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Rat Neuronal Leucine-Rich Repeat Protein-3: Cloning and Regulation of the Gene Expression

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Rat neuronal leucine-rich repeat protein-3 (rNLRR-3) gene was isolated and cloned from fibrosarcoma cells overexpressing c-Ha-ras. Stable expression of constitutively active forms of Ras (H-Ras^{V12} or v-H-Ras) led to a two- to fourfold increase in rNLRR-3 mRNA in rat normal fibroblasts (3Y1). When cells expressing H-Ras^{V12} were treated with mitogen activated protein kinase (MAPK) kinase inhibitors (U0126, PD98059), suppression of rNLRR-3 mRNA correlated well with a reduction in MAPK activity. Epidermal growth factor (EGF) led to elevation of rNLRR-3 gene expression about 4 h after stimulation of normal fibroblasts. U0126 completely suppressed the induction by EGF of rNLRR-3 mRNA with abrogation of MAPK phosphorylation. U0126 inhibited the basal transcription of rNLRR-3. LY294002, a PI3 kinase inhibitor, showed a lesser effect on expression of the gene. These results indicate that rNLRR-3 gene expression is regulated mainly through the Ras-MAPK signaling pathway in fibroblasts. © 2001 Academic Press

Key Words: **Ras; mitogen-activated protein kinase; MAPK/ERK kinase; leucine-rich repeat protein; cell adhesion; clathrin.**

The Ras/mitogen-activated protein kinase (MAPK) pathway is an evolutionarily conserved signaling pathway in eukaryotic cells. In mammals, the Ras family comprises Ha-Ras, Ki-Ras, and N-Ras that are cycling between the inactive GDP-bound and the signaling competent GTP-bound conformation. MAPKs (or extracellular signal-regulated kinases, ERKs) comprise a family of related protein kinases that are activated by phosphorylation on threonine and tyrosine residues. The MAPK-activating enzymes (MAPK/ERK kinases, or MEKs) are unusual in their potential to catalyze phosphorylation on both threonine and tyrosine residues (1, 2). MEKs are in turn activated by phosphorylation on serine residues by upstream kinases. These MEK kinases require the *ras* protooncogene product to become catalytically active (3, 4).

A protein with leucine-rich repeat (LRR) domains was first identified in an alpha-2-glycoprotein in human serum (5). LRR domains contain highly hydrophobic amino acids and a repeat structure consisting of about 24 residues (6). The LRR-motif provides an ideal conformation for binding to other proteins. Therefore, all LRR-containing proteins are thought to be involved in protein-protein interactions (7).

Functions of only a few of the LRR family proteins have been determined. Neuronal leucine-rich repeat protein (NLRR) genes were first isolated from a mouse brain cDNA library (8, 9), and three distinct isoforms (NLRR-1, -2 and -3) have been identified in fish, frog, mouse, and human (8–11). These isoforms constitute a novel LRR-family protein with 11 or 12 LRRs, one immunoglobulin-like domain and one fibronectin type III-like domain (10, 11). Although NLRRs have been proposed to function as a neuronal adhesion molecule or soluble ligand binding receptor, biochemical and cell biological analyses remained to be elucidated.

We isolated and cloned a newly identified member of the NLRR gene family, rat NLRR-3 (rNLRR-3), from fibrosarcoma cells overexpressing c-Ha-ras. rNLRR-3 mRNA is abundant in the adult rat brain. We provide evidence indicating that gene expression of rNLRR-3 in fibroblasts is regulated through the Ras-MAPK pathway.

MATERIALS AND METHODS

Cell culture. 3Y1 (12) cell was maintained in Dulbecco's modified Eagle medium containing 10% FCS. Stably transfected lines of 3Y1 cells that express wild or constitutively active H-ras were selected by G418 (GibcoBRL). HR-3Y1-2 cells transformed to 3Y1 by v-H-ras cDNA, were obtained from Health Science Research Resources Bank (HSRRB, Japan).

Construction of subtracted cDNA library. cDNA synthesis and subtraction were done using the PCR-select cDNA subtraction kits

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(Clontech) according to the manufacturer's directives. The subtracted cDNA fragments, purified using the NucleoSpin Extraction Kits (Clontech) were inserted 10 ng into the T/A cloning vector pCR2.1 (Invitrogen). Individual transformants carrying cDNA fragments were isolated from white colonies on X-gal/IPTG agar plates. The cDNA transformants were randomly picked up and subjected to sequence analysis.

Sequence. Nucleotide sequencing of the cDNA fragment was done using a Cy5 Thermo Sequenase Dye Terminator Kit and ALFexpress DNA sequencer (Amersham Pharmacia). Homology searches were done using the BLAST program at NCBI.

cDNA library screening. To isolate a rat NLRR-3 cDNA covering the entire open reading frame, a rat brain cDNA library constructed in λZAPII vector (Stratagene) was screened using as a probe the cDNA fragment obtained from subtraction and the standard methods. Several inserts in positive phages were excised by plasmid rescue of pBluescript SK after superinfection with Exassist helper phage (Stratagene).

Northern blot. Total RNA was isolated using ISOGEN (Nippon Gene, Japan). Total RNA (10 μg) was fractionated by electrophoresis through 1.2% agarose gels containing formaldehyde and blotted in 20× SSC onto nylon membrane (Amersham Pharmacia Biotech). Premade filter (rat Multiple Tissue northern Blot) purchased from Clontech was also used. cDNA fragments were labeled with [^{32}P]dCTP, using the ReadyPrime system (Amersham Pharmacia Biotech). Hybridization was performed in ExpressHyb hybridization buffer (Clontech) at 68°C for 1 h. Membranes were washed twice in 2× SSC/0.05%SDS at room temperature for 20 min, twice in 0.1× SSC/0.1% SDS at 50°C for 20 min. After washing, the blots were observed using by an image analyzer (Fuji Film BAS2000). The blot was stripped by boiling in 0.1% SDS, and then reprobed for β-actin or GAPDH, in some experiments.

RT-PCR. First strand cDNA synthesis from total RNA was done with the MMTV-RT (GibcoBRL), using the oligo(dT) primer (GibcoBRL). PCR amplifications were run for 25–35 cycles of 1 min at 95°C, 1 min at 60°C and 2 min at 72°C using the AmpliTaq Gold (Perkin–Elmer). The following primers were used for PCR: rat NLRR-3 forward, 5′-ATGCGAACACTCCCTTCATC-3′, reverse, 5′-TTCCGTCATGCTCCAGACTT-3′; H-ras forward, 5′-CCAGCTGATCCAGAACCATT-3′, reverse, 5′-AGCACACACTTGCAGCTCAT-3′; GAPDH forward, 5′-TTCAACGGCACAGTCAAGG-3′, reverse, 5′-CATGGACTGTGGTCATGAG-3′.

Plasmid constructs. Human c-H-ras expression vector was purchased from Upstate Biotechnology. Human H-ras^{V12} gene was a kind gift from Dr. Y. Kuchino and cloned into pUSEamp(+) (Upstate Biotechnology).

Treatment with EGF. 3Y1 cells were grown to confluency, then rendered quiescent by incubation in DMEM containing 0.5% FCS. After serum starvation for 48 h, the cells were treated with EGF (100 ng/ml) (Takara, Japan). In some experiments, 3Y1 cells were treated with PD98059 (50 μM) (Wako, Japan), U0126 (50 μM) (Calbiochem) or LY294002 (20 μM) (Calbiochem) for the last 10 h of serum starvation, followed by stimulation with EGF in the presence of reagents.

Western blot. Western blot analysis was done as described elsewhere (13). Anti-MAPK (16 ng/ml), anti-phospho MAPK (1 μg/ml) and anti-pan Ras (0.5 μg/ml) antibodies were purchased from Upstate Biotechnology.

RESULTS

Isolation and Sequence Analysis of a cDNA Clone Up-Regulated in Transformed Cells Overexpressing c-Ha-ras

To preferentially recover rare gene fragments up-regulated in fibrosarcoma cells developed in c-Ha-ras transgenic rat (14), by suppression subtractive hybridization, we used tester cDNA prepared from the tumor cells and excess driver cDNA from rat normal fibroblast (3Y1) cells. Of the 32 sequences isolated, one was assigned to be a known gene up-regulated in oncogenic H-ras-transformed cells (15). One fragment showed significant homology with about a 580 nucleotide stretch of the mouse neuronal leucine-rich repeat protein-3 gene (NLRR-3) (9). Northern analysis confirmed that a transcript of the rat gene was at least 13-fold overexpressed in the fibrosarcoma cells compared with findings in 3Y1 cells (Fig. 1A, top). Total mRNA level of c-Ha-ras (the endogenous gene and the transgene) in the former cells was at least 8-fold higher than in the latter cells (Fig. 1A, bottom). In adult rat tissues, a single 4.2-kb transcript of putative rat NLRR-3 gene (rNLRR-3) was detected, in the brain, lung and liver. The expression level was strong in the brain, weak in the lung and liver, and little was observed in the other tissues tested (Fig. 1B, a). The expression profile was the same as that of mouse NLRR-3 mRNA, except for low level expression in rat liver (9). In the c-Ha-ras transgenic rat, a single 4.2-kb transcript of the gene was also predominant in the brain. Tissue expression pattern, except for the liver was the same as that in the wild counterpart (Fig. 1B, b).

A rat brain cDNA library (1×10^6 plaques) was screened using as a probe the some 580 nt fragment to yield 5 positive clones. Partial nucleotide sequence analysis suggested that 3 of these clones covered the entire coding region. The deduced protein contained 707 amino acids and included two hydrophobic stretches. The one in the most N-terminal region was likely to represent a signal peptide (the possible cleavage site is between amino acid 22 and 23) while the other, close to the C-terminal region, was highly reminiscent of a transmembrane domain (Fig. 2A). In addition, the length of the latter stretch, 21 residues, was consistent with that of plasma membrane protein (16–18). Analysis of the 626 amino acid putative extracellular domain revealed the presence of 11 leucine-rich repeats encompassed by flanking cystein clusters (19), a single immunoglobulin C2 type domain and a fibronectin type III-like domain (Figs. 2A and 2B). The rat gene showed 94.9% and 87.5% similarities on the deduced amino acid level in the mouse (9) and in humans (GenBank Accession No. AC004142) NLRR-3, respectively (Fig. 2A), whereas it was 52.8, 52.8 and 50.5% to humans (20), mouse (8) and xenopus (10) NLRR-1, respectively. Furthermore, the RGD sequence, an integrin binding motif found in many adhesive extracellular matrix proteins such as fibronectin (21) and collagen I (22), common to human and mouse NLRR-3 was also present in the rat sequence. Direct sequencing of the gene originally identified in fibrosarcoma cells confirmed that it was identical to the gene

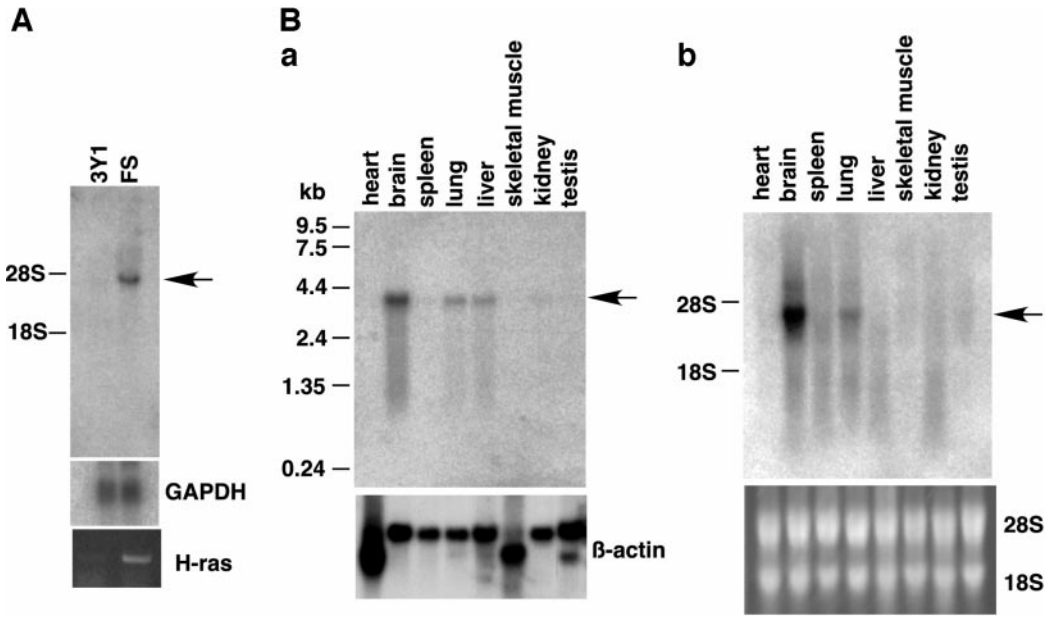

FIG. 1. A gene overexpressed in fibrosarcoma cells derived from c-Ha-ras transgenic rat is abundant in the adult rat brain. (A) Northern blot analysis of a cDNA fragment isolated by suppression subtractive hybridization technique. Total c-Ha-ras (the endogenous gene and the transgene) mRNA level was analyzed by RT-PCR using primers common to both genes (see Materials and Methods). FS, fibrosarcoma cells. (B) Expression profiles of mRNA of the isolated gene in the tissue of wild type (a) and c-Ha-ras transgenic (b) rats. Lower panels show the blot reprobed with a β-actin cDNA (a) and ethidium bromide-stained ribosomal RNA (b). Arrows indicate a major transcript detected at 4.2 kb in FS and rat tissues.

cloned from the brain library. Taken together, we concluded that the rat gene up-regulated in tumor cells overexpressing c-Ha-ras is rat NLRR-3.

Rat NLRR-3 Gene Expression Is Regulated through the Ras-MAPK Pathway

To demonstrate that rNLRR-3 is a downstream gene of the Ras-MAPK signaling pathway, 3Y1 cells stably expressing wild type H-Ras (3Y1Ras), constitutively active H-Ras^{V12} (3Y1RasV12) and v-H-Ras (HR-3Y1-2) were examined for rNLRR-3 expression levels and activation states of MAPK. The rNLRR-3 mRNA level was 3.7-, 2.1- fold higher in 3Y1RasV12, HR-3Y1-2 cells, respectively than in parent 3Y1 cells, determined in RT-PCR analysis. Little increase of the message was observed in 3Y1Ras cells. Similar results were obtained with northern blot analysis (Fig. 3). A three times longer exposure of the blot revealed little detectable rNLRR-3 signals in parent 3Y1 and 3Y1Ras cells, in good agreement with the RT-PCR data (data not shown). Phosphorylation levels of MAPK in 3Y1RasV12 and HR-3Y1-2 were 1.7 and 1.1 times higher, respectively, than that of 3Y1 or 3Y1Ras (Fig. 3). This result indicates causal relationships between activation of the Ras-MAPK pathway and up-regulation of rNLRR-3 gene expression.

PD98059 and U0126, inhibitors for MAPK kinase (MEK), and LY294002, an inhibitor for PI3 kinase, another downstream effector of Ras, were used to further demonstrate the specificity of the Ras-MAPK pathway on the regulation of rNLRR-3 gene expression (Fig. 4). After serum deprivation, 3Y1RasV12 cells were treated with each inhibitor for 24 h. U0126 (50 μM) completely suppressed rNLRR-3 mRNA level with complete inhibition of MAPK phosphorylation. PD98059 (50 μM) also inhibited the rNLRR-3 mRNA level (45% inhibition) with 38% inhibition of MAPK phosphorylation. The rNLRR-3 mRNA level was further inhibited (58% inhibition) when the cell was treated with PD98059 for 48 h (data not shown). LY294002 (20 μM) showed a weaker inhibitory effect (16% inhibition) on rNLRR-3 transcription compared with findings with PD98059 and U0126 (Fig. 4). These results suggest that the Ras-MAPK cascade is a major regulatory pathway for rNLRR-3 gene expression.

Physiological stimuli also regulates rNLRR-3 gene expression through the Ras-MAPK pathway. EGF stimulation of serum starved 3Y1 fibroblasts led to elevation of rNLRR-3 gene expression about 4 hr after the stimulation (Fig. 5A). When the cells were pretreated with U0126 for 10 h prior to EGF stimulation, elevation of rNLRR-3 gene expression by EGF was completely suppressed (compare lanes 2, 3 and 5 of Fig. 5B), and the expression level was reduced to below the level in a control unstimulated cells (compare lanes 2 and 5 of Fig. 5B). In contrast, another MEK inhibitor PD98059 had little effect on the rNLRR-3 mRNA level in 3Y1 cells, under the same condition. The inhibitory

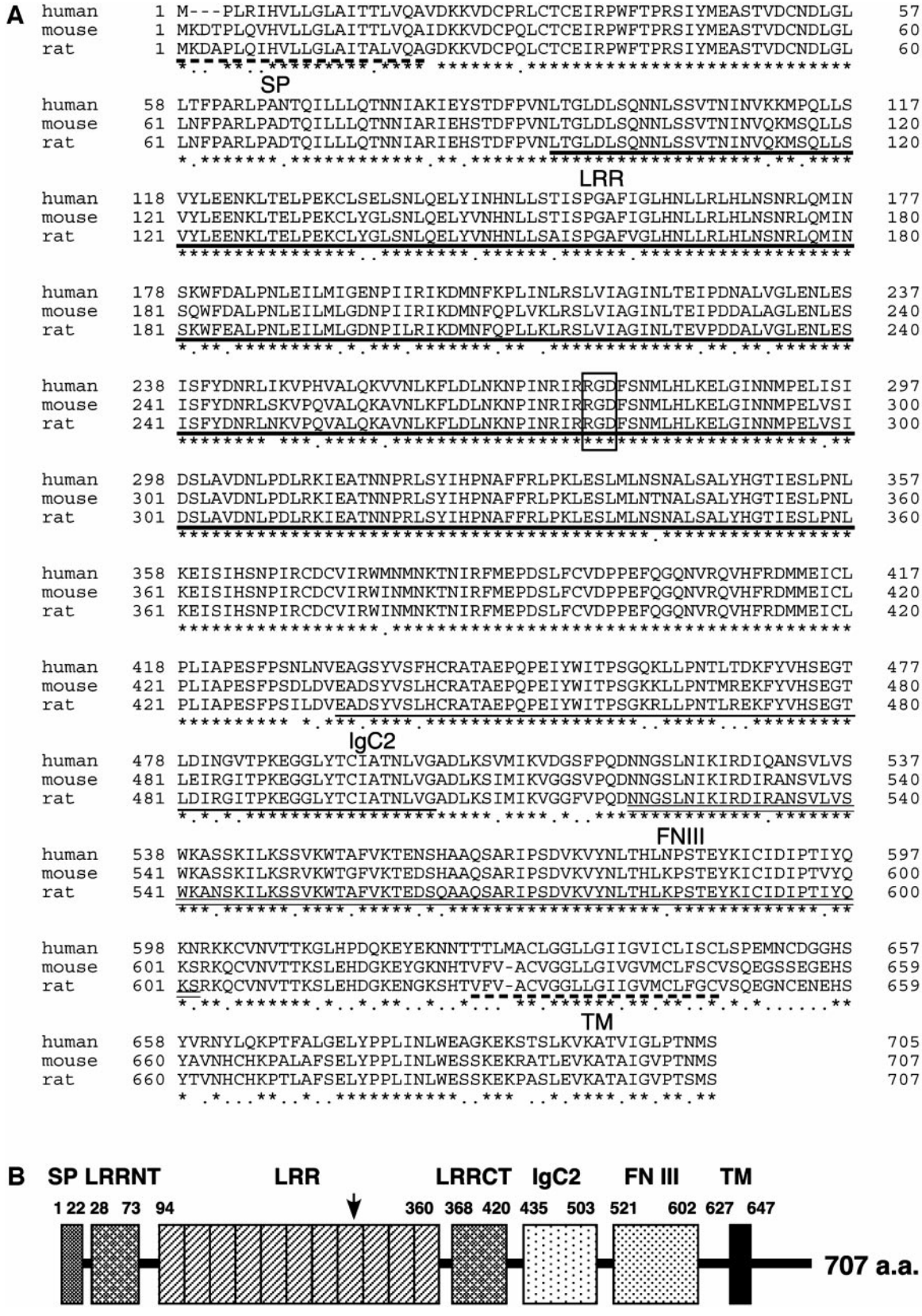

FIG. 2. Deduced amino acid sequence and schematic drawing of the structure of rat NLRR-3. (A) Amino acid sequence comparison of rNLRR-3 with human and mouse NLRR-3. Identical amino acids are indicated by asterisks. Signal peptide (SP) and transmembrane region (TM) are indicated by dotted underlines. Leucine-rich repeat (LRR), immunoglobulin-like C2 type domain (IgC2) and fibronectin type III-like domain (FNIII) are indicated by bold underline, underline and double underline, respectively. An integrin binding motif (RGD) is boxed. The nucleotide sequence for the rat NLRR-3 gene has been deposited in the GenBank database under Accession No. AF291437. (B) Schematic representation of rat NLRR-3. Arrow indicates position of the RGD sequence. LRRCT, leucine-rich repeat C-terminal domain; LRRNT, leucine-rich repeat N-terminal domain.

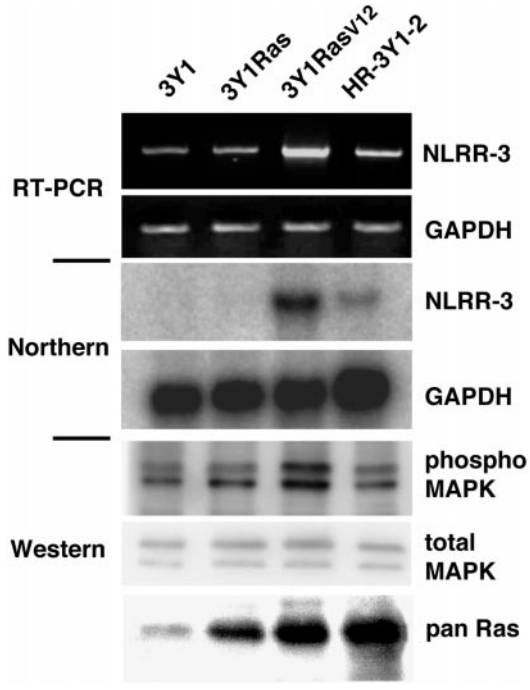

FIG. 3. Increase of rNLRR-3 mRNA in 3Y1 cells expressing constitutively active Ras. RT-PCR and Northern analyses of rNLRR-3 gene expression in 3Y1Ras, 3Y1RasV12 and HR-3Y1-2 cells, stably transfected with wild type H-ras, mutated H-ras^{V12} and v-H-ras, respectively. Activation of MAP kinase in these cell lines was assessed by Western blotting using a phospho-MAPK specific antibody. Ras protein level (pan Ras) represents total amount of H-, K- and N-Ras in the cells. Each lane was loaded with 10 μg RNA and 10 μg protein for northern and Western analyses, respectively. Anti phospho-MAPK (1 μg/ml), anti-MAPK (16 ng/ml) and anti-Ras (0.5 μg/ml) antibodies were used. NLRR-3; rat NLRR-3.

effect of LY294002 was much weaker than that of U0126 (Fig. 5B, lanes 5 and 6) although LY294002 did completely inhibit the EGF-induced up-regulation of rNLRR-3 expression (compare lanes 2, 3 and 6 of Fig. 5B). In the presence of U0126, MAPK phosphorylation was completely blocked at 10 min and 8 hr after EGF stimulation (Fig. 5C, lanes 5 and 10). In contrast, PD98059 and LY294002 showed much weaker effects on MAPK phosphorylation than did U0126; 9% inhibition for PD98059 and 35% inhibition for LY294002 at 10 min (Fig. 5C, lanes 4 and 6, see also lanes 9 and 11), findings comparable with data in Fig. 4. It is noteworthy that U0126 inhibits both active/inactive MEK1 and MEK2, whereas PD98059 only inhibits the inactive form of MEK1 (23, 24). Therefore, U0126 is more potent than PD98059 in inhibiting MAPK phosphorylation. Taken together, we concluded that rNLRR-3 gene expression in fibroblasts is mainly regulated through the Ras-MAPK signaling pathway.

DISCUSSION

We isolated and cloned rNLRR-3, a newly identified member of the NLRR gene family. Our evidence is the first that gene expression of rNLRR-3 is regulated through the Ras-MAPK pathway.

NLRRs are likely to function as cell adhesion molecules or signal transducing receptors because all LRR proteins with cysteine-rich carboxyl and amino flanking regions appear to be involved in cell adhesion or function as receptors (7). Although rNLRR-3 and other NLRRs have no signaling domain in the cytoplasmic region, they do share a well conserved stretch of 11 amino acids (ELYPPLIN/SLWE) with two clathrin mediated endocytosis motifs; YXXϕ, where ϕ is a bulky hydrophobic amino acid (25–27), and a dileucine-type motif (27). Endocytosis and recycling mechanisms are relevant for cell adhesion molecules (28, 29). The endocytosis motif in the β2 integrin has been shown to mediate its recycling to the plasma membrane and to be required for migration (30). Biochemical studies to demonstrate physical associations between rNLRR-3 and adaptins are currently ongoing in our laboratory.

Six members of NLRR family have to date been reported; mouse NLRR-1, -2 and -3 (8, 9), xenopus NLRR-1 (10), GAC1 (31), and zebrafish NLRR (11). Gene expressions of mouse NLRR-1, -2 and -3 are distinctly regulated during development (8, 9). Expression of mouse NLRR-3 mRNA is stronger in the brain from E17 to P7 than in adults. In the adult mouse, the highest expression of the gene is observed in the brain (9) as is the case in adult rats (Fig. 1B). The stronger expression is localized in the cerebral cortex of the adult mouse (9). It is interesting that the brain is one of the organs most abundantly expressing Ras (32; K. Fukamachi, unpublished data). Since we demonstrated that rNLRR-3 is a downstream target gene of

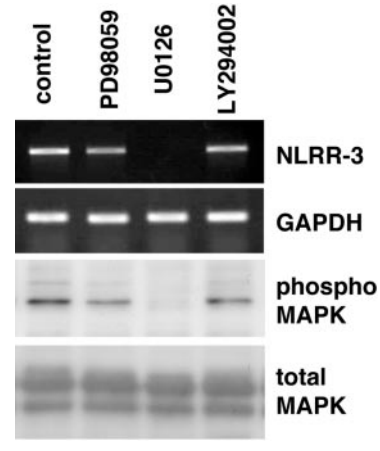

FIG. 4. Effects of MEK inhibitors and a PI3K inhibitor on the expression of rNLRR-3 gene in 3Y1RasV12 cells. rNLRR-3 (NLRR-3) mRNA level was assessed by RT-PCR. Serum starved cells were incubated in the absence (control) or presence of inhibitors (50 μM PD98059, 50 μM U0126, 20 μM LY294002) for 24 h and subjected to RNA or protein isolations. Each lane was loaded with 10 μg protein and probed with 1 μg/ml anti phospho-MAPK or 16 ng/ml anti-MAPK antibodies.

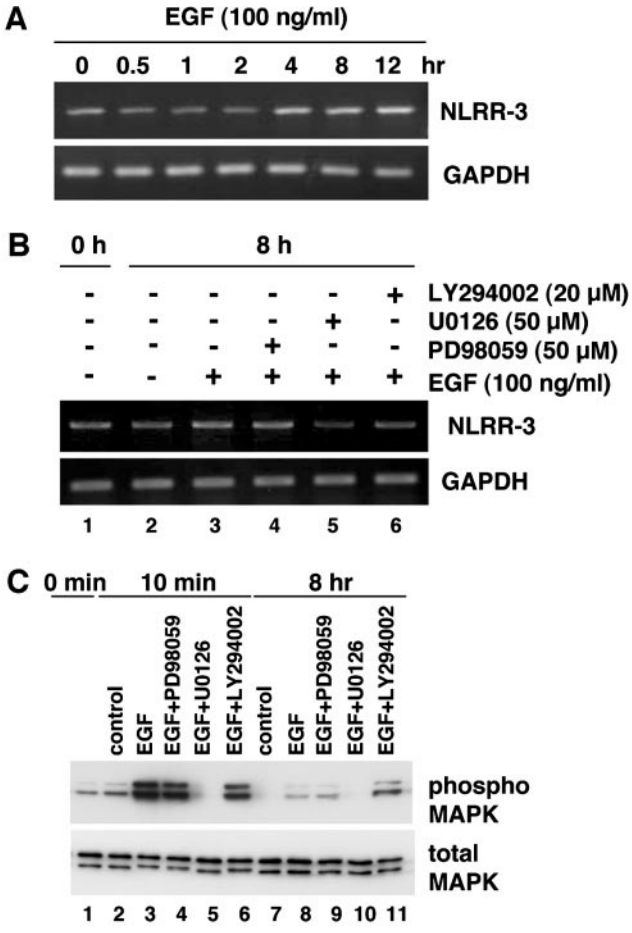

FIG. 5. Expression of rNLRR-3 is regulated through Ras-MAPK pathway in normal fibroblast. (A) RT-PCR analysis of rNLRR-3 (NLRR-3) expression in rat normal fibroblasts after EGF treatment. 3Y1 cells were serum starved for 48 h (0.5% FCS), then treated with 100 ng/ml of EGF for the indicated time. Gene expression of rNLRR-3 started to increase at around 4 h after EGF stimulation. (B) Effects of MEK inhibitors (PD98059, U0126) and PI3K inhibitor (LY294002) on expression of the rNLRR-3 gene in EGF-treated cells. Cells were serum starved in the absence (lanes 1–3) or presence (lanes 4–6) of inhibitors for 10 h prior to stimulation then treated without (lane 2) or with (lanes 3–6) EGF for 8 h, as described under Materials and Methods. The inhibitors were present during EGF treatment (lanes 4–6). rNLRR-3 mRNA level was determined by RT-PCR. (C) Effects of PD98059, U0126 and LY294002 on the activation of MAPK after addition of EGF. The cells were treated without (lanes 1–3, 7, 8) or with (lanes 4–6, 9–11) the same concentrations of inhibitors as in (B). After treatment without (lanes 1, 2, 7) or with 100 ng/ml of EGF for 0 min (lane 1), 10 min (lanes 2–6) and 8 h (lanes 7–11), cells were lysed and processed, as described under Materials and Methods. Phospho-MAPK was detected in lane 7 but hardly so in lanes 5 and 10 even after a longer exposure. Anti-phospho-MAPK (1 μg/ml) and anti-MAPK (16 ng/ml) antibodies were used.

Ras-MAPK signaling pathway in the fibroblasts, it may also be the case in the neuronal cells.

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