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Ras シグナル伝達系の活性化と遺伝子発現に関する研究

Studies on the intracellular signaling pathways and the gene expression regulated by Ras

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Contents

Abbreviations

EGF: epidermal growth factor

ERK: extracellular signal-regulated kinase

IPTG: isopropyl-1-thio-β-D-galactoside

JNK: c-Jun N-terminal kinase

LPA: lysophosphatidic acid

MAP kinase: mitogen-activated protein kinase

MEK: MAP kinase/ERK kinase

NLS: nuclear localization signal

PCR: polymerase chain reaction

RRM: RNA recognition motif

RT-PCR: reverse transcription PCR

Chapter 1.

General introduction

General introduction

GTP-binding protein functions as a molecular switch in intracellular signaling pathways. The GDP-bound form is an inactive conformation, and the GTP-bound form is an active conformation. The GDP-bound form is activated by the exchange of the bound guanine nucleotide from GDP to GTP. The exchange is stimulated with guanine nucleotide exchange factors (GEFs). The GTP-bound form of GTP-binding protein can interact with and activate its downstream molecules. The bound GTP is hydrolyzed into GDP and inorganic phosphate by the intrinsic GTPase activity with the aid of GTPase-activating proteins (GAPs), and the signal is turned off (Fig. 1) (Kaziro et al., 1991). GTP-binding proteins functioning in intracellular signal transduction pathways are grouped into two groups: heterotrimeric GTP-binding protein (G protein) and monomeric low-molecular weight GTP binding protein (small GTP-binding protein).

Heterotrimeric GTP-binding protein (G protein) consists of α, β, and γ subunits, and transduces the signals from seven-transmembrane receptors (STMRs) which are also called G protein-coupled receptors (GPCRs). The α subunit of G protein ($G\alpha$) is a regulatory subunit having a guanine nucleotide-binding domain and an intrinsic GTPase activity. The β and the γ subunits of G protein ($G\beta$ and $G\gamma$) tightly bind each other to form a heterodimer ($G\beta\gamma$) under the physiological conditions. The GDP-bound $G\alpha$ forms a

heterotrimeric complex with Gβγ. When GPCRs are activated by extracellular stimuli, the activated receptors induce the exchange of the guanine nucleotide on Gα, and the Gαβγ heterotrimer dissociates into the GTP-bound Gα and the Gβγ heterodimer. Each of the GTP-bound Gα and the Gβγ heterodimer activates the effector molecules such as adenylyl cyclase, phospholipase Cβ (PLCβ), and G protein-activated inwardly rectifying K^+ channel (GIRK) (Kaziro et al. 1991; Hepler and Gilman, 1992). The bound GTP is hydrolyzed into GDP by the intrinsic GTPase activity, in some cases with the aid of GAP. Recently, GAPs for Gα were characterized, and they are called regulators of G protein signaling (RGSs) (Fig. 2). RGSs enhance the hydrolysis of the GTP bound to certain types of Gα (Berman and Gilman, 1998).

The mammalian Gα cDNAs have cloned by the classical methods or polymerase-chain reaction (PCR), and are subdivided into 4 families (Gs., Gi, Gq and G12) based on the predicted amino acid sequences (Kaziro et al., 1991; Hepler and Gilman, 1992) (Fig. 3). Gαs stimulates adenylyl cyclases which increase intracellular cAMP concentration, and is a substrate for cholera toxin. Cholera toxin catalyzes the ADP-ribosylation of Gαs which induces the constitutive activation of Gαs. In pituitary tumors and thyroid adenomas, the point mutations of *Gαs* gene, *gsp*, were found (Landis et al., 1989; Lyons et al., 1990) (Table 1). The gene *gsp* encodes the constitutively active mutant of Gαs. In addition to *gsp*, the point mutations of *Gαi2* gene, *gip2*, were found in

tumors of adrenal cortex and ovary (Lyons et al., 1990) (Table 1). The gene *gip2* encodes the constitutively active mutant of Gαi2. Although it was shown that Gα of Gi family, excluding Gαt and Gαgust, can inhibit the activation of adenylyl cyclase, effector molecules of Gi family have not fully been characterized. Gαt and Gαgust activate cyclic nucleotide phosphodiesterase which decreases the intracellular concentration of cyclic-nucleotide. Gα of Gq family (Gαq) activates PLCβ which produces inositol triphosphate and diacylglycerol. In contrast to Gαs, Gαi, and Gαq, the effector molecules of Gα of G12 and G13 (Gα12 family) had not been characterized. However, recent studies revealed that p115RhoGEF, which has a GEF activity specific for Rho small GTP-binding protein, is regulated directly by Gα12 and Gα13 (Hart et al., 1998). p115RhoGEF contains the RGS box and Dbl homology (DH) domain. GTP-bound Gα12 and Gα13 can bind the RGS box of p115RhoGEF (Kozasa et al., 1998). The GTP-bound Gα13 activates Rho using the DH domain of p115RhoGEF. In contrast, the GTP-bound Gα12 competitively inhibits the Gα13-induced activation of p115RhoGEF (Hart et al., 1998). It is the first case in mammalian cells that the biochemical connections between a Gα-mediated signal and a small GTP-binding protein-mediated signal are revealed.

Small GTP-binding proteins expressed in mammalian cells are grouped into 5 groups: Ras family, Rho family, Rab family, Arf family, and Ran family. Mammalian Ras family consists of Ha-Ras, Ki-Ras, N-Ras, R-Ras, Ral,

Rap1A, Rap1B, Rap2, and Tc21 (Bos, 1997). Ha-, Ki-, and N-Ras proteins are well characterized. The genes Ha- and Ki-*ras* have been originally characterized as the cellular counterparts of v-*ras* genes encoded by the retroviruses that have been shown to induce sarcoma in rats or mice. The somatic mutations of these *ras* genes encoding the constitutively active mutants are frequently found in human tumors. For instance, in 90% of the pancreatic adenocarcinomas and in 50% of colon adenocarcinomas, the mutation of *ras* genes have been detected (Bos, 1989). Hence, Ras protien has been thought to function in certain mitogenic signaling pathways in mammalian cells. Many studies have revealed that Ras transmits the signals from tyrosine-kinase receptors, non-tyrosine-kinase receptors, and certain types of GPCRs to intracellular sigaling pathways (Kaziro et al., 1991; Bos, 1997). The GTP-bound Ras interacts with and activates various effector molecules including Raf, AF-6, Ral-GDS, and phosphatidylinositol-3 kinase (PI-3K). Raf kinase is a well characterized molecule, which activates the extracellular signaling-regulated kinase (ERK) cascade. However, the molecular mechanism of Ras-dependent activation of Raf is not fully understood, because the interaction between the GTP-bound Ras and Raf by itself is not sufficient to induce the activation of Raf. Thus, it has been suggested that additional Raf activators are required for the Ras-dependent activation (Bos, 1997). Recently, a protein factor for Ras-dependent Raf activation was partially purified from rat brain cytosol (Mizutani et al., 1998). In addition to the ERK cascade, the c-Jun N-terminal kinase (JNK) cascade was also activated by GTP-bound Ras through Rac small GTP-binding protein

(Minden et al., 1995; Nimnual et al., 1998). Activation of these mitogenic protein kinase cascades stimulates the transcription of early response genes, such as c-*fos* and c-*jun* (Minden et al., 1997), and induces cell proliferation or differentiation. In addition to the protein kinase cascades, Ras also activates the PI-3K/Akt pathway (Bos, 1997; Dudek et al., 1997; Kauffmann-Zeh et al., 1997). The activation of this pathway prevents cells entering an apoptotic processes.

In chapter 2 of this thesis, it is demonstrated that the expression of an oncogenic mutant of Gαi2 activates Ras and its downstream kinase cascades in Rat-1 fibroblast cells. In chapter 3, genes regulated by the activated Ras are analyzed by differential display.

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Table 1. Constitutively active mutations of Gα found in human tumors

Tumor type	Gαi2		Gαs	
	Tested	Mutation	Tested	Mutation
Pituitary adenoma				
GH	4	0	42	18
Prolactin	7	0	12	0
TSH	1	0	2	0
ACTH	2	0	7	0
Nonsecretor	2	0	3	0
Ovarian sex cord stromal tumors	10	3	6	0
Adrenal cortical tumor	11	3	9	0
Thyroid tumor	24	0	25	1
Other	197	0	201	0

This table is cited from the paper written by Lyons et al. (1990) wtih modifications.

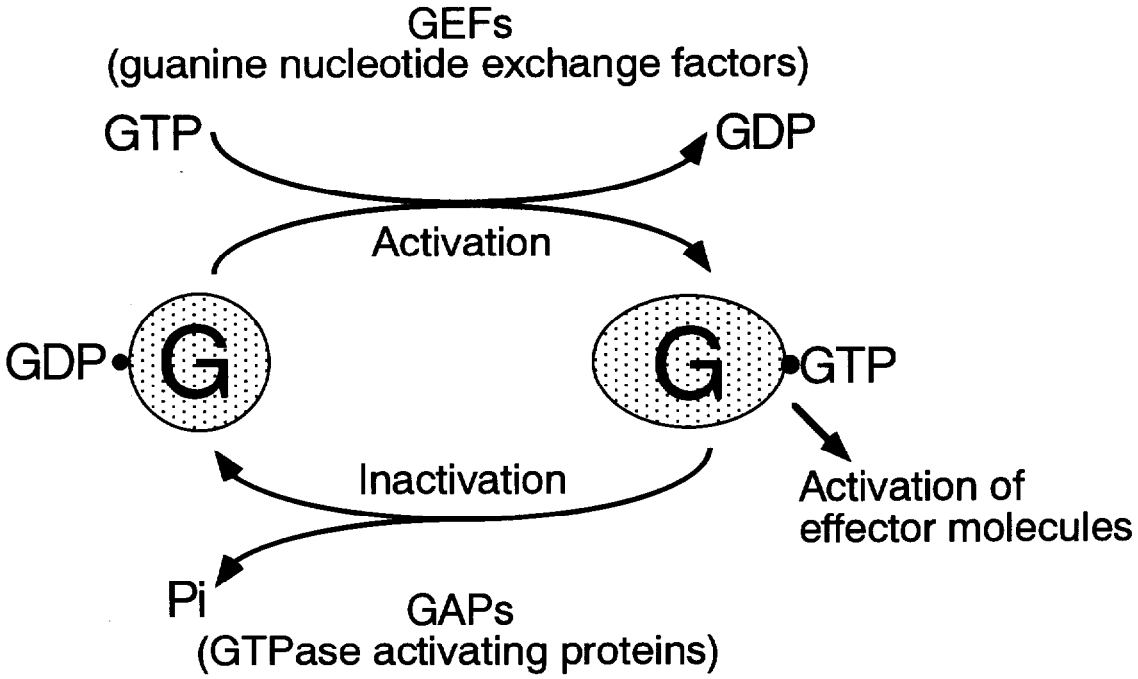

Fig. 1. Regulation of GTP-binding protein.

The activity of GTP-binding protein (G) is dependent on the bound ganine nucleotide. Guanine nucleotide exchange factors (GEFs) activate GTP-binding protein by exchanging the bound GDP with GTP. The GTP-bound GTP-binding protein activates the effector molecules. The bound GTP is hydrolyzed into GDP by an intrinsic GTPase activity with the aid of GTPase-activating proteins (GAPs).

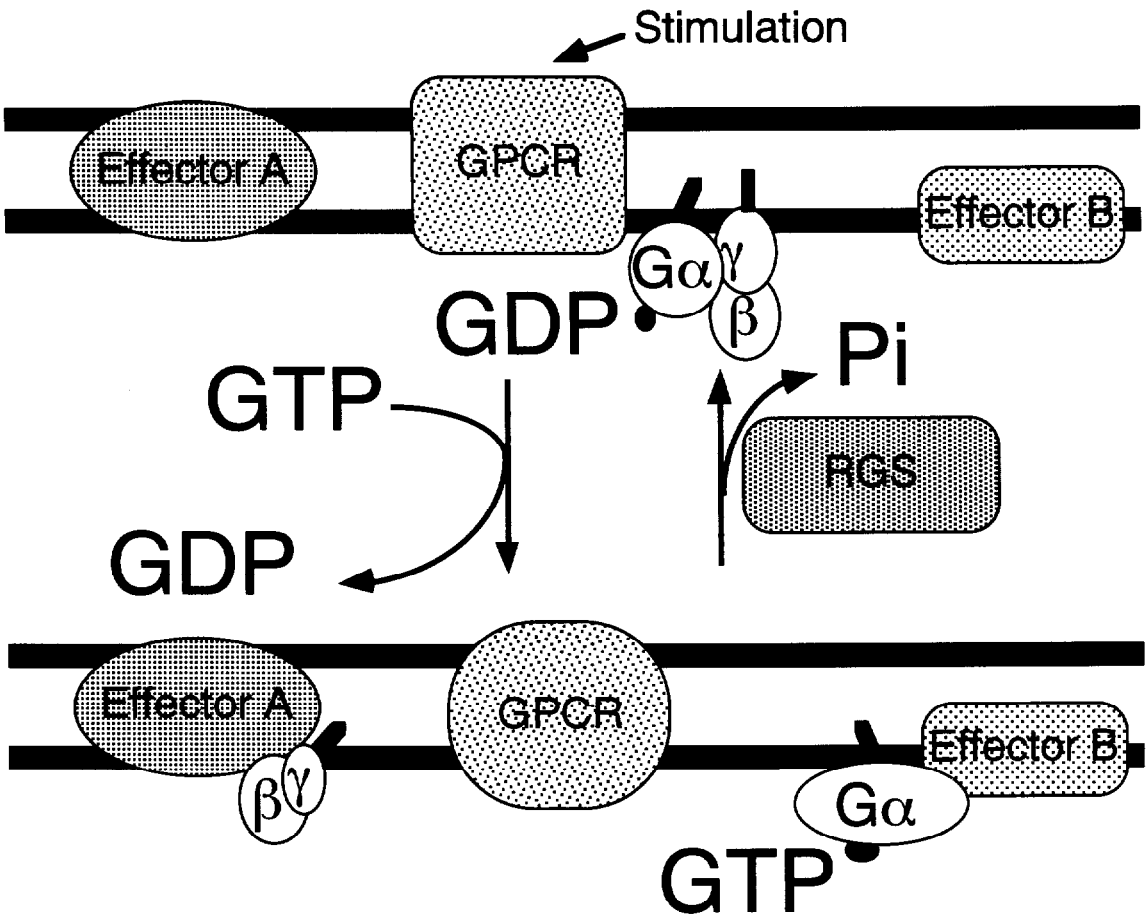

Fig. 2. Regulation of heterotrimeric GTP-binding protein (G protein) by receptors and RGS.

Heterotrimeric GTP-binding protein (G protein) is activated upon the stimulation with G protein-coupled receptors (GPCRs). Recent studies revealed that regulator of G protein signaling (RGS) functions as a GAP for Gα.

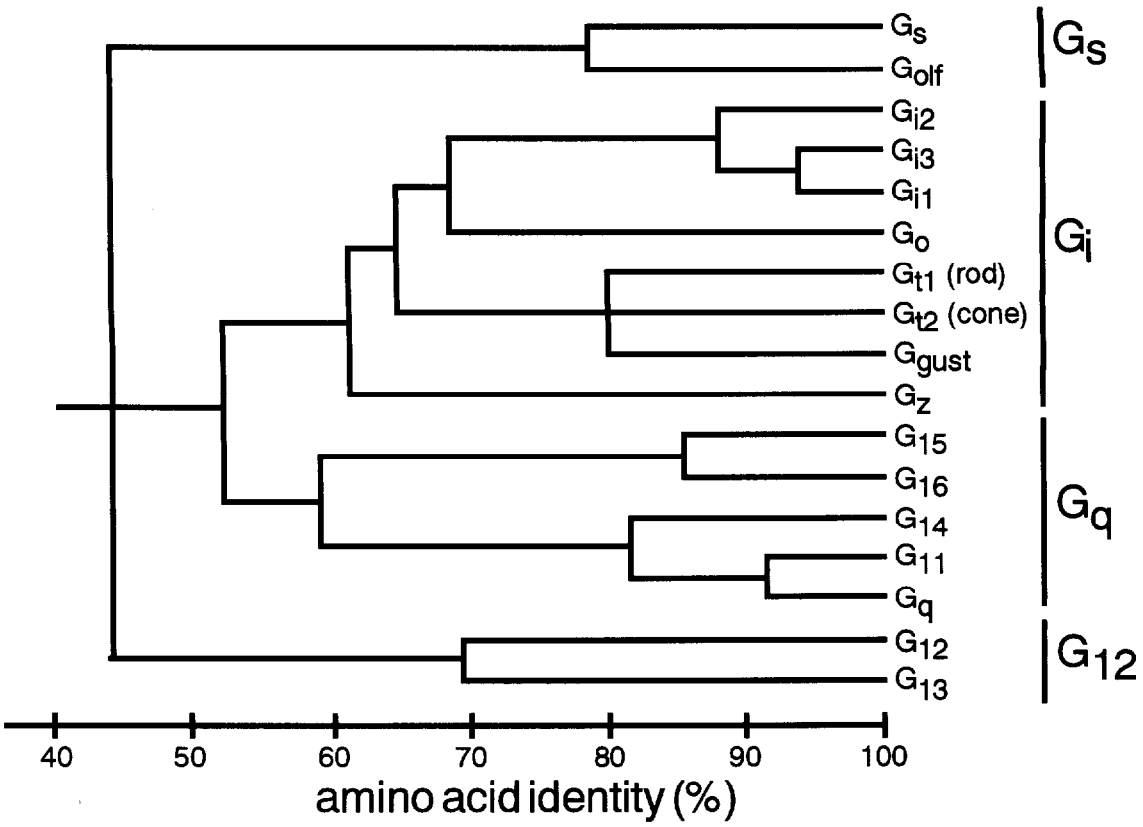

Fig. 3. Mammalian G protein α subunits.

About 20 mammalian Gα cDNAs have been cloned and subdivided into 4 groups based on the predicted amino acid sequences. It should be noted that 4 alternatively spliced variants of Gαs (Kozasa et al., 1988) and 2 variants of Gαo (Tsukamoto et al., 1991) are expressed in mammalian cells.

Chapter 2.

Expression of an oncogenic mutant Gαi2 activates Ras in Rat-1 fibroblast cells

Introduction

Heterotrimeric GTP-binding proteins (G proteins) play a key role in the intracellular signal transductions from the cell surface receptors which possess seven transmembrane domains. The α subunit of G proteins ($G\alpha$) has a guanine nucleotide-binding property and an intrinsic GTPase activity. The GDP-bound $G\alpha$ forms a complex with G protein $\beta\gamma$ subunits ($G\beta\gamma$). When the receptors are activated by stimuli, the activated receptors induce the exchange of the guanine nucleotide from GDP to GTP on $G\alpha$, and the $G\alpha\beta\gamma$ heterotrimer dissociates into the GTP-bound $G\alpha$ and the $G\beta\gamma$ heterodimer. The GTP-bound $G\alpha$ can interact with the effectors, such as adenylyl cyclase, phospholipase (PLC) β, and cGMP phophodiesterase. $G\beta\gamma$ can also interact with and regulate the effectors, such as certain types of adenylyl cyclases, PLC β and G protein-coupled inwardly rectifying K^+ channel (GIRK) (Kaziro et al., 1991; Hepler and Gilman, 1992). In addition, it has been reported that $G\beta\gamma$ can activate Ras-regulated mitogenic signaling cascades (Crespo et al., 1994; Faure et al., 1994; Koch et al., 1994; Ito et al., 1995).

In certain human tumor cells, GTPase-deficient mutations of $G\alpha$ which form a constitutively active form have been found. Mutation of the *$G\alpha s$* gene (designated *gsp* oncogene) was found in pituitary tumors and thyroid adenomas (Landis et al., 1989; Lyons et al., 1990). The *gsp* product constitutively activates adenylyl cyclase and elevates the intracellular cAMP

level. Mutation of the *Gαi2* gene (designated *gip2* oncogene) was found in tumors of adrenal cortex and ovary (Lyons et al., 1990). The *gip2* product inhibits cAMP accumulation (Wong et al., 1991). Expression of the mutant Gαi2 in Rat-1 cells induces transformation (Pace et al., 1991; Gupta et al., 1992b).

To clarify the mechanism of the Gαi2-mediated cell proliferation, several groups have investigated intracellular mitogenic signals and transcriptional regulation of immediate early genes. In Rat-1 cells expressing the constitutively active Gαi2, extracellular signal-regulated kinase (ERK) and its activator, MAPK/ERK kinases (MEK), are activated (Gardner et al., 1993). Microinjection of the antibody that interferes the function of Gαi2 inhibits the lysophosphatidic acid (LPA)-induced activation of c-*fos* promoter in mouse fibroblasts (Chuprun et al., 1997). In addition, transfection of the constitutively active Gαi2 activates c-*fos* promoter in CHO and Rat-1 cells (Ikezu et al., 1994), and enhances the activity of the collagenase promoter which is regulated by AP-1 in a c-Jun-dependent manner in Rat-1 cells (Gupta et al., 1992a). However, the upstream molecules of the MAP kinases or of AP-1 have not yet been determined.

To study the mitogenic signals mediated by Gαi2, a Rat-1 transfectant , which expresses an oncogenic mutant of Gαi2 (Gαi2(Q205L)) in an isopropyl-1-β-thio-D-galactoside (IPTG)-dependent manner, was constructed. In this

transfectant, Ras was activated by expression of Gαi2(Q205L). Moreover, activation of c-Jun N-terminal kinase (JNK) was found.

Materials and Methods

Plasmids

A plasmid pEF-LAC-Gi2(Q205L) was constructed by insertion of cDNA of rat Gαi2 (Itoh et al., 1986) whose glutamine-205 was substituted for leucine into the *Not* I site of pEF-LAC (Edamatsu et al., 1997). A plasmid pOPRSVI-Ras(V12) was constructed by insertion of cDNA of c-Ha-Ras whose glycine-12 was substituted for valine (Satoh et al., 1988) into the *Not* I site of pOPRSVI (Stratagene). A lactose repressor expression vector p3'SS was obtained from Stratagene. An expression vector for wild type c-Ha-Ras, pCMV5-Ras(WT) (Ito et al., 1995), was obtained from T. Satoh.

Glutathione *S*-transferase (GST)-fusion protein

GST-fused c-Jun (1-223) was produced as described elsewhere (Yamauchi et al., 1997). GST-fused c-Raf-1 Ras-binding domain (GST-RBD) produced in *E.coli* was provided by K. Tago.

Cell culture and stable transfectants

Rat embryonic fibroblast Rat-1 cells were grown in Dulbecco's modified Eagle's (DME) medium supplemented with 10% fetal calf serum (FCS). Rat-1 cells were transfected with 4 μg of pEF-LAC-Gi2(Q205L) and 16 μg of p3'SS (for Rat-1•QL112), or 10 μg of pOPRSVI-Ras(V12) and 10 μg of p3'SS (for Rat-1•RasValA1) by the calcium phosphate coprecipitation technique. Stable transfectants were selected with 1 mg/ml G418 and 0.4 mg/ml hygromycin B.

Analysis of the protein expression

Membrane proteins were prepared and analyzed by immunoblotting using a rabbit anti-Gαi1/2 antibody (AS/7, NEN Life Science) or a rabbit anti-Ras antibody. To detect Gαi2(Q205L), trypsin resistance assay was performed. Membrane proteins (20 μg) were solubilized in 8 μl of buffer (20 mM HEPES-NaOH (pH 8.0), 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 0.64% (w/v) Luburol PX). After the solubilized proteins were incubated with 111 μM GTP at 30°C for 30 min, TPCK-treated trypsin (Sigma) was added (200 μg/ml) and incubated at 30°C for 5 min. The reactions were terminated by adding trypsin-chymotrypsin inhibitor (2.2 mg/ml, Sigma) and Laemmli-sample buffer. The samples were analyzed by immunoblotting.

Colony formation in soft agar

Cells (3 x 10^4 cells) were seeded into the 60-mm dishes with DME medium supplemented with 0.27% agar and 10% FCS over the DME medium containing 0.53% agar and 10% FCS. After the cells were cultured for 12 days in the absence or presence of 5 mM IPTG, microphotographs were taken.

Analysis of Ras•GTP

The Ras-bound guanine nucleotide was analyzed as described previously (Ito et al., 1995; Satoh et al., 1995). An assay for Ras•GTP by determining the association between Ras•GTP and Ras-binding domain of c-Raf-1 *in vitro* was

performed as described elsewhere (de Rooij and Bos, 1997) with minor modifications. In brief, cells were transfected with 5 μg of pCMV5-Ras(WT) using Lipofectamine (GIBCO-BRL). After transfection, the cells were incubated with or without 5 mM IPTG for 24 h in the absence of serum. After stimulated with agonists, the cells were harvested and lysed with Ras•GTP IP buffer (50 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 150 mM NaCl, 0.5% Nonidet P-40, 20 μg/ml aprotinin, 1 mM Na_3VO_4). Ras•GTP associated with GST-RBD was collected with Glutathione-Sepharose 4B beads (Amersham-Pharmacia) and analyzed by immunoblotting with an anti-Ha-Ras antibody (Santa Cruz, sc-520).

ERK2 and JNK1 assays

In vitro kinase activity of ERK2 or JNK1 was assessed by *in vitro* kinase assay as described previously (Minden et al., 1995; Terada et al., 1997; Yamauchi et al., 1997). Phosphorylation of ERK2 was assayed by electrophoretic mobility shift as described previously (Ito et al., 1995).

Results

IPTG-induced expression of Gαi2(Q205L) in Rat-1 transfectants

To explore the function of Gαi2 on the mitogenic signaling pathways, we constructed Rat-1 transfectants which express Gαi2(Q205L) inducibly with IPTG treatment. Immunoblot analysis using an antibody for Gαi shows that endogenous Gαi is abundant and it is difficult to detect the IPTG-induced expression of the exogenous Gαi2(Q205L) (Fig. 1A). It has been reported that GTP-bound form of Gα is substantially protected from tryptic proteolysis (Hurley et al., 1984). To confirm the expression of Gαi2(Q205L), tryptic proteolysis of Gαi was performed. The exogenous Gαi2(Q205L) was cleaved into two fragments (2- and 38-kDa fragments) by trypsin, whereas the endogenous wild type Gαi was completely digested under the same conditions. The 38-kDa tryptic fragment of Gαi was detected by the anti-Gαi antibody for the C-terminus region of Gαi protein. Figure 1B shows that Gαi2(Q205L) was accumulated in one transfectant, designated Rat-1•QL112. Another type of Rat-1 transfectant, designated Rat-1•RasValA1, was also prepared. In this transfectant, constitutively active mutant Ras (Ras(G12V)) was accumulated after IPTG treatment (data not shown).

Colony formation of Rat-1 transfectants in soft agar

It has been reported that transfection of the mutationally activated Gαi2 mutants (R179C, R179H, and Q205L) induces an anchorage-independent growth of Rat-1 cells (Pace et al., 1991; Gupta et al., 1992b). To test the transforming activity of the inducibly expressed Gαi2(Q205L), colony formation in soft agar was assayed. Parental Rat-1 cells and their transfectants, Rat-1•QL112 and Rat-1•RasValA1, were seeded into soft agar and treated with IPTG. The parental cells did not form any remarkable colonies in the soft agar (Fig. 2A, B). On the other hand, IPTG treatment induced the colony formation of Rat-1•QL112 cells (Fig. 2C, D). However, the size of colonies of Rat-1•QL112 was smaller than that of Rat-1•RasValA1 (Fig. 2D, F). This experiment shows that, in Rat-1•QL112 cells, inducibly expressed Gαi2(Q205L) has a transforming activity and elicits certain mitogenic signals.

Ras activation in Gαi2(Q205L)-expressed Rat-1 cells

Ras is activated by various growth factors, cytokines, and certain types of G protein-coupled receptor agonists, and plays a key role in mitogenic signaling cascades (Kaziro et al., 1991; Bos 1997). To evaluate the activity of Ras in Gαi2(Q205L)-expressed Rat-1 cells, Ras-bound guanine nucleotide was assayed. Rat-1•QL112 cells were labeled with ^{32}P, and treated with IPTG in the serum-free medium. Ras was immunoprecipitated, and the bound guanine-nucleotide was analyzed. As shown in Fig. 3A, the ratio of GTP versus GTP+GDP in the anti-Ras immunoprecipitate was increased after the

cells were treated with IPTG for 24 h, or stimulated with epidermal growth factor (EGF) for 5 min. To confirm the Ras activation by expression of Gαi2(Q205L), the association of Ras•GTP with the Ras-binding domain (RBD) of c-Raf-1 was analyzed (de Rooij and Bos, 1997). Since the expression level of endogenous Ras is very low, exogenous Ha-Ras was transfected into Rat-1•QL112 cells. A small amount of Ras•GTP from the serum-deprived control cells was precipitated with GST-RBD *in vitro* (Fig. 3B). Treatment of the cells with EGF or LPA increased the complex formation of Ras and GST-RBD (Fig. 3B). In addition, IPTG treatment increased the amount of Ras precipitated from the lysates of Rat-1•QL112 cells (Fig. 3B), but not of parental Rat-1 cells (data not shown). These results indicate that Ras is activated in the Gαi2(Q205L)-expressed Rat-1 cells.

Activation of c-Jun N-terminal kinase (JNK) 1 in Gαi2(Q205L)-expressed Rat-1 cells

It has been reported that constitutive activation of Ras induces the activation of c-Jun N-terminal kinase (JNK) (Minden et al., 1994). In Rat-1•RasValA1 cells, IPTG treatment stimulated JNK1 activity (data not shown). To investigate whether the expression of Gαi2(Q205L) in Rat-1 cells induces JNK activation, *in vitro* kinase assay was performed. Rat-1•QL112 cells were starved of serum and treated with or without IPTG. Then, JNK1 was immunoprecipitated from the cell lysate, and *in vitro* kinase activity was assayed. As shown in Fig. 4A, JNK1 activity was enhanced in IPTG-treated

Rat-1•QL112 cells. Extracellular signal-regulated kinase (ERK) 2 was also activated as reported previously (Gardner et al., 1993) (Fig. 4B). Moreover, phosphorylation of ERK2 was detected after IPTG treatment of the cells for 12 h (Fig. 4C). It was suggested that long term activation of an ERK cascade might induce JNK activation through secretion of extracellular mediator (Minden et al., 1994). A recent study showed that the activation of an ERK cascade induces the JNK activation through the induction of heparin-binding epidermal growth factor (HB-EGF) (MaCarthy et al., 1995). To examine the possibility of the ERK-dependent JNK activation, Rat-1•QL112 cells were treated with a MEK1 inhibitor, PD98059 (Alessi et al., 1995; Dudley et al., 1995; Pang et al., 1995). Figure 4 shows that Gαi2(Q205L)-induced JNK1 activation was not inhibited by PD98059, whereas ERK2 activation was completely inhibited. These results indicate that the active Gαi2 activates JNK1 in an ERK-independent manner.

Discussion

This study shows that expression of the constitutively active mutant of Gαi2 induces activation of Ras in Rat-1 cells. This result suggests that Ras may be a key molecule in the Gαi2-mediated mitogenic signaling pathways. However, the mechanism of Ras activation by Gαi2 remains to be elucidated. Ras activation is regulated by the activation of Ras-guanine nucleotide exchanging factors (GEF) and/or the inhibition of Ras-GTPase activating proteins (GAP). It has been reported that oncogenic Gαi2-induced transformation of Rat-1 cells was not inhibited by the expression of dominant negative Ras (Ras(S17N)) which inhibits Ras activation by trapping GEFs in an inactive complex (Gupta et al., 1992b). In neutrophils, f-Met-Leu-Phe (FMLP)-induced Ras activation is associated with inhibition of p120-GAP activity (Zheng et al., 1997). These results suggest that Gαi2-induced Ras activation might be mediated by the inhibition of GAPs rather than by the activation of GEFs.

In Rat-1•QL112 cells, JNK1, as well as ERK2, was activated by induction of Gαi2(Q205L) (Fig. 4). It was previously reported that the long term activation of Raf and ERK resulted in the activation of JNK through secretion of HB-EGF (MaCarthy et al., 1995). However, the JNK1 activation was not inhibited by MEK1 inhibitor, PD98059 (Fig. 4A), whereas ERK2 activation was completely inhibited (Fig. 4B). The culture supernatant from

the IPTG-treated Rat-1•QL112 cells had no activity to induce JNK1 activation, whereas the culture supernatant from the IPTG-treated Rat-1•RasValA1 induced the JNK1 activation (data not shown). These results indicate that Gαi2-induced JNK1 activation is independent on ERK2 activation. It was previously reported that, in Rat-1 cells, Gαi2-induced activation of collagenase promoter which contains AP-1 binding sites was in a c-Jun-dependent manner (Gupta et al., 1992a). Present results suggest that this AP-1 activation may be mediated by the Gαi2-induced activation of JNK1. Recent studies have shown that Jun activity is essential for the transformation induced by oncoproteins, such as Ras and Bcr-Abl (Raitano et al., 1995; Johnson et al., 1996). Hence, activation of JNK, as well as activation of ERK, may be critical for the induction of the transformation of Rat-1 cells by constitutively active Gαi2.

Present results obtained from the IPTG-induced expression of Gαi2 revealed that Ras and JNK1 are activated in Gαi2-transformed Rat-1 cells. It was suggested that Gαi2, in addition to Gβγ, may activate Ras-mediated signaling cascades upon receptor activation. The activation of Ras and JNK1 may be cell-type dependent. Indeed, *gip2* mutations have been detected only in tumors of adrenal cortex and ovary (Lyons et al., 1990), whereas *ras* mutations have been detected in various types of human tumors (Bos, 1989). Clarification of the mechanism of Gαi2-mediated regulation of Ras activity should provide important findings that explain tumor induced by *gip2*

mutations and cell proliferation regulated by Gαi2.

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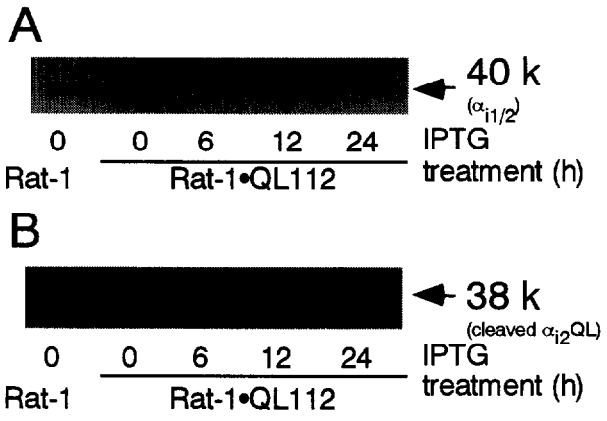

Fig. 1. IPTG-induced expression of Gαi2(Q205L) and Ras(G12V) in the Rat-1 transfectants.

(A) Immunoblot analysis of the crude membrane of Rat-1•QL112 cells. The cells were treated with 5 mM IPTG for the indicated hours and the membrane protein was immunoblotted with an anti-Gαi antibody (AS/7). (B) Tryptic proteolysis of Gαi. The membrane protein was treated with trypsin. The 38-kDa tryptic fragment was detected with AS/7 antibody.

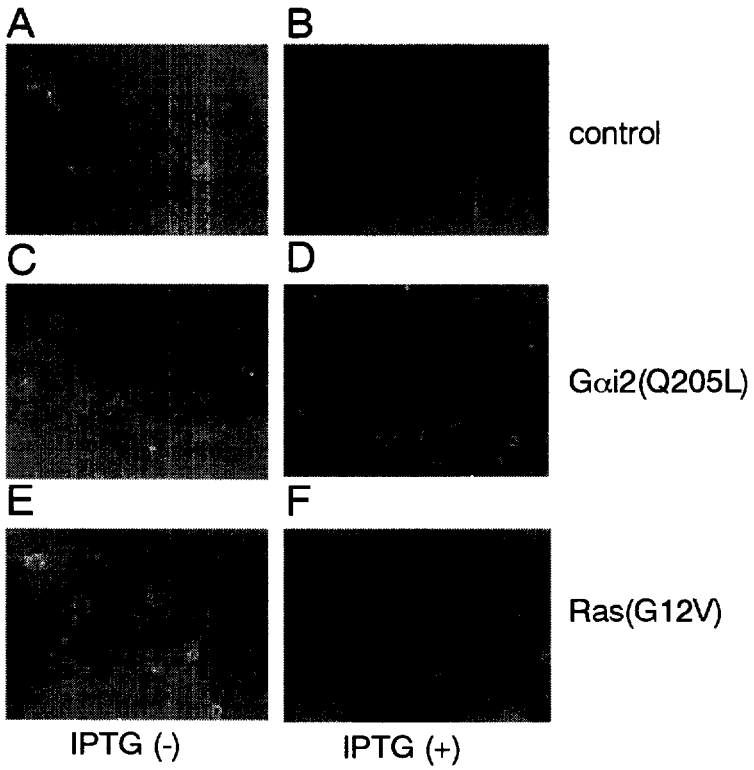

Fig. 2. Anchorage-independent growth of Rat-1 transfectants in soft agar.

Parental Rat-1 (A and B), Rat-1•QL112 (C and D), and Rat-1•RasValA1 (E and F) cells were seeded in 0.27% soft agar over 0.53% bottom agar without (A, C, and E) or with 5 mM IPTG (B, D, and F). Microphotographs were taken after culture for 12 days.

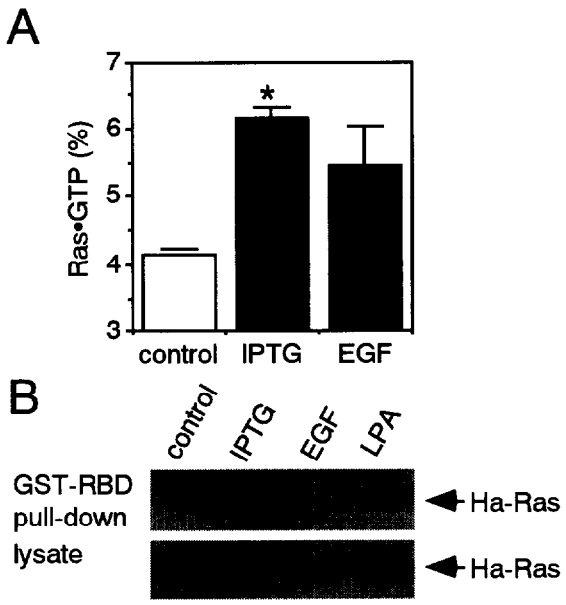

Fig. 3. IPTG-Induced Increase of GTP-bound Ras in Rat-1•QL112.

(A) Increase of Ras-bound GTP in Rat-1•QL112 cells. Confluent culture of Rat-1•QL112 cells was starved of serum and labeled with ^{32}P. The cells were treated with 5 mM IPTG for 24 h, or stimulated with 100 ng/ml EGF for 5 min. The Ras•GTP (%) was determined (GTP/(GDP+GTP) (%)). The bars show the mean ±S.E. of three to five independent experiments. An asterisk indicates $p<0.01$ as compared with the control. (B) *In vitro* association of Ras•GTP with c-Raf-RBD. Rat-1•QL112 cells were transfected with pCMV5-Ras(WT), and starved of serum. The cells were treated with 5 mM IPTG for 24 h, or stimulated with 100 ng/ml EGF or 100 µM LPA for 5 min. Ras•GTP associated with c-Raf-RBD (GST-RBD) was analyzed by immunoblotting with an anti-Ha Ras antibody.

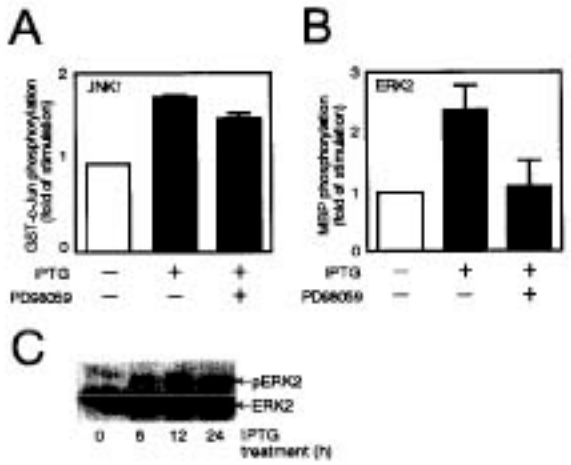

Fig. 4. Activation of JNK1 and ERK2 in the Gαi2(Q205L)-expressed cells.

Rat-1•QL112 cells were starved of serum, and treated with 5 mM IPTG in the presence or absence of 50 μM PD98059 for 36 h. *In vitro* kinase activity of JNK1 (A) and ERK2 (B) was assayed. The results are shown as the mean ±S.E. from three independent experiments. (C) ERK2 phosphorylation by expression of Gαi2(Q205L). Rat-1•QL112 cells were treated with 5 mM IPTG for the indicated hours. The phosphorylated ERK2 (pERK2) was detected by immunoblotting using an anti-ERK2 antibody.

Chapter 3.

Cloning and characterization of the oncogenic Ras-regulated gene that encodes an RNA-binding protein

Introduction

Proliferation of mammalian cells is controlled by various mitogenic signaling pathways. Ras is one of the key regulators which function as molecular switches of the mitogenic signaling pathways. The mammalian *ras* genes have been originally characterized as the cellular counterparts of v-*ras* genes encoded by the retroviruses that have been shown to induce sarcoma in rats or mice. They belong to a family of the genes encoding GTP-binding proteins. Generally, GTP-binding proteins are grouped into two groups: heterotrimeric GTP-binding proteins (G proteins) and low-molecular weight monomeric GTP-binding proteins (small GTP-binding proteins). Ras protein belongs to thefamily of small GTP-binding protein. The activity of GTP-binding proteins depends on the bound guanine nucleotide. The GTP-bound form is an active conformation which can bind and activate the downstream molecules, whereas the GDP-bound form is an inactive conformation. The bound GTP is hydrolyzed into GDP by the intrinsic GTPase activity with the aid of GTPase-activating proteins (GAPs) (Barbacid, 1987; Kaziro et al., 1991; Lowy and Willumsen, 1993).

In many tumors, mutations of *ras* gene have been found. These mutations inactivate the intrinsic GTPase activity and result in the constitutive activation of Ras protein (Bos, 1989; Bos, 1997). In addition to the mutation, it is also found that the expression of Ras protein is increased in tumors due to the activation of *ras* gene allele by insertion of a viral long terminal repeat (George et al., 1986; Westaway et al., 1986).

Constitutive activation of Ras protein without mutations has been detected in the oncogene-transformed fibroblast cells. For instance, in Bcr-Abl-transformed cells, Ras is constitutively activated (Puil et al., 1994). Recently, I reported the constitutive activation of Ras and Ras-regulated kinase cascades in Rat-1 cells transformed with an oncogenic mutant of Gαi2 (*gip2* oncogene product) (Edamatsu et al., 1998). These results suggest that constitutive activation of Ras may induce deregulated cell growth.

Ras activation induces the expression of early response genes such as c-*fos* and c-*jun* (Bos, 1997), and alters the set of the genes expressed in the cells. These gene products are involved in the regulation of cell proliferation. Thus, it is important to study the genes regulated by the Ras-mediated signaling cascades for understanding regulation of cell growth or tumorigenesis.

To study these genes, cells expressing an oncogenic mutant (G12V mutant) of Ras protein after treatment with isopropyl-1-β-thio-D-galactoside (IPTG) have constructed (Edamatsu et al., 1998). Messenger RNA was isolated from the cells before and after the IPTG treatment and subjected to differential display. Differential display is a method based on reverse transcription (RT) polymerase chain reaction (PCR), and is useful for making a finger print of the genes expressed in the cells and for the differential cloning (Liang and Pardee, 1992). Utilizing differential display, genes whose expression is up-regulated or down-regulated were isolated and analyzed. In this study, it was found that one of the down-regulated genes is a rat

homologue of a putative tumor suppressor gene, *LUCA15*, which is mapped on the short arm of human chromosome 3 (Wei et al., 1996). This region has been known as the tumor suppressor gene allele for lung, breast, and other cancers (Fondon III et al., 1998). In this chapter, the characterization of the *LUCA15* gene product, LUCA15, and the growth suppressing activity of LUCA15 in a human sarcoma cell line, HT1080 are described.

Materials and Methods

Cell culture

Rat embryonic fibroblast Rat-1 cells were maintained in Dulbecco's modified Eagle's (DME) medium (Sigma) supplemented with 10% fetal calf serum (FCS). Rat-1 transfectants (Rat-1•RasValA1, A2, and A5 cells) were prepared by the transfection with pOPRSVI-Ras(V12) and p3'SS (Stratagene), and maintained in DME medium supplemented with 10% FCS, 1 mg/ml G418 and 0.4 mg/ml hygromycin B (Edamatsu et al., 1998). Human sarcoma HT1080 cells (Raheed et al., 1974) were obtained from Health Science Research Resources Bank (HSRRB, Osaka, Japan), and maintained in DME medium supplemented with 10% FCS. Human embryonic kidney (HEK) 293 cells were maintained in DME medium supplemented with 10% FCS.

Total RNA

Total RNAs of cultured cell lines were extracted with guanidine thiocyanate and purified by the CsCl step gradient. Total RNAs of human breast cancer tissues were kind gifts from Dr. Lev Berstein (N. N. Petrov Research Institute of Oncology).

Oligo DNA primers

Oligo DNA primers used in this study were synthesized by OLIGO 1000 DNA synthesizer (Beckman). The sequences of the primers are listed in Table 1.

Differential display

Differential display was performed as described elsewhere (Liang and Pardee, 1992; Liang et al., 1994; Linskens et al., 1995) with minor modifications. First-strand cDNA was synthesized as follows. Total RNA (2.5 μg) was annealed with 50 pmol of an anchor primer (Table 1). Then, first-strand cDNA was synthesized with 200 U of SuperScript II reverse transcriptase (GIBCO-BRL) in 20 μl of 1 x first-strand buffer (50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$) containing 125 μM dNTPs and 10 mM DTT at 25°C for 10 min, followed by 42°C for 50 min. The reaction was terminated by inactivation of the reverse transcriptase at 70°C for 15 min. TE buffer (80 μl) was added and 2 μl of the cDNA solution was used for polymerase chain reaction (PCR). PCR was performed in 20 μl of 1 x PCR buffer with Mg^{2+} (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$) containing 1 U of recombinant Taq DNA polymerase (Takara syuzo), 7.5 μM non-radio-labeled dNTPs, radio-labeled dCTP ([α-^{32}P]dCTP, 74 kBq, 111 TBq/mmol, ICN), 10 pmol of an anchor primer (Table 1) and 10 pmol of an arbitrary primer (Table 1). The PCR was performed by PTC-100™ programmable thermal controller (MJ Research Inc.) at 94°C for 3 min, 37°C for 1 min, 72°C for 2 min for one cycle, at 94°C for 45 sec, 37°C for 1 min, 72°C for 2 min for three cycles, at 94°C for 45 sec, 55°C for 45 sec, 72°C for 2 min for 21 cycles, followed by a chase at 72°C for 5 min for one cycle. The PCR products (3 μl) were mixed with 2.5 μl of stop solution (95% formamide, 20 mM EDTA, 0.05%

Bromophenol Blue, 0.05% Xylene Cyanol) and denatured at 90°C for 3 min. Aliquots of the PCR samples (4 μl) were electrophoresed on a urea-denatured sequencing gel (6%). The gel was dried and exposed to X-lay films (NEN or Kodak). The bands of interest were cut out, and incubated in 50 μl of sterilized water at 60°C for 10 to 20 min to elute DNA fragments. The eluate was subjected to the reamplification of the DNA by PCR. The PCR was performed with the same primer pairs and recombinant Taq polymerase at 94°C for 1 min, 55°C for 1 min, 72 °C for 2 min for 25 cycles, followed by a chase at 72°C 7 min. The reamplified DNA was purified by agarose gel electrophoresis, digested with *Hind* III, subcloned into the *Hind* III site of pBluescript II SK (-) (Stratagene), and sequenced.

DNA sequencing

For DNA sequencing, DNA fragments were subcloned into appropriate cloning sites of pBluescript II SK (-) (Stratagene). DNA sequences were determined by using Thermo Sequenase™ cycle sequencing kit (Amersham Pharmacia) and an automated DNA sequencer (model 4000L, Li-Cor).

Digoxigenin (DIG)-labeled DNA probe synthesis

DIG-labeled DNA probes were synthesized as follows. To obtain the mouse β-actin probe, 1 μg of the 1.1-kb fragment of the mouse β-actin cDNA was prepared and labeled with DIG by Klenow fragment of DNA polymerase by using DIG-High prime kit (Boehringer Manheim). To obtain rat EST109795

probe, EST109795 fragment was cloned from total RNA of Rat-1 cells by RT-PCR using primers #86-P1 and #86-P2 (Table 1 and Fig. 2), and labeled with DIG by PCR using PCR DIG Probe Synthesis kit (Boehringer Manheim).

Northern Blot analysis

Total RNA (20 μg) was resolved by formaldehyde-containing 1.2% agarose gel electrophoresis, blotted onto a GeneScreen filter (Dupont/NEN), and fixed by baking at 80℃ for 2 h. After the filter was prehybridized in hybridization buffer (7% (w/v) sodium dodecyl sulfate (SDS), 50% (v/v) formamide, 5x SSC (0.75 M NaCl, 0.075 M sodium citrate (pH 7.0)), 2% blocking reagent (Boehringer Manheim), 50 mM sodium phosphate (pH 7.0), 0.1% *N*-lauroylsarcosine, 0.05 mg/ml yeast RNA (Boehringer Manheim)), the filter was hybridized with the probe in hybridization buffer overnight at 50℃ (for β-actin probe) or 42℃ (for rat EST 109795 probe). After hybridization, the filter was washed twice with 2x SSC, 0.1% SDS at room temperature for 5 min, and twice with 0.1x SSC, 0.1% SDS at 65℃ (for β-actin probe) or 0.5x SSC, 0.1% SDS at 55℃ (for rat EST 109795 probe) for 15 min. The hybridized probe was detected by DIG Luminescent Detection kit (Boehringer Manheim) according to the manufacturer's protocol.

Reverse transcription (RT)-PCR analysis

First-strand cDNA was synthesized with Moloney murine leukemia virus (M-MLV) reverse transcriptase (GIBCO-BRL) and an oligo $(dT)_{16}$ primer (Table 1).

To study the expression of *hLUCA15* in human breast cancer tissues, 0.5 μg of total cellular RNA was pretreated with RNase-free DNase (GIBCO-BRL) to digest contaminated chromosomal DNA, and subjected to first-strand cDNA synthesis. The primer pairs used in the PCR are described in the figure legends or the text. The PCR products (10 μl) were resolved by agarose-gel electrophoresis, stained with ethidium bromide, and detected by ultraviolet illumination.

Cloning of Rat EST and human LUCA15 cDNA

Rat cDNA clone RPNAU82 was cloned by RT-PCR using primers H33624-F and #86-P2 (Table 1 and Fig. 2). Human *LUCA15* (*hLUCA15*) cDNA was cloned by RT-PCR using primers hLUCA15-F and hLUCA15-R (Table 1 and Fig. 2) from total RNA of HEK293 cells. These PCR products were subcloned into the *Eco*R V site of pBluescript II SK (-) and sequenced.

Epitope tagging and expression vectors

To tag the human c-Myc epitope sequence (EQKLISEEDL) to the N-terminal of hLUCA15, a synthetic oligonucleotide EcoRI-N-Myc-hLUCA15-F encoding human c-Myc epitope (Table 1) was added at the 5' end of the cDNA of hLUCA15 by PCR. Complementary DNAs for hLUCA15 and c-Myc-tagged hLUCA15 (Myc-hLUCA15) were subcloned into a mammalian expression vector pCMV5 (Andersson et al., 1989) to generate pCMV5-hLUCA15 and pCMV5-Myc-hLUCA15, respectively.

Immunofluoresence analysis

HT1080 cells on a cover glass were transiently transfected with pCMV5-Myc-hLUCA15 with Lipofectamine™ (GIBCO-BRL). The transfected cells were fixed with 4% (v/v) paraformaldehyde in phosphate-buffered saline (PBS), and permeabilized with 0.1% (v/v) Triton-X100 in PBS. The permeabilized cells were incubated with an anti-c-Myc monoclonal antibody 9E10 (Babco). An antigen-antibody complex was visualized by a fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG antibody (Cappel).

Ribohomopolymer binding assay

Ribohomopolymer binding assay was performed as described elsewhere (Swanson and Dreyfuss, 1988; Dreyfuss et al., 1993; Macknight et al., 1997) with minor modifications. In brief, HEK293 cells were transfected either with pCMV5 (for mock transfection) or pCMV5-Myc-hLUCA15 (for Myc-hLUCA15 transfection) by the calcium phosphate coprecipitation technique as described previously (Edamatsu et al., 1997). The transfected cells were lysed in KHN buffer (150 mM KCl, 20 mM HEPES-KOH (pH 7.9), 0.01% (v/v) Nonidet P-40 (NP-40)). The cell lysates were mixed with polyadenylic acid (poly[rA])-, polycytidylic acid (poly[rC])-, polyguanylic acid (poly[rG])-, or polyuridylic acid (poly[U])-conjugated agarose beads (Sigma), or denatured DNA-conjugated cellulose beads (Amersham-Pharmacia) for 10 min at room temperature. These beads were washed three times with KHN buffer. The protein bound to the beads was eluted with Laemmli sample buffer, and analyzed by

immunoblotting with an anti-c-Myc antibody (9E10).

DNA transfection assay on neomycin resistance colony formation

HT1080 cells were transfected with pCMV5 (mock), pCMV5-Myc-hLUCA15 (Myc-hLUCA15), or pCMV5-hLUCA15 (hLUCA15) together with a neomycin resistant marker (pSV2-neo) with Lipofectamine™. The cells were treated with 0.5 mg/ml neomycin for 2 weeks, and the living cells were visualized by staining with 3-(4,5,-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT).

Results

Identification of oncogenic Ras-regulated genes by differential display

Rat-1 transfectants expressing an oncogenic mutant of Ras (Ras(G12V)) in an isopropyl-1-β-thio-D-galactoside (IPTG)-dependent manner have been constructed (Edamatsu et al., 1998). To study Ras-regulated genes in Rat-1 cells, total RNAs were isolated from these cells before and after the expression of Ras(G12V) with IPTG, and subjected to differential display. It was found that the expression of several genes, including activating transcription factor (ATF) 4, matrix metalloprotease (MMP) 13, a cell surface receptor for gibbon ape leukemia virus 1 (Glvr1), and isopentenyl diphosphate:dimethylallyl diphosphate isomerase (IPP isomerase), was up-regulated after the cells were treated with IPTG (data not shown). On the other hand, the expression of some genes was down-regulated after IPTG treatment. One of these genes, clone 86 (Fig. 1A), was isolated and sequenced to search for DNA databases. The search of the GenBank expressed sequence tag (EST) database by using the algorithm of Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) revealed that the sequence of clone 86 is identical to that of the 3' end of the rat cDNA clone RPNAU82 (EST109795, GenBank accession number is H33623) which has been originally cloned from the cDNA library of rat pheochromocytoma PC12 cells treated with nerve growth factor (NGF) (Lee et al., 1995). To confirm the

results of the differential display, Northern blot analysis using the 3' end fragment of this rat cDNA (rat EST109795) as the probe was performed. Figure 1B shows that the expression levels of clone 86 were decreased in three different Rat-1•RasVal cell lines after the cells were treated with IPTG. In addition to the Northern hybridization, RT-PCR using the specific primer pairs for rat cDNA clone RPNAU82 also shows that the expression of the gene was decreased after the IPTG-induced expression of Ras(G12V) (Fig. 1C). These results demonstrate that the rat cDNA clone RPNAU82 is one of the genes whose expression is down-regulated after the expression of Ras(G12V) in Rat-1 cells.

Rat EST clone RPNAU82 is one of the splicing variants of the rat homologue of a putative tumor suppressor gene, *LUCA15*

Since clone 86 is a partial cDNA fragment of the rat cDNA clone RPNAU82, the full-length of this rat cDNA was cloned from Rat-1 cells by RT-PCR using the primers H33624-F and #86-P2 (Table 1 and Fig. 2), and the PCR products were sequenced for searching for the DNA database. As shown in Fig. 2, search of the GenBank DNA database by the BLAST algorithm revealed that the sequence of each 5' and 3' region of this rat cDNA is identical to that of the human putative tumor suppressor gene *LUCA15* (GenBank accession number is U23946) (Wei et al., 1996). Furthermore, the sequence of the inner region of the rat cDNA, which is not identical to that of human *LUCA15* (*hLUCA15*) cDNA, is similar to the genomic sequence of *hLUCA15* locus (Fig. 2). As illustrated in Fig. 3, there are splicing consensus

sequences in the rat cDNA clone RPNAU82. In addition, Fig. 1B shows that several hybridized bands which look like smear were detected in this Northern hybridization. These suggest that alternatively spliced products which can be hybridized with rat EST109795 probe may be present in Rat-1 cells. Possibly, this rat cDNA may be one of the alternatively spliced products of *LUCA15*. To examine this possibility, RT-PCR analysis using the primer pairs (8602-225F/8602-976R for rat, hLUCA15-315F/hLUCA15-554R for human) which can detect two putative splicing variants of *LUCA15* mRNA was performed. As predicted, two alternatively spliced variants are present in Rat-1, human embryonic kidney (HEK) 293, and human sarcoma HT1080 cells (Fig. 5). One splicing variant encodes a putative RNA-binding protein having two RNA recognition motifs (RRMs) (Fig. 4). An RRM is a 90-amino-acid region containing a conserved RNP1 octapeptide, (K/R)G(F/R)(G/A)FVX(F/Y), and a less conserved RNP2 hexapeptide that is rich in aromatic and aliphatic amino acids (Dreyfuss et al., 1993; Burd and Dreyfuss, 1994; Oubridge et al., 1994; Nagai et al., 1995). In addition to the RRMs, this form of LUCA15 has two nuclear localization signals (NLSs) (Robbins and Dingwall consensus) which are rich in basic amino acids (Fig. 4) (Robbins et al., 1991). Another splicing variant encodes the truncated form of LUCA15. This truncated form has no RRMs (Fig. 2). It seems that this truncated form of LUCA15 cannot function in cells, because any functional motifs cannot be predicted. Thus, the putative gene product of *LUCA15* with two putative RRMs was studied.

LUCA15 is localized in nuclei

To investigate the function of LUCA15, cDNA encoding hLUCA15 was cloned by RT-PCR from the RNA of HEK293 cells. It should be noted that the nucleotide sequence of the cDNA cloned from HEK293 cells differs from the sequence that has already been registered in the GenBank DNA database (U23946). Differences with the replacement of the amino acids are Gly53(GGC) into Asp53(GAG), Ser54(TCC) into Tyr54(TAG), and Val354(GTC) into Gly354(GGC). Differences without the replacement of the amino acids are Ala330(GCC) into Ala330(GCT) and Tyr679(TAC) into Tyr679(TAT). To detect the exogeneously expressed hLUCA15, the human c-Myc epitope sequence (EQKLISEEDL) was tagged to the N-terminal of hLUCA15. The c-Myc-tagged hLUCA15 (Myc-hLUCA15) was transiently expressed in HT1080 cells and the protein was analyzed by immunoblotting using an anti-c-Myc monoclonal antibody (9E10). The immunoblot analysis shows that the molecular weight estimated by the electrophoretic mobility is about 120 kDa (Fig. 6A). As illustrated in Fig. 4, LUCA15 has two NLSs, suggesting that LUCA15 is localized in nuclei. To study the intracellular localization of LUCA15, Myc-hLUCA15 was transfected into HT1080 cells and stained with the anti-c-Myc antibody *in situ*. As shown in Fig. 6B, exogeneously expressed Myc-hLUCA15 was localized in the nuclei. This result suggests that endogeneous LUCA15 may be translocated into and function in nuclei.

LUCA15 binds RNA *in vitro*

In addition to the NLSs, the deduced amino acid sequence predicts that LUCA15 has two putative RRM domains, suggesting that LUCA15 may have RNA-binding activity. To confirm this property, *in vitro* binding activity to ribohomopolymer (poly[rN]) was tested. This assay has been widely used to study the properties of RNA-binding proteins including hnRNP (Swanson and Dreyfuss, 1988; Dreyfuss et al., 1993; Macknight et al., 1997). To obtain the recombinant hLUCA15, Myc-hLUCA15 was transiently expressed in HEK293 cells, and the crude cell extract containing Myc-hLUCA15 was prepared. Then, the extract was mixed with poly[rN]- or denatured DNA-conjugated beads, and the protein bound to the beads was precipitated and analyzed by immunoblotting using the anti-c-Myc antibody (9E10). As shown in Fig. 7, Myc-hLUCA15 bound to poly[rG], poly[rA], and poly[rC], but not to poly[U] *in vitro*. In addition to poly[rN], Myc-hLUCA15 also bound to denatured DNA. It has been reported that hnRNPs can also bind to denatured DNA *in vitro* (Wilk et al., 1983, Dreyfuss et al., 1993). These results suggest that LUCA15 can bind to RNA *in situ*. Taken together, LUCA15 may form a RNA-protein complex in nuclei.

Expression of *LUCA15* mRNA are reduced in human breast cancer tissue

The gene *LUCA15* has originally been cloned as a putative tumor suppressor gene mapped on the short arm of human chromosome 3 (3p21.3) (Wei et al., 1996). Since the 3p21.3 region has been found to be frequently

deleted in lung, breast, and other cancers (Fondon III et al., 1998), tumor suppressor genes have been thought to exist on this region. In addition, it was observed that the expression of *LUCA15* is reduced after the expression of Ras(G12V) in Rat-1 cells (Fig. 1). These results suggest that the reduction of the expression of *LUCA15* mRNA may contribute to induction of tumor. To study the expression levels of LUCA15 in cancer tissues, the mRNA levels of *LUCA15* in human breast cancer tissues from 13 patients were analyzed by RT-PCR using the primer pairs for *hLUCA15*. Figure 8 shows that the mRNA level of *LUCA15* of one patient (patient 8) was low compared with those of other 12 patients and of a normal tissue. The tumor of patient 8 has been recorded to be very aggressive and in higher pathological stage. These results suggest that the reduction of the expression of *LUCA15* might contribute to deregulated cell growth in certain tumors.

Elevated expression of LUCA15 suppresses tumor cell growth

As described above, the expression of *LUCA15* is decreased in Ras-transformed Rat-1 cells and human breast cancer tissues. These results suggest that the reduction of LUCA15 expression may promote the cell growth. Therefore, the elevation of the expression of LUCA15 might inhibit the tumor cell growth. To test this hypothesis, effect of the elevation of the expression of LUCA15 on the cell growth was studied by transfection with exogenous hLUCA15. The expression plasmids for hLUCA15 were transfected into HT1080 cells together with a neomycin resistant marker plasmid (pSV2-neo), and the cells stably harboring the plasmids were selected with neomycin.

As shown in Fig. 9, the transfection of hLUCA15 and Myc-hLUCA15 suppressed the formation of neomycin-resistant colonies compared with the transfection with the empty vector (mock). These results demonstrate that LUCA15 has a potent growth suppressing activity when overexpressed in HT1080 cells.

Discussion

In this study, genes whose mRNA levels are up-regulated or down-regulated after Ras(G12V)-induced transformation of Rat-1 fibroblast cells were analyzed by differential display. This differential display revealed that the expression of rat homologue of *LUCA15*, which has been originally cloned as a putative tumor suppressor gene for small cell lung cancer (SCLC) (Wei et al., 1996), is decreased after the expression of Ras(G12V) in Rat-1 cells.

The Northern blot analysis suggests that several splicing variants of *LUCA15* mRNA are expressed in Rat-1 cells (Fig. 1B). Especially, RT-PCR analysis demonstrated that at least two types of splicing variants are expressed in rat and human cell lines (Fig. 5). Sequencing of the cDNAs of the splicing variants of *LUCA15* revealed that one type of transcripts has two RRMs and the other does not have any RRMs because of the insertion of the nonsense codon upstream of the RRM coding region (Fig. 2). This style of alternative splicing is also seen in *Drosophila Melanogaster Sex-lethal* (*Sxl*) mRNA. In *Drosophila*, two types of the alternatively spliced *Sxl* mRNA are produced from the same allele. One is only in a male fly (male type) and the other is only in a female fly (female type). The female type *Sxl* mRNA encodes an active SXL protein that has two RRMs. On the other hand, the male type mRNA encodes an inactive and truncated SXL protein without RRMs, because of the insertion of the nonsense codon in the additional exon by the alternative splicing (Bell et al., 1988). These sex-specific SXL proteins promote the sex development of a fly by playing key roles in the post-

transcriptional regulation of sex-related gene expression (Cline, 1993). Although the significance of the alternative splicing of *Sxl* gene in a fly has been extensively studied, the significance of the alternative splicing of *LUCA15* is not clear. It is widely known that mRNAs of heterogeneous nuclear RNA-binding proteins (hnRNPs) are often alternatively spliced (Dreyfuss et al., 1993).

The intracellular localization of LUCA15 was studied by the Immunofluorescence of Myc-hLUCA15. This analysis demonstrated that Myc-hLUCA15 is localized in nuclei, suggesting that LUCA15 may function in nuclei. In addition to nuclei, weak signal was detected in the cytosol. This suggests another possibility that LUCA15 can shuttle between a nuclear and cytosol.

In addition to the nuclear localization, Myc-hLUCA15 produced in HEK293 cells binds ribohomopolymer (poly-[rN]) *in vitro*. These results suggest that LUCA15 can bind RNA in nuclei, and may function in post-transcriptional regulation in gene expression. However, how LUCA15 function in post-transcriptional regulation is unclear, because the ligand RNA molecules of LUCA15 have not yet been identified. Hence, identification of the ligand RNA molecules of LUCA15 by the Systematic Evolution of Ligand by Exponential enrichment (SELEX) (Tuerk and Gold, 1990; Tsai et al., 1991) is needed to understand the function in post-transcriptional regulation.

LUCA15 protein also contains the regions that are rich in particular amino acids. In particular, glutamine-rich (Q-rich) region from amino acid residue 372 to 385 is present next to the 2nd RRM (Fig. 4). Since a Q-rich

region is thought to serve as a protein-protein interaction site in certain RNA- and DNA-binding proteins (Courey and Tjian, 1988; Zhang et al., 1993), LUCA15 may interact with other proteins through this Q-rich region. Thus, identification of the proteins which can interact with LUCA15 may provide the information for understanding the function of LUCA15. It should be noted that, more recently, proteins contained in a spliceosome of HeLa cells was characterized by the mass spectrometry and EST-database searching (Neubauer et al., 1998). A spliceosome, which is a complex of RNA-binding proteins and RNA, functions in a process of RNA splicing. However, LUCA15 protein could not be detected in the spliceosome of HeLa cells by this analysis (Neubauer et al., 1998).

The gene *LUCA15* has originally been cloned as a putative tumor suppressor gene mapped on the short arm of human chromosome 3 (3p21.3). In this study, the growth suppressing activity of LUCA15 in human sarcoma HT1080 cells was detected (Fig. 9). When overexpressed in HT1080 cells, LUCA15 suppressed the neo-resistant colony formation (Fig. 9). This result shows that the elevation of the expression of LUCA15 suppresses certain types of tumor cell proliferation. In addition, in the breast cancer tissue tested, the mRNA level of *LUCA15* was reduced (Fig. 8). These results suggest that the gene *LUCA15* may be one of the class II tumor suppressor genes (Sager 1997; Zhang et al., 1998). Sager has grouped tumor suppressor genes into two classes: class I and class II. Class I genes are mutated or deleted in tumors. For instance, p53 is one of the most famous class I tumor suppressor gene products (Sager 1997; Zhang et al., 1998). On the other hand, class II

genes are altered at the mRNA expression levels rather than the mutation or the deletion of the DNA. It was observed that the mRNA level of *LUCA15* in the quiescent (serum-starved) Rat-1 cells is higher than that in the growing (not-starved) Rat-1 cells (data not shown). This result suggests that the expression of LUCA15 may be down-regulated when cells are growing, and that the expression may be up-regulated when cells are quiescent. To confirm this possibility and to evaluate the regulation of the expression of *LUCA15* gene, it is necessary to isolate and analyze the promoter region. In addition, it will be important to analyze the polymorphism and the mutation of *LUCA15* gene for determining whether *LUCA15* can be categorized into class I.

In this study, it was shown that the expression of mRNA of *LUCA15* is down-regulated after the expression of Ras(G12V) in Rat-1 cells, and the elevation of the expression of LUCA15 suppresses the proliferation of HT1080 sarcoma cells. In addition, LUCA15 has RNA-binding activity *in vitro*, and is located in the nuclei. These results provide the possibility that LUCA15 protein may function in the modulation of the expression of the proteins, which can regulate cell-cycle or cell proliferation, through the post-transcriptional regulation by assembling RNAs and proteins. Further studies on the structure and function of LUCA15 may help to understand the regulation of cell proliferation mediated by the *LUCA15* gene expression, and may clarify the function of LUCA15 as the tumor suppressor.

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Table 1. Sequences and names of oligo DNAs used in this study

oligo name	sequence (5' to 3')
Primers used for differential display	
Arbitrary primers (The underlines indicate *Hind* III restriction sites.)	
22A00	5'-CGG GAA GCT TAT CGA CTC CAA G-3'
22A01	5'-CGG GAA GCT TTA GCT AGC ATG G-3'
22A02	5'-CGG GAA GCT TGC TAA GAC TAG C-3'
22A03	5'-CGG GAA GCT TTG CAG TGT GTG A-3'
22A04	5'-CGG GAA GCT TGT GAC CAT TGC A-3'
22A05	5'-CGG GAA GCT TGT CTG CTA GGT A-3'
22A06	5'-CGG GAA GCT TGC ATG GTA GTC T-3'
22A07	5'-CGG GAA GCT TGT GTT GCA CCA T-3'
22A08	5'-CGG GAA GCT TAG ACG CTA GTG T-3'
22A09	5'-CGG GAA GCT TTA GCT AGC AGA C-3'
22A10	5'-CGG GAA GCT TCA TGA TGC TAC C-3'
22A14	5'-CGG GAA GCT TAT CTT TCT ACC C-3'
Anchor primers (The underlines indicate *Hind* III restriction sites.)	
HdT15A	5'-GCG CAA GCT TTT TTT TTT TTT TTA-3'
HdT15C	5'-GCG CAA GCT TTT TTT TTT TTT TTC-3'
HdT15G	5'-GCG CAA GCT TTT TTT TTT TTT TTG-3'
Primers used for RT-PCR	
oligo $(dT)_{16}$	5'-TTT TTT TTT TTT TTT T-3'
hLUCA15-F	5'-CAG TGG GAC AAT GGG TTC AGA C-3'
hLUCA15-R	5'-CTC TCA CTC CAT CTC AAT GAA CC-3'
hLUCA15-252R	5'-TCT GGA CTG TCA TAG TCT CG-3'
hLUCA15-315F	5'-TGG TGA GCA CGA CTA TAG GC-3'
hLUCA15-554R	5'-TTC TGA TTG GCT TCC ATC C-3'
H33624-F	5'-CGG TGA GAC AAT GGG TTC AG-3'
8602-408R	5'-TTC ATC AGC CTC ACA TCT GC-3'
8602-225F	5'-ATG GAG AAC ATG ACT ATA GAC-3'
8602-976R	5'-TGA TTG GCT TCC ATC CAG C-3'
#86-P1	5'-GTG GCA TTT AAT TCA GGT GG-3'
#86-P2	5'-AGT GAA GCA ACC TGA TTG GC-3'
L-Ha-Ras-F	5'-CTC GAG TAA GGA GAT CTG CG-3'
Ha-Ras-R	5'-TAG AAG GCA TCC TCC ACT CC-3'
βactF	5'-CTA CAA TGA GCT GCG TGT GG-3'
r/mβactR	5'-CAA CGT CAC ACT TCA TGA TGG-3'
hβactR	5'-CCA CGT CAC ACT TCA TGA TGG-3'
Primer used for epitope-tagging (The underline indicates an *Eco*R I restriction site.)	
EcoRI-N-Myc-hLUCA15-F	5'-CGGAATTC ATG GAG CAG AAG CTC ATC TCT GAG GAG GAC CTG GGT TCA GAC AAA AGA GTG AG-3'

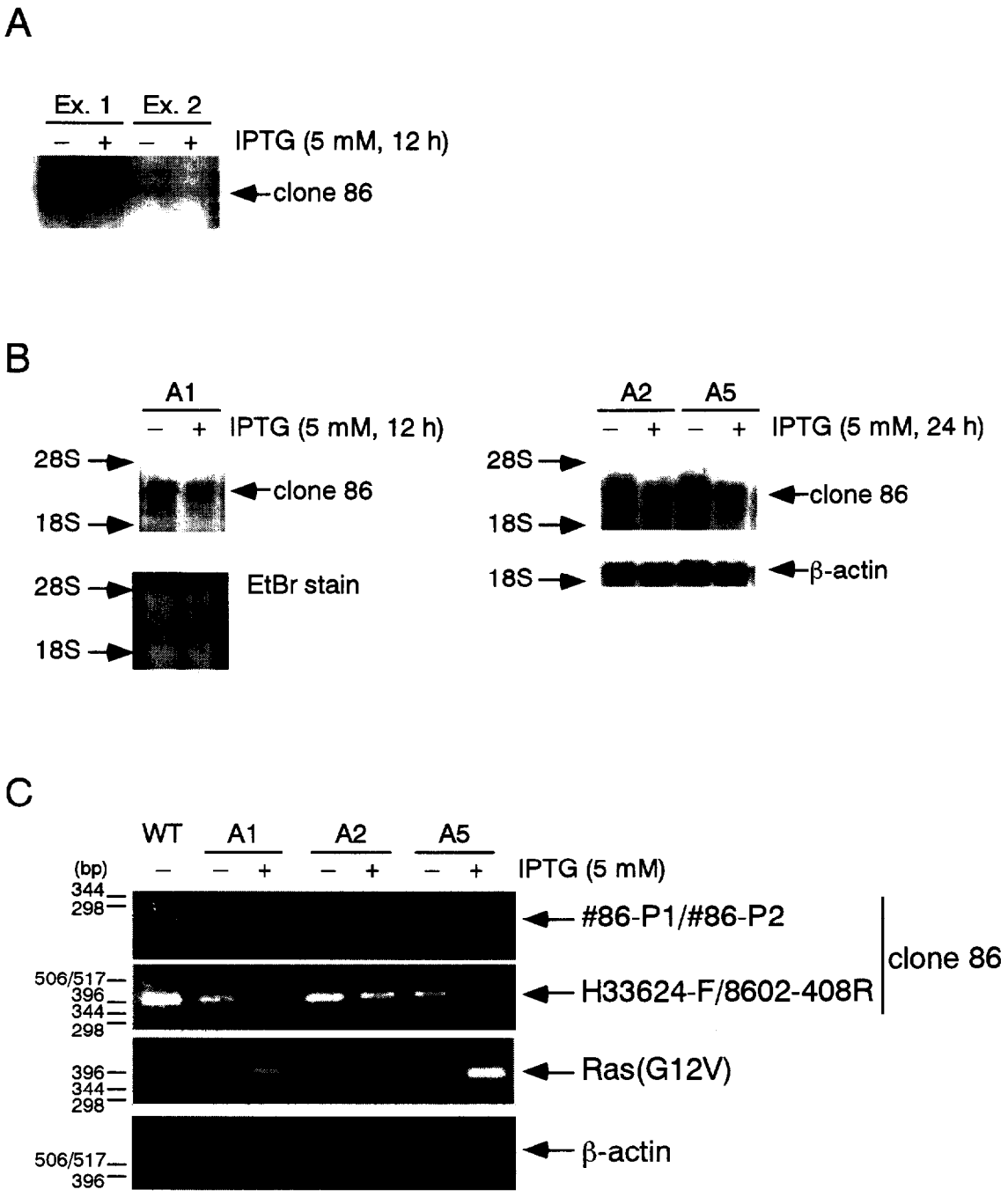

Fig. 1. Expression of clone 86 detected by differential display, Northern blotting, and RT-PCR

(A) Differential display of clone 86. Total RNA was prepared from Rat-1•RasVal A1 cells after treatment with or without 5 mM IPTG for 12 h. Differential display using primers HdT15C and 22A10 was performed as described in Materials and Methods. Two independent rots of

cDNAs were used for this differential display (Ex. 1 and Ex. 2). The arrowhead indicates the band of clone 86. (B) Northern blot analysis of clone 86. Total RNAs (20 μg) from three independent clones (A1, A2, and A5) were electrophoresed on a formaldehyde-containing 1.2% agarose gel, blotted onto a GeneScreen filter (Dupont/NEN), and hybridized with the DIG-labeled probes. The equivalency of the amount of RNA was checked by staining 18S and 28S ribosomal RNA with EtBr (lower panel) or by hybridization with the β-actin probe (lower panal). (C) RT-PCR analysis of clone 86. RT-PCR using the specific primer pairs (#86-P1/#86-P2 and H33624-F/8602-408R for clone 86, L-Ha-Ras-F/Ha-Ras-R for ras(G12V), βactF/r/mβactR for β-actin) was performed as described in Materials and Methods. The length of DNA indicated in these panels was determined by 1-kb Ladder DNA size marker (GIBCO-BRL). No bands were detected by using the templates without treatment with reverse-transcriptase (data not shown).

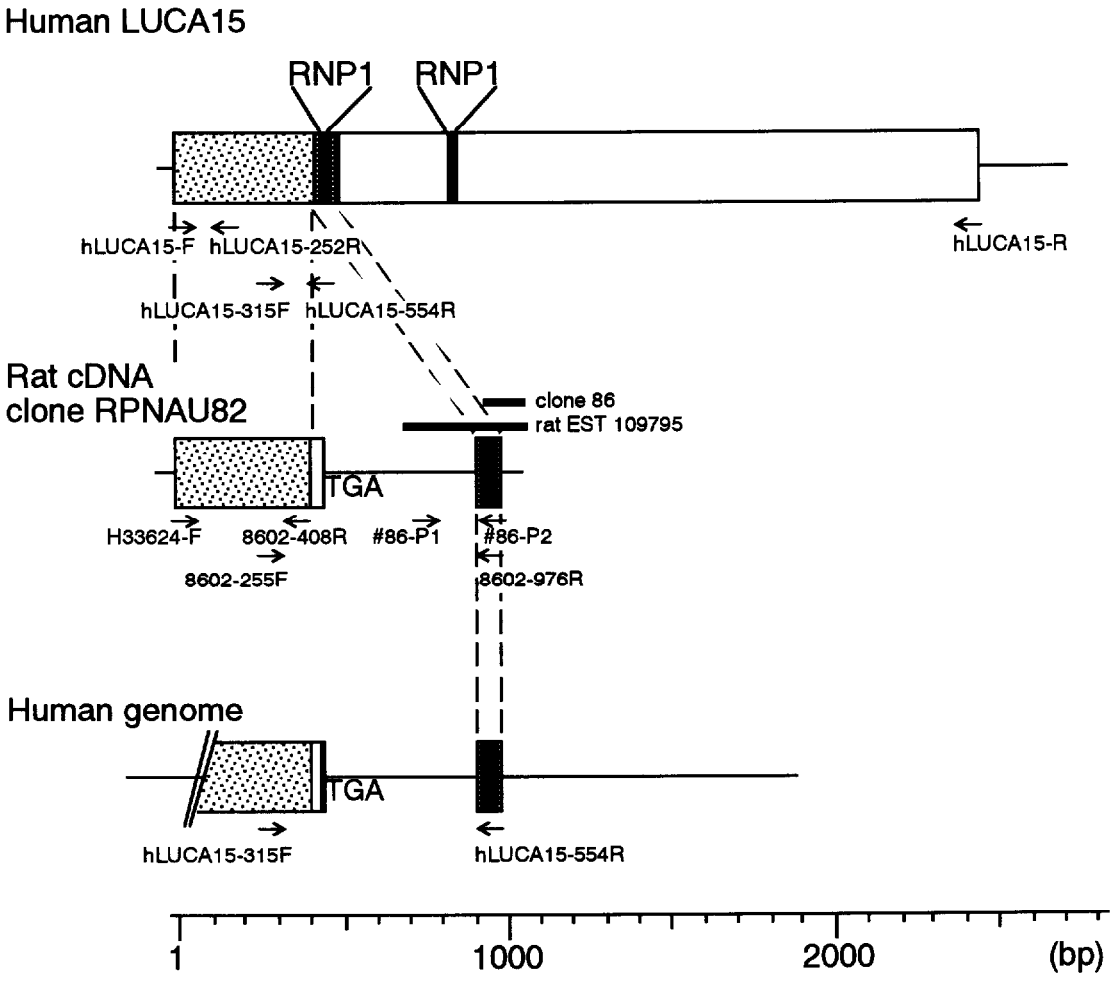

Fig. 2. Schematic representation of human *LUCA15* and rat cDNA clone RPNAU82

Structure of cDNAs and the positions of the primers used in this study are illustrated. Coding regions are shown as boxes. The regions identical to each other are shown as shadowed boxes. The closed boxes indicate the regions encoding RNP1 octapeptide. The positions of the DNA primers are shown as arrows. The positions of DNA fragments of clone 86 and rat EST 109795 are shown as thick lines.

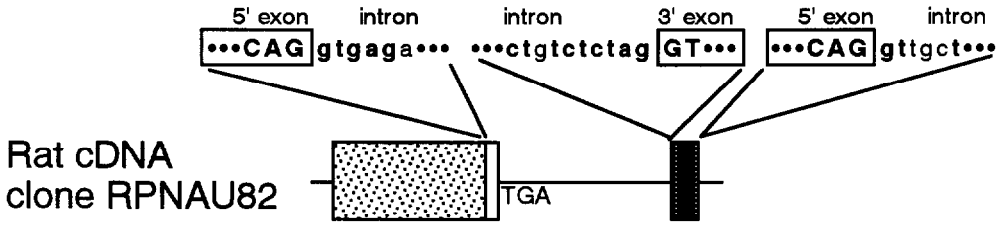

Fig. 3. Splicing consensus sequence is present in rat cDNA clone RPNAU82

Structure of rat cDNA clone RPNAU82 is illustrated. The boxes indicate the coding region. The capital letters and the small letters indicate the nucleotide sequence of the putative exon and the intron, respectively. The bold letters indicate the nucleotide sequences which are identical to the splicing consensus sequences.

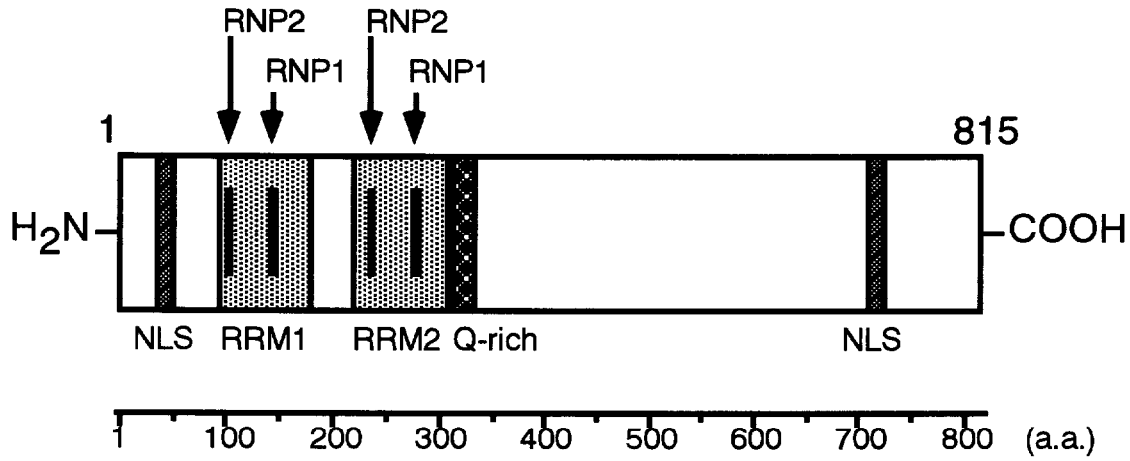

Fig. 4. Structure of human LUCA15 protein

Structure of human LUCA15 protein is illustrated. The regions of the nuclear localization signals (NLSs), the RNA recognition motifs (RRM1 and RRM2), and the glutamine-rich region (Q-rich) are indicated as boxes. The positions of RNP1 octapeptides and RNP2 hexapeptides are indicated as closed boxes.

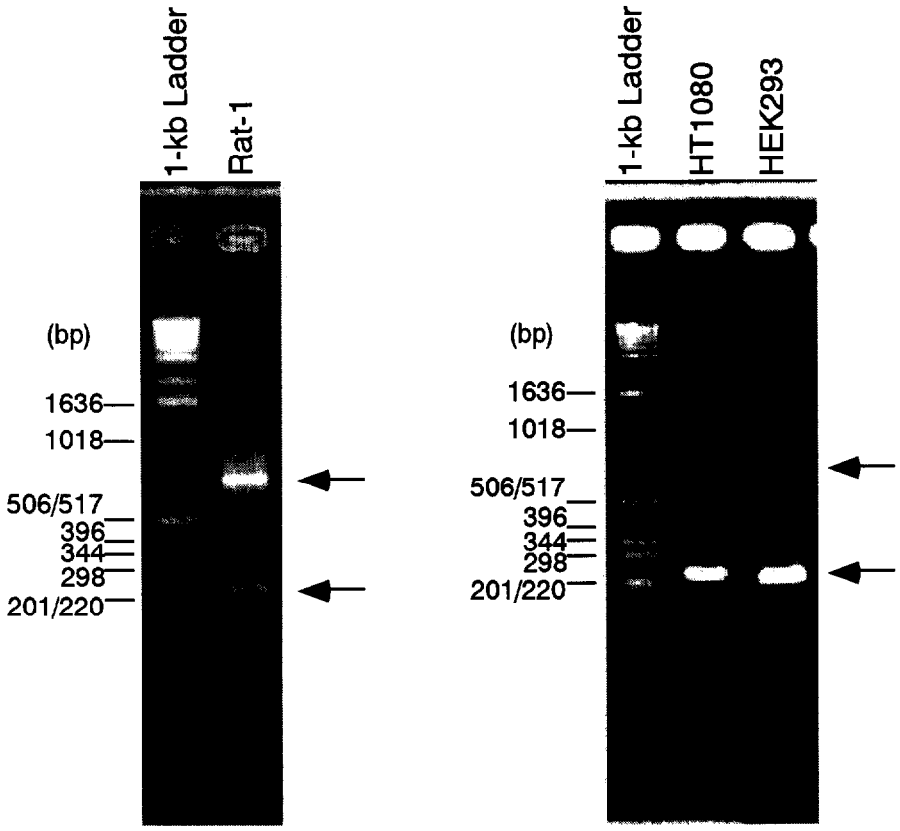

Fig. 5. Alternatively spliced products of *LUCA15* is present in rat and human cell lines

Total RNA samples were prepared from Rat-1 (left panel), HT1080 and HEK293 cells (right panel), and RT-PCR was performed as described in Materials and Methods. The length of DNA indicated in the panels was determined by 1-kb Ladder DNA size marker (GIBCO-RRL). No bands were detected by using the templates without treatment with reverse-transcriptase (data not shown). The arrows indicate short and long PCR products of *LUCA15*.

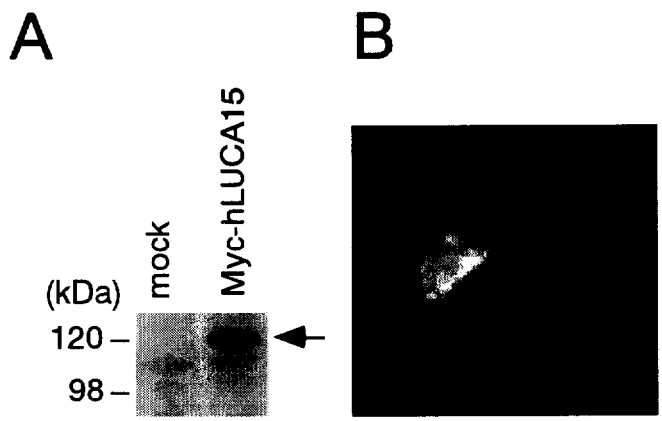

Fig. 6. Immunoblot analysis and Immunofluorescence analysis of Myc-hLUCA15 in the transfected HT1080 cells

(A) Immunoblot analysis of Myc-hLUCA15. HT1080 cells were transiently transfected with pCMV5 (mock) or pCMV5-Myc-hLUCA15 (Myc-hLUCA15) and lysed. The cellular protein was electrophoresed and immunoblotted with an anti-c-Myc monoclonal antibody 9E10 (Babco). The arrow indicates Myc-hLUCA15. (B) Immunofluoresence analysis of Myc-hLUCA15 in the transfected HT1080 cells. HT1080 cells were transfected with pCMV5-Myc-hLUCA15, fixed, and permiabilized as described in Materials and Methods. Then, the cells were treated with an anti-c-Myc antibody 9E10, and an antigen-antibody complex was visualized by a fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG antibody (Cappel).

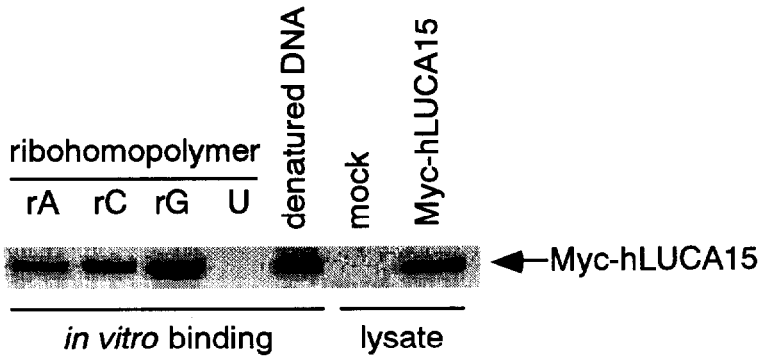

Fig. 7. RNA-binding activity of Myc-hLUCA15 *in vitro*

HEK293 cell lysate containing Myc-hLUCA15 was mixed with ribohomopolymer-conjugated agarose beads or denatured DNA-conjugated cellulose beads. Protein bound to the beads was analyzed by immunoblotting with an anti-c-Myc antibody 9E10. Lysates of the mock-transfected cells (mock) and the Myc-hLUCA15-transfected cells (Myc-hLUCA15) were also blotted.

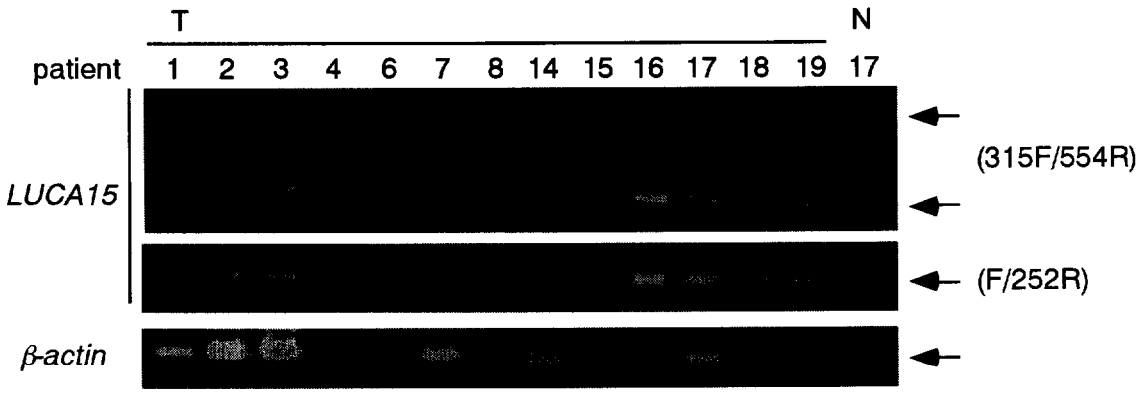

Fig. 8. RT-PCR analysis of *LUCA15* from human breast cancer tissues

Total RNA samples from breast cancer tissues (T) and a normal tissue (N) were subjected to RT-PCR as described in Materials and Methods. To detect the mRNA of *hLUCA15*, two primer pairs, hLUCA15-315F/hLUCA15-554R (top panel) and hLUCA15-F/hLUCA15-252R (middle panel) were used.

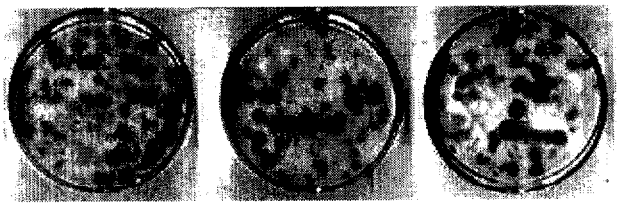

Fig. 9. Effect of the elevated expression of LUCA15 in neo-resistance colony formation of HT1080 cells

HT1080 cells were transfected with pCMV5 (mock), pCMV5-Myc-hLUCA15 (Myc-hLUCA15), or pCMV5-hLUCA15 (hLUCA15 (tag minus)). After the selection with neomycin for 2 weeks, living cells were stained with MTT.

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