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Thromboxane A_2 promotes interleukin-6 biosynthesis mediated by an activation of cyclic AMP-response element binding protein in 1321N1 human astrocytoma cells.

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Abbreviations:

CREB, cyclic AMP-response element-binding protein; MAPK, mitogen-activated protein kinase; PI-PLC, phosphatidylinositol-specific phospholipase C; ERK, extracellular signal-regulated kinase; PC-PLC, phosphatidylcholine-specific phospholipase C; ELISA, enzyme-linked immunosorbent assay; RT-PCR, reverse transcription-polymerase chain reaction; PMA, phorbol-12-myristate-13-acetate; HRP, horseradish peroxidase; GFAP, glial fibrillary acidic protein; FITC, fluorescein isothiocyanate; DMEM, Dulbecco's modified Eagle's medium; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; MSK, mitogen and stress-activated protein kinase; TBST, Tris-buffered saline supplemented with 0.1% Tween-20; C/EBP-β, CCAAT enhancer binding protein-β; CaMK, calmodulin-dependent kinases; PI3K, phosphatidylinositol 3-kinase; EGFR, epidermal growth factor receptor.

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ABSTRACT

1321N1 human astrocytoma cells express thromboxane A_2 (TXA_2) receptors (TP). However, physiological consequences of TXA_2 signaling in glial cells remain unclear. Here, we show that TXA_2 promotes interleukin-6 (IL-6) biosynthesis in glial cells. A TP agonist, U46619, enhanced IL-6 production in both 1321N1 cells and cultured mouse astrocytes. It has been shown that IL-6 gene expression is regulated by various transcription factors. Among them, we found a significant increase in cyclic AMP-response element-binding protein (CREB) activity with its phosphorylation at Ser 133 by U46619 in 1321N1 cells. While U46619 increased IL-6 promoter activity, a mutation at CRE on the promoter clearly suppressed the effect, suggesting that CRE is involved in U46619-induced IL-6 expression. Furthermore, both CREB and IL-6 promoter activities were suppressed by SB203580, a p38 mitogen-activated protein kinase (MAPK) inhibitor and H89, a protein kinase A (PKA) inhibitor, indicating involvements of p38 MAPK and PKA in CREB activation and IL-6 expression. To determine which G-proteins are implicated in the U46619-induced IL-6 synthesis, the interfering mutants of $G\alpha_q$, $G\alpha_{12}$ or $G\alpha_{13}$ were overexpressed in 1321N1 cells by adenoviral approach. Interestingly, the $G\alpha_q$ or $G\alpha_{13}$ mutant blocked the IL-6 production by U46619. The constitutively active mutant of $G\alpha_q$, $G\alpha_{12}$ or $G\alpha_{13}$ enhanced IL-6 production, indicating that $G\alpha_q$ and $G\alpha_{13}$ were involved in U46619-induced IL-6 production. In conclusion, TXA_2 enhances the IL-6 biosynthesis via PKA, p38 MAPK/CREB pathway in 1321N1 cells. IL-6 induction depends on $G\alpha_q$ and $G\alpha_{13}$ as well. This is the first report showing TP-mediated IL-6 production in glial cells.

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The central nervous system (CNS) consists mainly of neurons and glial cells. Neurons play a central role in signal transduction by releasing neurotransmitters, whereas glial cells support the neuronal functions. Glial cells in CNS are generally classified into astrocytes, oligodendrocytes and microglia. Astrocytes form the blood-brain barrier cooperating with endothelial cells, metabolize neurotransmitters, cause gliosis after brain injury and regulate extrasynaptic ion and H^+ concentrations (Ransom et al., 2003; Somjen, 1988). In addition to these roles, they produce neurotrophic factors to promote neuronal survival (prevention of apoptosis), differentiation, or regeneration. It has been shown that they secrete neurotrophic factors such as neurotrophin family members (Althaus and Richter-Landsberg, 2000), interleukin-6 (IL-6) (Schwaninger et al., 1997; Van Wagoner and Benveniste, 1999), and glial cell line-derived neurotrophic factor (Lin et al., 1993). Previously, we have shown that 1321N1 human astrocytoma cells produce nerve growth factor (NGF) and IL-6 (Obara et al., 2001). These neurotrophic factors have been suggested as potential therapeutic agents for the treatment of neurodegenerative diseases such as Alzheimer's and Parkinson's diseases.

Thromboxane A_2 (TXA_2), a metabolite of arachidonic acid produced by cyclooxygenase and TXA_2 synthetase, is known to induce many cellular responses, including platelet aggregation and contraction in various smooth muscle types (Huang et al., 2004). Since TXA_2 is associated with various pathema such as thrombogenesis, hypertension, bronchial asthma and nephrotic syndrome, the antagonist for TXA_2 receptor and inhibitor of its synthesis have been suggested as therapeutic targets (Huang et al., 2004).

We found an existence of TXA_2 receptors (TPs) in 1321N1 human astrocytoma cells and rabbit cultured astrocytes (Nakahata et al., 1992; Nakahata et al., 1989). 1321N1 human astrocytoma cells express both TP isoforms (TP-α □ □ □ TP-β) (Honma et al., 1998), which are alternative spliced variants from the same gene. They are seven-transmembrane receptors and

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have been shown to associate with heterotrimeric GTP-binding proteins such as $G_{q/11}$, $G_{\square}$, G_{12}, G_{13} and G_h (Allan et al., 1996; Gao et al., 2001; Nakahata et al., 1995; Offermanns et al., 1994; Vezza et al., 1999). In response to TXA_2 analog, an elevation of intracellular Ca^{2+} concentration is observed as a result of phosphatidylinositol-specific phospholipase C (PI-PLC) activation in 1321N1 cells. TP-mediated extracellular signal-regulated kinase (ERK) activation in differentiated 1321N1 cells depends on the activation of phosphatidylcholine-specific phospholipase C (PC-PLC) (Kobayashi et al., 2000). It has also been shown that TXA_2 analog elevates cyclic AMP concentration in several cell types such as schwann cells (Muja et al., 2001) even though there is currently no evidence for the interaction between TPs and $G\alpha_s$. In that report, cyclic AMP-response element binding protein (CREB) is activated, which is assumed to be by a cyclic AMP-dependent mechanism. Thus, the downstream pathway of TPs is complex and has not been clarified in detail, yet.

In the screening for compounds which enhance neurotrophic factor production in 1321N1 cells, we found that TXA_2 analog, U46619, significantly promoted IL-6 secretion. Here, we show the mechanism of IL-6 production by TXA_2 in detail.

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Materials and Methods

Materials. U46619 (9,11-Dideoxy-9α,11α-methanoepoxyprostaglandin $F_{2\alpha}$) is obtained from Cayman Chemical (Ann Arbor, MI). Prostaglandin E_2, Ro20-1724 (4-(3-Butoxy-4-methoxybenzyl)imidazolidin-2-one), isoproterenol, carbachol, dibutyryl cyclic AMP (dbcAMP), H89 (N-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline), wortmannin, and KN62 (1-[N,O-bis(5-Isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine) were purchased from Sigma Aldrich (St. Louis, MO). IL-6 enzyme-linked immunosorbent assay (ELISA) kit was from Amersham Pharmacia (Piscataway, NJ). RNA extraction kit was from Nippon Gene (Toyama, Japan) and reverse transcription-polymerase chain reaction (RT-PCR) kit was from Toyobo (Osaka, Japan). Phorbol-12-myristate-13-acetate (PMA), SB203580 (4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole) and forskolin were from Wako Pure Chemicals (Tokyo, Japan). SQ29548 ([1S- [1α, 2α(Z), 3α, 4α]] -7- [3-[[2- [(phenylamino)carbonyl] hydrazino] methyl] -7-oxabicyclo(2,2,1)hept-2-yl] -5-heptenoic acid) was from Squibb (Tokyo, Japan). KT5720 ((9S,10S,12R)-2,3,9,10,11,12-Hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3',2',1'-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid hexyl ester) was from Calbiochem (La Jolla, CA). CRE-luciferase reporter plasmid was from Clontech (Palo Alto, CA). FuGENE 6 transfection reagent was from Roche (Indianapolis, IN). PD98059 (2-(2'-Amino-3'-methoxyphenol)-ox-anaphthalen-4-one), anti-phospho-ERK antibody, anti-phospho-p38 mitogen-activated protein kinase (MAPK) antibody, anti-p38 MAPK antibody, anti-phospho-CREB antibody, anti-CREB antibody and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody were from Cell Signaling (Beverly, MA).

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Anti-ERK 2 antibody was from Santa Cruz (Santa Cruz, CA). Anti-glial fibrillary acidic protein (GFAP) antibody was from PROGEN (Heidelberg, Germany). Fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibody was from Kirkegaard & Perry Laboratories Inc. (Gaithersburg, MD). Cholera toxin was from List Biological Laboratories Inc. (Campbell, CA).

Cell culture. 1321N1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS) (Cell Culture Laboratory, Cleveland, OH), penicillin (50 units/ml), and streptomycin (50 μg/ml), in a 5% CO_2 incubator at 37°C. Mouse astrocytes were prepared from neonatal mouse brains, then cultivated in DMEM supplemented with 10% fetal calf serum, penicillin and streptomycin (Muller et al., 1984).

IL-6 sandwich ELISA. IL-6 ELISA was performed as the protocol of IL-6 ELISA kit (Amersham Pharmacia). Briefly, 1321N1 cells were seeded onto 6-well plates (1 x 10^6 cells/well) and cultivated overnight in the absence of FCS. The cells were cultured for 24 h in the presence of U46619, then the conditioned media were collected, followed by centrifugation to remove contaminating cells. These conditioned media were regarded as samples for ELISA, then added to 96-well plates pre-coated with IL-6 antibody. After incubation, IL-6 antibody conjugated with biotin was added, followed by the subsequent addition of avidin-horseradish peroxidase.

Semi-quantitative RT-PCR. Total RNA from 1321N1 cells was extracted by using a total RNA extraction kit, and semi-quantitative RT-PCR was carried out by using a RT-PCR kit. IL-6 mRNA expression was examined as previously described (Obara et al., 2001). For analysis of human IL-6 mRNA, the sense primer (5'-AAA TTC GGT ACA TCC TCG AC-3' of human IL-6 cDNA) and the antisense primer (5'-CAG GAA CTG GAT CAG GAC TT-3'), which were complementary to conserved regions of cDNA from human IL-6,

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were used. For analysis of mouse IL-6 mRNA, the sense primer (5'-CAA GAG ACT TCC ATC CAG TTG C-3') and the antisense primer (5'-TTG CCG AGT AGA TCT CAA AGT GAC-3') were used. The human IL-6 cDNA of 295 base pairs was amplified 26-30 cycles (95°C for 60 s, 56°C for 60 s, 74°C for 60 s) and mouse IL-6 cDNA of 614 base pairs was amplified 28 cycles (94°C for 30 s, 57°C for 60 s, 72°C for 60 s). Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) transcripts of 700 base pairs were used as an internal control. The number of cycles that yielded a quantitative amount of product was determined in preliminary experiments. The densities of the bands corresponding to IL-6 and G3PDH were analyzed by densitometry (NIH image) and the data were expressed as a ratio to G3PDH.

Immunostaining. Mouse astrocytes were fixed with 4% paraformaldehyde, stained with anti-GFAP antibody (1:10 dilution) and FITC-anti-mouse antibody (1:80 dilution).

Plasmids, transfection and reporter gene assay. Plasmids containing IL-6 promoter (wild type or CRE mutated) linked to luciferase gene (Funakoshi et al., 1999; Tokunou et al., 2001) were kindly given from Dr. Toshihiro Ichiki (Kyushu University, Fukuoka, Japan). Human mitogen and stress-activated protein kinase 1 (MSK1) wild type and the kinase-dead mutant (MSK1 D195A/D565A) (Arthur et al., 2004) were from Dr. Soren Impey (Vollum Institute, Oregon Health Sciences University, Portland, OR). A plasmid encoding PKI was from Dr. Philip Stork (Vollum Institute, Oregon Health Sciences University). Plasmids encoding constitutively active mutants of G-proteins ($G\alpha_q$Q209L, $G\alpha_{12}$Q231L and $G\alpha_{13}$Q226L) were kindly provided from John Scott laboratory (Vollum Institute, Oregon Health Sciences University). For transfection, 1321N1 cells were seeded onto 24-well plates at 1×10^5 (cells/well). Next day, plasmids (0.6-1.2 μg/well) and FuGene 6 (0.75-1.8 μl/well) were mixed gently in DMEM (25 μl) and added to culture plates. For luciferase assay, media were replaced by serum-free media 24 h after transfection. The cells were incubated with the

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indicated drugs at 37°C for 6 h, the following day. Luciferase assay was performed by using dual luciferase assay kit (Promega, Madison, WI) according to its instruction. *Renilla* luciferase (phRL-TK, Promega) activity was measured for normalizing the transfection efficiency. The luciferase activity was measured using a luminometer (GENE LIGHT 55, Microtech Nition, Funabashi, Japan).

cAMP assay. Intracellular cAMP concentration was measured as follows. 1321N1 cells on 24 well-plate were labeled with 2 μCi/ml of [^{3}H]adenine (23 Ci/mmol) (Amersham Pharmacia Biotech) in the growth medium for 3 h. The labeled cells were washed twice with DMEM-HEPES (20 mM, pH 7.4). The cells were incubated for 10 min with U46619 (or with SQ29548) in the DMEM-HEPES containing phosphodiesterase inhibitor, Ro20-1724 (100 μM). After aspirating the incubation buffer, the reactions were stopped by adding 0.4 mL of 2.5% $HClO_4$. Acid-extracts were mixed with 1/10 volume of 4.2 M KOH to neutralize and deposit potassium perchlorate. [^{3}H]cAMP in the clear supernatant was separated by Dowex 50W-X8 (Bio-Rad, Hercules, CA)/alumina (Merck, Darmstadt, Germany) double columns, and determined by liquid scintillation counting. [^{3}H]cAMP increase was expressed as the fold over the control (no drug).

Adenoviral infection. Adenoviruses encoding carboxyl terminal regions of $G\alpha_q$ ($G\alpha_q$–ct, amino acids 305-359), $G\alpha_{12}$ ($G\alpha_{12}$–ct, amino acids 325-379) and $G\alpha_{13}$ ($G\alpha_{13}$–ct, amino acids 322-378) were created in our laboratory. The expressions of those genes are regulated by CMV promoter, their products putatively block G protein coupling with their corresponding receptors (Arai et al., 2003). Each adenoviral vector contains both G-protein-ct and GFP that is upstream of the G-protein-ct, and each entity is under a control of CMV promoter. 1321N1 cells were infected with the indicated virus at 75 MOI for a day. Then the medium was replaced with serum-free medium the next day, and the cells were cultured

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overnight. At the time of drug incubation, more than 90% of the cells were GFP-positive.

SDS-polyacrylamide gel electrophoresis and immunoblotting. Samples used for immunoblotting were prepared as follows. 1321N1 cells were seeded onto 6-well plates at a density of 1 x 10^6 (cells/well). The cells were cultured overnight in serum-free DMEM, then drugs were added for various periods of time. The incubation medium was aspirated after the reaction, and the cells were dissolved in Laemmli sample buffer (final concentration, Tris-HCl 75 mM, SDS 2%, glycerol 15%, 2-mercaptoethanol 3%, pH 6.8), and boiled at 95°C for 5 min.

Electrophoresis was performed on 11% acrylamide gels. Proteins were transferred electrically from the gel onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA) by the semi-dry blotting method. The blots were blocked for 40 min with 5% skim-milk in Tris-buffered saline supplemented with 0.1% Tween-20 (TBST), and incubated with primary antibodies (anti-phospho-ERK (Thr 202/Tyr 204) antibody, anti-ERK2 antibody, anti-phospho-p38 MAPK (Thr 180/Tyr 182) antibody, anti-p38 MAPK antibody, anti-phospho-CREB (Ser 133) antibody or anti-CREB antibody (1:1000 dilution)) overnight at 4°C. The blots were washed several times and then incubated at 25°C for 2 h with a 1:3,000 dilution of secondary antibody (HRP-conjugated anti-rabbit IgG antibody). Blots were developed using an enhanced chemiluminescence assay kit (Amersham Biosciences), and visualized by chemiluminescence on Hyper-film ECL. The densities of the bands corresponding to CREB, ERK, p38 MAPK were analyzed by densitometry (NIH image) and the data were expressed as a ratio to their total proteins.

Statistical methods. Data were expressed as the mean values $\pm$ S.E.M, and the significant differences were analyzed by Student *t*-test or Tukey's method for multiple-comparisons.

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Results

U46619 enhances IL-6 production in 1321N1 cells. We investigated the biosynthesis of IL-6. 1321N1 cells were stimulated with TP agonist, U46619, then IL-6 secretion was examined by sandwich-ELISA (Fig. 1A). U46619 significantly increased IL-6 secretion from 1321N1 cells in a concentration-dependent manner, that was inhibited by TP antagonist, SQ29548. Adenylyl cyclase activator, forskolin and cAMP analog, dbcAMP also promoted IL-6 secretion 1.6 fold and 2.9 fold, respectively (data not shown). U46619 (10 μM) also enhanced IL-6 gene expression (5.8 fold), and this effect was abolished by SQ29548 (1 μM) again (Fig. 1B). Carbachol (10 μM), did not affect IL-6 gene expression. Forskolin and a PKC activator, PMA, enhanced IL-6 expression 4.5 fold and 7.0 fold in the same condition, respectively (Fig. 1B). U46619 and SQ29548 have low affinity to other prostanoid receptors such as EP_4 (Kiriyama et al., 1997), and it is also known that prostaglandin E_2 induces IL-6 release in U373 MG human astrocytoma cells (Fiebich et al., 2001). To avoid the possibility that the effect of U46619 on IL-6 induction is mediated by EPs, we tested prostaglandin E_2. U46619-induced IL-6 expression was completely blocked by SQ29548 (3% of U46619 alone), while most part of the effect of prostaglandin E_2 was not blocked by SQ29548 (82% of prostaglandin E_2 alone) (Fig. 1C). Cholera toxin as a positive control enhanced IL-6 expression 13.2 fold (Fig. 1C). This result indicates that effect of U46619 is mediated by TPs. To examine the IL-6 promoter activity, plasmid encoding wild type IL-6 promoter (-1039~+1) linked to luciferase gene was transfected into 1321N1 cells, and luciferase activity was measured (Fig. 1D). U46619 (10 μM) as well as forskolin (10 μM) significantly enhanced IL-6 promoter activity. Furthermore, GFAP-positive cultured mouse astrocytes (Fig. 2A) were

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stimulated with U46619 to determine whether IL-6 enhancement is 1321N1 cell-specific or not. As expected, U46619 (10 μM) similarly promoted IL-6 gene expression (2.7 fold) in astrocytes as dbcAMP (2.1 fold), and the effect of U46619 was mostly reversed by SQ29548 (12% of dbcAMP alone) (Fig. 2B). Taken together, these results suggest that U46619 enhances the secretion of IL-6 from 1321N1 cells accompanied with an increase in IL-6 gene expression.

CRE regulates the IL-6 gene expression by U46619 in 1321N1 cells. IL-6 gene expression is regulated by multiple transcription factors including CREB, activator protein-1 (AP-1), nuclear factor-κB (NF-κB), CCAAT enhancer binding protein-β (C/EBP-β) and glucocorticoid receptor in complex ways (Obara and Nakahata, 2002). Since CREB activation by U46619 has been reported (Muja et al., 2001), we determined if CREB is involved in the U46619-induced IL-6 gene expression. CRE activity was measured by reporter gene assay. pCRE-luciferase plasmid was transfected into 1321N1 cells, then newly induced luciferase activity was regarded as the CREB transcriptional activity. U46619 (10 μM) and forskolin (10 μM) significantly increased CRE activity (Fig. 3A). Next, to confirm the specificity of CRE in enhanced IL-6 gene expression, we utilized the IL-6 promoter-luciferase plasmid harboring a mutant CRE in the IL-6 promoter region (from ATGACGTCA to ATCGATCCA). The effect of U46619 on IL-6 promoter activity was completely abolished by mutation of CRE as in the case of forskolin (Fig. 3B). These results indicate that CRE activation is essential for U46619–induced IL-6 gene expression in 1321N1 cells.

PKA and p38 MAPK/MSK pathways are involved in CREB activation and IL-6 gene expression by U46619 in 1321N1 cells. It has been shown that CREB activity is regulated by phosphorylation at Ser 133, where various kinases have been proposed to

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phosphorylate, including protein kinase A (PKA), Akt (or protein kinase B), calmodulin-dependent kinases (CaMKs), ribosomal S6 kinases (RSKs) and MSKs (Lonze and Ginty, 2002). Akt is downstream effecter of phosphoinositide 3-kinase (PI3K), and RSKs and MSKs can be activated by ERK 1/2, ERK 5 or p38 MAPK. To investigate the U46619 signaling to CREB in detail, pharmacological tools were utilized to identify the CREB kinase. 1321N1 cells were pretreated with H89 (PKA inhibitor; 10 μM), PD98059 (MEK inhibitor; 30 μM), SB203580 (p38 MAPK inhibitor; 30 μM), wortmannin (PI3K inhibitor; 1 μM) or KN62 (CaMK inhibitor; 10 μM) for 15 min, then, they were stimulated with U46619 (10 μM) for 6 h (Fig. 4A). The U46619-induced CRE activation was significantly blocked by H89 and SB203580. Carbachol (10 or 100 μM) did not increase the CRE activity. Since H89 has been reported to inhibit MSK as well as PKA (Davies et al., 2000), another PKA inhibitor KT5720 was tested. KT5720 (2 μM) also inhibited the CRE activity significantly (Fig. 4B). Increase in CRE activity by U46619 was also blocked by SQ29548 (2 μM) and lower concentration of SB203580 (3 μM) (Fig. 4C). Furthermore, MSK 1 wild type and the kinase-dead mutant (D195A and D565A) were overexpressed in 1321N1 cells, and CRE activity was measured (Fig. 4D). The MSK 1 kinase-dead mutant partially but significantly attenuated the ability of U46619 to increase the CRE activity. CREB family members including CREB, activating transcription factor 1 and CRE modulator binds to CRE. To investigate whether CREB is involved in this process, CREB phosphorylation at Ser 133 was monitored. This phosphorylation site can be regarded as an index of CREB activation. The CREB phosphorylation by U46619 via TPs was observed (Fig. 5A), confirming that CREB is activated by U46619. This phosphorylation was completely blocked by H89 (10 μM), SB203580 (30 μM) and KT5720 (2 μM) again as in Fig. 4A and 4B (Fig. 5B). These results

suggest that CREB activation by U46619 is mediated by PKA and p38 MAPK/MSK pathways. Next, the effects of these kinase inhibitors on IL-6 gene expression were investigated. H89 (10 μM) and SB203580 (30 μM) decreased IL-6 promoter activity accelerated by U46619 (10 μM) (Fig. 6A), similar to the inhibition of CRE activity shown in Fig. 4A. The effect of U46619 was also significantly inhibited by SQ29548 (2 μM), SB203580 (3 μM) and overexpressing a physiological PKA pseudosubstrate peptide, PKI (Fig. 6B). Furthermore, when 1321N1 cells were pretreated with SB203580 (30 μM), IL-6 gene expression induced by U46619 (10 μM) was largely suppressed (21% of U46619 alone), as determined by RT-PCR (Fig. 6C). Since the involvement of p38 MAPK in TP-mediated IL-6 expression was suggested, we examined whether p38 MAPK was activated by U46619. When 1321N1 cells were incubated with U46619 (10 μM) for 5-20 min, transient phosphorylation of ERK 1/2 was observed (2.2 fold at 5 min, 1.9 fold at 10 min, 0.97 fold at 20 min)(Fig. 7). In addition to ERKs, p38 MAPK phosphorylation was induced by U46619 (10 μM) for 5-20 min (1.8 fold at 5 min, 2.1 fold at 10 min, 2.8 fold at 20 min). CREB phosphorylation at Ser 133 in the same lysates was also monitored. As expected, U46619 induced CREB phosphorylation, correlated with p38 MAPK phosphorylation (5.6 fold at 5 min, 9.1 fold at 10 min, 2.9 fold at 20 min) (Fig. 7). $G\alpha_s$ coupling to TPs has not currently been reported although an intracellular cAMP concentration by TP agonist is elevated in many cell types. We measured cAMP level after U46619 treatment (Fig. 8). β-Adrenergic receptor agonist, isoproterenol (10 μM) strongly elevated cAMP level. Comparing with isoproterenol, the effect of U46619 on cAMP accumulation was much weaker, but the significant cAMP increase via TPs was observed. Taken together, these results suggest that PKA and p38 MAPK/MSK pathways are involved in CREB activation and IL-6 gene expression induced by U46619 in 1321N1 cells.

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$G\alpha_q$ and $G\alpha_{13}$ mediate TXA_2 signaling to IL-6 expression. TPs couple with various kinds of G-proteins such as $G\alpha_q$, $G\alpha_{12}$ and $G\alpha_{13}$ (Huang et al., 2004). We have shown that TPs associate with at least $G\alpha_q$ and $G\alpha_{12}$ in 1321N1 cells (Honma et al., 1998). To determine which G-proteins are involved in the signaling pathway leading to IL-6 production, we took an adenoviral approach to interfere with endogenous G-protein functions. The products from adenoviruses that encode the C-terminal regions of $G\alpha_q$, $G\alpha_{12}$ or $G\alpha_{13}$ inhibited the coupling between these G-proteins and their associated receptors (Arai et al., 2003). GFP gene is introduced to monitor the infection efficiency of those G-protein interfering mutants. 1321N1 cells infected with those viruses were stimulated with U46619 (10 μM) for 4 h, then RT-PCR was carried out. IL-6 gene expression was promoted with U46619 treatment in 1321N1 cells infected with control virus, and the enhancement was blocked by overexpressing C-terminal regions of $G\alpha_q$ or $G\alpha_{13}$, (45% and 53% of U46619 (GFP), respectively) but not by $G\alpha_{12}$ (Fig. 9A). Furthermore, to ask if these G-proteins can affect IL-6 production, the cells were transfected with constitutively active mutants ($G\alpha_q$Q209L, $G\alpha_{12}$Q231L and $G\alpha_{13}$Q226L). $G\alpha_q$Q209L and $G\alpha_{13}$Q226L promoted IL-6 gene expression 15 fold and 7.9 fold, respectively, determined by RT-PCR, while $G\alpha_{12}$Q231L marginally promoted it (2.6 fold) (Fig. 9B). In addition, these active mutants could significantly enhance both CRE and IL-6 promoter activities (Fig. 9C). Taken together, the results suggest that $G\alpha_q$ and $G\alpha_{13}$ are necessary and sufficient for U46619-induced IL-6 gene expression.

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Discussion

In the present study, we showed that U46619 promoted IL-6 biosynthesis in 1321N1 human astrocytoma cells and GFAP-positive mouse astrocytes. In addition, U46619 increased CREB activity that was accompanied by its phosphorylation at Ser 133 by PKA and p38 MAPK/MSK, and this CREB activation is essential for IL-6 induction in 1321N1 cells (Fig. 10). This is the first report showing TP-mediated IL-6 production in glial cells.

The amount of arachidonic acid metabolites are increased dramatically following brain injury, viral infection or inflammation. These metabolites, including TXA_2 are assumed to play important roles in the CNS under such pathologic conditions. In fact, astrocytes and microglia have the ability to release TXA_2 following activation by bacterial lipopolysaccharide and ATP (Bruner and Murphy, 1993; Minghetti and Levi, 1995; Pearce et al., 1989). Thus, the elevation of adenine nucleotide concentration or microglial activation after CNS injury or infection may cause the secretion of TXA_2, leading to activation of TPs on glial cells via autocrine or juxacrine mechanisms. Therefore, a clarification of the physiological roles of TPs in astrocytes will help in identifying therapeutic targets in various CNS disorders.

IL-6 is a pleiotropic factor involved in the regulation of various inflammatory and endocrine functions in both central and periphery nervous systems. In the CNS, although various cell types can produce IL-6, astrocytes play a dominant role in IL-6 production (Gruol and Nelson, 1997). Under normal physiological conditions, IL-6 expression levels in CNS remain low, while IL-6 is up-regulated upon stress, diseases, inflammation or injury. To date, it

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has been shown that numerous physiological receptor agonists or pharmacological reagents influence IL-6 production (Sehgal, 1992; Van Wagoner and Benveniste, 1999). Cytokines and proinflammatory factors, including lipopolysaccharide, IL-1β, tumor necrosis factor (TNF)-α □□□□□□□□□□□□ and prostaglandin E_2 are known as IL-6 inducers, and play important roles in CNS diseases. In addition, various kinds of neurotransmitters and neuropeptides, including catecholamines, adenosine, serotonin, histamine, substance P, calcitonin, vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide, also influence IL-6 production. Abnormal physiological condition often changes the induction of IL-6, such as hypoxia/reoxygenation, generation of reactive oxygen species, viral infections and irradiation. Not surprisingly, compounds that increase cyclic AMP concentration such as cholera toxin, cyclic AMP analogs and forskolin also lead to an induction of IL-6 in astrocytes (Norris et al., 1994). Furthermore, phorbol ester and Ca^{2+} ionophore, A23187, induced IL-6 production, and both of these reagents caused synergistic production, suggesting that PKC and Ca^{2+} signaling are responsible for IL-6 production in rat astrocytes (Norris et al., 1994). Fiebich et al. (1995) showed that ceramide induced IL-6 gene expression in U373-MG human astrocytoma cells. In terms of transcription factors, IL-6 promoter contains various consensus sequences of transcription factors, indicating that multiple transcription factors influence its gene expression. For example, CREB, AP-1, NF-κB, C/EBP β (or NF-IL6) and glucocorticoid receptor are known as regulators, and stimuli leading to the activation of these transcription factors are assumed to modulate IL-6 gene expression (Obara and Nakahata, 2002; Sehgal, 1992).

In the present study, it was demonstrated that U46619 increased the CRE activity by reporter gene assay, accompanied by the phosphorylation of CREB at Ser 133, which is a key regulatory site for its transcription activity (Fig. 3A, 4, 5 and 7). Furthermore, this CREB

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activation is required for IL-6 gene expression (Fig. 3B). To date, it has been shown that the phosphorylation of this site occurs via multiple kinases including PKA, CaM-kinase, Akt, RSKs and MSKs (Lonze and Ginty, 2002). RSKs and MSKs are known as down-stream effecters of ERK 1/2, ERK 5 and p38 MAPK (Lonze and Ginty, 2002). In our study, to clarify the mechanism leading to CREB activation by U46619, various pharmacological tools were tested. U46619-induced CREB activation was significantly blocked by H89, KT5720 and SB203580 (Fig. 4). In addition, MSK 1 kinase-dead mutant blocked CRE activity (Fig. 4D), suggesting the involvements of both PKA and p38 MAPK/MSK pathways. Although the mechanism remains unclear, cyclic AMP elevation and PKA activation have been shown in response to TP agonist (Miggin and Kinsella, 2002; Muja et al., 2001). On the other hand, p38 MAPK activation by TXA_2 has been reported in platelets and isolated rat mesenteric resistance arteries (Bolla et al., 2002; Minuz et al., 2002). In the present study, we confirmed the cAMP elevation and activation of p38 MAPK by U46619 in 1321N1 cells (Fig. 7 and 8). Taken together, this study strongly indicates that PKA and p38 MAPK participate in CREB activation by TXA_2. However, as mentioned above, IL-6 gene expression is regulated by multiple transcription factors. Although it was demonstrated in this study that CREB activity was critical for IL-6 induction, we do not deny the possibility that other transcription factors such as AP-1 and NF-κB activated by U46619 (Huang et al., 2004) may work cooperatively with CREB.

We previously demonstrated that TPs coupled with the α subunits of at least two heterotrimeric GTP-binding proteins, G_q and G_{12} in 1321N1 cells (Honma et al., 1998). By stimulation with TP agonists, phosphoinositide hydrolysis is promoted, followed by increase in intracellular Ca^{2+} concentration and PKC activation (Nakahata et al., 1989). In

dbcAMP-treated 1321N1 cells, phosphatidylcholine-specific phospholipase C rather than phosphatidylinositol-specific phospholipase C is predominantly activated by TP agonist, followed by subsequent ERK activation (Kobayashi et al., 2000). Because carbachol, an agonist of M_3 receptor which couples to G_q, did not increase CRE activity nor induce IL-6 expression in this study, it was assumed that the other signaling pathway such as G_{12} or G_{13} other than G_q-mediated PLC pathway may be essential for U46619-induced IL-6 expression. In fact, although all of constitutively active G-protein mutants (G_q, G_{12} and G_{13}) could activate CRE and IL-6 promoter, interfering mutants, G_q-ct and G_{13}-ct, but not G_{12}-ct blocked IL-6 gene expression by TP stimulation (Fig. 6), indicating that TPs utilize G_q and G_{13} pathways for IL-6 production. Even though G_{12} and G_{13} belong to the same heterotrimeric G-protein family, the functional difference between those two G-proteins remain unclear. Nevertheless, in our study, G_{13}, but not G_{12}, is essential for TP-signaling. Identification of direct down-stream effecter of $G\alpha_{13}$ leading to CRE activation and IL-6 gene expression is necessary.

In vascular smooth muscle cells, thrombin induces IL-6 expression through the CRE as in the case of TXA_2 (Tokunou et al., 2001). In that report, involvements of ERK, p38 MAPK and epidermal growth factor receptor (EGFR) are also implicated in thrombin-induced IL-6 production. EGFR transactivation that lies up-stream of ERK and p38 MAPK is essential for this thrombin-induced IL-6 induction. EGFR transactivation by TXA_2 has been also reported (Gao et al., 2001; Miggin and Kinsella, 2001), which was probably mediated by either EGF-like ligand release by metalloproteinases or direct EGFR trans-phosphorylation by unidentified tyrosine kinases. Thus, it is possible that this cross-talk between G protein-coupled receptors and receptor tyrosine kinases is involved in p38 MAPK and CREB activation leading to IL-6 gene expression.

It has been shown that IL-6 acts as a neurotrophic factor as well as cytokine, and numerous studies have demonstrated both beneficial and unfavorable effects of IL-6 in CNS (Van Wagoner and Benveniste, 1999). A variety of *in vitro* and *in vivo* studies provide evidence for involvement of IL-6 in neuronal survival, differentiation and regeneration. IL-6 directly affects neurons, while it also affects glial cells, i.e. IL-6 promotes proliferation of astrocytes and production of NGF by autocrine mechanism. In contrast to the benefits above, IL-6 sometimes cause destructive effects. For example, in IL-6 transgenic mouse models, high level of IL-6 led to neurodegeneration, blood-brain barrier breakdown, abnormal angiogenesis, increased level of complement proteins and impaired learning. In those mice, it has been demonstrated that icv injection of LPS resulted in higher level productions of proinflammatory factors such as TNF-α and IL-1β. IL-6 overproduction in the CNS may trigger unnecessary inflammatory responses and confusion of neuronal network. Thus, both beneficial and detrimental effects of IL-6 in the CNS have to be considered for the further study.

In conclusion, we showed that TXA_2 analog induced a production of IL-6 mediated via PKA, p38 MAPK/CREB pathway through $G\alpha_q$ and $G\alpha_{13}$ in 1321N1 cells. This observation indicates one of physiological roles of TPs in astrocytes and raises a possibility as a therapeutic target in neuronal diseases.

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FIGURE LEGENDS

Fig. 1. Induction of IL-6 by U46619 in 1321N1 cells. (A) Effect of U46619 on the secretion of IL-6 from 1321N1 cells. After incubation with the indicated concentrations of U46619 for 24 h, IL-6 released from 1321N1 cells was measured by ELISA. Values are the means ± S.E.M. of three determinations. U46619 (10 μM) significantly increased IL-6 secretion compared with the corresponding control ($^{*}P < 0.05$), and this effect was blocked by SQ29548 (1 μM) ($^{\#}P < 0.05$). (B) Effect of U46619 on IL-6 gene expression in 1321N1 cells. 1321N1 cells were incubated with U46619 (U, 10 μM), U46619+SQ29548 (SQ, 1 μM), carbachol (Carb, 10 μM), forskolin (FK, 10 μM) or PMA (100 nM) for 4 h in serum-free condition, then RT-PCR was performed as described in Experimental Procedures. (C) Specificity of U46619 and SQ29548 to TPs. 1321N1 cells were incubated with U46619 (U, 10 μM), U46619+SQ29548 (SQ, 1 μM), prostaglandin E_2 (E2, 1 μM), prostaglandin E_2 +SQ29548 or cholera toxin (CTX, 100 μg/ml) for 4 h in serum-free condition, then RT-PCR was performed as described in Experimental Procedures. (D) Effect of U46619 on IL-6 promoter activity in 1321N1 cells. 1321N1 cells were transfected with plasmid encoding IL-6 promoter-luciferase gene. After overnight serum-starvation, the cells were incubated with U46619 (10 μM) or forskolin (10 μM) for 6 h. Luciferase activity was measured as described in Experimental Procedures. Values are the means ± S.E.M. of three determinations. U46619 (10 μM) and forskolin (10 μM) significantly increased IL-6 promoter activity compared with the corresponding control ($^{*}P < 0.05$).

Fig. 2. Induction of IL-6 by U46619 in mouse astrocytes. (A) Identification of astrocytes.

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Mouse astrocytes prepared from mouse brains were identified by immunostaining with anti-GFAP antibody. (B) IL-6 induction by U46619. Mouse astrocytes were incubated with SQ29548 (SQ, 1 μM), U46619 (U, 10 μM), U46619+SQ29548 or dbcAMP (0.5 mM) for 4 h in the serum-free condition, then RT-PCR was performed.

Fig. 3. Involvement of CRE in IL-6 induction by U46619 in 1321N1 cells. (A) Increase in CRE activity by U46619. After 1321N1 cells were transfected with CRE-luciferase reporter gene plasmid, they were stimulated with U46619 (10 μM) or forskolin (10 μM) for 6 h in the serum-free condition. After the incubation, luciferase assay was performed. Values represent the means $\pm$ S.E.M for three determinations. U46619 (10 μM) and forskolin (10 μM) significantly increased the CRE activity ($^{*}P < 0.05$). (B) CRE is essential for IL-6 expression. After 1321N1 cells were transfected with plasmid encoding wild type (WT) IL-6 promoter-luciferase gene or its CRE-mutant, they were stimulated with U46619 (10 μM) or forskolin (10 μM) for 6 h in the serum-free condition. After the incubation, luciferase assay was performed. Values represent the means $\pm$ S.E.M for three determinations. U46619 (10 μM) and forskolin (10 μM) significantly increased the IL-6 promoter activity ($^{*}P < 0.05$), and mutation at CRE significantly suppressed both activities ($^{\#}P < 0.05$).

Fig. 4. Involvements of PKA and p38 MAPK in CRE activation by U46619 in 1321N1 cells. (A) Effects of pharmacological inhibitors on U46619-induced CRE activation. After 1321N1 cells were transfected with CRE-luciferase reporter gene plasmid, they were pretreated with H89 (10 μM), PD98059 (PD, 30 μM), SB203580 (SB, 30 μM), wortmannin (Wort, 1 μM) or KN62 (KN, 10 μM) for 15 min. Then, they were stimulated with U46619 (10 μM),

carbachol (10 or 100 μM) or forskolin (10 μM) for 6 h in the serum-free condition. After the incubation, luciferase assay was performed. Values represent the means ± S.E.M for three determinations. U46619 (10 μM) and forskolin (10 μM) significantly increased the CRE activity ($^{*}P < 0.05$), which was inhibited by H89 (10 μM) and SB203580 (30 μM) ($^{\#}P < 0.05$). (B) Effect of KT5720 on CRE activated by U46619. 1321N1 cells were pretreated with KT5720 (2 μM), then stimulated with U46619 (10 μM) for 6 h as performed above. Values represent the means ± S.E.M for three determinations. U46619 significantly increased the CRE activity ($^{*}P < 0.05$), which was blocked by KT5720 (2 μM) ($^{\#}P < 0.05$). (C) Effects of SQ29548 and SB203580 on CRE activated by U46619. 1321N1 cells were pretreated with SQ29548 (2 μM) or SB203580 (3 μM), then stimulated with U46619 (10 μM) for 6 h as performed above. Values represent the means ± S.E.M for three determinations. U46619 significantly increased the CRE activity ($^{*}P < 0.05$), which was blocked by SQ29548 (2 μM) and SB203580 (3 μM) ($^{\#}P < 0.05$). (D) Involvement of MSK in CRE activated by U46619. MSK1 wild type (WT) or the kinase-dead mutant (KD) (D195A/D565A) was overexpressed in 1321N1 cells, then the cells were stimulated with U46619 (10 μM) for 6 h as performed above. Values represent the means ± S.E.M for three determinations. U46619 significantly increased the CRE activity in the cells overexpressing MSK1 wild type ($^{*}P < 0.05$). The effect was blocked by mutations in two kinase-domains ($^{\#}P < 0.05$).

Fig. 5. CREB phosphorylation by U46619 in 1321N1 cells. (A) CREB at Ser 133 is phosphorylated by U46619 via TPs. 1321N1 cells were stimulated with U46619 (10 μM) for 10 min in the presence or absence of SQ29548 (SQ, 2 μM), followed by Western blotting, using antibodies against phospho-CREB and CREB. (B) CREB at Ser 133 is phosphorylated by

U46619 via PKA and p38 MAPK. 1321N1 cells were stimulated with U46619 (10 μM) for 10 min in the presence or absence of H89 (10 μM), SB203580 (SB, 30 μM) and KT5720 (KT, 2 μM), followed by Western blotting, using antibodies against phospho-CREB and CREB.

Fig. 6. Involvements of PKA and p38 MAPK in IL-6 expression in 1321N1 cells. (A) Effects of several kinase inhibitors on IL-6 promoter activity. After 1321N1 cells were transfected with plasmid encoding IL-6 promoter-luciferase reporter gene plasmid, the cells were pretreated with H89 (10 μM), PD98059 (PD, 30 μM), SB203580 (SB, 30 μM), wortmannin (Wort, 1 μM) or KN62 (KN, 10 μM) for 15 min. Then, they were stimulated with U46619 (10 μM), carbachol (Carb, 10 μM) or forskolin (FK, 10 μM) for 6 h in the serum-free condition. After the incubation, luciferase assay was performed. Values represent the means ± S.E.M for three determinations. U46619 (10 μM) and forskolin (10 μM) significantly increased the IL-6 promoter activity ($^{*}P < 0.05$), which was inhibited by H89 (10 μM) and SB203580 (30 μM) ($^{\#}P < 0.05$). (B) Involvements of TPs, p38 MAPK and PKA in U46619-activated IL-6 promoter activity. After 1321N1 cells were co-transfected with plasmid encoding IL-6 promoter-luciferase reporter gene plasmid and PKI, the cells were pretreated with SQ29548 (SQ, 2 μM) or SB203580 (SB, 3 μM) for 15 min. Then, they were stimulated with U46619 (10 μM) or forskolin (FK, 10 μM) for 6 h in the serum-free condition. After the incubation, luciferase assay was performed. Values represent the means ± S.E.M for three determinations. U46619 (10 μM) and forskolin (10 μM) significantly increased the IL-6 promoter activity ($^{*}P < 0.05$), which was inhibited by SQ29548 (2 μM), SB203580 (3 μM) and PKI ($^{\#}P < 0.05$). (C) Inhibitory effect of SB203580 on IL-6 gene expression. 1321N1 cells were incubated with U46619 (U, 10 μM) in the presence or absence of SB203580 (SB, 30 μM)

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for 4 h in the serum-free condition. Then, RT-PCR was performed.

Fig. 7. Effect of U46619 on phosphorylation of ERK 1/2, p38 MAPK or CREB in 1321N1 cells. 1321N1 cells were stimulated with U46619 (U, 10 μM) for 5, 10 or 20 min, followed by performing Western blotting, using antibodies against phospho-ERK, ERK 2, phospho-p38 MAPK, p38 MAPK, phospho-CREB and CREB.

Fig. 8. U46619 promotes cAMP accumulation in 1321N1 cells. 1321N1 cells were labeled with [^{3}H]adenine (2 μCi/ml) for 3 h, then stimulated with U46619 (3 or 10 μM), U46619 (10 μM)+SQ29548 (2 μM) or isoproterenol (10 μM) for 10 min in the presence of phosphodiesterase inhibitor, Ro20-1724 (100 μM). Values represent the means $\pm$ S.E.M for three determinations. U46619 (10 μM) and isoproterenol (10 μM) significantly accumulated cAMP ($^*P < 0.05$), which was blocked by SQ29548 ($^\#P < 0.05$).

Fig. 9. Involvements of $G\alpha_q$ and $G\alpha_{13}$ in the U46619-enhanced IL-6 production in 1321N1 cells. (A) Inhibition of IL-6 gene expression by C-terminal peptides of Gα proteins. 1321N1 cells were infected with adenovirus encoding GFP, GFP/$G\alpha_q$-ct, GFP/$G\alpha_{12}$-ct or GFP/$G\alpha_{13}$-ct at 75 MOI for a day, then the cells were serum-starved overnight. The cells were stimulated with U46619 (10 μM) for 4h, then RT-PCR was performed to examine IL-6 gene expression. (B) $G\alpha_q$, $G\alpha_{12}$ and $G\alpha_{13}$ are sufficient for IL-6 gene expression. 1321N1 cells were transfected with constitutively active mutants of $G\alpha_q$, $G\alpha_{12}$ or $G\alpha_{13}$ ($G\alpha_q$QL, $G\alpha_{12}$QL and $G\alpha_{13}$QL, respectively). The cells were serum-starved overnight 24 h after transfection, then RT-PCR was performed to examine basal IL-6 gene expression. (C) $G\alpha_q$, $G\alpha_{12}$ and $G\alpha_{13}$ are

sufficient for activation of CRE and IL-6 promoter. 1321N1 cells were cotransfected with constitutively active mutants of $G\alpha_q$, $G\alpha_{12}$ or $G\alpha_{13}$ ($G\alpha_q$QL, $G\alpha_{12}$QL and $G\alpha_{13}$QL, respectively) and pCRE- or pIL-6-luciferase, then luciferase activity was measured. Values represent the means $\pm$ S.E.M for three determinations. $G\alpha_q$QL, $G\alpha_{12}$QL and $G\alpha_{13}$QL significantly increased the CRE and IL-6 promoter activity ($^{*}P < 0.05$).

Fig. 10. Diagram of putative signaling pathway of TXA_2.

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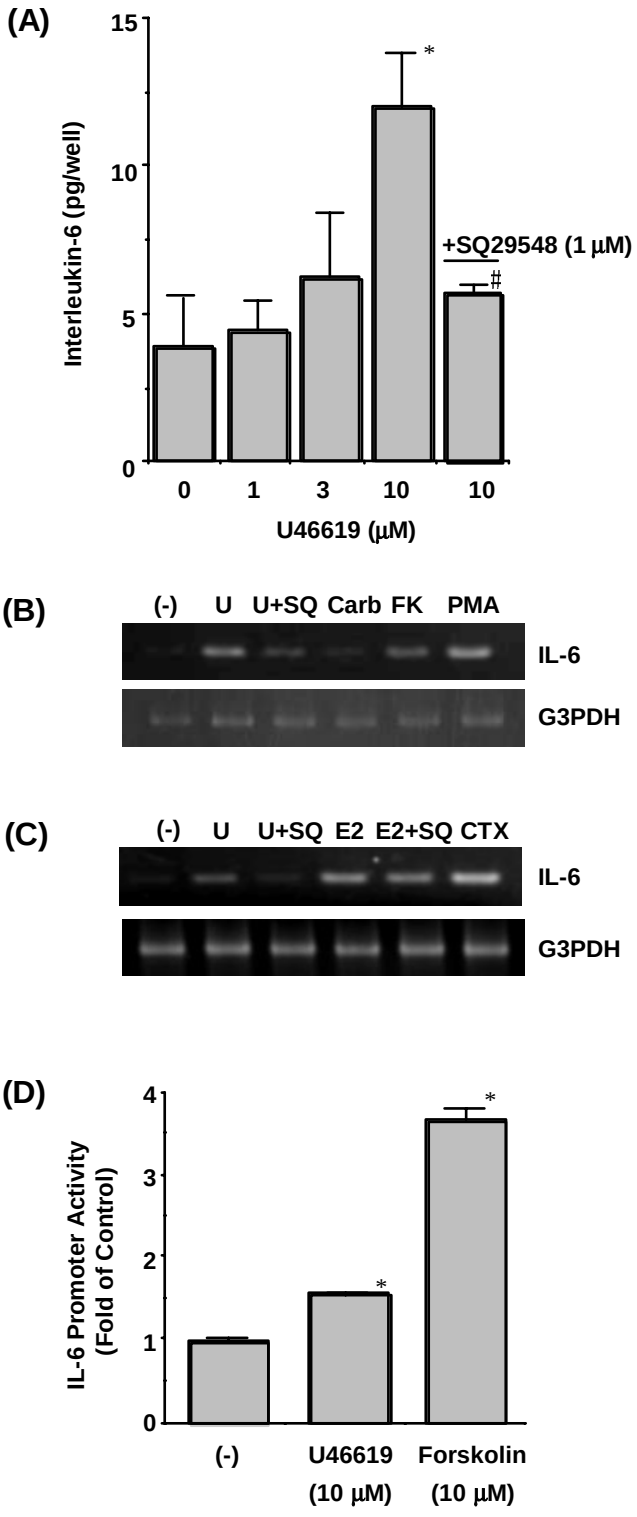

Fig. 1

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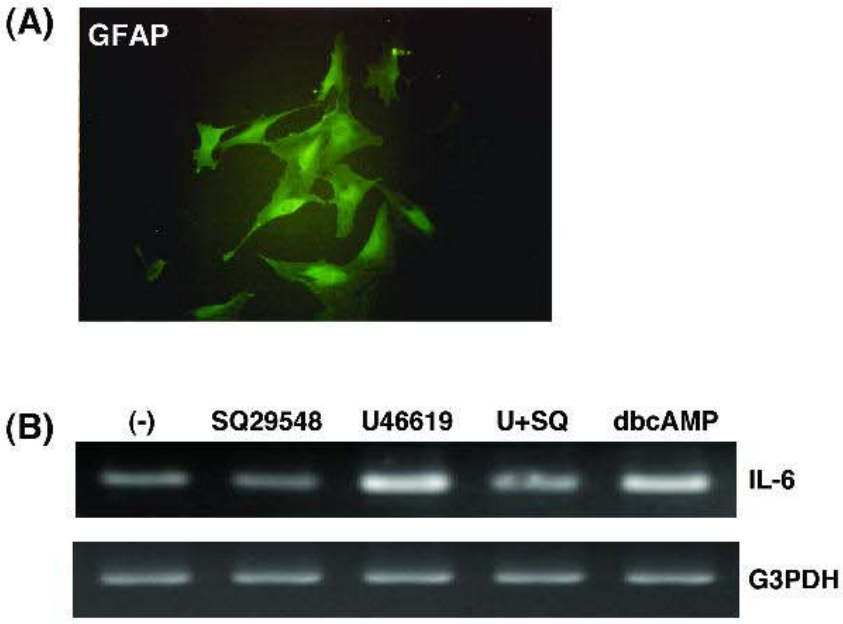

Fig. 2

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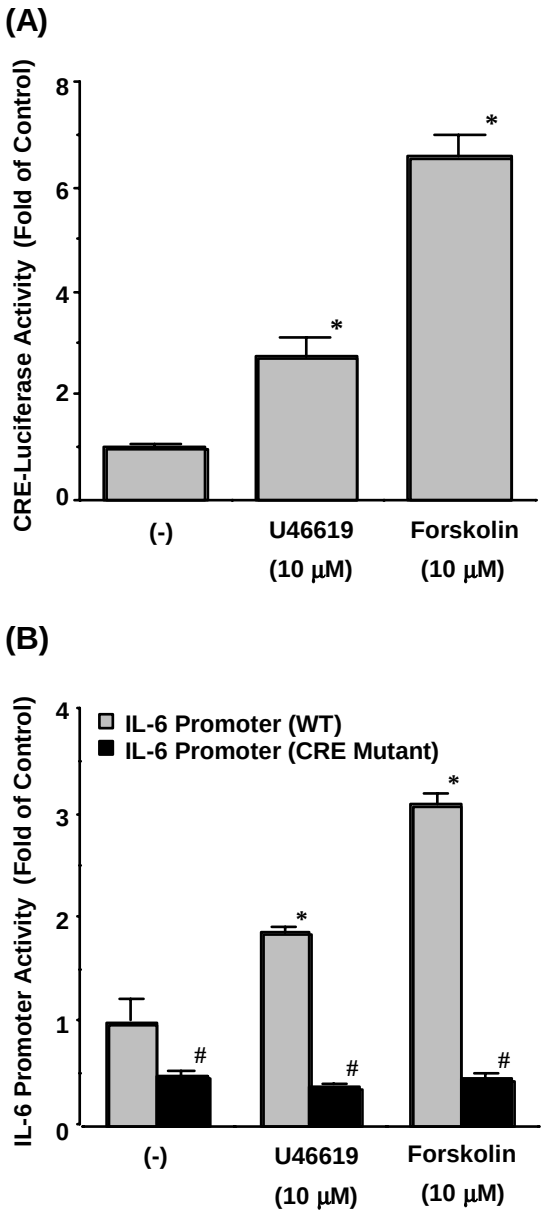

Fig. 3

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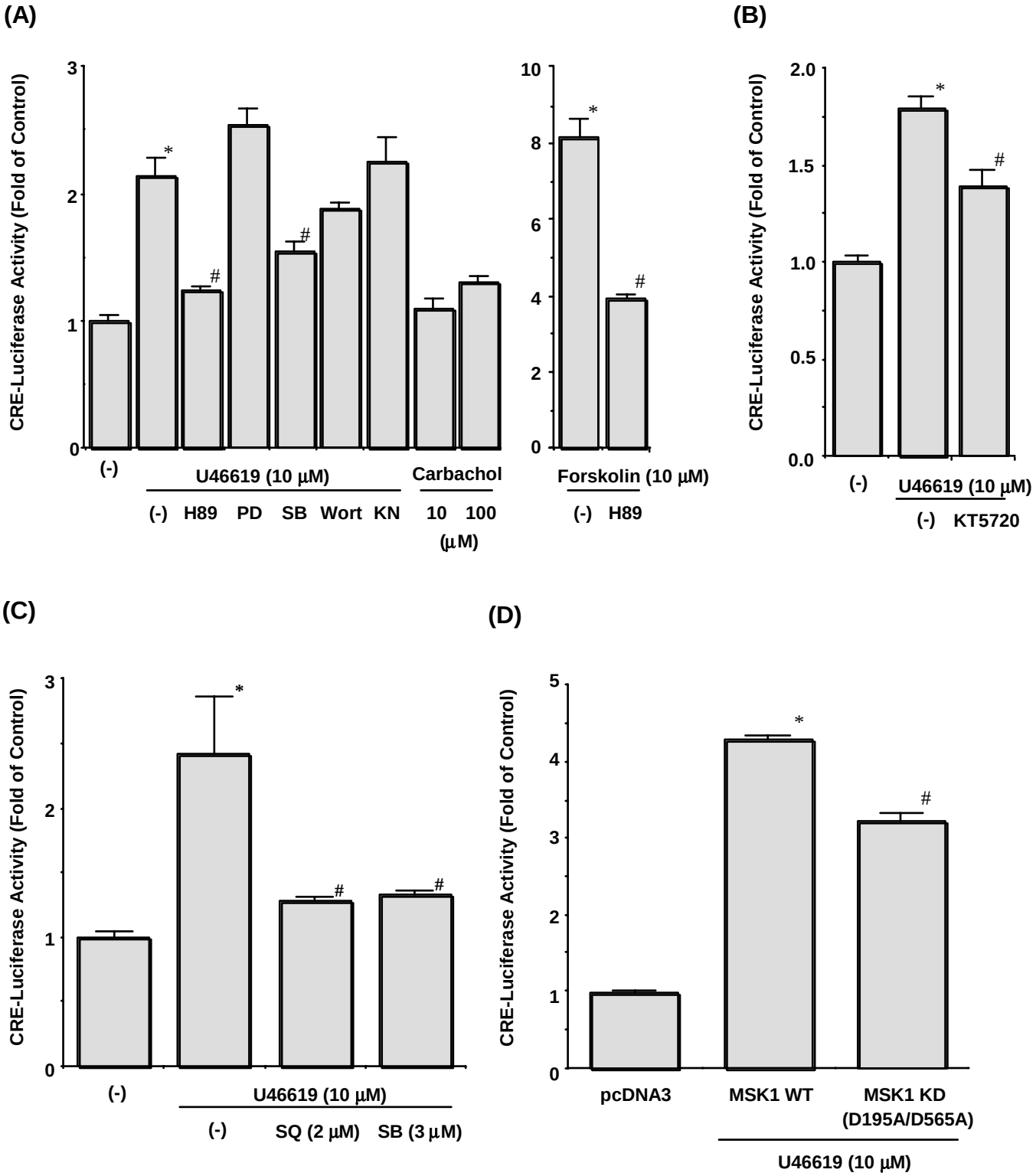

Fig. 4

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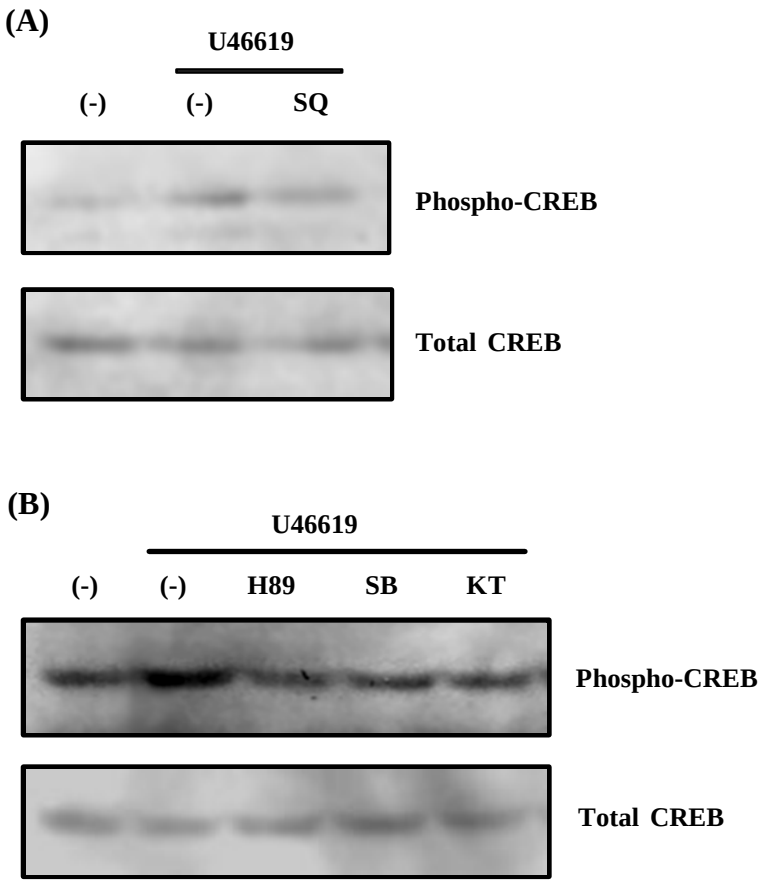

Fig. 5

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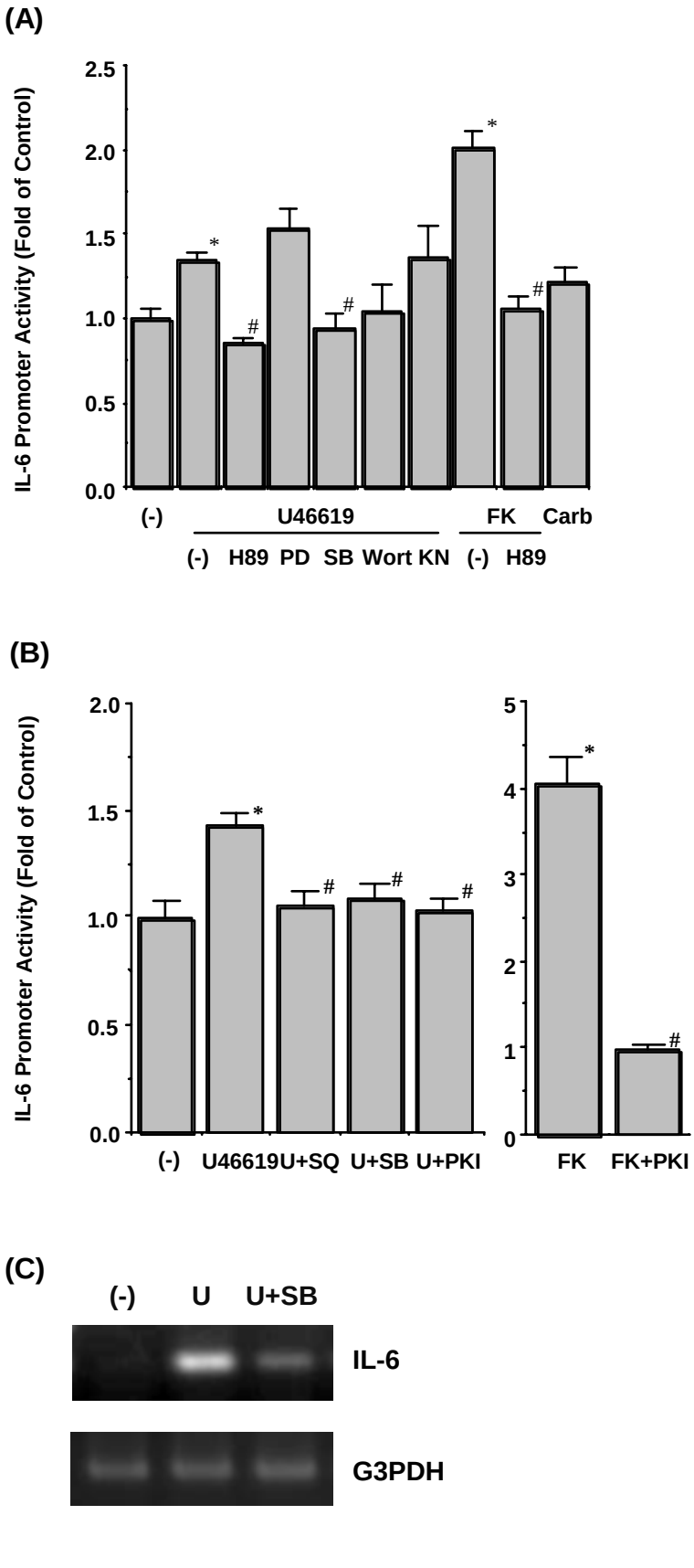

Fig. 6

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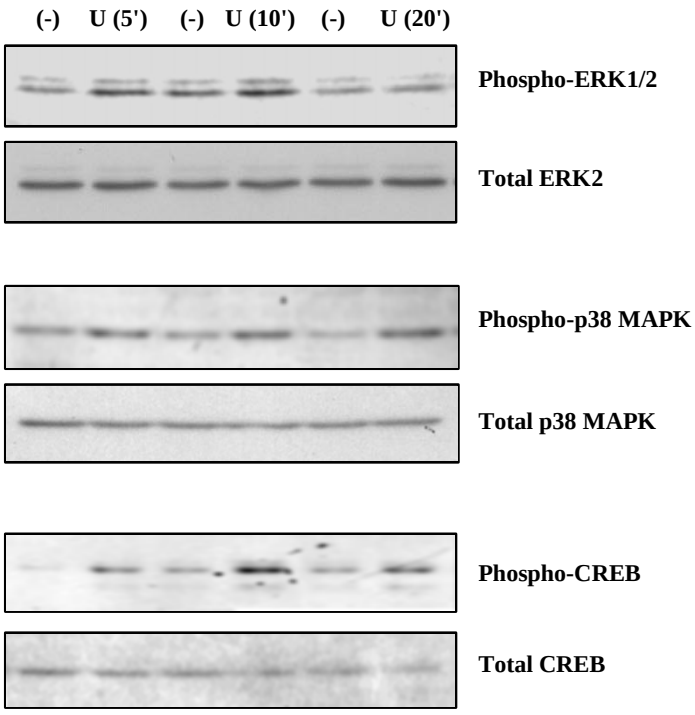

Fig. 7

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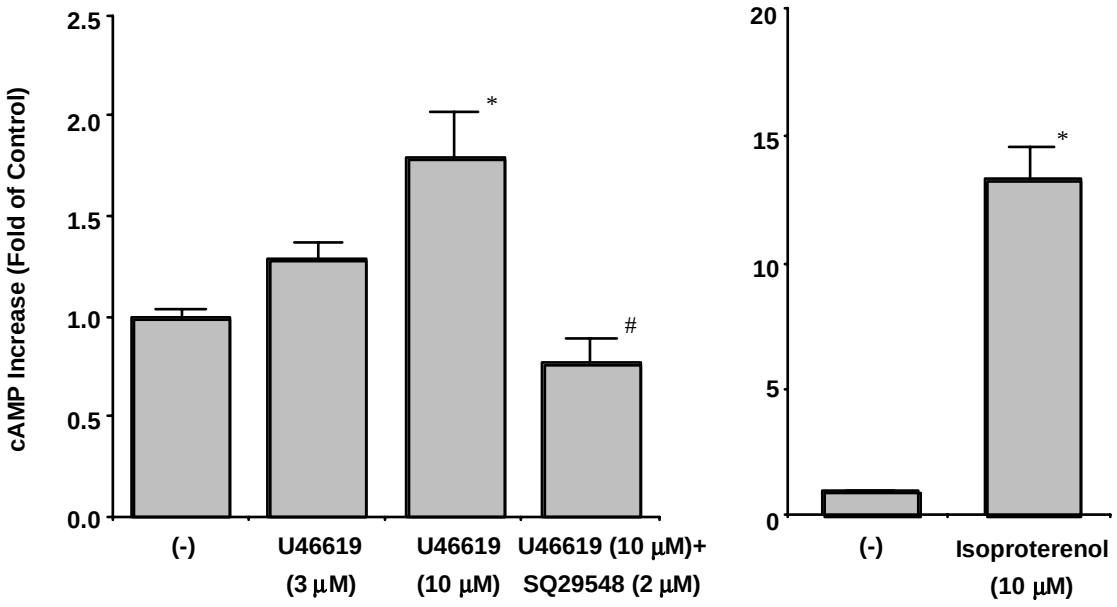

Fig. 8

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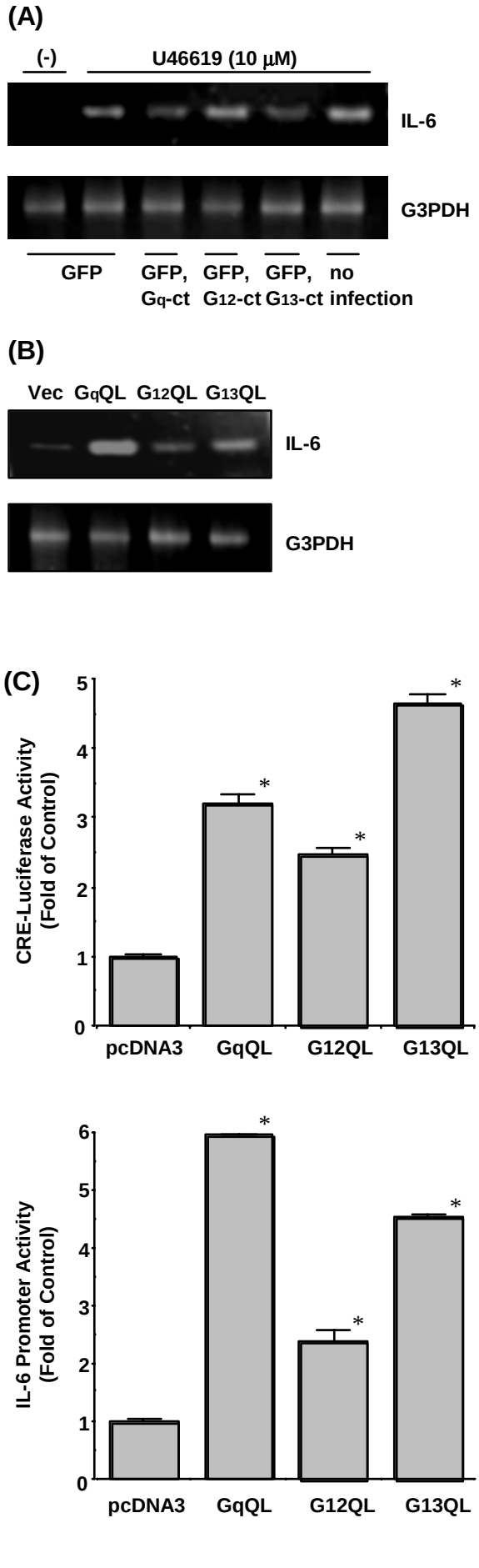

Fig. 9

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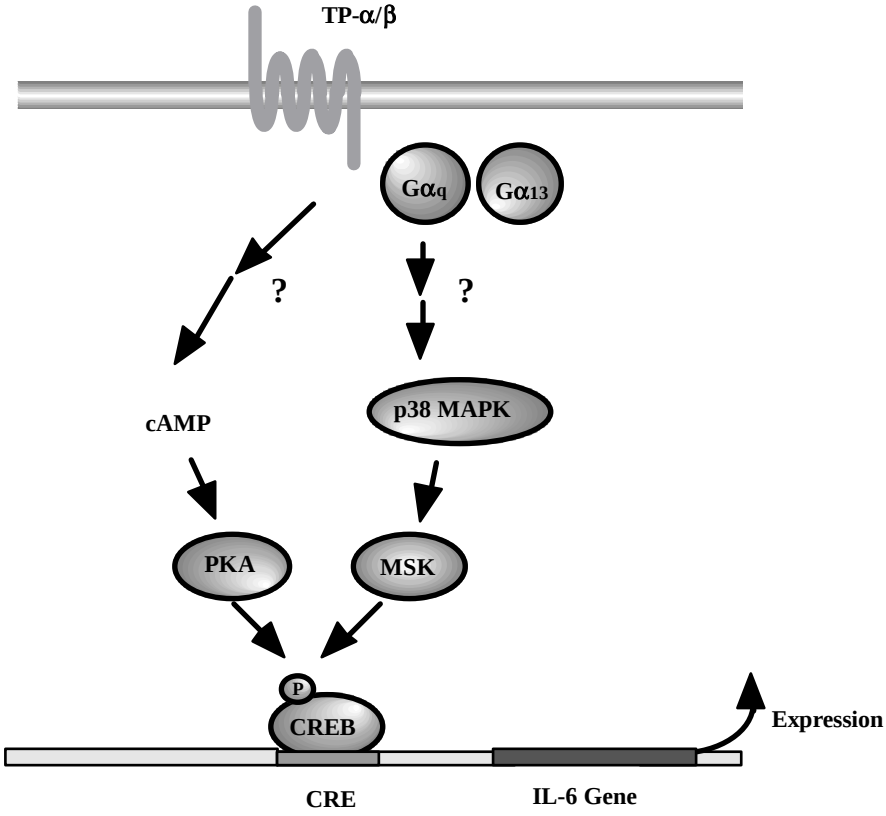

Fig. 10