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Signal Transduction Mechanism Leading to Enhanced Proliferation of Primary Cultured Adult Rat Hepatocytes Treated with Royal Jelly 57-kDa Protein

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A 57-kDa protein in royal jelly (RJ) was previously shown to stimulate hepatocyte DNA synthesis and prolongs the proliferation of hepatocytes as well as increasing albumin production [Kamakura, M., Suenobu, N., and Fukushima, M. (2001) *Biochem. Biophys. Res. Commun.* 282, 865–874]. In this study, I investigated the signal transduction mechanisms involved in the induction of hepatocyte DNA synthesis and the promotion of cell survival by this 57-kDa protein in primary cultures of adult rat hepatocytes. Hepatocyte DNA synthesis induced by the 57-kDa protein was not influenced by several α- and β-adrenoceptor antagonists, but was dose-dependently abolished by an inhibitor of a tyrosine-specific protein kinase, genistein. A phospholipase C inhibitor (U-73122) and a protein kinase C (PKC) inhibitor (sphingosine) inhibited 57-kDa protein-stimulated hepatocyte DNA synthesis, whereas a protein kinase A inhibitor (H-89) did not. The 57-kDa protein also activated PKC in rat hepatocytes. Various inhibitors of intracellular signal transduction elements (PD98059, p21 ras farnesyltransferase inhibitor, wortmannin and rapamycin) also blocked hepatocyte DNA synthesis induced by the 57-kDa protein. Furthermore, the 57-kDa protein activated mitogen-activated protein (MAP) kinase in rat hepatocytes. The activation of MAP kinase by the 57-kDa protein was inhibited by PD98059 and sphingosine. The 57-kDa protein also activated protein kinase B, which is a key regulator of cell survival. These results suggest that, like growth factors, the 57-kDa protein activates several important intracellular signaling factors involved in the stimulation of hepatocyte DNA synthesis and the protection of cells from apoptosis.

Key words: anti-apoptotic action, hepatocyte DNA synthesis, 57-kDa royal jelly protein, MAP kinase, protein kinase B.

The liver plays a central role in many bodily activities and has diverse functions that are regulated by various hormones. Moreover, liver parenchymal cells have the potential to proliferate under certain conditions, such as after partial hepatectomy or chemical liver injury (*1*). The elaborate regulation of their proliferation and functions can be studied *in vitro* by the use of primary cultured hepatocytes, which retain many *in vivo* liver functions and respond to various hormones (*2–4*). Adult rat hepatocytes in primary culture are in the resting state under usual culture conditions, even in the presence of serum. In contrast, when cultured at low cell density, they proliferate upon the addition of growth factors, such as epidermal growth factor (EGF) or hepatocyte growth factor (HGF) (*5–10*), and, during this growing state, their differentiated functions are suppressed. Hepatocytes cultured at low density correspond to liver that is regenerating after partial hepatectomy *in vivo* (*9*). Growth factors regulate liver regeneration by modulating not only cell proliferation, but also programmed cell death (*11–13*). The ability to induce cellular proliferation often correlates with the ability to promote survival (*14–16*). Mitogens such as EGF or HGF bind to their specific receptors to initiate a protein kinase cascade that phosphorylates and activates a mitogen-activated protein (MAP) kinase termed MEK. MEK in turn phosphorylates and activates other MAP kinases, p44 and p42, also called extracellular signal-regulated kinase 1 (ERK-1) and ERK-2, which phosphorylate cytosolic and nuclear targets and transduce proliferative differentiation signals (*17, 18*). This MAP kinase cascade is a key signaling pathway in the regulation of cell-cycle progression in hepatocytes (*19*). The activation of ERKs by growth factors has been shown to lead to DNA synthesis in primary rat hepatocyte culture (*20, 21*). Phosphatidylinositol 3-kinase (PI3K) and its downstream signaling kinase, protein kinase B (PKB) (also known as Akt), have been reported to be major regulators of cell survival in response to growth factors (*22–25*). Recently, the activation of PKB serine-threonine kinase has been shown to cause the phosphorylation and inactivation of proapoptotic pro-

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Abbreviations: DAG, diacylglycerol; EGF, epidermal growth factor; ERK, extracellular signal–regulated kinase; Ftase, farnesyltransferase; HGF, hepatocyte growth factor; IGF-I, insulin-like growth factor–I; IGF-II, insulin-like growth factor–II; IL-1, interleukin-1; IL-6, interleukin-6; MAP kinase, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PLC, phospholipase C; PDGF, platelet-derived growth factors; PKA, protein kinase A; PKB, protein kinase B; PKC, protein kinase C; p70S6K, p70 ribosomal protein S6 kinase; RJ, royal jelly; TGF-α, transforming growth factor–α.

teins such as BAD (*26*) and caspase-9 (*27*). Thus, growth factors play an important role in the regulation of hepatic growth and survival.

A 57-kDa protein in royal jelly (RJ) was previously found to stimulate hepatocyte DNA synthesis and prolongs the proliferation of hepatocytes, as well as increasing albumin production (*28*). The stimulatory effect of this 57-kDa protein on hepatocyte DNA synthesis was cell density-dependent, and more potent at lower than at higher cell densities, suggesting that the 57-kDa protein might promote liver regeneration. Thus, the 57-kDa protein exhibited various growth factor–like effects on hepatocytes. However, the mechanism leading to the promotion of cell proliferation by the 57-kDa protein has not yet been established.

In the present study, to clarify how the 57-kDa protein induces hepatocyte DNA synthesis and proliferation, I examined the effects of specific inhibitors of signal transducers and several antagonists of adrenoceptors on 57-kDa protein–stimulated DNA synthesis in primary cultures of adult rat hepatocytes. I also examined whether the 57-kDa protein activates MAP kinase and PKB of hepatocytes cultured in serum-free medium.

MATERIALS AND METHODS

Materials—Royal jelly (RJ) was purchased from Hangzhou Green Forever Apiculture Company (Hangzhou, China). Leibovitz L-15 medium was purchased from ICN Biochemicals (Costa Mesa, CA). Collagenase, trypsin, HEPES, dexamethasone, aprotinin, bovine serum albumin (BSA), hydroxyurea, RNase A, proteinase K, metoprolol, yohimbine, prazosin, U-73122 (1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrol-2,5-dione), PD98059 (2′-amino-3′-methoxyflavone), wortmannin, genistein and rapamycin were purchased from Wako Pure Chemicals (Osaka). HGF was from Toyobo (Osaka). Penicillin-streptomycin was from Gibco (Grand Island, NY, USA). H-89·2HCl (*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride) was from Calbiochem-Behring (La Jolla, CA). Butoxamine, (–)-epinephrine, propranolol, D-sphingosine and p21 ras farnesyltransferase (Ftase) inhibitor (Cys-Val-2-naphthyl-3-alanyl-Met) were from Sigma (St. Louis, MO, USA). [*methyl*-^{3}H]Thymidine (20 Ci/mmol) was from Dupont–New England Nuclear (Boston, MA, USA). A HiLoad 16/10 Superdex 200 column, and an LMW electrophoresis calibration kit were from Amersham Pharmacia Biotech (Uppsala, Sweden). *N*-Glycosidase F was purchased from Boehringer Mannheim (Mannheim, Germany). All other reagents were of analytical grade.

Isolation and Culture of Hepatocytes—Parenchymal cells were isolated from adult male Wistar rats (SLC, Shizuoka), weighing 150–250 g, by two-step *in situ* collagenase perfusion based on the method of Seglen (*29*). The viability of hepatocytes was monitored in terms of trypan blue dye exclusion. Routinely, more than 90% of the cells remained intact. The isolated hepatocytes were suspended at a density of 3.0×10^5 cells/ml in L-15 pre-culture medium containing 5% new calf serum, 1×10^{-6} M dexamethasone, 0.7 μg/ml aprotinin and antibiotics, and plated into type I collagen-coated 35-mm diameter plastic dishes (Sumitomo Bakelite, Tokyo) at 2.0 ml/dish, then incubated at 37°C under an atmosphere of 5% CO_2 in humidified air. Three hours later, the medium was changed to serum-free L-15 medium containing 1×10^{-6} M dexamethasone, 0.7 μg/ml aprotinin and antibiotics for the measurement of DNA synthesis, analysis of DNA fragmentation and assays of MAP kinase and PKB. The purified 57-kDa protein and HGF were added to the culture medium. Furthermore, antagonists of α-, β-adrenoceptors and the various specific inhibitors of signal transducers were added directly to the culture medium containing the purified 57-kDa protein, and the cells were cultured for 24 h.

Measurement of DNA Synthesis—The isolated hepatocytes were plated on 12-well collagen-coated plates and the incorporation of [^{3}H]thymidine into DNA was measured by the method of Kimura and Ogihara with some modifications (*30*). Hepatocytes were pulsed at 22 h after plating for 2 h with [^{3}H]thymidine (2.0 μCi/well), and the cells were washed twice with ice-cold phosphate-buffered saline and immersed in 1 ml of 10% trichloroacetic acid for 1 h at 4°C. Thereafter, they were solubilized by incubation with 1 ml of 0.5 N NaOH at 37°C for 1 h. The solubilized cells were neutralized with 0.5 N HCl and the amount of [^{3}H]thymidine incorporation into DNA was measured using a liquid scintillation counter (Aloka, LSC-700). Replicative DNA synthesis was calculated as the difference between the values in the presence and absence of 10 mM hydroxyurea. DNA synthesis activity is given as dpm/h/mg protein. Cellular protein was determined by the method of Lowry *et al.* using BSA as the standard (*31*).

Purification of the 57-kDa Protein—RJ produced by honey bees (*Apis mellifera*) fed with nectar and pollen from rape (*Brassica napus*) was used in this study. The purification of the 57-kDa protein was conducted as described previously (*32*), with monitoring by native PAGE. The purified 57-kDa protein was dialyzed against distilled water and lyophilized.

Polyacrylamide Gel Electrophoresis (PAGE)—Native PAGE was performed in a 5–20% gradient polyacrylamide gel by the method of Davis (*33*). SDS-PAGE was performed in a 5–20% gradient polyacrylamide gel by the method of Laemmli (*34*). To estimate the molecular mass, the following marker proteins were used: lysozyme (14.4 kDa), soybean trypsin inhibitor (20.1 kDa), bovine carbonic anhydrase (30 kDa), ovalbumin (43 kDa), serum albumin (67 kDa), and phosphorylase b (94 kDa). The protein in the gel was stained with Coomassie Brilliant Blue R-250 (CBB).

Treatment of the Purified 57-kDa Protein—The 57-kDa protein (0.5 mg) was treated with trypsin (4 μg) at 37°C for 6 h, after which the trypsin was inactivated by heat treatment at 100°C for 15 min. The resulting product was analyzed by SDS-PAGE and native PAGE.

Deglycosylation of the 57-kDa Protein—The purified 57-kDa protein (1.6 mg) was denatured by heating at 100°C for 3 min in 25 mM phosphate buffer (pH 7.0) containing 1% SDS and 2% (by volume) 2-mercaptoethanol. The denatured protein was incubated with 60 U of *N*-glycosidase F for 24 h at 37°C. The resulting product was dialyzed against distilled water, and analyzed by SDS-PAGE. Glycoprotein was detected by periodic acid Schiff staining.

Antibody—A polyclonal antibody against the 57-kDa protein was prepared as described previously (*28*).

Detection of DNA Fragmentation by Gel Electrophoresis—Rat hepatocytes were cultured for 48 h. The cells were lysed in 100 μl of cell-lysis buffer (10 mM Tris-HCl pH 8.0,

10 mM EDTA, and 0.5% Triton X-100), stored at 4°C for 10 min, and centrifuged at 1,500 ×*g* for 5 min. The supernatant was incubated with 200 μg/ml RNase A at 37°C for 1 h and then for an additional 30 min with 200 μg/ml proteinase K. DNA was precipitated by the addition of an equivalent volume of isopropanol. The DNA was resuspended in 20 μl of preservation solution (1 mM EDTA, 10 mM Tris-HCl pH 8.0), and electrophoresed in a 1.5% agarose gel at 50 V for 2 h.

Analysis of Cell Cycle and DNA Fragmentation by Flow Cytometry—Rat hepatocytes were cultured for 48 h. Cells were detached from the dishes by the addition of 0.05% collagenase. After 2–3 min, the cells were centrifuged at 500 ×*g* for 5 min and then collected. The DNA content per nucleus was evaluated in a FACS Calibur™ flow cytometer (Becton-Dickinson, San Jose, CA) after staining the nuclei with propidium iodide using a Cycle Test DNA reagent kit (Becton-Dickinson). Data were acquired using selective gating to exclude doublet cells, and the amount of degraded DNA was analyzed with CELLQuest software. DNA fragmentation was recognized as the sub-G_1 population. In this experiment, data for 10,000 cells were collected, stored and an-alyzed.

Determination of MAP Kinase Activity—Adult hepatocytes were pre-incubated for 60 min with 75 μM PD98059, 5 μM sphingosine, or no additive and then left untreated or stimulated with the 57-kDa protein (0.1 mg/ml) or HGF (20 ng/ml). After 10 min of stimulation, the cells were collected, washed with cold phosphate-buffered saline, and lysed at 4°C in lysis buffer A containing 50 mM Tris-HCl (pH 7.0), 2 mM EDTA, 1 mM Na_3VO_4, 150 mM NaCl, 1% mercaptoethanol, 50 mM NaF, 5 mM sodium pyrophosphate, 10 mM sodium β-glycerophosphate, 1 mM PMSF, 10 μg/ml of aprotinin and leupeptin and 3 μg/ml pepstatin A. Cell lysates were used for MAP kinase assay and aliquots of 10 μg of soluble protein were separated by SDS-PAGE in 5–20% gradient polyacrylamide gels and transferred to a polyvinylidene disulfide (PVDF) membrane. For phospho-MAP kinase Western blot analysis, the membrane was incubated with anti-active MAP kinase antibody (Promega, USA), followed by peroxidase-conjugated anti–rabbit IgG polyclonal antibody (Amersham Pharmacia Biotech). The blot was analyzed by the enhanced chemiluminescence method using an ECL Western blotting detection kit (Amersham Pharmacia Biotech). For p44/p42 MAP kinase Western blot analysis, a similar procedure was followed, using a polyclonal anti–rat MAP kinase 1/2 (Erk1/2-CT) antibody (Upstate Biotechnology, USA) that recognizes p44 and p42 MAP kinase isoforms to verify equal protein loading. Bands were quantified using NIH image Ver. 1.62 (NIH, USA).

Determination of PKB Activity—Isolated hepatocytes were untreated, or stimulated with the 57-kDa protein (0.1 mg/ml) or HGF (20 ng/ml). After 10 min of stimulation, the cells were collected, washed with cold phosphate-buffered saline, and lysed at 4°C in lysis buffer A. Aliquots of 6 μg of soluble protein in cell lysates were subjected to SDS-PAGE in 5–20% gradient polyacrylamide gels and transferred to PVDF membranes. For phospho PKB (Akt-1) Western blot analysis, the membranes were incubated with a polyclonal anti-phospho-Akt1/PKBα (Ser 473) antibody (Upstate Biotechnology). Western blot analysis of Akt-1 was carried out using anti-Akt1 (D-17) antibody (Santa Cruz Biotechnology, Santa Cruz, CA), which acts independently of the phosphorylation state, to verify equal protein loading. Blots were developed by the same method as described above. Bands were quantified using NIH image Ver. 1.62.

Determination of PKC Activity—Adult hepatocytes were pre-incubated for 60 min with 5 μM genistein, 5 μM U-73122, 5 μM sphingosine, or no additive and then were left untreated or stimulated with the 57-kDa protein (0.1 mg/ml) and HGF (20 ng/ml). After 10 min of stimulation, the cells were collected, washed with cold phosphate-buffered saline, and lysed at 4°C in lysis buffer A. Cell lysates were used for PKC assay. PKC activity was assessed by measuring the phosphorylation of a synthetic fluorescent substrate, PepTag C1 peptide (P-L-S-R-T-L-S-V-A-A-K; Promega). One unit of kinase is the number of nanomoles of phosphate transferred to a substrate per minute per milliliter.

Statistics—Values are expressed as mean±SEM. Data were analyzed by one-way analysis of variance (ANOVA). A level of $p < 0.05$ was used as the criterion of statistical significance.

RESULTS

Purification and Deglycosylation of the 57-kDa Protein—As shown in Fig. 1, the 57-kDa protein purified by column chromatography showed a single band on native PAGE and SDS-PAGE. The band of the native 57-kDa protein shifted to a position equivalent to 48 kDa after enzyme treatment. The 57-kDa protein was stained by the periodic acid Schiff reagent, but the deglycosylated 57-kDa protein was not (Fig. 2). These results indicate that the 57-kDa protein is a glycoprotein and that the oligosaccharide chains are removed by *N*-glycosidase F.

Induction of Hepatocyte DNA Synthesis by the 57-kDa Protein and Deglycosylated 57-kDa Protein—To investigate whether carbohydrate moieties covalently linked to the 57-kDa protein are responsible for the stimulatory effect of 57-kDa protein on hepatocyte DNA synthesis, rat hepatocytes

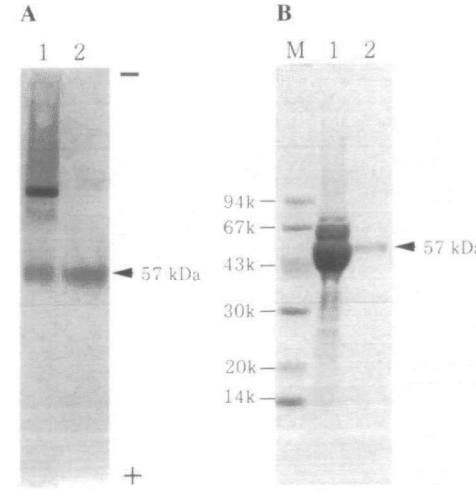

Fig. 1. **The gel electrophoresis pattern of RJ and the purified 57-kDa protein.** The 57-kDa protein was purified from RJ as described previously (*32*). RJ and the purified 57-kDa protein were subjected to native PAGE (A) and SDS-PAGE (B). Lane M, marker proteins (sizes in kDa); lane 1, 3% (w/v) RJ solution; lane 2, purified 57-kDa protein. The position of the 57-kDa protein is indicated by an arrowhead.

were cultured with HGF, the purified 57-kDa protein, or the deglycosylated 57-kDa protein, or were untreated, and hepatocyte DNA synthesis was measured at 24 h after plating. The results show that the deglycosylated 57-kDa protein enhances hepatocyte DNA synthesis as effectively as the 57-kDa protein (Fig. 3). The mitogenic effect of the 57-kDa protein on hepatocytes was lost after treatment with trypsin. The addition of an anti–57-kDa protein antibody to the culture medium blocked hepatocyte DNA synthesis induced by the 57-kDa protein.

Effects of Specific Adrenoceptor Antagonists on 57-kDa Protein–Induced Hepatocyte DNA Synthesis—The 57-kDa protein may act nonspecifically on various cell surface receptors of rat hepatocytes to activate intracellular signal transduction factors involved in the stimulation of DNA synthesis and cell division. Adrenoceptors that mediate signals from catecholamines are implicated in hepatocyte DNA synthesis and the control of liver growth (*35, 36*). Therefore, the influences of α_1-, α_2-, β_1-, and β_2-adrenoceptor antagonists on hepatocyte DNA synthesis stimulated by the 57-kDa protein were examined. The stimulatory effect of the 57-kDa protein on hepatocyte DNA synthesis was not influenced by the specific α_1-adrenoceptor antagonist prazosin or the specific α_2-adrenoceptor antagonist yohimbine at doses up to 3×10^{-6} M (data not shown). Furthermore, the specific β_1-adrenoceptor antagonist metoprolol (3×10^{-6} M), the specific β_2-adrenoceptor antagonist butoxamine (3×10^{-6} M) and the β-adrenoceptor antagonist propranolol (3×10^{-6} M) did not influence the 57-kDa protein–induced hepatocyte DNA synthesis (data not shown). None of these adrenoceptor antagonists alone had any direct effect on DNA synthesis in untreated hepatocytes.

Effects of a Specific Inhibitor of Tyrosine Kinase on 57-kDa Protein–Induced Hepatocyte DNA Synthesis—To investigate whether or not 57-kDa protein–induced hepatocyte DNA synthesis is mediated by tyrosine kinases, such as growth factor receptors, hepatocytes were treated for 24 h with the 57-kDa protein in the presence and in the absence of a specific inhibitor of tyrosine kinase, genistein, which has little effect on the activities of several serine and threonine kinases and other ATP analogue-related enzymes (*37*). As shown in Table I, genistein blocks the stimulatory effect of the 57-kDa protein on hepatocyte DNA synthesis in a concentration-dependent manner as well as HGF, while it

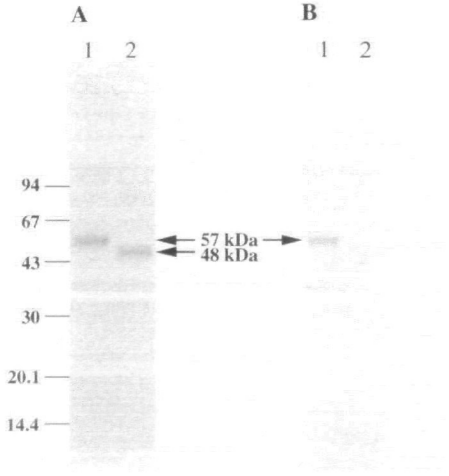

Fig. 2. **Deglycosylation of the 57-kDa protein by treatment with *N*-glycosidase F.** The purified 57-kDa protein (1.6 mg) was incubated with 60 units of *N*-glycosidase F. The resulting product was subjected to SDS-PAGE, and stained with CBB (A) or periodic acid Schiff reagent (B). Lane 1, purified 57-kDa protein; lane 2, 57-kDa protein treated with *N*-glycosidase F. The gel was calibrated with molecular mass markers as described in "MATERIALS AND METHODS." The positions of the intact 57-kDa protein and the product are indicated by arrowheads.

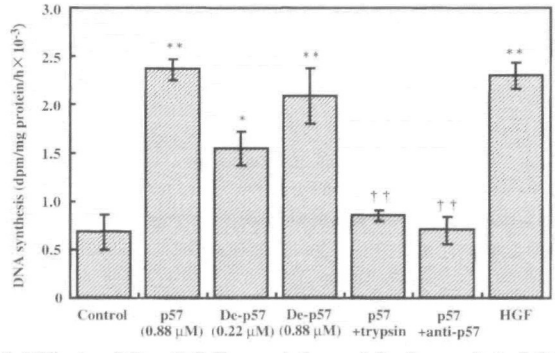

Fig. 3. **Effects of the 57-kDa protein and deglycosylated 57-kDa protein on DNA synthesis in rat hepatocytes.** Hepatocytes at a cell density of 3.0×10^4 cells/cm² were plated and harvested at 24 h after plating. [³H]Thymidine (2.0 μCi/well) was added 2 h before harvest. [³H]Thymidine incorporation was measured as described in "MATERIALS AND METHODS." Each value is the mean ± SEM of triplicate independent experiments. Rat hepatocytes were incubated without treatment (Control), or with the 57-kDa protein [p57, 0.05 mg/ml (0.88 μM)], the 57-kDa protein deglycosylated by *N*-glycosidase F [De-p57, 0.01 mg/ml (0.22 μM) or 0.04 mg/ml (0.88 μM)], the 57-kDa protein treated with trypsin at 37°C for 6 h (p57+trypsin, 0.05 mg/ml), the 57-kDa protein treated with 0.15 mg/ml anti–57-kDa protein antibody (p57+anti-p57, 0.05 mg/ml) or HGF (10 ng/ml). Values significantly different from the control are indicated by $^*p<0.05$, $^{**}p<0.01$. Values significantly different from the 57-kDa protein alone are indicated by $^{\dagger\dagger}p<0.01$.

TABLE I. **Effects of genistein on hepatocyte DNA synthesis induced by the 57-kDa protein, HGF or epinephrine.** Hepatocytes were plated at a density of 3.0×10^4 cells/cm² and cultured. Genistein was added with or without 0.05 mg/ml 57-kDa protein, 10 ng/ml HGF, or 10 μM epinephrine immediately after medium change, and cells were cultured for a further 24 h. Values are expressed as mean ± SEM of three independent experiments. Values significantly different from the control, the 57-kDa protein alone and HGF alone are indicated by $^{**}p<0.01$, $^{\dagger\dagger}p<0.01$, and $^{\sharp}p<0.05$, respectively.

Treatment	DNA synthesis (dpm/mg protein/h × 10^{-3})			
	Control	57-kDa protein	HGF	Epinephrine
—	0.453±0.153	2.361±0.111**	2.101±0.293**	0.931±0.086**
Genistein (5 μM)	0.524±0.101	1.261±0.233††	1.202±0.006♯	0.927±0.033
Genistein (10 μM)	0.450±0.051	0.882±0.053††	0.880±0.104♯	0.960±0.036
Genistein (20 μM)	0.477±0.146	0.651±0.097††	0.654±0.233♯	0.970±0.199

does not influence DNA synthesis in untreated hepatocytes or epinephrine-treated hepatocytes in the concentration range tested.

Effects of Specific Inhibitors of Phospholipase C, Protein Kinase C, Protein Kinase A, and PMA on 57-kDa Protein–Induced Hepatocyte DNA Synthesis—Next, the role of phospholipase C (PLC)/PKC system or protein kinase A (PKA) was investigated in hepatocyte DNA synthesis induced by the 57-kDa protein. The isoquinoline sulfonamide, H-89, is a specific inhibitor of PKA in some cell types. As shown in Table II, H-89 (3×10^{-7} M) had no significant effect on hepatocyte DNA synthesis induced by the 57-kDa protein, suggesting that PKA is not involved in the 57-kDa protein signal transduction pathway. On the other hand, the specific PLC inhibitor, U-73122 (5×10^{-6} M), significantly reduced the action of the 57-kDa protein on hepatocyte DNA synthesis. Furthermore, the PKC inhibitor, sphingosine (1×10^{-6} M), also significantly decreased the 57-kDa protein–induced hepatocyte DNA synthesis. U-73122, sphingosine and H-89 alone did not affect DNA synthesis of untreated hepatocytes.

Effect of the 57-kDa Protein on PKC Activity in Rat Hepatocytes—Furthermore, whether the 57-kDa protein stimulates PKC activity in rat hepatocytes was investigated. As shown in Fig. 4, the 57-kDa protein or HGF activated PKC in hepatocytes. The activation of PKC caused by the 57-kDa protein or HGF was significantly inhibited by genistein, U-73122 or sphingosine.

Effect of Specific Inhibitors of Signal-Transducing Elements on Hepatocyte DNA Synthesis Induced by the 57-kDa Protein—Whether or not the mitogenic responses of hepatocytes to the 57-kDa protein are mediated by such signal transducers as Ras, phosphatidylinositol 3-kinase (PI3K), MAP kinase and p70 ribosomal protein S6 kinase (p70S6K) was investigated. As shown in Table III, the stimulatory effect of the 57-kDa protein on hepatocyte DNA synthesis was significantly inhibited by the specific inhibitor of p21 ras farnesyltransferase (Ftase) (1×10^{-5} M) (*38*). Furthermore, a specific inhibitor of PI3K, wortmannin (1×10^{-7} M), and a specific inhibitor of MAP kinase kinase, PD98059 (5×10^{-5} M), produced significant attenuations of the 57-kDa protein–induced hepatocyte DNA synthesis. Additionally, rapamycin (10 ng/ml), a specific inhibitor of p70S6K, blocked the stimulation of hepatocyte DNA synthesis induced by the 57-kDa protein. None of the specific inhibitors of signal transducers (namely, genistein, wortmannin, PD98059, or rapamycin) alone had any significant effect on DNA synthesis in untreated hepatocytes.

Effect of the 57-kDa Protein on MAP Kinase Activity in

TABLE II. **Effects of H-89, U-73122, and sphingosine on hepatocyte DNA synthesis induced by the 57-kDa protein.** Hepatocytes were plated at a density of 3.0×10^4 cells/cm^2 and cultured. H-89, U-73122, and sphingosine were added with or without 0.05 mg/ml 57-kDa protein immediately after medium change, and cells were cultured for a further 24 h. Values are expressed as mean ± SEM of three independent experiments. The value of hepatocyte DNA synthesis stimulated by 10 ng/ml HGF (positive control) was 2.297 ± 0.138 dpm/mg protein/h × 10^{-3}. Values significantly different from the control are indicated by $^{**}p<0.01$. Values significantly different from the 57-kDa protein alone are indicated by $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$.

Treatment	Concentration	DNA synthesis (dpm/mg protein/h × 10^{-3})
Control	—	0.676±0.119
+H-89	3×10^{-7} M	0.737±0.028
+U-73122	1×10^{-5} M	0.688±0.068
+Sphingosine	5×10^{-6} M	0.690±0.167
57-kDa protein	0.05 mg/ml	2.361±0.111**
+H-89	1×10^{-7} M	2.219±0.142**
	3×10^{-7} M	2.284±0.330**
+U-73122	3×10^{-6} M	1.267±0.126††
	1×10^{-5} M	1.009±0.196††
+Sphingosine	1×10^{-6} M	1.457±0.180†
	5×10^{-6} M	1.085±0.220††

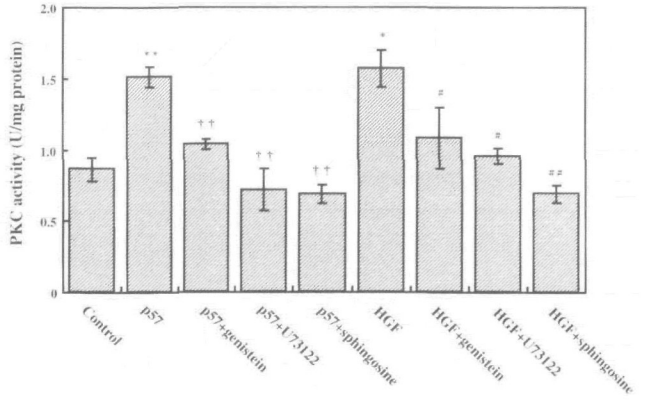

Fig. 4. **Effects of the 57-kDa protein and HGF on PKC activity.** Hepatocytes were pre-incubated for 60 min with or without 5 μM genistein, 5 μM U-73122 or 5 μM sphingosine and then were treated or stimulated with 57-kDa protein (p57, 0.1 mg/ml) or HGF (20 ng/ml). After 10 min of stimulation, cell lysates were examined for protein kinase C activity. Each value is the mean ± SEM of triplicate independent experiments. Values significantly different from the control are indicated by $^{*}p<0.05$, $^{**}p<0.01$. Values significantly different from the 57-kDa protein alone are indicated by $^{\dagger\dagger}p<0.01$. Values significantly different from HGF alone are indicated by $^{\sharp}p<0.05$, $^{\sharp\sharp}p<0.01$.

TABLE III. **Effects of specific inhibitors of signal-transducing elements on hepatocyte DNA synthesis induced by the 57-kDa protein.** Hepatocytes were plated at a density of 3.0×10^4 cells/cm^2 and cultured. Specific inhibitors of signal-transducing elements were added with or without 0.05 mg/ml 57-kDa protein immediately after medium change, and cells were cultured for a further 24 h. Values are expressed as mean ± SEM of three independent experiments. The value of hepatocyte DNA synthesis stimulated by 10 ng/ml HGF (positive control) was 2.297 ± 0.138 dpm/mg protein/h × 10^{-3}. Values significantly different from the control are indicated by $^{**}p<0.01$. Values significantly different from 57-kDa protein alone are indicated by $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$.

Treatment	Concentration	DNA synthesis (dpm/mg protein/h × 10^{-3})
Control	—	0.676±0.119
+Ftase inhibitor	3×10^{-6} M	0.730±0.172
+PD98059	2×10^{-4} M	0.635±0.020
+Wortmannin	3×10^{-7} M	0.659±0.092
+Rapamycin	30 ng/ml	0.746±0.068
57-kDa protein	0.05 mg/ml	2.361±0.111**
+Ftase inhibitor	1×10^{-5} M	1.359±0.172††
	3×10^{-5} M	0.982±0.014††
+PD98059	5×10^{-5} M	1.185±0.022††
	2×10^{-4} M	0.717±0.139††
+Wortmannin	1×10^{-7} M	1.237±0.060††
	3×10^{-7} M	1.010±0.263††
+Rapamycin	10 ng/ml	1.234±0.329†
	30 ng/ml	1.111±0.088††

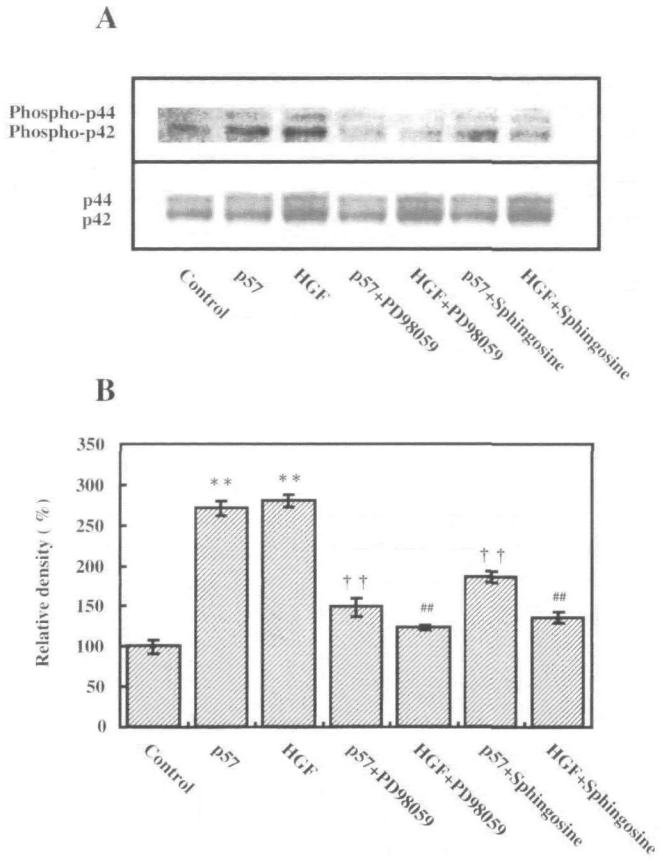

Fig. 5. **Effects of the 57-kDa protein and HGF on MAP kinase activity.** Hepatocytes were pre-incubated for 60 min with or without 75 μM PD98059 or 5 μM sphingosine and then treated or stimulated with the 57-kDa protein (p57, 0.1 mg/ml) or HGF (20 ng/ml) After 10 min of stimulation, cell lysates were examined for MAP kinase activity, and aliquots of 10 μg of soluble protein were separated by SDS-PAGE and transferred to PVDF membranes. Antibodies against phospho-MAP kinase (active forms) and total MAP kinase were used sequentially. (A) Representative immunoblot. (B) Bar graphs represent relative intensities of both phospho p42 and phospho p44 MAP kinase quantified by scanning densitometry of blots from three independent experiments. Values significantly different from the control, the 57-kDa protein alone and HGF alone are indicated by $^{**}p<0.01$, $^{\dagger\dagger}p<0.01$, and $^{\#\#}p<0.01$, respectively.

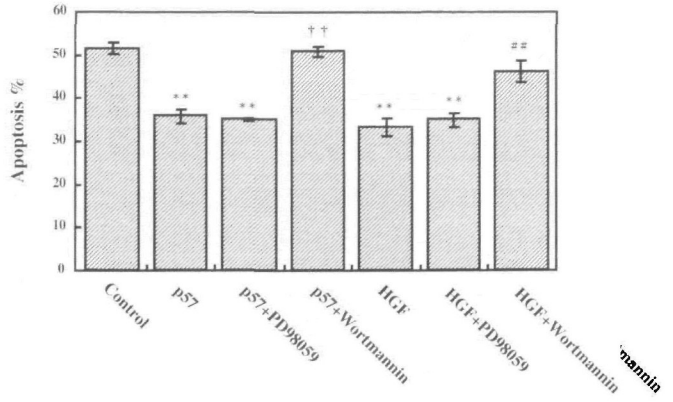

Fig. 6. **Suppression of hepatocyte DNA fragmentation in serum-free primary culture by the 57-kDa protein.** Isolated hepatocytes were treated with or without 50 μM PD98059 or 10 μM wortmannin for 30 min before the addition of the 57-kDa protein (p57, 0.1 mg/ml), HGF (20 ng/ml) or no additive (Control). Cells were cultured for 48 h. The DNA content per nucleus was evaluated by flow cytometry. DNA fragmentation (apoptosis) was recognized as the sub-G_1 population. Each value is the mean ± SEM of triplicate independent experiments. Values significantly different from the control, the 57-kDa protein alone and HGF alone are indicated by $^{**}p<0.01$, $^{\dagger\dagger}p<0.01$, and $^{\#\#}p<0.01$, respectively.

Rat Hepatocytes—MAP kinases are important intermediates in the signaling pathway that transduces extracellular signals into intracellular responses, and have been implicated in the modulation of DNA synthesis and cell differentiation (*39*). Therefore, whether HGF (positive control) and the 57-kDa protein modulate these activities was examined. As shown in Fig. 5, both HGF and the 57-kDa protein caused increases in the active forms of MAP kinase. Pretreatment of hepatocytes with PD98059 or sphingosine effectively blocked the activation of MAP kinase caused by the 57-kDa protein or HGF.

Suppression of Apoptosis of Hepatocytes by the 57-kDa Protein—Flow-cytometric analysis and agarose gel electrophoresis were used to investigate whether the 57-kDa protein suppresses the apoptosis of hepatocytes induced by serum deprivation. As shown in Fig. 6, apoptosis was clearly observed in untreated hepatocytes at 48 h by flow-cytometric analysis, but was significantly suppressed in hepatocytes cultured with the 57-kDa protein or HGF (positive control). Similarly, apoptosis of hepatocytes cultured in serum-free medium for 48 h appeared to be suppressed by the 57-kDa protein on agarose gel electrophoresis (data not shown). The protective effect of the 57-kDa protein on serum deprivation-induced apoptosis of hepatocytes was inhibited by wortmannin. However, PD98059 did not impair the ability of the 57-kDa protein to suppress the apoptosis of hepatocytes. The same results were observed in hepatocytes treated with HGF.

Effect of the 57-kDa Protein on Protein Kinase B of Rat Hepatocytes—Furthermore, I investigated whether PKB, which is an important mediator of cell survival in primary cultured rat hepatocytes, is modulated by the 57-kDa protein. As shown in Fig. 7, the 57-kDa protein, like HGF, activates PKB in hepatocytes.

DISCUSSION

In the present study, the mechanism by which the 57-kDa protein enhances the proliferation of primary cultured rat hepatocytes was investigated. The 57-kDa protein is a glycoprotein whose polypeptide chain has a molecular size of 48 kDa. The deglycosylated 57-kDa protein has a stimulatory effect on hepatocyte DNA synthesis, as does the 57-kDa protein. The mitogenic effect of the 57-kDa protein on hepatocytes is lost after treatment with trypsin. These results suggest that the polypeptide chain of the 57-kDa protein, but not its sugar chains, is responsible for 57-kDa protein–induced DNA synthesis in hepatocytes.

The 57-kDa protein enhances hepatocyte proliferation and improves cell viability through anti-apoptoic action, as in the case of growth factors such as EGF and HGF, suggesting that the 57-kDa protein may promote liver regeneration (*28*). So far, it has been reported that growth factor receptors and adrenoceptors, which mediate signals from catecholamines, in various hepatocyte receptors may be

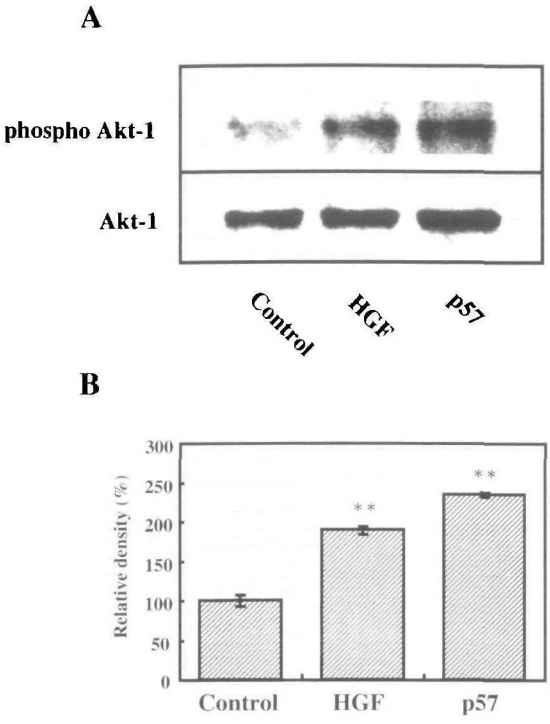

Fig. 7. **Effects of the 57-kDa protein and HGF on PKB activity.** Hepatocytes were untreated, or stimulated with the 57-kDa protein (p57, 0.1 mg/ml) or HGF (20 ng/ml). After 10 min of stimulation, cell lysates were assayed for PKB activity and aliquots of 6 μg of soluble protein were separated by SDS-PAGE and transferred to PVDF membranes. Antibodies against phospho-PKB (Akt-1) and total PKB were used sequentially. (A) Representative immunoblot. (B) Bar graphs represent relative intensities of both phospho Akt-1 quantified by scanning densitometry of blots from three independent experiments. Values significantly different from the control are indicated by $^{**}p<0.01$.

involved in liver regeneration and the stimulatory regulation of liver growth (*11–13, 35, 36*). Therefore, I investigated the influences of adrenoceptor antagonists and an inhibitor of tyrosine kinase, which are required for the autophosphorylation of growth factor receptors activated by growth factor binding, on hepatocyte DNA synthesis induced by the 57-kDa protein. Several antagonists of α_1-, α_2-, β_1-, and β_2-adrenoceptors had no influence or hepatocyte DNA synthesis induced by the 57-kDa protein. However, a specific inhibitor of tyrosine kinase, genistein, dose-dependently inhibited 57-kDa protein–induced DNA synthesis in rat hepatocytes as well as HGF. Genistein inhibits receptor tyrosine kinases such as the EGF receptor and c-*met*, and nonreceptor tyrosine kinases, the Src family tyrosine kinases (*37, 40*). The Src family tyrosine kinases are activated by cytokines such as interleukin (IL)-1 and IL-6, but these cytokines inhibit DNA synthesis in hepatocytes in primary culture (*41, 42*). They are also associated with downstream signaling in mitogenesis after stimulation by growth factors, such as EGF, HGF, and platelet-derived growth factor (PDGF) (*43–45*). Therefore, these results suggest that the 57-kDa protein may act through a tyrosine kinase receptor on a growth factor in hepatocytes to exert the mitogenic effect.

Next, I investigated the influence of the 57-kDa protein on signal transduction enzymes involved in the induction of hepatocyte DNA synthesis. U-73122 (an inhibitor of PLC) and sphingosine (an inhibitor of PKC) attenuated the stimulatory effect of the 57-kDa protein on hepatocyte DNA synthesis. The 57-kDa protein also activated PKC in hepatocytes, and the activation of PKC by the 57-kDa protein was inhibited by pre-treatment of hepatocytes with genistein, U-73122 or sphingosine. These results suggest that the 57-kDa protein may activate the PLC/PKC cascade through a receptor tyrosine kinase to induce hepatocyte DNA synthesis. On the other hand, a specific inhibitor of PKA, H-89, did not influence hepatocyte DNA synthesis stimulated by the 57-kDa protein. Therefore, it is considered that PKA is not implicated in the enhancement of hepatocyte DNA synthesis by the 57-kDa protein.

Furthermore, to investigate the possible mechanisms involved in the activation of hepatocyte DNA synthesis induced by the 57-kDa protein, hepatocytes were cultured with specific inhibitors of other signal transducers. An inhibitor of p21 ras Ftase, which farnesylates the Ras protein to make it functionally active (*46*), abolished hepatocyte DNA synthesis induced by the 57-kDa protein. Accordingly, Ras may be involved in hepatocyte DNA synthesis induced by the 57-kDa protein. PD98059, which specifically blocks the activation of MAP kinase kinase, inhibited 57-kDa protein-induced hepatocyte DNA synthesis. Furthermore, the 57-kDa protein activated p42/p44 MAP kinase in hepatocytes. This activation of MAP kinase by the 57-kDa protein was blocked by PD98059. These findings indicate that the Ras/MAP kinase pathway is involved in the enhancement of hepatocyte DNA synthesis by the 57-kDa protein. Sphingosine reduced the activation of MAP kinase by the 57-kDa protein or HGF. PKC has been reported to function as an activator of c-Raf-1 (*47*). Therefore, these results suggest that PKC may act as an upstream mediator of MAP kinase in signal transduction of 57-kDa protein-induced hepatocyte DNA synthesis. It has been reported that PI3K can mediate mitogenic signaling and that the PI3K-dependent pathway required for mitogenic signaling involves p70S6K (*48, 49*). In the present study, specific inhibitors of PI3K and p70S6K, wortmannin and rapamycin, respectively, abolished 57-kDa protein–induced DNA synthesis in rat hepatocytes; therefore, PI3K and p70S6K seem to be involved in the enhancement of hepatocyte DNA synthesis by the 57-kDa protein. The inhibitions of 57-kDa protein–induced hepatocyte DNA synthesis by wortmannin and rapamycin are not as potent as that by PD98059. Therefore, the 57-kDa protein seems to stimulate hepatocyte DNA synthesis mainly through the Ras/MAP kinase pathway and, to a lesser extent, through PI3K/p70S6K.

In this study, this anti-apoptotic action of the 57-kDa protein at 48 h was investigated by both flow cytometry and agarose gel electrophoresis. Significant suppression of apoptosis of hepatocytes in serum-free culture by the 57-kDa protein was observed in both analyses. The anti-apoptotic effect of the 57-kDa protein on hepatocytes was inhibited by wortmannin, but not by PD98059. Furthermore, the 57-kDa protein activated PKB, which promotes survival by phosphorylating pro-apoptotic proteins and anti-apoptotic mediators (*26, 27*), as well as HGF. These results suggest that the protective effect of the 57-kDa protein against apoptosis of hepatocytes occurs *via* the PI3K/PKB pathway and is not dependent on the Ras/MAP kinase pathway.

Various growth factors have been reported to stimulate DNA synthesis in primary cultured rat hepatocytes. Hepatocyte DNA synthesis induced by insulin-like growth factor–I (IGF-I) and IGF-II is not influenced by an inhibitor of PKC, and PI3K is not associated with IGF-II–stimulated hepatocyte DNA synthesis (*50*). Hepatocyte DNA synthesis induced by the 57-kDa protein is strictly cell density–dependent, like that induced by EGF and HGF (*28, 30, 51*), whereas that induced by transforming growth factor–α (TGF-α), PDGF or insulin does not depend exclusively on initial plating density (*52, 53*). From these findings and the results demonstrated above, the 57-kDa protein seems to activate intracellular signal transduction factors involved in the stimulation of DNA synthesis and cell division through EGF receptor or c-*met* of rat hepatocytes. Further studies will be required to identify the specific hepatocyte receptor on which the 57-kDa protein acts.

In conclusion, it was found that the 57-kDa protein stimulates hepatocyte DNA synthesis by the activation of the Ras/MAP kinase pathway. Other intracellular signal transducers, PLC, PKC, PI3K, and p70S6K are also involved in the induction of hepatocyte DNA synthesis by the 57-kDa protein. Moreover, the 57-kDa protein activates PKB in hepatocytes. Thus, the 57-kDa protein stimulates both the Ras/MAP kinase pathway, which is closely involved in cell proliferation, and the PI3K/PKB pathway, which is essential for protecting cells from apoptosis. Its action appears to be similar to that of growth factors such as HGF and EGF.

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