Guanine Nucleotide Exchange Factor, Tiam1, Directly Binds to c-Myc and Interferes with c-Myc-mediated Apoptosis in Rat-1 Fibroblasts

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The transcription factor c-Myc is important for the control of cell growth, cell cycle progression, neoplasia, and apoptotic cell death. Recently, c-Myc-binding proteins, which bind either to the N-terminal domain or the C-terminal domain of c-Myc, have been proposed as the key molecules to realize the mechanisms of these multiple c-Myc functions. We report in the present study on another protein, Tiam1, which is a specific guanine nucleotide exchange factor of Rac1 and which binds to c-Myc and modulates several of its biological functions. We were able to detect the direct binding and in vivo association between c-Myc and Tiam1. The necessary role in this interaction of the Myc box II of c-Myc was revealed in the cell extracts. The additional discovery of the intranuclear localization of Tiam1 in Rat1 cells and in neuronal cells of the mouse brain suggests this interaction may occur in the nucleus. Overexpression of Tiam1 repressed the luciferase activity of c-Myc and also inhibited the c-Myc apoptotic activity through this protein-protein interaction. Taken together, we concluded that Tiam1 is another c-Myc regulator, working in the nuclei to control c-Myc-related apoptosis.

The c-Myc gene was discovered as the cellular homologue of the retroviral v-Myc oncogene and has been proven to have a crucial role in various pathological and physiological events in cellular biology. In human cancers, alterations of the c-Myc gene have in fact been reported, which have included chromosomal translocation, point mutation, gene amplification, and heterotopic expression (1). On the other hand, the levels of c-Myc oncprotein increase and remain elevated in normal cells that have been induced to divide, indicating it is required throughout the cell cycle for proliferation. Deregressed c-Myc expression is asserted to be sufficient to drive quiescent cells into the S phase to prevent cell cycle exit (2–4).

Previous studies have revealed one of these actions is through the modulation of transcription of various genes, i.e. c-Myc recognizes the E-box sequence in the genome together with Max as a partner and activates transcription. In this case the C-terminal basic/helix-loop-helix/leucine zipper (B-HLH-LZ) domain of c-Myc mediates binding to a specific area of DNA. c-Myc also interacts directly with a wide variety of other proteins through this C-terminal B-HLH-LZ domain. For example, dimerization with a protein known as Max is essential for the binding of Myc to DNA (5). In addition, the C-terminal domain of c-Myc binds to other transcription factors, TFII-I, YY1, and AP-2, which regulate the DNA binding and transcriptional activities of c-Myc (reviewed in Ref. 6). On the other hand, the N-terminal domain of c-Myc (amino acids (aa) 1–144) has both transcriptional activation and repression activities. This domain contains two evolutionarily conserved regions termed Myc box (MB) I (aa 47–62) and MB II (aa 106–143). A c-Myc mutant deleted within MB I has been shown to diminish Myc-mediated transcriptional activity (7). MB II has been reported to mediate several functions, including transformation, induction of apoptosis, blocking differentiation, and transcriptional repression (8–13). These studies show MB II is a crucial domain for diverse c-Myc functions. Along these lines, there is a growing interest in c-Myc-binding proteins to realize the mechanisms of multiple c-Myc functions.

In this paper, we report on Tiam1, which has been already reported as a specific guanine nucleotide exchange factor (GEF) for Rac1, a Rho family p21 low molecular weight G protein, which also binds to c-Myc (14). Tiam1 can modify actin cytoskeleton and cell migration, and can activate the mitogen-activated protein kinase cascade through activating Rac1. Although some papers have recently reported that Tiam1 or Rac1 is also related to apoptosis, both these proteins show various effects for apoptosis depending on the cell type (15–26). As far as we know, no previous paper has ever addressed a potential interaction between Tiam1 and c-Myc. We here report the direct interaction between c-Myc and Tiam1, and the negative regulation of Tiam1 of c-Myc transactivation and its apoptosis activity. This interaction with c-Myc may provide a new facet of Tiam1, demonstrating various unexpected functions.

MATERIALS AND METHODS

Plasmids and Constructs—A full-length human c-Myc was cloned in pGEX-4T3 (Amersham Biosciences), and that with a flag epitope at the C terminus was cloned in pCS2+ (provided by D. Turner, Hutchinson Cancer Research Center, Seattle, WA) for transfection into 293T cells. Full-length human Tiam1 cDNA (a gift from K. Nakaya, Showa University, Tokyo, Japan) was generated as described (15) and cloned into pCS2+. Deletion mutants of c-Myc (1–142, 1–128, 129–439, and 143–355; these numbers refer to the amino acids included in the encoded c-Myc protein) and those of Tiam1 (C1199, C682, and N392; nomenclature refers to the number of C-terminal or N-terminal amino acids of leucine zipper; FBS, fetal bovine serum; GEF, guanine nucleotide exchange factor; TRITC, tetramethylrhodamine isothiocyanate; GST, glutathione S-transferase; PBS, phosphate-buffered saline; MB, Myc box; aa, amino acid(s); HA, hemagglutinin; WT, wild-type.
the encoded Tiam1 proteins) were generated by two-step PCR mutagenesis, as previously described (27). Tiam1(N392) was tagged by hemagglutinin (HA) at the C terminus. Plasmids encoding c-DNAs of human β-Pix CH domain deleted mutant and human Vav1 were gifts from T. Nagase (Kazusa DNA Research Institute, Kisarazu, Japan) and M. Shibuya (Institute of Medical Science, University of Tokyo, Japan), respectively. Oncogenic Vav1 was generated by two-step PCR mutagenesis (28). The dominant negative mutant of Rac1 (N17Rac1), tagged with HA and cloned into pcDNA3+, was provided by B. J. Mayer (University of Connecticut Health Center, Farmington, CT) (29).

Antibodies—The monoclonal antibodies for the HA epitope tag, c-Myc, and nucleopiperin p62 were from Berkeley Antibody (Richmond, CA), Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and Transduction Laboratories (Lexington, KY), respectively. The monoclonal antibodies for the flag tag and β-tubulin were from Sigma. The polyclonal antibodies for Tiam1(1-6) and Tiam1(1-15) (Tiam1C and Tiam1N, respectively) were from Santa Cruz Biotechnology, Inc. The polyclonal antibody anti-c-Myc was from Upstate Biotechnology, Inc. (Lake Placid, NY) Anti-mouse or anti-rabbit immunoglobulin conjugated or un conjugated with alkaline phosphatase and TRITC (tetramethylrhodamine isothiocyanate)-conjugated secondary antibodies were from DAKO (Copenhagen, Denmark).

Cell Culture, Transfection, and Immunoprecipitation—Rat1/CM cells were kindly supplied by Y. Kuchino (National Cancer Center Research Institute, Tokyo, Japan) (30). 293T human embryonal kidney cells and Rat1 fibroblasts were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum. Rat-1/CM cells were maintained as previously described (30). For transient expression assays, 293T cells were transfected with a maximum of 5 μg of plasmid DNA/6-cm diameter dish by a calcium phosphate coprecipitation method with concurrent treatment with 25 μg chloroquine, essentially as described previously (27). 293T cells and Rat1 cells were also transfected using LipofectAMINE 2000 (Invitrogen) with a maximum of 3.5 μg of plasmid DNA/6-cm diameter dish. Cells were harvested 48 h after transfection. To monitor the effect of fibronectin on the interaction of the following molecules, we transfected Rat1 and Rat1/CM after 20 or 50 min, after their placement on fibronectin-coated dishes with a diameter of 6.0 cm. Cell lysates were prepared using protease inhibitors in TXB buffer (10 mM Tris (pH 7.6), 150 mM NaCl, 5 mM EDTA (pH 8.0), 10% glycerol, 1 mM Na4VO4, and 1% Triton X-100). Whole brain tissue from 5-week-old ICR mice was also lysed in TXB buffer with a Dounce homogenizer. Lysates were precleared by incubation with protein G-agarose (Roche Molecular Biochemicals) for 3 h at 4 °C. The monoclonal antibodies for the HA epitope tag, c-Myc, and nucleoporin p62 were from Berkeley Antibody (Richmond, CA), respectively. The monoclonal antibody for GST was from Calbiochem, and the monoclonal antibody for c-Myc was from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-mouse or anti-rabbit immunoglobulin conjugated or unconjugated with alkaline phosphatase and TRITC (tetramethylrhodamine isothiocyanate)-conjugated secondary antibodies were from DAKO (Copenhagen, Denmark).

Immunohistochemistry of Mouse Brain—Whole brain tissue from 5-week-old ICR mice was fixed with PBS containing 4% paraformaldehyde for 20 min at 25 °C and then permeabilized with Protein G-agarose for 4 h at 4 °C. Immunoprecipitates were extensively washed with TXB buffer, separated by SDS-PAGE (27).

In Vitro Binding Assay—293T cells lysates transiently expressing Tiam1 and the deletion mutants were prepared as described above. Glutathione S-transferase (GST)-c-Myc, GST deletion mutants of c-Myc, and GST were purified from Escherichia coli transformed with wild-type and deletion mutants of c-Myc cloned into pGEX-4T3 and pGEX-4T3 alone, incubated, and analyzed as described in Ref. 31.

Direct Binding Assay—The proteins of Tiam1(FL) and Tiam1(N392) were produced in in vitro reticulocyte lysate, by TNT quick-coupled translation/transcription systems (Promega, Madison, WI), according to the instructions from the manufacturer, and incubated with wild-type and deletion mutants of GST-c-Myc, and GST alone as described above. The solution was purified by gluthathione-Sepharose beads (Roche Molecular Biochemicals), separated by SDS-PAGE. The results were visualized and quantitated with a Bio-Imaging Analyzer (BAS1000, Fuji). c-Myc, NLS, Rbm, NLS, and C-terminal pleckstrin homology domain; wt, wild-type.

Anti-Tiam1 (Tiam1N) antibody for 1 h at 25 °C. The cells were then rinsed in PBS containing 1% bovine serum albumin. The cells were further incubated with a TRITC-conjugated secondary antibody for 1 h at 25 °C. Cells were examined with an Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY).

Luciferase Assay—293T cells in a 3.5-cm dish were transiently transfected with 0.02 μg of pRL-TK (Toyo Ink, Tokyo, Japan), 1 μg of p4x(WT)/E-SVP-Luc (a gift from H. Ariga, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan), and various plasmids described under "Results" using LipofectAMINE 2000 (Invitrogen). Two days after transfection, whole cell extracts were prepared and the luciferase activity caused by the reporter plasmid was determined by using the Pica Gene dual kit (Toyo Ink).

Apopitosis Assay—To induce apoptosis in Rat-1 cells and their transfectants, 2 days after transfection, the cells were seeded 24 h before serum deprivation and then transferred to medium containing 0.3% FBS unless otherwise indicated, after treatment or no treatment of transient transfection. The cells were judged to be apoptotic when they were nonadherent and showed typical nuclear changes (definite chromatin condensation and/or nuclear fragmentation) (30). After photography, the cells were harvested by trypsinization and fixed in PBS containing 4% paraformaldehyde for 20 min at 25 °C. The cells were stained with 4′,6-diamidino-2-phenylindole and observed as described in Ref. 33.

RESULTS

C-Myc Specifically Associates with Guanine Nucleotide Exchange Factor Tiam1—We explored the binding between c-Myc and a GEF of Rac1, Tiam1. First, to determine the location of the Tiam1 binding site in the c-Myc protein, we made truncated c-Myc cDNA constructs (Fig. 1a). Bacterially extracted GST fusioned wild-type (WT) and the truncated form of c-Myc, which were purified by glutathione-Sepharose beads, were incubated with the cell lysate of Tiam1(WT)-transfected 293T cells. In this in vitro condition, Tiam1(WT) could associate with some of these c-Myc constructs that contain the intact N-terminal MB II domain (Fig. 2a). We also prepared truncated Tiam1 cDNA constructs to determine the c-Myc-binding region
A. c-Myc specifically associates with the guanine nucleotide exchange factor Tiam1. A, 293T cells were transiently transfected with Tiam1(WT) construct. Bacterially purified GST-fusioned wild-type and truncated c-Mycs were incubated with the 293T cell lysates and affinity-precipitated (AP) by glutathione beads, according to the in vitro binding assay. Bound proteins were immunoblotted (IB) with anti-Tiam1C (lanes 1–6), which reacted with epitopes located at the C terminus of Tiam1. GST fusion proteins are shown at the bottom. 293T cell total lysates are shown in lane 7.

B. 293T cells were transiently transfected with Tiam1(WT), Tiam1(C1199), Tiam1(C682), and HA-tagged Tiam1(N392) constructs. Bacterially purified GST-fusioned wild-type c-Myc was incubated with the 293T cell lysates, according to the in vitro binding assay. Bound proteins were immunoblotted with anti-Tiam1C (lanes 1–3) and anti-HA (lane 4). GST-c-Myc is shown at the right.

C. Tiam1 Binds to c-Myc and Blocks c-Myc-mediated Apoptosis

D. 5134

E. 5134

F. 5134

Fig. 2. c-Myc specifically associates with the guanine nucleotide exchange factor Tiam1. A, 293T cells were transiently transfected with Tiam1(WT) construct. Bacterially purified GST-fusioned wild-type and truncated c-Mycs were incubated with the 293T cell lysates and affinity-precipitated (AP) by glutathione beads, according to the in vitro binding assay. Bound proteins were immunoblotted (IB) with anti-Tiam1C (lanes 1–6), which reacted with epitopes located at the C terminus of Tiam1. GST fusion proteins are shown at the bottom. 293T cell total lysates are shown in lane 7. B, 293T cells were transiently transfected with Tiam1(WT), Tiam1(C1199), Tiam1(C682), and HA-tagged Tiam1(N392) constructs. Bacterially purified GST-fusioned wild-type c-Myc was incubated with the 293T cell lysates, according to the in vitro binding assay. Bound proteins were immunoblotted with anti-Tiam1C (lanes 1–3) and anti-HA (lane 4). GST-c-Myc is shown at the right. Expressions of Tiam1s
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in Tiam1 (Fig. 1b). GST-c-Myc(WT) extracted from bacteria could associate only to the WT and N392 (aa 1–392) truncated mutants of Tiam1, but not to the C1199 and C682 mutants in the in vitro condition described above (Fig. 2b). These observations imply that MB II in the N-terminal domain of c-Myc and N-terminal 1–392 amino acids of Tiam1 may be the principal regions for binding in vitro with each other.

We next examined the Tiam1 association with c-Myc under another condition. Coexpression and coprecipitation analyses in 293T cells revealed that immunoprecipitated Tiam1(WT) could associate to c-Myc(WT) tightly in vivo, but not to the aa 143–355 truncated mutant of c-Myc, which could not associate to Tiam1(WT) in vitro (Fig. 2c). Conversely, immunoprecipitated c-Myc(WT) could also associate to Tiam1(WT), but c-Myc(143–355) could not (Fig. 2c).

Then we were interested in whether the other Rac1-GEFs, including α-Pix and Vav1, could associate to c-Myc in vivo. Immuno precipitated c-Myc(WT) overexpressed in 293T cells could specifically associate to Tiam1(WT), but not to the C1199 truncated mutant of Tiam1, other Rac1-GEFs and their mutants; the wild-type Vav1 and CH domain truncated α-Pix (Fig. 2d).

Next we tested the direct binding between Tiam1 and c-Myc by the in vitro translation and transcription reticulocyte lysate system (described under “Materials and Methods”). GST-fusion c-Myc(WT) could directly bind to in vitro transcribed Tiam1(N392) constructs, but GST fusion mutated c-Myc(143–355) and GST alone could not directly bind them (Fig. 2e).

Among native mouse tissues, Tiam1 has been reported to be highly expressed in the brain and the testis (14). The expression of c-Myc is also reported to be detected in the mouse brain and to be enhanced by methamphetamine-induced apoptotic processes in neurons (34). In the protein extracted from mouse brain, c-Myc was coprecipitated with immunoprecipitated Tiam1 by the specific Tiam1 antibody but was not coprecipitated by preimmune rabbit immunoglobulins (Fig. 2f). Despite the weak expression of c-Myc in the brain, we believe we could prove the considerable interaction between c-Myc and Tiam1 in the mouse brain.

Nuclear Localization of Tiam1—We next attempted to investigate the intracellular location of both proteins. A transcription factor c-Myc is known to be present in the nucleus (34). We tested whether Tiam1 is mainly present in the same location as c-Myc. First, we applied the immunohistochemical technique for the mouse brain tissue in which c-Myc and Tiam1 associated with each other (Fig. 2f). As shown in Fig. 3, the expression of c-Myc was mainly and diffusely located at the nucleus of the cerebral neuronal cells in the cortex and the basal ganglia by the c-Myc-specific antibody. The immunoreactivity of Tiam1 was also demonstrated in the nuclei of cerebral neuronal cells, but only in those of the thalamus, hypothalamus, and dentate nucleus (Fig. 3). That immunoreactivity disappeared by absorption with peptide used as the immunogen for the Tiam1-specific antibody (Fig. 3).

Previously, several investigators including us reported that an F-actin-containing membrane ruffle was observed at 15 min after stimulation (adherence of Rat-1 fibroblasts to the extracellular matrix, fibronectin) and a Tiam1 localization on that membrane ruffle was detected (27, 35). When Rat-1 cells cultured on uncoated slides were analyzed, we found that endogenous Tiam1 was demonstrated in the nucleus, but endogenous c-Myc was barely detectable by immunofluorescence with the specific antibody (Fig. 4, a and b). On the other hand, both endogenous Tiam1 and stably transected c-Myc were detected clearly and abundantly in the nucleus, when Rat-1/CM (Rat-1 cells stably transfected with c-Myc), were used in the same
conditions as Rat-1 cells (Fig. 4, d and e). A negative control against anti-Tiam1 antibody using preimmune rabbit immunoglobulins as the primary antibody revealed no immunoreactivity in both Rat-1 and Rat-1/CM (Fig. 4, c and f).

We next attempted to record the time-course profile of Tiam1 localization in Rat-1 and Rat-1/CM placed on fibronectin-coated slides. In this experiment, we observed immunofluorescence with a specific antibody in these cells after various periods from plating, as described under "Materials and Methods." In Rat-1 cells, endogenous Tiam1 was localized both in the nucleus and in the cell membrane ruffle at 30, 50, and 70 min after cells were placed on fibronectin (Fig. 4, g-i). However, by 90 min, endogenous Tiam1 gradually localized in the nucleus and disappeared from the membrane ruffle (Fig. 4j).
Rat-1/CM, endogenous Tiam1 was also localized both in the nucleus and in the cytoplasmic membrane ruffle at 50 and 70 min, and mainly in the nucleus but not in the membrane ruffle at 90 min after plating (Fig. 4, lane n). In Rat-1/CM, granular expression of endogenous Tiam1 in cytoplasm was detected but was weak in nucleus for the first 30 min (Fig. 4k). During the course of these experiments, c-Myc expression was constantly detected in the nucleus in Rat-1/CM (data not shown).

The influence of fibronectin stimulation on the interaction between c-Myc and Tiam1 was examined using a time-course experiment as described under "Materials and Methods." The in vivo binding between Tiam1 and c-Myc in Rat1/CM cells was detected at 50 min (Fig. 4p, lane 2) but not at 20 min (Fig. 4p, lane 1) after the plating on fibronectin.

To confirm these results, we examined and checked the intracellular localization of both Tiam1 and c-Myc in Rat-1 and Rat-1/CM by subcellular fractionation and Western blotting. We could also detect Tiam1 expression in the nuclear fraction, but weakly in the cytoplasmic fraction in both Rat-1 and Rat-1/CM (Fig. 4o). In contrast, c-Myc expression of Rat-1/CM was detected only in the nuclear fraction (Fig. 4o, lane 1). In Rat-1 cells, endogenous c-Myc was not detected by this method (Fig. 4o, lanes 4–6). In addition, stably overexpressed c-Myc did not affect the location of endogenous Tiam1 in Rat-1 cells in this subcellular fractionation assay. Tiam1 represses the transcriptional activity of c-Myc. To determine whether the association with Tiam1 affects the transcription properties of c-Myc, 293T cells were transiently transfected with the expression vectors harboring c-Myc and Tiam1 together with the reporter plasmid containing the luciferase gene linked to the tetramerized E-box sequence (4XE) followed by the SV40 promoter, using a cationic lipid (36). First, we examined the single luciferase activity of c-Myc or Tiam1. As shown in Fig. 5a, c-Myc stimulated the E-box-dependent luciferase activity. The relative luciferase activity of c-Myc is ~3 times that of pCS2 mock control vector (Fig. 5a, lane 3). On the other hand, Tiam1 showed no effect on the luciferase activity by transfection of Tiam1 with the reporter plasmid (Fig. 5a, lane 4). We next checked the influence of Tiam1 toward c-Myc luciferase activity. When 293T cells were transfected with c-Myc, pCS2 mock control vector and reporter plasmid, the luciferase activity was stimulated (Fig. 5b, lane 2), whereas the mock vector alone showed no effect on that activity (Fig. 5b, lane 1). The luciferase activity was enhanced by c-Myc, and was repressed by co-transfection of Tiam1 with c-Myc and the reporter plasmid, in a dose-dependent manner (Fig. 5b, lanes 4 and 5). Co-expression of N17Rac1, a dominant negative mutant of Rac1, under the same conditions as in Fig. 5b (lane 4) had no Tiam1-mediated suppression of the transcriptional activity of c-Myc (Fig. 5b, lane 3). In the case of Tiam1(C682), Vav1, and oncogenic Vav1, which is constitutive active mutant of Vav1, none of these constructs showed any effect on the c-Myc-enhanced luciferase activity (Fig. 5b, lanes 6–8). Because the Tiam1 deletion mutant (C682), which could not associate with c-Myc, did not affect the luciferase activity in this experimental system, and because another Rac1-GEF (here Vav1) did not, either, the repression activity of Tiam1 that we observed here is dependent on its binding capability to c-Myc and may be specific to Tiam1.

Tiam1 Interfered with c-Myc-mediated Apoptosis in Rat-1 Cells—As mentioned above, c-Myc has been implicated in the regulation of apoptosis in certain settings. To clarify the effect of Tiam1 on the apoptotic activity of c-Myc, we used Rat-1 cells and Rat-1/CM cells, which were stably expressed with c-Myc. In this experimental system, Rat-1/CM causes c-Myc-related apoptosis in a serum-deprived cultured medium (30). Beforehand, we checked the ratio of apoptotic cells in Rat-1 and Rat-1/CM. As previously reported, both Rat-1 and Rat-1/CM were cultured in 10 or 0.3% FBS-containing medium for 24 h, respectively (30), and we counted cells with apoptotic phenotypes. When Rat 1/CM were harvested in 10 and 0.3% FBS, approximately 8 and 46% cells showed apoptotic phenotypes, respectively (Fig. 6a, lanes 3 and 4). Rat-1 in 10% FBS grew normally and showed the low apoptotic ratio (Fig. 6a, lane 1). Rat-1 in 0.3% FBS exhibited a ratio of ~14% apoptotic cells (Fig. 6a, lane 2). These results showed c-Myc-induced apoptosis is related to the status of the serum deprivation.

Next, we examined whether Tiam1 interfered with the apoptosis inducing activity of c-Myc in this experimental system. Constructs of Tiam1(WT), Tiam1(C682), Vav1, or mock control vector were transiently transfected with or without N17Rac1 in Rat-1/CM by using cationic lipid (Fig. 6, b and c). We had already monitored the transfection efficiency and obtained the results that more than 90% of cells showed expression of the construct when Rat-1/CM cells were trans-
Fig. 6. **Tiam1 interfered with c-Myc-mediated apoptosis in Rat1 cells.** A, induction of c-Myc-mediated apoptosis by serum deprivation. Rat1 cells and Rat1/CM cells were seeded at a density of $1 \times 10^5$ cells in 60-mm dishes and cultured for 12 h in medium containing 10% FBS. The cells were transferred to the medium containing 10 or 0.3% FBS. The percentage of apoptotic cells was determined 24 h later as described under “Materials and Methods.” B–E, Rat1/CM cells were transiently transfected with 2 μg of GEFs and 1 μg of HA-tagged N17Rac1 or 1 μg of flag-tagged Tiam1.
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fected by this transfection protocol (data not shown). These transfected cells after serum deprivation for 24 h showed that the exogenous expression of Tiam1(WT) reduced the number of apoptotic cells (Fig. 6, b and c, lane 3), whereas Tiam1(C682) and Vav1, which could not bind to c-Myc, did not act on apoptosis compared with the mock control (Fig. 6, b and c, lanes 5 and 6). However, the expression of Tiam1(C1199) increased the apoptotic cell ratio compared with the mock control. In the case of transfection by Tiam1 together with N17Rac1, which is a dominant negative Rac1, the number of apoptotic cells also reduced (Fig. 6, b and c, lane 2). These results showed that Tiam1 inhibited the apoptotic activity of c-Myc that was dependent on the binding capacity of Tiam1 to c-Myc, whereas N17Rac1 showed no effect on this inhibition. On the other hand, Tiam1(C1199), which is known to be an active form for GEF activity and does not bind with c-Myc, had no effect on this inhibition (Fig. 6, b and c, lane 4). This observation probably reflects the induction of apoptosis by Tiam1(C1199) through the activated mitogen-activated protein kinase cascade by its dominant GEF activity for Rac1, not through c-Myc interaction.

Next, we examined whether the ectopic expression of the MBII domain of c-Myc, which binds Tiam1, could reverse the inhibition of c-Myc apoptosis observed upon the co-expression of Tiam1. We used a deletion mutant of c-Myc, c-Myc(1–142), which contains the MB II domain but not the nuclear localization signal, basic, HLH, and LZ domains. We expected that c-Myc(1–142) would bind to Tiam1, as shown in Fig. 2a, but would not be translocated to the nucleus. We transiently transfected both Tiam1(WT) and c-Myc(1–142) in a Rat-1/CM cell line and performed an apoptosis assay. When both Tiam1 and the mock control vector were transfected, the apoptotic activity of c-Myc was inhibited (by the interaction of Tiam1; Fig. 6, d and e, lane 1), but when both Tiam1 and c-Myc(1–142) were transfected, the apoptotic activity of c-Myc was only partially inhibited (Fig. 6, d and e, lane 2). We interpreted this finding as implying that the coexpression of excess c-Myc(1–142) partially rescued the Tiam1(WT)-mediated inhibition of c-Myc-induced apoptosis.

**DISCUSSION**

The mechanisms through which the effects of c-Myc mediate on the fate of cells (for example cell transformation or apoptosis) have been reviewed (37, 38). A broad body of work argues convincingly that c-Myc is a transcription factor, which activates and represses many target genes (39). However, a large number of studies have recently identified cellular factors that, through their interaction with c-Myc in cells, have provided a deeper insight into other possible functions. Many proteins that interact with the c-Myc N-terminal domain have, in particular, been identified, and their respective effects on various c-Myc functions have been analyzed, including transactivation, cell transformation, and apoptosis. As described in the Introduction, MB II seems to be the most crucial domain in N-terminal domain for several c-Myc functions, and thus identification of MB II-binding proteins has been thought to significantly advance our understanding of the mechanisms of c-Myc. Several MB II-binding proteins, which are indispensable to control several c-Myc functions, have in fact been identified, such as Bin1, TRRAP, Pam, and so on (reviewed in Ref. 6). Tiam1, on which we report here, is a new N-terminal domain and MB II-binding protein. As shown in the results, the direct interaction of Tiam1 and c-Myc induces repression of the transactivation and apoptotic activities of the latter.

The question may be raised whether Tiam1 can directly associate with c-Myc. Recently it was reported that Cdc24, a GEF for Cdc42 that is one of the Rho family GTPases (40, 41), was sequestered in the cell nucleus by the adapter protein Far1. It was then relocated to the cytoplasm by degradation of Far1 by the G1 cyclin-dependent kinase Cdc28-Cln at the actin cytoskeletal change, and by the importin β-family member Min5, which is required for nuclear export (40, 41). These reports demonstrated an important process; a GEF for the Rho-family GTPases could be sequestered in the nucleus and relocated in the cytoplasm by other proteins (41). Our observations of the Tiam1 localization we have presented in our study here remind us of the situation of Cdc24, whereby Tiam1 was mobilized to the cytoplasm in Rat-1 cells at the first response to contact with the extracellular matrix fibronectin, was recruited to both the membrane ruffle and nucleus during the next phase, and then was finally relocated to the nucleus (Fig. 4, g–n). There was no difference between Rat-1 and Rat-1/CM in that Tiam1 was present in the nucleus when plated on the uncoated slides (Fig. 4, b and c). On the contrary, the membrane ruffle localization of Tiam1 was detected as early as 30 min later in Rat-1 cells, whereas it takes 50 min in Rat 1/CM (Fig. 4, g–n). These phenomena may show that overexpression of c-Myc itself has no effect on the stable localization of Tiam1, but it affects the Tiam1 mobilization between the cytoplasm and the nucleus. c-Myc seems to slow down the Tiam1 mobility to the membrane ruffle and the cytoplasm from the nucleus, but we could not conclude at what point in this nuclear import system the c-Myc had its effect. At this moment, information about the nuclear localization of Rho-GTPases-GEFs is only available about Tiam1 and Cdc24. We could not find other endogenous Rac1 GEFs, Vav1 and α-Pix, expressed in the Rat-1 and Rat-1/CM nucleus by immunofluorescence when we examined them under the same experimental conditions as Tiam1 (data not shown). The nuclear localization of Tiam1 may be important in terms of enabling direct interaction with c-Myc. Specific interaction of c-Myc with Tiam1, but not with Vav1 and α-Pix, was validated by coexpression and coprecipitation in our in vivo association analysis (Fig. 2d). On the other hand, Tiam1 has recently been reported to interact with other proteins related to cell migration and cytoskeletal modification (27). However, until now, there has been no report that has proved any interaction between Tiam1 and any nuclear protein. We have been able to show for the first time the direct interaction of Tiam1 with the c-Myc nuclear protein. As shown in Fig. 4p, the attachment of the cells to the fibronectin changed the degree of binding between Tiam1 and c-Myc, probably by altering the localization of Tiam1. We think that these data are also consistent with the idea that the direct binding of these proteins takes place in the nucleus. Naturally, further investigation regarding the influences of cell attachment on apoptosis and Myc function is needed. We delineated the c-Myc-binding locus in Tiam1 as the N terminus of Tiam1 (aa 1–392). Although Tiam1 (aa 1–392) contains two PEST domains (aa 58–92 and 100–132), which are considered to be predictors of protein instability (14, 42) (Fig. 2, b and e), the presence of a c-Myc binding motif in Tiam1 should be elucidated in further experiments.

c-Myc is known to act as a transcription factor in its classical function. Many genes, in which c-Myc activates or represses...
expression, have been identified (reviewed in Refs. 37 and 43). As mentioned under expression, Rac1 and Rac1(N17Rac1) failed to relieve Tiam1-mediated inhibition of the transactivation and apoptosis inducing activities of c-Myc (Fig. 5b, lane 3), suggesting that Rac1, a known effector of Tiam1, may not be involved in the regulation of c-Myc functions by Tiam1.

In terms of the ultimate effector of the apoptotic function of c-Myc, many transactivated target genes have also been reported, including p53, ornithine decarboxylase, and cyclin A (44–47). Recently several genes indirectly induced by c-Myc during apoptosis have also been reported. The CD95/Fas ligand is additionally known as an indirect target gene of c-Myc. c-Myc controls apoptosis through regulation of CD95/Fas ligand expression in some cells, but not in the others (48). Along these lines, we monitored the expression of CD95/Fas ligand in both Rat-1 and Rat 1CM by reverse transcription-PCR, but we did not detect any change in expression (data not shown). Soucie et al. (49) also reported that c-Myc activates Bax and elicits cytochrome c release from mitochondria into the cytoplasm during apoptosis, and this effect is inhibited by Bcl-2. It is not, however, clear what kind of proteins were directly transactivated by c-Myc in this situation. In our results, Tiam1(WT) inhibited c-Myc-related apoptosis in Rat-1/CM cells, but the Tiam1 mutant and Vav1, which lacked any binding activity to c-Myc, could not modify c-Myc-induced apoptosis in some cells, but not in the others (48).

Acknowledgments—We acknowledge the generosity of Drs. D. Turc-Carel, K. Nakaya, T. Nagase, M. Shibuya, B. J. Meyer, and H. Ariga for providing the plasmids used in these experiments.

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