An Alternative Transcript Derived from the Trio Locus Encodes a Guanosine Nucleotide Exchange Factor with Mouse Cell-transforming Potential*

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By screening cDNA expression libraries derived from fresh leukemic cells of adult T-cell leukemia for the potential to transform murine fibroblasts, NIH3T3, we have identified a novel transforming gene, designated Tgat. Expression of Tgat in NIH3T3 resulted in the loss of contact inhibition, increase of saturation density, anchorage-independent growth in a semisolid medium, tumorigenicity in nude mice, and increased invasiveness. Sequence comparison revealed that an alternative RNA splicing of the Trio gene was involved in the generation of Tgat. The Tgat cDNA encoded a protein product consisting of the Rho-guanosine nucleotide exchange factor (GEF) domain of a multifunctional protein, Trio, and a unique C-terminal 15-amino acid sequence, which were derived from the exons 38–46 of the Trio gene and a novel exon located downstream of its last exon (exon 58), respectively. A Tgat mutant cDNA lacking the C-terminal coding region preserved Rho-GEF activity but lost the transforming potential, indicating an indispensable role of the unique sequence. On the other hand, treatment of Tgat-transformed NIH3T3 cells with Y-27632, a pharmacological inhibitor of Rho-associated kinase, abrogated their transforming phenotypes, suggesting the coinvolvement of Rho-GEF activity. Thus, alternative RNA splicing, resulting in the fusion protein with the Rho-GEF domain and the unique 15 amino acids, is the mechanism generating the novel oncogene, Tgat.

Human cancer develops as a consequence of multiple genetic alterations that allow cells to escape normal cell-growth regulation (1). These genetic alterations include chromosomal amplification, deletion, translocations, and somatic point mutation, leading to proto-oncogene activation or suppressor oncogene inactivation. One of the approaches to identifying the gain-of-function of oncogenes in cancer cells has been phenotypic screening of cultured cells transfected with cancer cell-derived genomic DNAs or cDNA libraries. Indeed, a number of cellular oncogenes that are susceptible to single-hit oncogenic transformation have been identified in transfected murine fibroblasts NIH3T3 (2–6). The oncogenic potential of certain oncogenes have been easily identified in NIH3T3 cells by loss of contact inhibition and anchorage-independent growth in vitro as well as tumorigenicity in vivo. Theoretically, phenotypic screening has advantages in detecting subtle genomic change, such as the point mutation of ras genes, and the alterations involved in post-transcriptional processes, leading to the gain-of-function of oncogenes.

Adult T-cell leukemia (ATL) is associated with prior infection with HTLV-I, but the mechanisms by which leukemogenesis occurs, are not fully defined. HTLV-I persists as a proviral DNA in T-cells of infected individuals, and a minor population of carriers develops ATL after a long latency (7, 8). The virus does not carry any host-derived oncogenes, nor does it activate a proto-oncogene upon proviral integration at a common site. Accumulating evidence has indicated an important role of TAX, the transactivator of HTLV-I, with a potential to immortalize T-cells in vitro in the early stages of leukemogenesis. The diverse functions of TAX, including transcriptional transactivation of various growth-related genes (9–12), deregulation of cell cycle progression (13–16), and initiation of DNA damage (17, 18), are implicated in the leukemogenesis. On the other hand, the expression of TAX or its transcript is barely detectable by conventional methods, in freshly isolated ATL cells. Moreover, ATL cells occasionally harbor intact HTLV-I proviruses encoding nonfunctional TAX (19, 20) or lacking 5'-long terminal repeat essential for TAX expression (21), arguing against the role of TAX in malignant ATL phenotypes in patients. In severe combined immunodeficient mice, the expression of TAX alone failed to support neoplastic growth of T cells (22). The long latency of ATL also suggests the age-dependent accumulation of leukemogenic events within HTLV-I-infected T-cells (23). Recently, abnormalities in tumor suppressor genes such as p53, p15, or p16 have been identified with high frequency in ATL cells. Additional changes in the cellular genome and/or transcriptome are likely to be involved in leukemogenesis and malignant phenotypes of ATL cells in vivo.

In the present study, we constructed cDNA expression libraries using a retroviral vector from fresh ATL cells and screened

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The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBankTM/EBI Data Bank with accession number(s) AB115332.

§ To whom correspondence should be addressed. Tel.: 81-95-849-7058; Fax: 81-95-849-7060; E-mail: ryozo@net.nagasaki-u.ac.jp.

1 The abbreviations used are: ATL, adult T-cell leukemia; Tgat, trio-related transforming gene in ATL tumor cells; GEF, guanine nucleotide exchange factor; HTLV-I, human T-cell lymphotrophic virus, type I; DMEM, Dulbecco’s modified Eagle’s medium; GST, glutathione S-transferase; RBD, rhoetekin Rho binding domain; ROCK, Rho-associated kinase; LPA, lysophosphatidic acid; DH, Dbl homology; PH, pleckstrin homology.

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for cDNAs with the potential to transform NIH3T3 cells. The identified novel transforming gene, designated trio-related transforming gene (Tgat), was generated by alternative RNA splicing between a part of the Trio gene encoding Rho-GEF (from exons 38 to 46) and a novel exon located downstream of the last exon of Trio (exon 58). Both the Rho-GEF activity and the C-terminal region of Tgat were required for its transforming activity. A potential role of Tgat in ATL is also discussed.

EXPERIMENTAL PROCEDURES

DNA Constructs—A retroviral vector, pDON/SfiI/PacI, and pBluescript/SfiI/PacI were constructed by modifying pDON-AI (Takara) and pBluescript SR (+) (Stratagene), respectively, as described previously (24). To make TgatΔC and TgatPH2, PCR-amplified DNA fragments were ligated into pDON-AI and confirmed by DNA sequencing.

Cells and Cell Lines—Peripheral blood mononuclear cells were obtained from two acute-type ATL patients. The experimental protocol was approved by the Ethics Review Committee for Human Experimentation of our institution, and informed consent was obtained from all subjects. The rodent fibroblast NIH3T3 cell line was maintained in Dulbecco’s modified Eagle medium (DMEM) (Invitrogen) supplemented with 5% calf serum. The 293 10A-1 packaging cell line (IMGENEX) was maintained in DMEM supplemented with 10% fetal bovine serum in the presence of blasticidin S (20 μg/ml).

Library Construction and Transduction to NIH3T3 Cells—cDNA was made from 1 μg of total RNA using a SMART cDNA construction kit (Clontech) according to the manufacturer’s protocol. Fractions of cDNA greater than 1 kb were used for the library construction. Supernatants were recovered 48 h after transfection of the plasmid DNA library into the 293 10A-1 packaging cell line. This library produced viral titers of 3 × 10⁷ colony-forming units/ml. Recombinant retrovirus libraries were infected to NIH3T3 cells (multiplicity of infection = 0.1–1). The infected NIH3T3 cells were cultivated for 2–4 weeks.

cDNA Rescue—Genomic DNAs were purified from transformed foci and used for cDNA rescue by nested PCR using vector primers. The thermal cycles were as follows: 95 °C for 3 min; 25 cycles at 95 °C for 0.5 min, 62 °C for 0.5 min, then 68 °C for 3 min. The amplified DNA was then digested by SfiI enzyme (Promega) and subcloned into pBluescript/SfiI/PacI vector for further screening. All sequences were compared against the GenBank™ databases by using the Basic Local Alignment Search Tool (BLAST).

Tumor Growth in Nude Mice—Four- to six-week-old athymic BALB/c nu/nu mice were injected with 1 × 10⁶ cells in 200 μl of phosphate-buffered saline from transformed clones. Tumors were measured after 4 weeks.

Immunofluorescence—For actin localization, stable cell lines were cultured for 2 days on LabTek chamber microscopy slides (Nalge Nunc) in normal media and then serum-starved for 16 h and fixed in 4% paraformaldehyde. Slides were then sequentially incubated with anti-vinculin antibodies (Sigma), fluorescein isothiocyanate-conjugated antimouse IgG (Vector Laboratory Inc.), rhodamine-phalloidin, and 4',6-diamidino-2-phenylindole (Molecular Probes). Images were obtained using an Axioscan microscope and the Axiovision capture system (Carl Zeiss).

Affinity Precipitation of Cellular GTP-Rho—We used a Rho activation assay kit (Upstate Biotechnology) to detect cellular GTP-Rho according to the manufacturer’s protocol. Cell lysates were incubated with GST-RBD ( rhoetekin Rho binding domain, 40 μg)-agarose beads at 4 °C for 45 min. The beads were washed four times with wash buffer, and bound Rho protein was detected by Western blotting using a polyclonal antibody against Rho A.

In Vitro Invasion Assay—A Biocoat Matrigel Invasion Chamber (BD Biosciences Labware) was used for monitoring the in vitro cell invasion assay, which was performed as described previously (25). The lower chamber was filled with DMEM, supplemented with 5% calf serum. NIH3T3 cells (1 × 10⁶), stably expressing Tgat, TgatΔC, TgatPH2, and V12ras and suspended in DMEM supplemented with 0.1% bovine se-
rum albumin, were placed in the upper components of the chamber. After incubation for 22 h at 37°C in a 95% air and 5% CO2 atmosphere, the filters were removed, fixed with methanol, and stained with toluidine blue. Each assay was set up in triplicate, and all data were analyzed statistically by t tests.

RESULTS

Cloning of Transforming Genes Expressed in ATL Cells—Infection of NIH3T3 cells with the recombinant viruses containing cDNAs derived from ATL leukemic cells gave a number of foci 2 weeks post-infection, about one-third of which showed anchorage-independent growth in a semisolid medium. Sequence analysis identified a particular cDNA species, designated Tgat (trio-related transforming gene in ATL tumor cells), which was shared by 6 of the 10 independent anchorage-independent colonies analyzed (Tgat sequence was submitted to DDBJ; accession number, AB115332). Unfortunately, another four colonies contained multiple cDNAs, and all rescued cDNA could not independently transform NIH3T3 cells. Interestingly, Tgat was also identified in the foci of NIH3T3 cells infected with two other cDNA libraries derived from independent patients with acute ATL. To confirm the transforming potential of Tgat, the cDNA amplified from an anchorage-independent colony was recloned in pDON/SfiI/PacI and expressed in NIH3T3 cells. The morphology of foci induced by Tgat showed extensive aggregation of the cells at the center of the focus leading to an overall punctate appearance, and the number of foci was dose-dependent on the transfected DNA (Fig. 1). The transformed NIH3T3 cells exhibited reduced cell doubling time and grew to a higher cell density. Moreover, subcutaneous inoculation of the transformed cells produced tumors in athymic nude mice within 30 days. In contrast, none of the mice inoculated with control cells with empty vectors developed tumors during the 3-month observation period.

Tgat Encodes Rho-GEF followed by 15 Unique Amino Acids—The nucleotide sequencing of the Tgat cDNA (1,574 bp) and searches using the GenBank nucleotide sequence data bank revealed a homology to the cDNA for the multifunctional protein Trio, which comprises spectrin-like domains, two functional guanosine nucleotide exchange factor (GEF) domains, and a protein serine/threonine kinase domain (26). The Trio gene consists of 58 exons, which are distributed in the 380-kb region of chromosome 5p14–15.1. The 5'H11032-H11032-half of the Tgat cDNA, 771 bp, was completely identical to the Trio exons 38–46 encoding the GEF-D2 sequence, but the following 803-bp sequence appeared to be unique. A genome data base search revealed that this unique sequence was derived from a novel exon located 1.6 kb downstream of the poly(A) site on the last exon (exon 58) of Trio (Fig. 2a), indicating the involvement of alternative RNA splicing between exon 46 and the novel downstream exon. 5'H11032-Rapid amplification of cDNA ends experiments suggested that Tgat expression was driven by a putative cryptic promoter located upstream of exon 38 of the Trio genome (data not shown). The Tgat cDNA contained an open reading frame, which was initiated from the ATG codon conforming to the Kozak consensus sequence (27) located at 48-bp downstream from the 5'H11032-terminus of the cDNA and encoding a protein product with 255 amino acids. Sequence comparisons revealed that N-terminal 241 amino acids corresponding to the Trio GEF-D2 domain were followed by the unique C-terminal 15-amino acid sequence encoded by the novel downstream exon (Fig. 2b).

The C-terminal 15 Amino Acids Encoded by Tgat Are Crucial for Its Transforming Activity—To explore the role of the unique C-terminal region of the Tgat protein, we examined the transforming potential of TgatΔC lacking the C-terminal 15 amino acids. Deletion of the 15 amino acids completely abrogated the

![Fig. 3. Transforming activities of Tgat and Tgat mutants.](image)

|          | Focus forming activity (f.f.u. / 10^3 c.f.u.) | Tumorigenicity (tumors / injection) |
|----------|--------------------------------------------|------------------------------------|
| Tgat     | 58.3 ± 14.6                                 | 5/5                                |
| TgatΔC   | 0                                           | 0/4                                |
| TgatPH2  | 0                                           | 0/4                                |

Tgat comprises the Rho-GEF-D2 domain of Trio and the unique C-terminal 15-amino acid sequence. The light gray box represents the unique 15-amino acid sequence shown in Fig. 2b. Focus formation assay (left) and colony formation assay (middle) in soft agar, and tumorigenicity in nude mice (right). Both TgatΔC- and TgatPH2-expressing NIH3T3 cells lack the transforming activities in vitro and in vivo. f.f.u., focus forming units; c.f.u., colony forming units.
transforming activity of Tgat in vitro and in vivo (Fig. 3). TgatPH2, in which the C-terminal 15 amino acids were replaced by the PH2 domain of Trio, also failed to transform NIH3T3 cells. These results indicate that the C-terminal 15 amino acids of Tgat play a crucial role in the transforming potential and that the Trio PH domain cannot replace its function.

**Tgat Activates Cellular Rho-GTPases**—The Trio GEF-D1 domain is specific for Rac, whereas the GEF-D2 domain is specific for Rho (26). Rho activation usually leads to the formation of contractile actin-myosin stress fibers and associated focal adhesion complexes. ROCKs (Rho-associated kinases) and Dia activated by binding to Rho-GTP are thought to be involved in the Rho-induced assembly of stress fibers and focal adhesions (28). As shown in Fig. 4a, expression of Tgat, as well as TgatPH2 or TgatΔC, in Swiss 3T3 fibroblasts induced clear contractile actin stress fibers and focal adhesion complexes, which are characteristic of Rho activation and not of Rac or cdc42 activation. To confirm Rho activation in the cells, the quantity of Rho-GTP was directly measured by a GST-RBD pull-down assay (Fig. 4b). The level of Rho-GTP induced by Tgat was similar to that in the cells treated with lysophosphatidic acid (LPA) or transformed by TgatPH2, whereas TgatΔC failed to fully activate Rho. The PH domain is thought to mediate membrane localization through lipid binding and directly affect the activity of the DH domain (26, 29, 30); therefore, the C-terminal region of Tgat would mimic the function of the PH domain of Trio, keeping the GEF activity.

**ROCK Inhibitor Abrogates the Transforming Activity of Tgat**—To elucidate the role of Rho-GEF activity in the Tgat-induced transformation, Tgat was expressed in NIH3T3 cells in the presence of a pharmacological ROCK inhibitor, Y-27632 (Calbiochem). As shown in Fig. 4c, Y-27632 completely abrogated focus formation by Tgat. These data support the conclusion that both
the DH domain and the unique C-terminal region of Tgat are necessary for the transforming activity.

**Tgat Expression Induces Invasion Capability**—Tiam1 (T-lymphoma invasion and metastasis 1), another member of the DH protein family, is reported to induce an invasive phenotype in otherwise noninvasive lymphoma cell lines (32, 33). We therefore investigated whether the expression of Tgat could affect the invasiveness of NIH3T3 cells using an *in vitro* invasion assay (25). The invasiveness of Tgat-expressing cells was about 12-fold higher than that of TgatPH2- or TgatΔC-expressing cells. Moreover, Tgat had an edge over the activated oncogene (V12ras) in invasion-inducing ability. Y-27632 treatment of Tgat-expressing cells reduced its invasiveness, but the effect was insufficient. These results suggest that the existence of the unique C-terminal region outweighs the Rho-GEF activity for the invasion-inducing ability of Tgat (Fig. 5, a and b).

**DISCUSSION**

Rho family proteins were initially cloned on the basis of their similarity to the Ras oncoproteins. Like Ras, Rho family proteins have lipid modifications that target them to cell membranes, and cycle between GTP- and GDP-bound states. Binding to GTP is promoted by Rho-GEFs, and GTP hydrolysis is catalyzed by Rho-GTPase-activating proteins. Unlike Ras, there are no reports of mutated, constitutively active forms of Rho proteins in tumors. However, recent work has shown that Rho proteins are overexpressed in several human tumors (34).

The Dbl proteins, a family of Rho-GEFs that transduce diverse intracellular signals leading to the activation of Rho family GTPases, contain a tandem Dbl homology (DH) domain/pleckstrin homology (PH) domain structure. The DH domain is a catalytic region of the protein, whereas the PH domain regulates the DH domain as well as the subcellular localization of the Dbl protein (35). Many Dbl family proteins, such as Dbl (6), Vav (36), Ect2 (37), Lfc (38), and Lsc (39), were originally identified in gene transfer screening studies as novel oncoproteins that cause transformation of NIH3T3 cells. For a number of Dbl proteins, activation of these transforming activities was a result of either N-terminal or C-terminal truncation of sequences that flank the conserved DH/PH domains. The rearrangements that led to activation of transforming activity were due to artifacts of the transfection procedure, although some of these screenings involved the analysis of DNA from...
tumor cell lines. Thus no DbI family protein has been found to be aberrantly activated in human cancers except BCIR, rearranged in the leukemia-associated BCR-AbI translocation gene product, and LARG, a fusion partner with MLL in a patient with acute myeloid leukemia (40).

In the case of Tgat, the PH domain is spliced out and the unique C-terminal 15 amino acids follow the Rho-GEF domain. Because TgatC containing the DH domain alone and TgatPH2 showed no transforming activity in the \textit{in vitro} and \textit{in vivo} assays, the unique C-terminal 15 amino acids are essential for the transforming activity of Tgat (Fig. 3). Moreover, a Tgat mutant, which lacked GEF activity due to a single amino acid substitution, also showed no transforming activity (data not shown), and inhibition of ROCK using a pharmacological inhibitor (Y-27632) caused the loss of the foci produced by Tgat (Fig. 4c). These results suggest that both the DH domain and C-terminal 15-amino acid sequence of Tgat, but not the PH domain, are required for its transforming function.

Tgat expression induced not only transforming activity but also invasion capability in NIH3T3 cells (Fig. 5). Rho family proteins are known to be associated with changes in the membrane-linked cytoskeleton. Activation of Rac1, Cdc42, and RhoA has been shown to produce specific structural changes in the plasma membrane cytoskeleton associated with membrane ruffling, lamellipodia, filopodia, and stress fiber formation (35). The coordinated activation of these Rho family proteins is thought to be a possible mechanism underlying cell motility, an obvious prerequisite for metastasis or invasion. It has also been shown that Tiam1, which is Rac1-GEF identified by retroviral insertional mutagenesis and selected for its invasive cell behavior \textit{in vitro}, promotes Rac1 signaling and metastatic breast tumor cell invasion and migration by interaction with ankyrin (41). In the case of Tgat, the existence of the unique C-terminal region is indicated to be more important than Rho-GEF activity for the invasion-inducing ability, because Y-27632 treatment or ROCK inhibition using a pharmacological inhibitor (Y-27632) caused the loss of the foci produced by Tgat (Fig. 4c).

In three different cDNA libraries derived from independent patients with ATL, we examined the Tgat expression in ATL patients by semi-quantitative reverse transcription-PCR using a primer set that specifically amplified Tgat, but not authentic Trio transcripts (data not shown). So far, the reverse transcription-PCR has detected Tgat mRNA in peripheral blood mononuclear cells of 14 of 21 ATL patients, in contrast to 0 of 4 control subjects. To determine whether Tgat affects the ability of human cells to transform, we have tried to examine the growth behaviors of the human peripheral blood mononuclear cells or Jurkat cell line expressing Tgat. Unfortunately, we have not been able to demonstrate the effect of Tgat on human cell transformation or growth yet. Although the roles of Tgat in T-cell leukemogenesis and the malignant phenotypes of ATL remain to be elucidated, it would be conceivable that Tgat is involved in some aspects of malignant phenotypes \textit{in vivo} in a significant proportion of ATL patients. Acute ATL progresses rapidly, and the median survival period after diagnosis is approximately 6 months (42). The resistance of ATL to conventional chemotherapy is mainly due to the induction of drug-resistant related genes (43) and leukemic infiltration into various organs (44, 45). The invasive character of ATL tumor cells is recognized to be an important factor in the poor prognosis. The expression of Tgat in ATL tumor cells may explain this malignant profile of leukemic cells, although it remains to be examined whether Tgat influences the invasive phenotype of ATL cells. A potential role of Tgat in ATL may provide new insights into our understanding of ATL biology and strategies for developing therapeutics.
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**A Novel Oncogene Identified in ATL**

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