) ROG interacts specifically with GATA-3. The indicated prey vectors and bait vectors were used to transform an EGY48 yeast strain. The transformed yeast was then plated in the dropout selecting plates, Gal/Raf-UHWL and Glu-UHWL, or nonselecting plates, Glu-UHW. Only the yeast strain containing the GATA-3 bait and the ROG prey was able to grow in the selecting plates in a galactose-dependent fashion. "Glu," "Gal," and "Raf" stand for glucose, galactose, and raffinose, respectively. U, H, W, and L are single letter amino acid code.
(B) Coimmunoprecipitation of ROG and GATA-3. Jurkat cells were transfected with the indicated expression vectors. Cell extracts were immunoprecipitated with anti-GATA-3 antibody or control IgG. The immunoprecipitates were then probed with anti-GFP antibody in Western blot analysis.
(C) The amino acid sequence of full-length ROG. The BTB domain is underlined and the three zinc fingers are boxed.
(D) Schematic diagrams of ROG and PLZF. The percentages represent the homology between the indicated domains. "Zn" stands for zinc finger. The diagrams are not to scale.
(E) In vitro transcription/translation of ROG. In vitro transcription/translation was programmed with either a full-length ROG cDNA or empty plasmid in the presence of ^{35}S-methionine. The products were fractionated on a SDS-PAGE gel and subjected to autoradiography.

tion as transcriptional repressors in vitro. For example, Bcl-6 can compete with Stat6 for its DNA-binding site and thus negatively regulate the expression of Stat6-inducible genes (Chang et al., 1996; Seyfert et al., 1996). Therefore, it was proposed that Bcl-6 attenuated the differentiation of Th2 cells, a process dependent on Stat6 signaling. Indeed, mice rendered deficient in Bcl-6 display unopposed Th2 inflammatory responses, strongly supporting a role for Bcl-6 as a negative regulator of the Th2 pathway (Dent et al., 1997, 1998; Ye et al., 1997).

Here we report the cloning of a GATA-3 interacting protein that we have named ROG, repressor of GATA. ROG is a new member of the POZ family and its expression is induced rapidly in T cells by stimulation via the TCR. In vitro, ROG represses the function of GATA-3 in part by preventing the binding of GATA-3 to its cognate DNA target sequence. Overexpression of ROG in Th cells specifically inhibits the production of cytokines, including both Th1 and Th2 cytokines, but does not inhibit the upregulation of T cell activation markers such as FasL or CD69. Taken together, these data suggest that ROG may be an important component of a negative feedback mechanism that regulates the effector function of Th cells.

Results

Molecular Cloning of ROG
In order to identify proteins that interact with and modulate the function of GATA-3, we used a truncated GATA-3 protein (amino acid residues ∼96–444) encompassing the zinc finger domain as a bait to screen a Th2 cDNA yeast expression library in a yeast two-hybrid system. One clone thus isolated interacted with the GATA-3 bait but not with other nonrelated baits containing Max or c-maf (Figure 1A). Of note, this clone also interacted with the zinc finger domain of GATA-4, suggesting it was capable of interacting with other GATA family members (data not shown). Sequence analysis revealed that this clone encoded a novel open reading frame that contains three C2H2-type zinc fingers in its C terminus. Subsequently, the insert of the yeast clone was used to isolate 1.7 kb and 2 kb cDNA clones from a Th2 cDNA phage library. Both phage cDNA clones encoded proteins with identical open reading frames of 465 amino acid residues (Figure 1C). The 2 kb cDNA phage clone had an additional 5′ untranslated region of ∼300 bp. In vitro transcription and translation assays using this novel full-length cDNA clone produced a distinct protein of ∼60 kDa (Figure 1E). This novel protein was subsequently named ROG for repressor of GATA.

ROG Is a Member of the POZ Family
Sequence analysis revealed that the N terminus of ROG contained a BTB domain, missing from the yeast clone, thus identifying ROG as a new member of the BTB/zinc finger (or POZ) protein family. A search of the GenBank database, revealed that ROG was highly homologous to another POZ protein, PLZF. The amino acid sequence of ROG is 36% and 71% homologous to the BTB and zinc finger domains, respectively, of PLZF (Figure 1D). Outside the BTB and zinc finger domains, ROG did not

match to any known protein sequence. These data confirm that ROG is a novel POZ family member.

ROG Can Be Coimmunoprecipitated with GATA-3 in Mammalian T Cells

To confirm the interaction between GATA-3 and ROG, in vivo coimmunoprecipitation experiments were performed. Jurkat cells were transfected with plasmid expression constructs expressing GATA-3 and green fluorescence protein (GFP), or GATA-3 and a GFP-ROG fusion protein. Cell lysates prepared from transfected cells were subjected to immunoprecipitation with anti-GATA-3 antibody. As seen in Figure 1B, an ~100 kDa protein corresponding to the molecular weight of the GFP-ROG fusion protein was immunoprecipitated from GFP-ROG transfected cells by anti-GATA-3 antibody, but not by control antibody. Furthermore, no anti-GFP antibody reactive protein was immunoprecipitated from cells transfected only with the GATA-3 and GFP expression vectors (data not shown). This result confirms the physical interaction between ROG and GATA-3 in mammalian T cells.

Mapping the Physical Domains Mediating the Interaction between ROG and GATA-3

To determine the physical domains mediating the interaction between ROG and GATA-3, we first generated a series of truncation mutants of ROG and examined their interaction with the original GATA-3 bait in the yeast two-hybrid system. The optimal interaction required the zinc finger domain and the C-terminal region of ROG. While a substantial decrease in the GATA-3–ROG interaction was observed when the last 20 amino acid residues of ROG were deleted, it was the first two zinc fingers of ROG that appeared to be essential for the interaction (Figure 2A). Several truncation mutants of the GATA-3 bait were then generated and examined for their ability to interact with the original ROG yeast clone. As shown in Figure 2B, the zinc finger domain (including N and C fingers) of GATA-3 was sufficient to mediate the interaction with ROG. Further deletion of the C-terminal zinc finger completely abrogated the interaction, demonstrating that the C-terminal zinc finger of GATA-3 is essential for the interaction.

ROG Is Preferentially Expressed in Lymphoid Cells and Is an Early Response Gene in T Cells

To delineate the tissue distribution of ROG in the adult animal, organ blot analysis was performed. Among the organs examined, only the testis expressed a transcript, ~7 kb rather than 2 kb in size, that was recognized by a ROG-specific probe (Figure 3A). No ROG expression was detected in lymphoid organs such as thymus or spleen. Thus, the expression of ROG was examined in a variety of cell lines, either unstimulated or stimulated. As shown in Figure 3B, the 2 kb ROG transcript was exclusively detected in lymphoid cells, both B and T cells, but not in other hematopoietic cells, such as monocytes and mastocytoma cells, or in nonhematopoietic cells, such as hepatocytes, chondrocytes, and fibroblasts. In addition to the major 2 kb transcript, an ~4 kb transcript with a similar expression pattern was also detected by the ROG-specific probe, suggesting the presence of an alternative splice form or a closely related
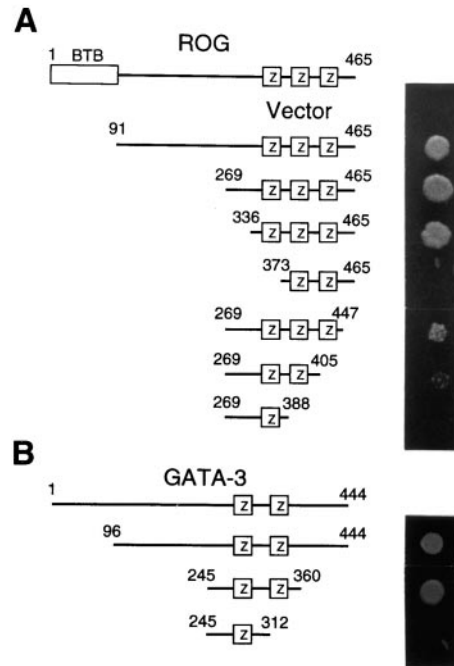

Figure 2. Mapping the ROG/GATA-3 Interaction Domains

The EGY48 yeast strain was transformed with (A) the GATA-3 bait, along with a series of truncated ROG preys or empty pJG4-5 (vector), or (B) the ROG prey and the indicated truncation mutants of the GATA-3 bait. The transformed yeast was selected in leucine dropout plates. Growth indicates an interaction between baits and preys. The indicated wild-type and mutant proteins are shown schematically. The boxed Z stands for zinc finger. The numbers indicate the amino acid residues. The diagrams are not to scale.

gene. In resting T cells, ROG is expressed at very low levels, if at all. Upon stimulation with anti-CD3, the expression of ROG is substantially induced in Th1 (AE7 and D1.1) clones, Th2 (D10 and CDC35) clones, and EL4 (a murine thymoma cell line), whereas the expression of GATA-3 is limited to Th2 clones and EL4 cells (Figure 3B). In contrast to T cells, ROG was expressed at comparable levels in resting and activated B cells. Overall, these results indicate that the gene encoded by the 2 kb transcript is lymphoid-specific and is an early response gene in T cells.

To determine how the pattern of ROG expression compares with that of GATA-3 itself in primary Th cells, splenic CD4$^+$ T cells derived from BALB/c mice were purified and differentiated in vitro under Th1 or Th2 polarizing conditions. The completeness of polarization was confirmed by cytokine ELISA (Figure 3D). At different time points, total RNA was prepared and subjected to Northern analysis using a ROG-specific or a GATA-3-specific cDNA probe. As shown in Figure 3C, very low levels, if any, of GATA-3 and ROG transcripts were detected in naive T cells, whereas high levels of GATA-3 and ROG transcripts were induced within 24 hr post primary stimulation under Th2 skewing conditions. Interestingly, levels of ROG transcripts decreased gradually to almost undetectable levels 4–5 days post stimulation and were rapidly induced within 3 hr post restimulation with anti-CD3. This is in dramatic contrast to the kinetics of GATA-3 expression. Once induced in Th2 cells, levels of GATA-3 transcripts were maintained and were not
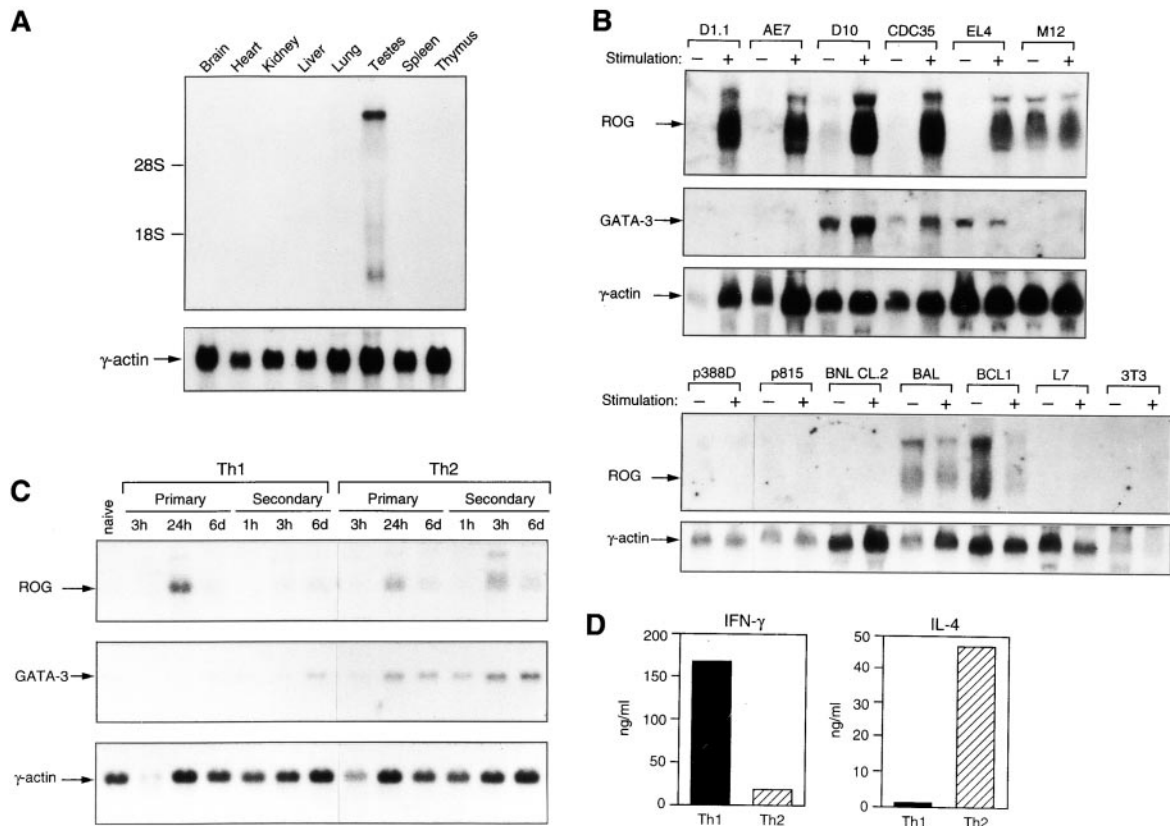
Figure 3. Northern Analysis of ROG

(A) Northern analysis of the expression of ROG in indicated organs.

(B) The indicated cell clones or cell lines were left unstimulated or stimulated with plate-bound anti-CD3 (D1.1, AE7, D10, and CDC35) or 50 ng/ml PMA/1 μM ionomycin (others) for 6 hr. Total RNA was then prepared from harvested cells.

(C) CD4⁺ Th cells derived from BALB/c mice were differentiated in vitro under Th1 skewing (Th1) or Th2 skewing (Th2) conditions. At indicated time points, total RNA was prepared from harvested cells. The concentration of IL-4 and IFN-γ in the supernatant of cultured Th cells was determined by ELISA 24 hr after secondary stimulation and was shown in (D).

In (A), (B), and (C) the expression of ROG and GATA-3 was examined by Northern analysis using a ROG or GATA-3-specific probe. The expression of γ-actin was used as a control for the amount of RNA loaded per lane.

responsive to restimulation with anti-CD3. A similar pattern of ROG induction was detected when primary CD4⁺ T cells were skewed toward the Th1 pathway. We conclude that while both GATA-3 and ROG are rapidly induced upon activation of primary Thp cells, both their tissue specificity and their expression during the course of Th differentiation is markedly different. The discordant expression of ROG and GATA-3 in both primary Th cells and Th clones suggests that the physical interaction of ROG with GATA-3 is only one of its functions.

ROG Is a Repressor of GATA-3-Induced Transactivation

GATA-3 has been shown to be a transcriptional activator of the IL-5 and IL-4 promoters. To examine whether ROG can modulate the function of GATA-3, in vitro cotransfection experiments were performed. As shown in Figures 4A and 4B, overexpression of GATA-3 in a murine B cell line, M12, resulted in an ~35-fold induction of an IL-5 promoter and a 5-fold induction of an IL-4 promoter. The GATA-3-induced transactivation of both promoters was almost completely abrogated by overexpression of ROG. Similar results were obtained when a

reporter construct driven by a minimal TCR α enhancer, also GATA-3 responsive, or by the overlapping GATA-3-binding sites derived from the IL-5 promoter was used (data not shown). As a control, overexpression of ROG did not affect the transactivation of the IL-4 promoter induced by NF-ATp (Figure 4C). Similar results were obtained when EL-4 thymoma cells were used (Figure 4E). Furthermore, overexpression of ROG in the absence of GATA-3 did not affect the basal activity of the IL-5 and IL-4 promoters, nor did it repress the activity of a SV40 promoter/enhancer (Figure 4D). These results demonstrate that ROG is a potent repressor of GATA-3-induced transactivation.

The Middle Region and Zinc Finger Domains Are Essential for the Repressor Effect of ROG

In order to determine the functional domains of ROG responsible for its repressor activity, a series of ROG deletion mutants were generated and used in cotransfection assays. In vitro transcription/translation was used to show the successful generation of various deletion mutants (Figure 5, middle panel). Deletion of the
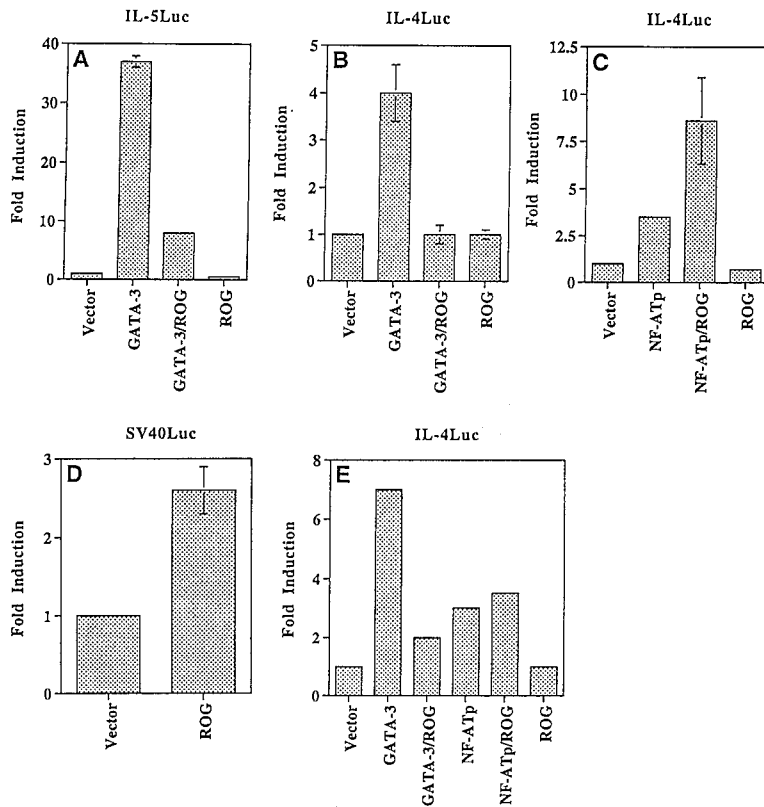
Figure 4. ROG Represses GATA-3-Induced Transactivation

M12 (A–D) or EL4 (E) cells were transfected with the indicated expression vectors and luciferase reporter plasmid driven by the IL-5 promoter (IL-5Luc [A]), IL-4 promoter (IL-4Luc [B, C, and E]), or an SV40 promoter/enhancer (SV40Luc [D]), and were stimulated with 50 ng/ml PMA/1 μM ionomycin 2 hr later. The luciferase activities were normalized against the activity, which was arbitrarily set as 1, obtained from cells cotransfected with the respective reporter plasmid and empty expression vectors. The data shown is representative of at least three independent experiments. All the luciferase assays were also normalized against the internal control pRL-TK.

BTB domain did not affect the function of ROG. In contrast, deletion of the middle region (amino acid residues ~105–291) or the zinc finger domain and the C-terminal region completely abrogated repressor function (Figure 5). Since the zinc finger domain of ROG is sufficient to mediate the interaction between GATA-3 and ROG, these results suggest that the repression of GATA-3-induced transactivation requires multiple functional domains of ROG.

ROG Prevents GATA-3 from Binding to DNA

ROG may repress the function of GATA-3 by several mechanisms. ROG might, for example, recruit the histone deacetylase complex (HDAC) to the IL-4 or IL-5 promoter. Several other POZ proteins, such as PLZF and Bcl-6, have been shown to repress transcription by recruiting nuclear corepressor (SMRT) and HDAC (Dhordain et al., 1997; Hong et al., 1997; David et al., 1998). Alternatively, ROG might contain intrinsic repressor activity. These two possibilities are unlikely because the presence of Trichostatin A (TSA), a potent inhibitor of HDAC, in the culture media, even at a concentration as high as 1000 nM, did not inhibit ROG repressor activity. Furthermore, recruiting ROG to a SV40 promoter via GAL4 DNA-binding domain and GAL4 DNA-binding site did not affect the activity of the SV40 promoter (data not shown). The third possibility was that ROG might prevent GATA-3 from binding to the IL-4 or IL-5 promoter
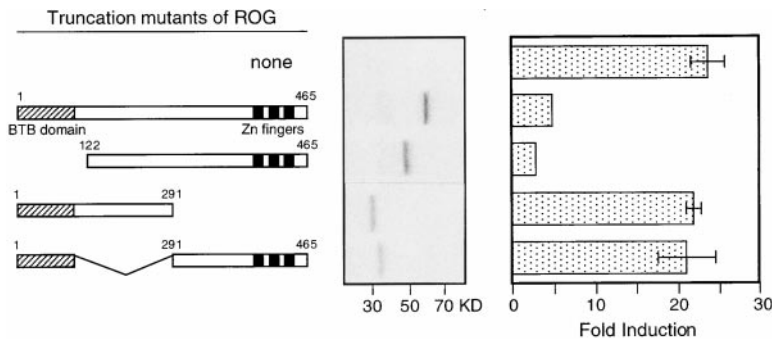

Figure 5. Mapping the Functional Domains of ROG

M12 cells were cotransfected with the IL-5 Luc reporter plasmid and the GATA-3 expression vector (none) or together with the wild-type or truncated ROG expression vectors. The luciferase activities were normalized against the activity, which was arbitrarily set as 1, obtained from M12 cells transfected with IL-5 Luc plasmid alone. All the luciferase assays were also normalized against the internal control pRL-TK. The wild-type and truncated ROGs are shown schematically. The numbers indicate the amino acid residues. The diagrams are not to scale. The in vitro transcribed/translated wild-type or truncated ROG proteins were fractionated in a 12% SDS-PAGE gel and shown in the middle panel.
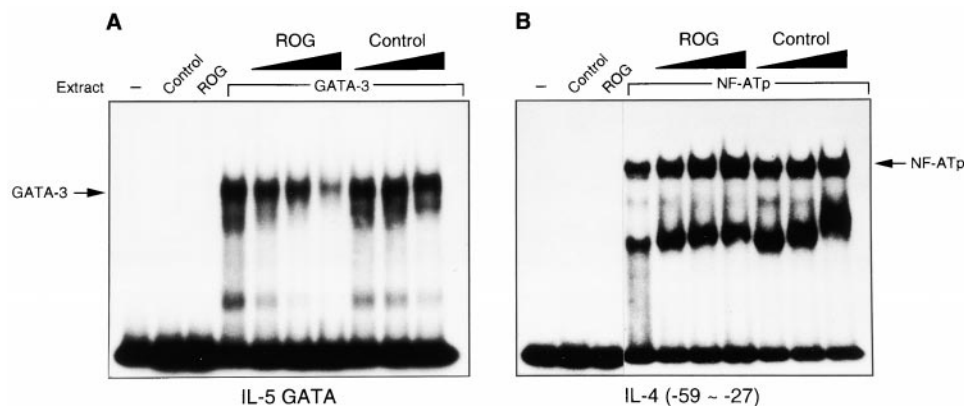
Figure 6. ROG Prevents GATA-3 from Binding to DNA

EMSA was performed by using the radiolabeled GATA-binding sequence derived from the IL-5 promoter (A) or the NF-AT-binding site derived from the IL-4 promoter (B), along with 2 μg or increments of 2 μg of indicated recombinant proteins.

or that ROG might simply inactivate GATA-3 by forming a nonfunctional complex on DNA. To distinguish between these two mechanisms, electrophoretic mobility shift assays were performed using recombinant GATA-3 and ROG proteins that had been partially purified from *E. coli*. Consistent with previous reports, GATA-3 but not ROG recombinant protein or control extract bound to the overlapping GATA sites derived from the IL-5 promoter. Addition of ROG protein into the reaction did not generate any higher order complex. Instead, ROG protein but not control extract attenuated the formation of the GATA-3/DNA complex in a dose-dependent fashion (Figure 6A). As a control, recombinant ROG protein did not affect the binding of recombinant NF-ATp protein to the IL-4 promoter (Figure 6B). These results suggest that ROG exerts its repressor effect, at least in part, by preventing GATA-3 from binding to DNA.

ROG Inhibits the Production of Cytokines by both Th1 and Th2 Cells

Overexpression of anti-sense GATA-3 in Th2 cells results in downregulation of Th2 cytokine genes, including IL-5, IL-4, and IL-10 (Zheng and Flavell, 1997; Zhang et al., 1998). Since ROG negatively modulates the function of GATA-3, it was possible that overexpression of ROG might also downregulate the expression of Th2 cytokines. To test this hypothesis, D10 Th2 cells were transfected with the GFP-ROG fusion protein expression plasmid. This plasmid retained 100% of the repressor activity of ROG when examined in in vitro transfection assays (data not shown). The transfected D10 cells were stimulated with anti-CD3 and analyzed for cytokine expression by intracellular cytokine staining. The percentage of cells producing IL-4, IL-5, or IL-10 was comparable between untransfected or GFP vector–transfected populations. In contrast, the percentage of cytokine-producing cells was dramatically reduced in the subset of cells that expressed GFP-ROG proteins (Figures 7A–7C).

Since ROG is also expressed in Th1 clones, it was important to know whether ROG also repressed the production of Th1 cytokines. Therefore, a similar experiment was performed by using the Th1 clone AE7, and

the production of IFN-γ was examined. As shown in Figure 7D, the production of IFN-γ by AE7 Th1 cells was also significantly reduced by overexpression of GFP-ROG. Although ROG repressed production of both Th1 and Th2 cytokines, this repression did not extend to other T cell response genes. The repressor activity of ROG appeared to be specific for cytokine genes because overexpression of ROG did not inhibit the upregulation of several activation markers such as FasL (Figure 7E) or CD69 (data not shown) in the same experiments. Taken together, these results demonstrated that overexpression of ROG in vitro specifically inhibits the production of both Th1 and Th2 cytokines.

Discussion

To date, several GATA interacting proteins have been identified and demonstrated to be involved in a variety of functions ranging from erythropoiesis to T cell leukemogenesis. For example, the GATA-1 interacting protein FOG is coexpressed with GATA-1 during embryogenesis (Tsang et al., 1997). Mice lacking FOG die during midembryonic development due to a profound defect in erythropoiesis similar to that seen in GATA-1-deficient mice (Tsang et al., 1998). The interaction between GATA-1 and PU.1 helps determine whether a differentiating hematopoietic stem cell will become an erythroblast or a myeloblast (P. Zhang et al., 1999). Both the RBTN2 and Tal transcription factors are induced and activated by chromosomal translocations in T cell leukemias and have been shown to physically and functionally interact with GATA-1 (Warren et al., 1994; Osada et al., 1995; Wadman et al., 1997). However, neither gene is normally expressed in T cells. While most of these GATA interacting proteins can interact with almost all GATA members in vitro, their roles in regulating the function and development of T cells remain unclear. As described in this report, we have identified and cloned a GATA-3 interacting protein, ROG, a tightly regulated early response gene in T cells. Our functional analysis revealed that ROG is a specific repressor of GATA-3-induced transactivation and is a potent negative regulator of both
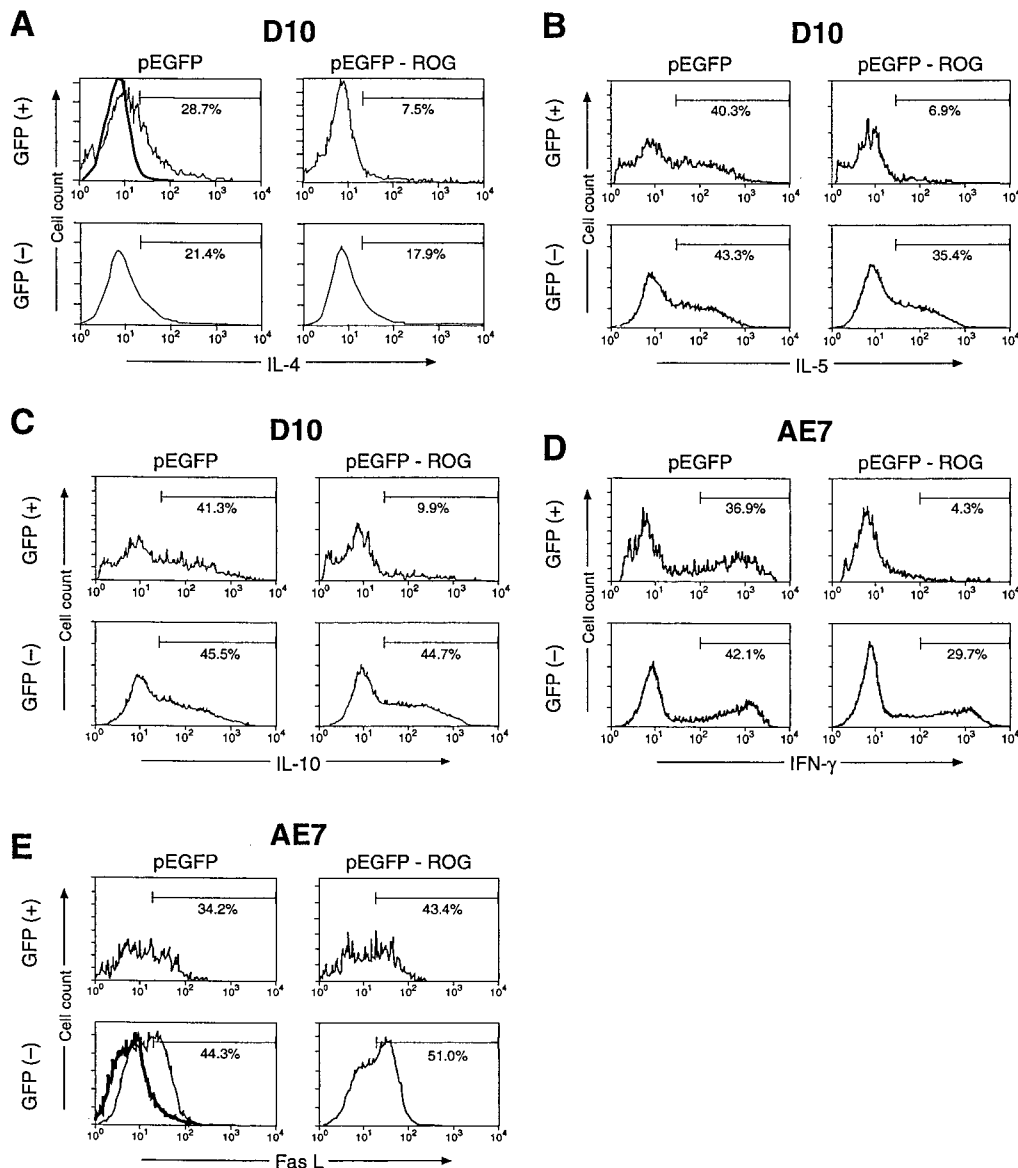
Figure 7. Overexpression of ROG Represses the Production of Cytokines by Th Clones

D10 (A–C) or AE7 (D and E) cells were transfected with expression vectors encoding GFP (pEGFP) or the GFP-ROG fusion protein (pEGFP-ROG). The transfected cells were rested overnight, stimulated with plate-bound anti-CD3 for 6 hr, and subjected to intracellular cytokine staining with PE-conjugated anti-IL-4 (A), anti-IL-5 (B), anti-IL-10 (C), or anti-IFN-γ (D) antibody, or surface staining with PE-conjugated anti-FasL antibody (E). The numbers stand for the percentages of cells that stained positive for PE among GFP-positive (+) or GFP-negative (−) populations. The bold line in (A) represents the background staining with a PE-conjugated control antibody. The bold line in (E) represents the baseline FasL staining of unstimulated AE7 cells.

Th1 and Th2 cytokine genes. ROG is thus identified as a lymphoid-specific GATA interacting protein that may play a critical role in regulating the differentiation and function of Th cells.

How Does ROG Repress the Function of GATA-3?
The majority of POZ proteins function as transcriptional repressors. The mechanisms of repression have been well characterized for Bcl-6 and PLZF. In mammalian cells, transcriptional repression by Bcl-6 or PLZF is mediated by interaction of their BTB domains with SMRT/

HDAC complex as evidenced by the enhancement of repressor activity by SMRT overexpression and inhibition of repressor activity by compounds such as TSA that block HDAC (Dhordain et al., 1997, 1998; David et al., 1998). We found no evidence that ROG interacts with SMRT/HDAC complex as evidenced by the lack of effect of TSA on ROG-mediated repression. It is still possible that ROG might recruit a novel HDAC isoform that is resistant to TSA. However, this possibility is unlikely since recruitment of ROG to an SV40 promoter via a GAL4 DNA-binding domain did not affect transcriptional

activity. While we showed that interference with the formation of the GATA-3/DNA complex is one mechanism by which ROG mediates repression, it may not be the only one. The zinc finger domain of ROG is inactive as a GATA-3 repressor but can still interact with and prevent GATA-3 from binding to DNA (data not shown). Furthermore, the middle region of ROG is essential for repression but is dispensable to the interaction with GATA-3. Thus, in addition to preventing GATA-3 from binding to DNA, ROG might itself be recruited to the promoter and inactivate the function of GATA-3 or block the interaction between GATA-3 and the basal transcriptional machinery. This latter function might require the middle region of ROG. Furthermore, it has also been demonstrated that BTB domains can alter chromatin structure once bound to DNA (Raff et al., 1994; Igarashi et al., 1998). It is thus possible that the BTB domain of ROG, by altering chromatin structure and accessibility to other factors, might provide another level of repression in vivo.

Is ROG a DNA-Binding Protein?
Many POZ family members have been shown to be DNA-binding proteins. For example, Bcl-6 recognizes a DNA sequence that is almost identical to the Stat6 target site (Chang et al., 1996; Seyfert et al., 1996). The zinc finger domain of ROG is ~70% homologous to the C-terminal three zinc fingers of PLZF, which was shown to recognize a consensus sequence with a core of TAAA (Li et al., 1997). While a near consensus PLZF-binding site was found next to the GATA site in the IL-5 promoter, the recombinant ROG protein did not bind to this sequence as tested by EMSA (data not shown). Our experiments have demonstrated that ROG can repress GATA-3 function independent of a direct interaction between ROG and DNA; however, it is still possible that ROG might bind to a DNA sequence other than the PLZF consensus sequence. The direct binding of ROG to DNA may play a critical role in regulating the transcription of cytokine and noncytokine genes. Identification of the consensus ROG-binding sequence should facilitate the isolation of additional ROG target genes.

How Does ROG Inhibit the Expression of Cytokine Genes?
One simple explanation for the ROG-mediated repression of Th2 cytokine genes is that ROG might lower the functional levels of GATA-3. A similar effect was achieved by using anti-sense GATA-3 (Zheng and Flavell, 1997; Zhang et al., 1998). However, it is obvious that ROG also represses the expression of Th1 cytokine genes, suggesting the presence of GATA-3-independent mechanisms. For instance, as discussed previously, ROG might repress the expression of both Th1 and Th2 cytokine genes by directly binding to the cytokine promoters. Furthermore, ROG might negatively modulate the function of a transcription factor in addition to GATA-3 that is critical for the expression of both Th1 and Th2 cytokine genes. Alternatively, and more intriguingly, ROG might repress the function of a yet to be discovered cell lineage-specific transcription factor that is essential for the expression of Th1 cytokine genes. Recently, it was demonstrated that overexpression of a dominant-negative form of GATA-3 protein in

vivo significantly attenuated the production of both Th2 and Th1 cytokines (D. H. Zhang et al., 1999). While it remains unclear how the dominant-negative GATA-3 represses Th1 cytokine production, it is very possible that the dominant-negative GATA-3 protein and ROG share the same mechanism to downregulate the expression of both Th2 and Th1 cytokine genes.

What Is the In Vivo Functional Role of ROG in Regulating the Th Immune Response?
Our in vitro data showed that ROG is an early response gene and overexpression of ROG downregulates the Th cytokine genes. It is tempting to postulate that ROG might play a critical role in a negative feedback mechanism that serves to terminate the Th immune response. Several negative feedback mechanisms that modulate the cytokine production and signaling have been identified. For example, the same stimuli that induce the production of TNF-α, a major mediator for both acute and chronic inflammation, can also induce a zinc finger protein, Tristetraprolin (TTP), which destabilizes TNF-α transcripts by binding to an AU-rich element (Carballo et al., 1998; Lai et al., 1999). In addition, several cytokines, such as IL-3 and IL-2, can induce a group of SH-2-containing proteins, such as CIS, that specifically inhibit the cytokine-induced Jak-Stat signaling pathway (Yoshimura et al., 1995; Starr et al., 1997). Thus, ROG might provide a mechanism by which activated Th cells can become quiescent once the antigenic stimulus has been removed. If this is true, why is ROG induced rapidly at a time when large amounts of cytokine are being produced by Th cells? Similar scenarios were observed for both TTP and CIS. Both TTP and CIS are dramatically induced within 5 to 20 min by the same stimuli, such as LPS and cytokines, that activate the cells (Yoshimura et al., 1995; Starr et al., 1997; Carballo et al., 1998). These observations suggest that for a cell to avoid overactivation, these negative feedback mechanisms have to be turned on at a very early stage. This may explain why ROG, a negative regulator of cytokine gene expression, is induced rapidly by anti-CD3 stimulation. However, it is also true that ROG is induced in naive Th cells within 24 hr post anti-CD3 stimulation, a stage when insignificant amounts of cytokines are produced and a time when termination of the cytokine response would seem to be premature. This observation suggests that ROG might play a role in the early stage of Th cell differentiation in addition to acting as a negative regulator of cytokine genes at later time points. The generation and analysis of mice lacking ROG should provide insight into the functional role of ROG in vivo and is underway.

Experimental Procedures

Cell Cultures
The mouse thymoma line EL4, B lymphoma cell lines M12, Bal17, and BCL1, monocyte/macrophage line P388D, and mastocytoma line P815 were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS). The mouse hepatoma line BNL CL.2, chondrocyte line L7 (gift of Dr. Laurie H. Glimcher), and fibroblast 3T3 line were cultured in DMEM supplemented with 10% FCS. Murine Th1 (AE7 and D1.1) and Th2 (D10 and CDC35) clones used in this study have been previously described (Ho et al., 1996). All Th clones were cultured in RPMI 1640 supplemented with 10% FCS and 10% Con

A–stimulated rat splenocyte supernatant and maintained by bi-weekly stimulation with appropriate antigens and APCs.

Reporter and Expression Vectors

The IL-5 and IL-4 promoter reporter constructs were reported previously (Kim et al., 1999). The expression vector of human GATA-3, pcDNA-GATA-3, was the gift of Dr. Jeffrey M. Leiden. The expression vector for ROG, pCI-ROG, was constructed by inserting the full-length ROG into the NotI site of pCI vector (Promega, Madison, WI). The expression vector for GFP-ROG fusion protein was generated by cloning a SpeI restriction fragment containing the full-length cDNA of ROG into the XbaI site of the pEGFP vector (Clontech, Palo Alto, CA). The NF-ATp expression vector pREP4-NFATp was previously reported (Ho et al., 1996).

Transfection and Luciferase Assays

Per transfection, five million cells were washed once with RPMI 1640, resuspended in 0.4 ml RPMI 1640, transferred to a 0.4 cm electrocuvette (Bio-Rad, Richmond, CA), and incubated with 10 μg of each plasmid DNA for 10 min at room temperature. For each transfection, 0.2 μg of Renilla luciferase reporter plasmid, pRL-TK (Promega), was added to serve as internal controls. Samples were then electroporated using a Bio-Rad Gene Pulser set at 975 μF, 280 V, and rested for 10 min at room temperature. The transfected cells were allowed to recover overnight in complete medium. Twenty-four hours post transfection, cells were harvested and equal amounts of cell extract were subjected to luciferase assay by using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. The luciferase activities were normalized against the Renilla luciferase activity.

Yeast Two-Hybrid Interaction

Briefly, a cDNA fragment containing the zinc finger domain of human GATA-3 (corresponding to amino acid residues 96–444), was cloned into the yeast bait vector pEG202. The GATA-3 bait vector (pEG-GATA-3) and a LacZ reporter vector, pSH18-34, were used to transform the yeast strain EGY48. A Th2 yeast prey library, constructed in the pJG4-5 vector, gift of Dr. Laurie H. Glimcher, was introduced into the pEGGATA-3/pSH18-8 doubly transformed EGY48. The transformed yeast was then selected in the appropriate selecting media. The yeast clones thus selected were lysed with breaking buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris–Cl [PH 8.0], 1 mM EDTA [PH 8.0]), and the yeast extract was used to transform the E. coli strain DH10B to recover the prey vectors. The control bait pEGMax was a gift of Dr. Roger Brent, and the pEGMaf was previously reported (Ho et al., 1996).

Intracellular Cytokine Staining

Transfected AE7 or D10 cells were stimulated with plate-bound anti-CD3 (2C11 at 1 μg/ml in PBS). Two hours post stimulation, monensin (2 μg/ml) was added into the culture. Four hours post addition of monensin, cells were fixed with 4% paraformaldehyde at 4°C overnight, permeabilized with saponin, stained with PE-conjugated anti-cytokine, anti-FasL, anti-CD69, or control antibodies for 30 min on ice, washed with 0.1% BSA in PBS, subjected to flow cytometric analysis on a FACS (Becton Dickinson and Co., Mountain View, CA), and analyzed with Cellquest software. All antibodies were purchased from PharMingen (San Diego, CA).

In Vitro Differentiation of Th Cells

Splenic CD4^{+} T cells derived from BALB/c mice were isolated by using CD4 MiniMACS columns (Miltenyi Biotec, Auburn, CA) and stimulated in vitro with plate-bound anti-CD3 antibody, along with anti-IL-12 antibody (5C3) at 20 μg/ml (Th2 skewing conditions) or anti-IL-4 antibody (11B11) at 5 μg/ml (Th1 skewing conditions). Twenty-four hours post stimulation, IL-2 at 50 units/ml was added to all cultures. In addition, IL-4 at 500 units/ml or IL-12 at 50 units/ml was added into Th2 or Th1 cultures, respectively. Six days post stimulation, cells were harvested, washed thoroughly, and restimulated with plate-bound anti-CD3. Under these conditions, we find that ∼80% of the population at the end of the secondary stimulation are CD4^{+} T cells. All antibodies were purchased from PharMingen.

Northern Analysis

Total RNA was prepared from stimulated or unstimulated cells with TRIZOL reagent (GIBCO–BRL, Gaithersburg, MD) according to the manufacturer's instructions. Ten micrograms of each RNA sample was fractionated on 1.2% agarose gels, transferred to nitrocellulose membranes, and hybridized with the indicated cDNA probes in QuickHyb buffer (Stratagene, La Jolla, CA). The cDNA probes used were a 200 bp SmaI restriction fragment of the murine ROG cDNA, the full-length human GATA-3 cDNA, and the full-length murine γ-actin cDNA. The organ blot was kindly provided by Dr. Andrea Wurster.

Electrophoretic Mobility Shift Assay

T4 polynucleotide kinase was used to end-label 100 ng of double-stranded oligonucleotides with ^{32}P-dATP (DuPont NEN Research Product, Wilmington, DE). The labeled double-stranded oligonucleotides were fractionated in 15% polyacrylamide gels, eluted overnight at 37°C in 1× TE, and precipitated in ethanol. Binding assays were performed at room temperature for 20 min using the indicated amount of partially purified recombinant ROG, GATA-3, a control protein, and purified recombinant truncated NF-ATp (gift of Dr. Timothy Hoey) prepared from E. coli, along with 500 ng poly(dI-dC) and 20,000 cpm of probe in 15 μl of 20 mM HEPES (pH 7.9), 100 mM KCl, 5% glycerol, 1 mM EDTA, 5 mM DTT, and 0.1% NP-40. The samples were then fractionated in 4% nondenaturing polyacrylamide gels containing 0.5× TBE at room temperature.

The sequence of the IL-5 GATA oligonucleotide is 5'-GGACTCGC CTTTATTAGGTGTCCTCTATCTGATTGTTAG-3'. The sequence of the IL-4 (approximately −59 to −27) oligonucleotide is 5'-CTCATTT TCCCTTGGTTTCAGCAACTTTAACTC-3'. All oligonucleotides were annealed with their reverse-complementary strands to form double-stranded oligonucleotides.

Coimmunoprecipitation Assays

Jurkat cells (5 × 10⁶ cells per transfection) were transfected with the GATA-3 expression vector, along with the GFP or GFP-ROG fusion protein expression plasmids. Twenty-four hours post transfection, coimmunoprecipitation was performed using the Immuno-catcher kit (Cytosignal, Irvine, CA). Briefly, cells were harvested and lysed with 180 μl of mild lysis buffer in the presence of 1× protease inhibitors and precleared with 20 μl of protein A/G bead. The precleared samples were incubated with 5 μl of monoclonal antibody against GATA-3 (Santa Cruz Biotechnology, Santa Cruz, CA) for 45 min at room temperature, captured with 10 μl of protein A/G bead, and washed three times with 1.5 ml mild lysis buffer. The captured proteins were then eluted with 30 μl 1× sample buffer, fractionated in 8% SDS-PAGE gels, and transferred to Optitran membranes (Schleicher and Schuell, Keene, NH). The membranes were incubated with anti-GFP rabbit serum (1:1000 dilution; Clontech), and the GFP-containing proteins were detected with the ECL kit (Amersham Pharmacia, Piscataway, NJ).

In Vitro Transcription/Translation

In vitro transcription/translation of the full-length ROG and its truncated mutants was performed by using the TNT rabbit reticulocyte lysate kit (Promega) programmed with indicated plasmid DNA in the presence of ^{35}S-Methionine (Dupont-NEN). The transcribed/translated products were fractionated in 10%–12% SDS-PAGE gels and subjected to autoradiography.

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GenBank Accession Number