Oncogenic K-Ras and Basic Fibroblast Growth Factor Prevent Fas-mediated Apoptosis in Fibroblasts through Activation of Mitogen-activated Protein Kinase

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Abstract. By an expression cloning method using Fas-transgenic Balb3T3 cells, we tried to obtain inhibitory genes against Fas-mediated apoptosis and identified proto-oncogene c-K-ras. Transient expression of K-Ras mutants revealed that oncogenic mutant K-Ras (RasV12) strongly inhibited, whereas dominant-inhibitory mutant K-Ras (RasN17) enhanced, Fas-mediated apoptosis by inhibiting Fas-triggered activation of caspases without affecting an expression level of Fas. Among the target molecules of Ras, including Raf (mitogen-activated protein kinase kinase kinase [MAPKKK]), phosphatidylinositol 3 (PI-3) kinase, and Ral guanine nucleotide exchange factor (RalGDS), only the constitutively active form of Raf (Raf-CAAX) could inhibit Fas-mediated apoptosis. In addition, the constitutively active form of MAPKK (SDSE-MAPKK) suppressed Fas-mediated apoptosis, and MKP-1, a phosphatase specific for classical MAPK, canceled the protective activity of oncogenic K-Ras (K-RasV12), Raf-CAAX, and SDSE-MAPKK. Furthermore, physiological activation of Ras by basic fibroblast growth factor (bFGF) protected Fas-transgenic Balb3T3 cells from Fas-mediated apoptosis. bFGF protection was also dependent on the activation of the MAPK pathway through Ras. All the results indicate that the activation of MAPK through Ras inhibits Fas-mediated apoptosis in Balb3T3 cells, which may play a role in oncogenesis.

Key words: basic fibroblast growth factor • Fas • mitogen-activated protein kinase • oncogenesis • Ras

Introduction

Apoptosis is a form of cell death fundamental to the embryonic development and maintenance of homeostasis (Rinkenberger and Korsmeyer, 1997). Disorders of the process of apoptosis cause various pathologies, including autoimmune diseases and neurodegenerative diseases. Apoptosis is especially important to inhibit oncogenesis because (a) tumor-suppressor genes can induce apoptosis that is triggered by an abnormal progression of the cell cycle promoted by cellular or viral oncogenes; and (b) defects of proapoptotic genes frequently result in the tumorigenesis (Williams, 1991; Yin et al., 1997).

Apoptosis is also induced by the stimulation of death receptors, members of the tumor necrosis factor receptor superfamily (Nagata, 1997). These death receptors are characterized by the presence of a death domain within their cytoplasmic regions and can induce apoptosis triggered by binding of their ligands. Fas (CD95/APO-1) is the best-characterized death receptor, having been identified by preparing agonistic anti-Fas mAb with cell-killing activity (Yonehara et al., 1989; Itoh et al., 1991). Fas is involved in the elimination of self-reactive lymphocytes and tumor cells (Zornig et al., 1995; Peng et al., 1996; Maeda et al., 1999). Stimulation of Fas with agonistic antibodies or Fas ligand leads to the clustering of Fas. This enables both the adapter molecule Fas-associated death domain (FADD)[1]/MORT1 (Boldin et al., 1995; Chinnaiyan et al., 1995) and the complex of caspase-8 (FADD-like interleukin-1β–converting enzyme [FLICE]/MACH/MCH5) (Boldin et al.,

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[1]*Abbreviations used in this paper:* bFGF, basic FGF; EF1α, elongation factor 1α; FADD, Fas-associated death domain; FLICE, FADD-like interleukin-1β–converting enzyme; FLIP, FLICE-inhibitory protein; GFP, green fluorescence protein; IGF, insulin-like growth factor; K-RasV12, oncogenic K-Ras; MAP , mitogen-activated protein; MAPK, MAP kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; MKP-1, MAPK phosphatase 1; MST1, mammalian STE-20–like protein kinase; MST1-KD, kinase-defective MST1; PE, phycoerythrin; PI-3, phosphatidylinositol 3; PKB, protein kinase B; Raf-CAAX, constitutively active Raf; RalGDS, Ral guanine nucleotide exchange factor; RT, reverse trancription; SDSE-MAPKK, constitutively active MAPKK.

1996; Muzio et al., 1996; Srinivasula et al., 1996) and FLICE-associated huge protein (FLASH) (Imai et al., 1999) to be recruited to the cytoplasmic region of the receptor, forming the death-inducing signaling complex (DISC) (Kischkel et al., 1995; Imai et al., 1999). In DISC, caspase-8 is proteolytically activated and then initiates activation of the other caspases, which results in the induction of apoptosis (Medema et al., 1997).

Some types of apoptosis are known to be inhibited by the activation of Akt/protein kinase B (PKB) or mitogen-activated protein kinase (MAPK)/extracellular signal regulatory kinase (ERK). Activation of Akt/PKB was reported to prevent the apoptosis induced by withdrawal of survival factors such as insulin-like growth factor (IGF)-I or NGF in neurons (Dudek et al., 1997; Philpott et al., 1997), and interleukin-3 in hematopoietic cells (Songyang et al., 1997). Akt/PKB also inhibits the apoptosis induced by activation of Myc in the absence of serum in fibroblasts (Kauffmann-Zeh et al., 1997; Kennedy et al., 1997) and by detachment of epithelial cells from the extracellular matrix (Khwaja et al., 1997). MAPK was reported to inhibit the apoptosis induced by withdrawal of NGF in neurons (Xia et al., 1995) and by the expression of Hid in *Drosophila* (Bergmann et al., 1998; Kurada and White, 1998). In the case of the death receptors, Fas-mediated apoptosis was reported to be inhibited by a cellular gene, c–FLICE-inhibitory protein (FLIP) (Irmler et al., 1997), the expression of which was suggested to be upregulated by activated MAPKK in T lymphocytes (Yeh et al., 1998). However, it was also reported that activated T cells in early phase are resistant to Fas stimulation independently of c-FLIP.

The key regulator upstream of both Akt/PKB and MAPK is a small G protein Ras, known as an oncogene product. GTP-bound active Ras recruits its effector molecules, including Raf and phosphatidylinositol 3 (PI-3) kinase, under the plasma membrane and then activates the Raf/MAPK pathway and the PI-3 kinase/Akt pathway, respectively. Here, we report that c-K-Ras suppresses Fas-mediated apoptosis, and oncogenic Ras strongly protects cells against Fas-mediated apoptosis through the activation of the MAPK pathway in Fas-transgenic Balb3T3 cells. In addition, we found that basic FGF (bFGF) but not EGF confers resistance on the fibroblasts against Fas-mediated apoptosis. This protective ability of bFGF was also shown to be mediated by the activation of the Ras/MAPK pathway. Although it was recently reported that oncogenic Ras downregulates the expression of Fas through activation of the PI-3 kinase/Akt pathway (Peli et al., 1999), the MAPK pathway inhibited Fas-mediated apoptosis without affecting the expression level of Fas. Our results indicate that the activation of MAPK inhibits Fas-triggered apoptotic signaling in fibroblasts, which may play a role in oncogenesis.

Materials and Methods

Cell Lines

Mouse embryonic fibroblast Balb3T3 cells were kindly provided by K. Nagata (Kyoto University, Kyoto, Japan). The cells were maintained in DME supplemented with 10% FBS and 100 μg/ml kanamycin at 37°C in 5% CO_2. Balb3T3 cells were transfected with the expression vector of mouse Fas driven by human β-actin promoter (Gunning et al., 1987), together with hygromycin B phosphotransferase gene inserted into the BamHI/HindIII sites of pRc/CMV (Invitrogen). The transfected cells were selected in DME with 10% FBS containing 200 μg/ml of hygromycin B (Sigma Chemical Co.). Stably Fas-expressing Balb3T3, designated FH2, was cloned based on the high-level expression of Fas analyzed by flow cytometry after the staining with FITC-conjugated anti-Fas antibody RMF-6 (Nishimura et al., 1995), or phycoerythrin (PE)-conjugated anti-Fas antibody Jo-2 (PharMingen).

cDNA Library and Plasmid Constructs

cDNA was prepared by using time saver cDNA synthesis kit (Amersham Pharmacia Biotech) from polyA^+ RNA of Balb3T3 cells purified by oligo-dT column (Amersham Pharmacia Biotech), and then subcloned into pME18S expression vector (Sakamaki et al., 1992). Various mutants of mouse Ras, K-RasV12, K-RasN17, K-RasS35, K-RasG37, and K-RasC40 (Kauffmann-Zeh et al., 1997), were prepared from c-K-Ras by using Quick-Change site-directed mutagenesis kit (Stratagene). pJ7Ω-lacZ (Morgenstern and Land, 1990) was used for the expression of β-galactosidase. Flag-tagged mammalian STE-20–like protein kinase (MST1) (Lee et al., 1998) was mutagenized to be kinase-defective (MST1-KD) by replacement of lysine 59 with arginine (K59R). The proper construction of all the mutants was confirmed by DNA sequencing. cDNAs encoding constitutively active Raf (Raf-CAAX), Δp85, Akt, constitutively active Akt (HA-mΔ4-129 Akt), and RalN28 were kind gifts from J.F. Hancock (University of Queensland Medical School, Brisbane, Australia), W. Ogawa (Kobe University, Kobe, Japan), U. Kikkawa (Kobe University), R. Roth (Stanford University, Stanford, CA), and L.A. Feig (Tufts University, Medford, MA), respectively. cDNAs for constitutively active MAPKK (SDSE-MAPKK) were provided by E. Nishida (Kyoto University), and an expression vector for green fluorescence protein (GFP) was from K. Umesono (Kyoto University).

Antibodies and Reagents

Agonistic anti–mouse Fas mAb RK-8 (Nishimura et al., 1995) was provided by Medical and Biological Laboratories (Nagoya, Japan). mAbs against Ras (clone 18) and phospho-p42/44 MAPK (E10) were purchased from Transduction Laboratories and New England Biolabs, respectively. Polyclonal antibody against phospho-Akt was purchased from New England Biolabs. EGF purified from mouse submaxillary glands was from Sigma Chemical Co. Recombinant human IGF-I and recombinant human bFGF were from GIBCO BRL. Fluorescent substrates acetyl-Asp-Glu-Val-Asp-α-(4-methyl-coumaryl-7-amide) (Ac-DEVD-MCA) and acetyl-Ile-Glu-Thy-Asp-α-(4-methyl-coumaryl-7-amide) (Ac-IETD-MCA) for caspase-3/7 and caspase-8/6, respectively, were purchased from Peptide Institute. For staining β-galactosidase–positive cells, 5-bromo-4-chloro-3-indolyl-b-D-(−)-galactopyranoside (X-Gal) was purchased from Wako.

Expression Cloning

Subconfluent FH2 cells in five 10-cm dishes were transfected with pME18S encoding the cDNA library described above by the calcium-phosphate method (Sambrook et al., 1989). In brief, the cells were incubated first in a culture medium containing 10% FBS with calcium-phosphate–DNA complex for 24 h, and then in fresh medium with 10% FBS for another 24 h. After the incubation, cells were stimulated with 0.1–1.0 μg/ml RK-8 for 4–6 h. Apoptotic cells were removed by washing with PBS three times. The surviving cells that adhered to the dishes were collected and episomal plasmids in the collected cells were recovered according to the method described by Itoh et al. (1991). The recovered plasmids were amplified in ElectroMAX DH10B cells (GIBCO BRL) and transfected into FH2 cells. This cycle was repeated six times with the amplified plasmids obtained in the previous cycle.

Transient Transfection of Expression Vectors

For transient transfection, FH2 cells were seeded at 1×10^5 cells per well in 6-well plates. Cells were cultured for 1 d and then transfected with various expression vectors (0.4 μg/each vector) by using Lipofectamine plus (GIBCO BRL) according to the manufacturer's protocol.

Assay of Fas-mediated Apoptosis in the Transfected Cells

FH2 cells transfected with expression vectors (0.4 μg/each vector) and 0.4

μg pJ7Ω-lacZ were cultured for 2 d and then transferred to 24-well plates at 2×10^4 cells per well. After cultivation for 12–16 h, cells were stimulated with 200 ng/ml RK-8 for 0, 4, or 8 h, and fixed with PBS containing 2% formaldehyde and 0.2% glutaraldehyde for 5 min. After the removal of apoptotic cells by washing with PBS, attached cells were stained with PBS containing 1 mg/ml X-Gal, 0.02% NP-40, 5 mM K-ferricyanide, 5 mM K-ferrocyanide, and 2 mM $MgCl_2$ for 1 h at 37°C. The total number of β-galactosidase–positive cells per well was counted for each independent transfection ($n = 3$) under a microscope. Cell viability was represented by the percentage of the number of β-galactosidase–positive cells after treatment with anti-Fas mAb against that before the treatment with anti-Fas mAb.

In Vivo Analysis of Caspase-3 Activation

Cells were transfected with various expression vectors together with an expression vector of Flag-tagged MST1-KD as a substrate for caspase-3. 2 d later, cells were stimulated with 0.2 μg/ml RK-8 and lysed in 50 mM Tris-HCl, pH 7.5, containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 40 mM $Na_2P_2O_7$, 50 mM NaF, 5 mM $MgCl_2$, 100 μM Na_3VO_4, 10 mM EDTA, and protease inhibitor cocktail (Sigma Chemical Co.) at 4°C. For Western blotting, cell lysates were separated by 10% SDS-PAGE and transferred to PVDF membrane (Millipore). The membrane was blocked with 5% skim milk in PBST (PBS + 0.05% Tween 20) for at least 1 h, and then incubated with anti-Flag antibody M2 (Kodak) for detection of intact and cleaved MST1-KD.

Cytotoxic Assay by Staining with Amido Black

FH2 cells (1×10^5 cells/well) in 96-well plates were treated with anti-Fas mAb RK-8 and stained with 0.05% amido black in 9% CH_3COOH with 0.1 M CH_3COONa for 30–60 min at room temperature. Cells were washed with water, dried, and dissolved in 100 μl/well of 25 mM NaOH. Cell density was determined by measuring optical absorbance at 560 nm with a microplate reader (Molecular Devices).

Quantification of FLIP mRNA by Reverse Transcription PCR

One step reverse trancription (RT)-PCR with 2 μg total RNA was carried out by using ready-to-go RT-PCR beads (Amersham Pharmacia Biotech) supplemented with extra 1.25 U Pfu turbo (Stratagene). Primers used for mouse cellular FLIP and elongation factor 1α (EF1α) were as follows: mouse $FLIP_L$, 5′-GAG CCA AGA TTT GTG GAA TAC CG-3′; mouse $FLIP_L$, 5′-TCT TCC AAC TGG CTA CCT AAC GAC T-3′; mouse EF1α, 5′-TCC TAC CAC CAA CTC GTC CAA C-3′; and mouse EF1α, 5′-CAG CTT CTT ACC AGA ACG ACG ATC-3′.

Results

Cloning of c-K-Ras as a Gene Inhibiting Fas-mediated Apoptosis

To search for inhibitory genes against Fas-mediated apoptosis, we prepared the cells sensitive to the stimulation of Fas from Balb3T3 cells by transfecting an expression vector of mouse Fas under the control of human β-actin promoter. FH2 was sensitive to the stimulation of Fas. By using FH2 cells, we carried out expression cloning to obtain cDNA that confers resistance to Fas-mediated apoptosis in FH2 cells as described in Materials and Methods, and finally obtained c-K-Ras. To confirm the protective activity of c-K-Ras, we cotransfected expression vectors encoding c-K-Ras and β-galactosidase, and quantified β-galactosidase–positive cells after stimulation of Fas (Fig. 1). Treatment with agonistic anti-Fas mAb RK-8 dose-dependently induced apoptosis in FH2 cells, and transient expression of c-K-Ras suppressed this apoptosis (Fig. 1). We then observed the morphologies of cells that were transfected with an empty vector or an expression vector of c-K-Ras,

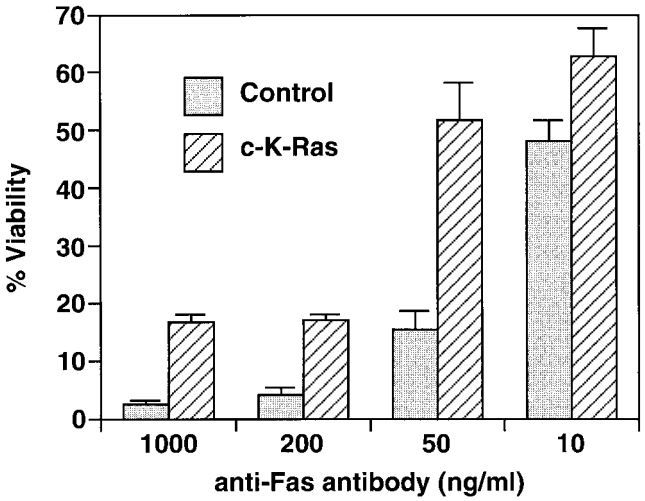

Figure 1. c-K-Ras inhibits Fas-mediated apoptosis in FH2 cells. FH2 cells were transiently transfected with an empty vector (Control) or an expression vector of c-K-Ras together with pJ7Ω-LacZ. Cells were treated with indicated concentrations of agonistic anti-Fas mAb RK-8 for 8 h. The percentages of viable cells after Fas-stimulation were determined as described in Materials and Methods.

and treated with or without anti-Fas mAb for 4 h (Fig. 2, A, B, E, and F). Control cells treated with anti-Fas mAb showed an apoptotic morphology with a rounded form and were detached from the culture dish (Fig. 2 E). In contrast, a significant number of the c-K-Ras–transfected cells kept an extended morphology even after the stimulation of Fas (Fig. 2 F). These results indicate that the overexpression of c-K-Ras decreases the sensitivity of fibroblasts to Fas-mediated apoptosis.

Activated K-Ras Inhibits Fas-mediated Apoptosis

To analyze whether the inhibitory effect of c-K-Ras on Fas-mediated apoptosis is reflected by the effects of active (GTP-bound) or inactive (guanosine diphosphate–bound) Ras, FH2 cells were transfected with an expression vector of Ras mutant together with that of β-galactosidase, constitutively active Ras (K-RasV12), or dominant-inhibitory Ras (K-RasN17). We counted the β-galactosidase–positive cells transfected with K-RasV12 or K-RasN17 before and after the treatment with anti-Fas mAb (Fig. 3 A) and found that K-RasV12 strongly inhibited, whereas K-RasN17 enhanced, Fas-mediated apoptosis. Fig. 2 G shows that most of the cells transfected with K-RasV12 displayed an intact morphology after 4 h of stimulation with anti-Fas mAb. In contrast, almost all the cells transfected with K-RasN17 were completely detached from the culture dish (Fig. 2 H). These results show that Fas-mediated apoptosis is suppressed by activated K-Ras and enhanced by dominant-inhibitory K-Ras in FH2 cells.

Activated K-Ras Inhibits Activation of Caspases

Caspases play a central role in apoptosis by cleaving intracellular proteins, including DNA fragmentation factor (DFF) 45/Inhibitor of caspase-activated DNase (ICAD), poly (ADP-ribose) polymerase (PARP), and protein ki-

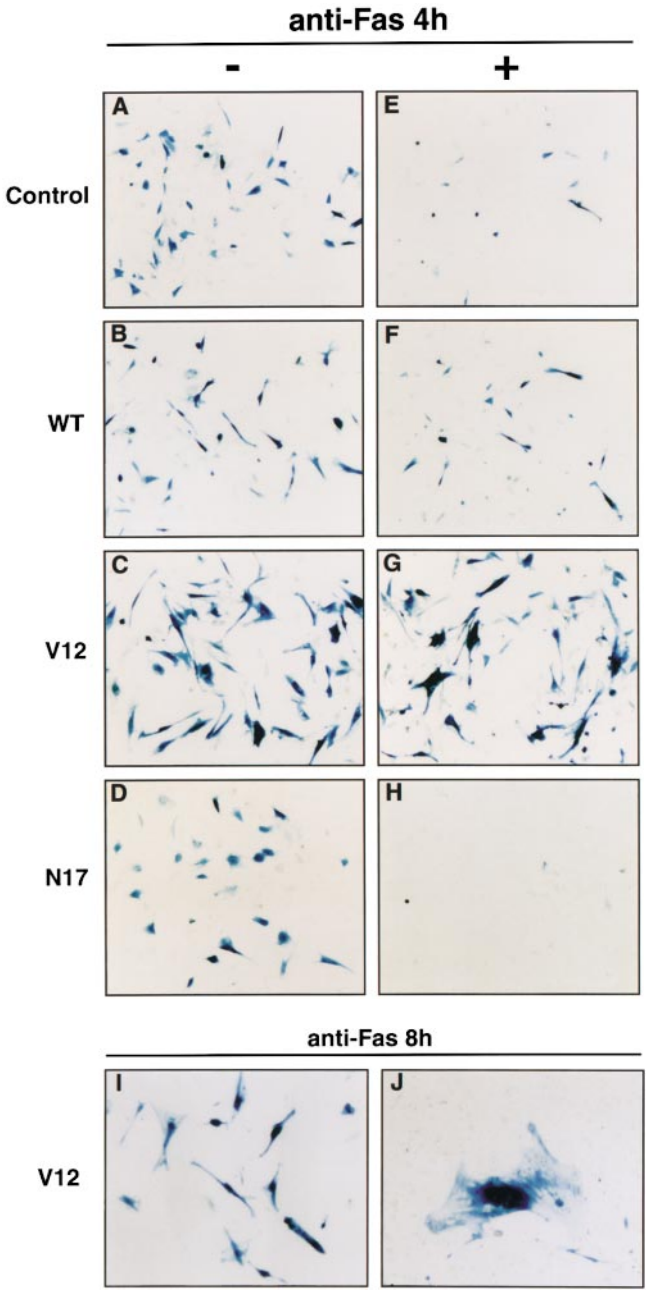

Figure 2. Oncogenic K-Ras inhibits morphological changes in Fas-mediated apoptosis. FH2 cells were transiently transfected with an empty vector (Control), an expression vector of c-K-Ras, constitutively active Ras (K-RasV12), or dominant-inhibitory Ras (K-RasN17) together with pJ7Ω-LacZ and stained with X-Gal before (A–D) or after (E–J) the stimulation with 200 ng/ml anti-Fas mAb RK-8 for 4 (E–H) or 8 h (I and J). (A and E) Cells were transfected with a control vector. After a 4-h stimulation of Fas, almost all cells were detached from culture dishes. (B and F) Cells were transfected with an expression vector of c-K-Ras. About 40% of the c-K-Ras–transfected cells survived even after a 4-h stimulation of Fas (F). (C, G, I, and J) Cells were transfected with an expression vector of K-RasV12. Most of the cells survived after 4 h of stimulation (G). Even after 8 h of stimulation, many of the K-RasV12–transfected cells survived (I) and developed filamentous structure (J). (D and H) Cells were transfected with an expression vector of K-RasN17. All the cells stimulated with 200 ng/ml RK-8 for 4 h were detached from culture dishes (H).

nase MST (Lee et al., 1998). MST was directly cleaved by caspase-3 both in vitro and in vivo (Lee, K.K., and S. Yonehara, unpublished data). To investigate whether K-RasV12 inhibits Fas-mediated apoptosis upstream or downstream of caspase activation, we cotransfected K-RasV12 with MST1-KD tagged with Flag as a substrate for activated caspase-3, and analyzed the cleavage of MST1-KD by caspase-3 after stimulation with anti-Fas mAb by Western blotting. In control cells, MST1-KD began to be cleaved after 1 h of stimulation and was almost completely cleaved within 4 h (Fig. 3 C). In the cells cotransfected with K-RasV12, we could not observe cleaved MST1-KD after 1 h of stimulation. In addition, most of the MST1-KD remained full-length even after 4 h of stimulation (Fig. 3 C), suggesting no activation of caspase-3. These results indicate that K-RasV12 inhibits Fas-triggered apoptotic signaling at a point upstream of caspase-3.

Activation of the MAPK Pathway Inhibits Fas-mediated Apoptosis

GTP-bound active Ras was reported to transduce various signals by activating multiple intracellular target molecules, including Raf, PI-3 kinase, and Ral guanine nucleotide exchange factor (RalGDS) (Downward, 1998). To investigate which target molecule of Ras is involved in the protection against Fas-mediated apoptosis, we analyzed the effects of three partial loss-of-function mutants derived from K-RasV12, K-RasS35, K-RasG37, and K-RasC40, which were reported to activate only Raf, RalGDS, and PI-3 kinase, respectively (Kauffmann-Zeh et al., 1997). Fig. 3 A shows that K-RasS35 and K-RasG37 could inhibit Fas-mediated apoptosis but that their protective activities were significantly lower than that of K-RasV12. K-RasC40 did not show significant suppressing activity against Fas-mediated apoptosis in FH2 cells (Fig. 3 A), although the expression was confirmed (Fig. 3 B). These results suggest that the protective activity of K-Ras against Fas-mediated apoptosis depends on the activation of Raf and/or RalGDS.

Raf is an activator of MAPKK, which is an activator of MAPK. To confirm whether the activation of the MAPK pathway is involved in the inhibition of Fas-mediated apoptosis, FH2 cells were transfected with Raf-CAAX (Stokoe et al., 1994) or SDSE-MAPKK (Fukuda et al., 1997). Both Raf-CAAX and SDSE-MAPKK protected FH2 cells against Fas-mediated apoptosis (Fig. 4 A) and inhibited caspase-dependent cleavage of MST1-KD (Fig. 4 B). To further confirm the activation of MAPK to be essential for Ras-dependent protection against Fas-mediated apoptosis, we examined the effect of MKP-1, a phosphatase specific for activated MAPK, on the protective activity of K-RasV12, Raf-CAAX, or SDSE-MAPKK in FH2 cells. MKP-1, the expression of which was confirmed by Western blotting (Fig. 4 C), completely canceled the protective activity of K-RasV12, Raf-CAAX, and SDSE-MAPKK against Fas-mediated apoptosis (Fig. 4 A) and caspase-dependent cleavage of MST1-KD (Fig. 4 B).

Then we examined whether the RalGDS pathway is involved in Ras-dependent protection, and dominant-inhibitory mutant of Ral, RalN28, transfected with K-RasV12 into FH2 cells could not cancel Ras-dependent protection

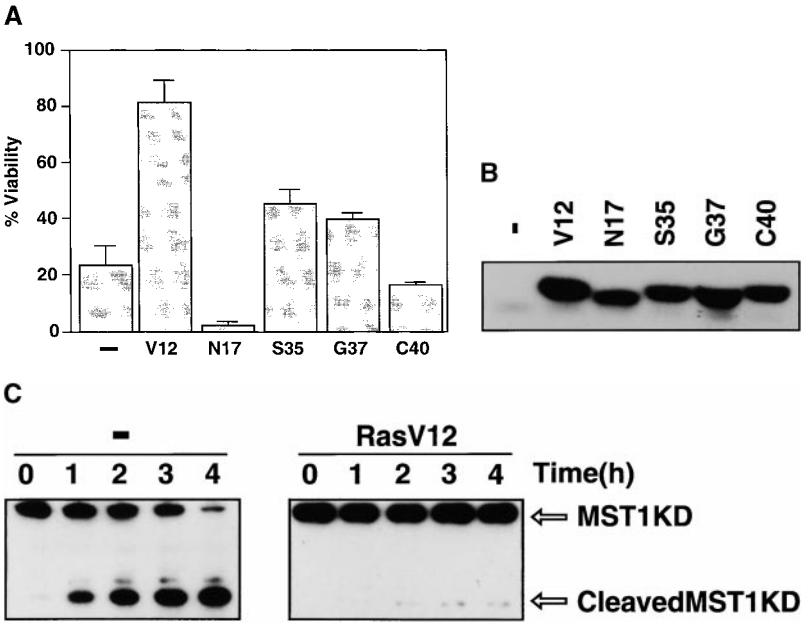

Figure 3. Oncogenic K-Ras prevents, and dominant-inhibitory K-Ras enhances, Fas-mediated apoptosis. (A) FH2 cells were transiently transfected with an empty vector (control) or an expression vector of K-RasV12, K-RasN17, K-RasS35, K-RasG37, or K-RasC40 together with pJ7Ω-LacZ. K-RasS35, K-RasG37, and K-RasC40 possess RasV12 background. The percentages of viable cells after 4 h of treatment with 200 ng/ml agonistic anti-Fas mAb RK-8 were determined as described in Materials and Methods. B, Transient expression of Ras mutants was examined by Western blotting with anti-Ras antibody. C, FH2 cells were co-transfected with an empty vector (control) or an expression vector of K-RasV12 with that of MST1-KD tagged with Flag as a substrate of caspase-3 to monitor the Fas-triggered activation of caspases. After stimulation of Fas for the indicated times, intact and cleaved Flag-tagged MST1-KD in total cell lysates were detected by Western blotting with anti-Flag antibody.

against Fas-mediated apoptosis (data not shown). Recently, H-Ras–dependent activation of PI-3 kinase, which is an activator of Akt/PKB, was reported to prevent Fas-mediated apoptosis by downregulating the expression level of Fas (Peli et al., 1999). To test the effect of PI-3 kinase activated by Ras on Fas-mediated apoptosis in FH2 cells, we transfected a mutant of PI-3 kinase subunit Δp85 (Sakaue et al., 1995), which was reported to dominantly inhibit Ras-dependent activation of PI-3 kinase (Rodriguez-Viciana et al., 1997). Overexpression of Δp85, which was confirmed by Western blotting (Fig. 4 D), did not prevent the protective effect of K-RasV12 against Fas-mediated apoptosis in FH2 cells, although the inhibitory effect of Δp85 on phosphorylation of Akt/PKB by K-RasV12 was confirmed (Fig. 4 D). Moreover, overexpression of constitutively active Akt/PKB (Kohn et al., 1996) also did not suppress Fas-mediated apoptosis (Fig. 4, A and E). All the results indicate that the MAPK pathway but not the Akt/PKB pathway plays an important role on K-Ras–dependent protection against Fas-mediated apoptosis in FH2 cells.

To analyze whether K-RasV12, Raf-CAAX, and SDSE-MAPKK regulate Fas-expression in FH2 cells that express exogenous Fas under the control of human β-actin promoter, we analyzed the expression levels of Fas by flow cytometry on the cells that were cotransfected with K-RasV12, Raf-CAAX, or SDSE-MAPKK together with GFP expression vector. Control cells highly expressed Fas (Fig. 5 A), and the expression levels of Fas on GFP-intensive cells were as high as those on GFP-negative cells (Fig. 5 B). These results indicate that K-RasV12, Raf-CAAX, and SDSE-MAPKK do not influence the Fas-expression enforced by human β-actin promoter in FH2 cells, because GFP-intensive cells were considered to highly express K-RasV12, Raf-CAAX, or SDSE-MAPKK. In addition, the expression level of endogenous Fas in parental Balb3T3 cells was also unaffected by overexpressed K-RasV12, Raf-CAAX, or SDSE-MAPKK (Fig. 5 C). These results indicate that activation of the MAPK pathway by K-Ras does not regulate the expression level of Fas.

Pretreatment with bFGF Inhibits Fas-mediated Apoptosis

To examine whether physiological activation of MAPK is sufficient to inhibit Fas-mediated apoptosis, FH2 cells were pretreated with several growth factors, including EGF, IGF, and bFGF, which are known to activate MAPK, and then stimulated with anti-Fas mAb. After pretreatment with bFGF for >12 h, FH2 cells showed a resistant phenotype to Fas-mediated apoptosis, although the cells pretreated with either EGF or IGF were as sensitive as nontreated cells (Fig. 6 A). Then we compared the kinetics of the phosphorylation of MAPK after the treatment with EGF, IGF, and bFGF. bFGF treatment induced a strong and sustained phosphorylation of MAPK (Fig. 6 B). EGF treatment induced a relatively transient phosphorylation of MAPK (Fig. 6 B). These results suggest that strong and constitutive activation of MAPK is necessary to inhibit Fas-mediated apoptosis.

We then investigated Fas-triggered activation of caspases in FH2 cells pretreated with or without bFGF by using fluorescence tetrapeptides, IETD-MCA and DEVD-MCA, as specific substrates for caspase-8/6 and caspase-3/7, respectively. Protease activity of caspases specific for both IETD and DEVD in control cells increased markedly after a 2-h stimulation of Fas (Fig. 6, C and D). However, in bFGF-treated cells, the protease activity for IETD was completely suppressed even after a 4-h stimulation of Fas (Fig. 6 C). The protease activity for DEVD was also distinctly suppressed by the pretreatment with bFGF (Fig. 6 D), although it slightly increased from 3 h after the stimulation of Fas. These results show that bFGF suppressed Fas-triggered apoptotic signaling at a point upstream of caspases the same as oncogenic K-Ras.

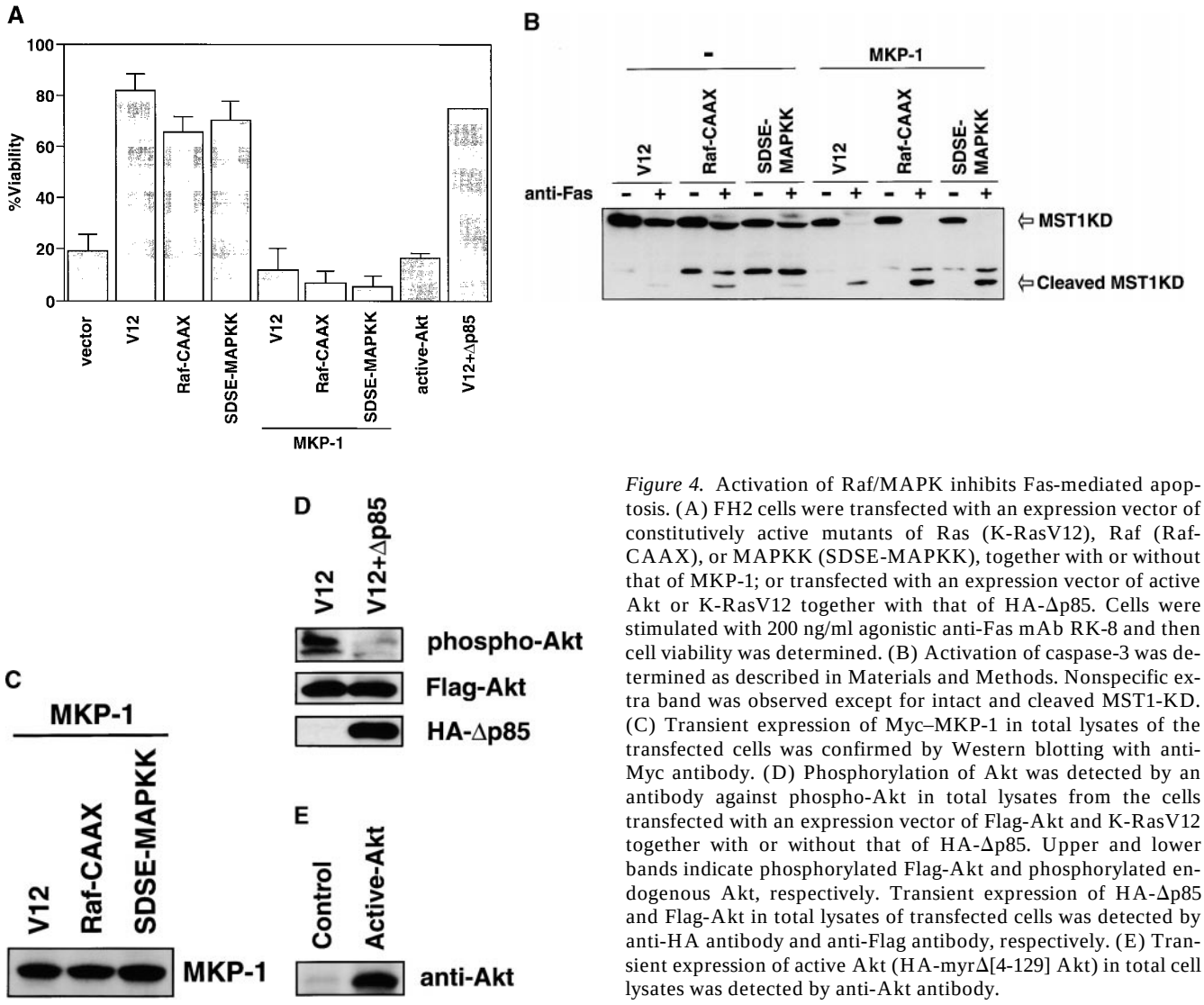

Figure 4. Activation of Raf/MAPK inhibits Fas-mediated apoptosis. (A) FH2 cells were transfected with an expression vector of constitutively active mutants of Ras (K-RasV12), Raf (Raf-CAAX), or MAPKK (SDSE-MAPKK), together with or without that of MKP-1; or transfected with an expression vector of active Akt or K-RasV12 together with that of HA-Δp85. Cells were stimulated with 200 ng/ml agonistic anti-Fas mAb RK-8 and then cell viability was determined. (B) Activation of caspase-3 was determined as described in Materials and Methods. Nonspecific extra band was observed except for intact and cleaved MST1-KD. (C) Transient expression of Myc–MKP-1 in total lysates of the transfected cells was confirmed by Western blotting with anti-Myc antibody. (D) Phosphorylation of Akt was detected by an antibody against phospho-Akt in total lysates from the cells transfected with an expression vector of Flag-Akt and K-RasV12 together with or without that of HA-Δp85. Upper and lower bands indicate phosphorylated Flag-Akt and phosphorylated endogenous Akt, respectively. Transient expression of HA-Δp85 and Flag-Akt in total lysates of transfected cells was detected by anti-HA antibody and anti-Flag antibody, respectively. (E) Transient expression of active Akt (HA-myrΔ[4-129] Akt) in total cell lysates was detected by anti-Akt antibody.

bFGF Prevents Fas-mediated Apoptosis by Activating the Ras/MAPK Pathway

To investigate whether the activation of MAPK is involved in the inhibition of Fas-mediated apoptosis by bFGF, FH2 cells were transfected with an expression vector of K-RasN17, MKP-1, or Δp85, and then treated with bFGF followed by the stimulation with anti-Fas mAb. Fig. 7 A shows that K-RasN17 and MKP-1 prevented the protective effect of bFGF against Fas-mediated apoptosis, although Δp85 did not have any inhibitory effect on the activity of bFGF. These results indicate that bFGF inhibits Fas-mediated apoptosis in FH2 cells through activation of MAPK.

We analyzed expression levels of endogenous and stably expressed exogenous Fas on Balb3T3 cells and FH2 cells, respectively, before and after the treatment with bFGF. bFGF treatment did not downregulate expression levels of Fas on either Balb3T3 cells (Fig. 7 B) or FH2 cells (data not shown). These results show that the protective effect of bFGF on Fas-mediated apoptosis is mediated by the Ras/MAPK pathway without downregulating Fas expression.

It was reported previously that c-FLIP, which can inhibit Fas-induced apoptosis (Irmler et al., 1997), is upregulated by MAPKK activation in lymphocytes (Yeh et al., 1998). We analyzed c-FLIP expression in FH2 cells pretreated with or without bFGF by Northern hybridization. However, we could not detect specific expression of c-FLIP mRNA by Northern hybridization, which indicates that the expression level of c-FLIP mRNA in FH2 cells treated with or without bFGF is low. Then we carried out RT-PCR with c-FLIP–specific primers and detected the expression of c-FLIP mRNA (Fig. 7 C). The PCR product of c-FLIP from the bFGF-treated cells was detected about one cycle earlier than that from the nontreated cells. However, the PCR product of control EF1α was observed seven cycles earlier than c-FLIP (Fig. 7 C). These data suggest that c-FLIP expression was upregulated by the bFGF treatment about twice, but the expression level of c-FLIP was low.

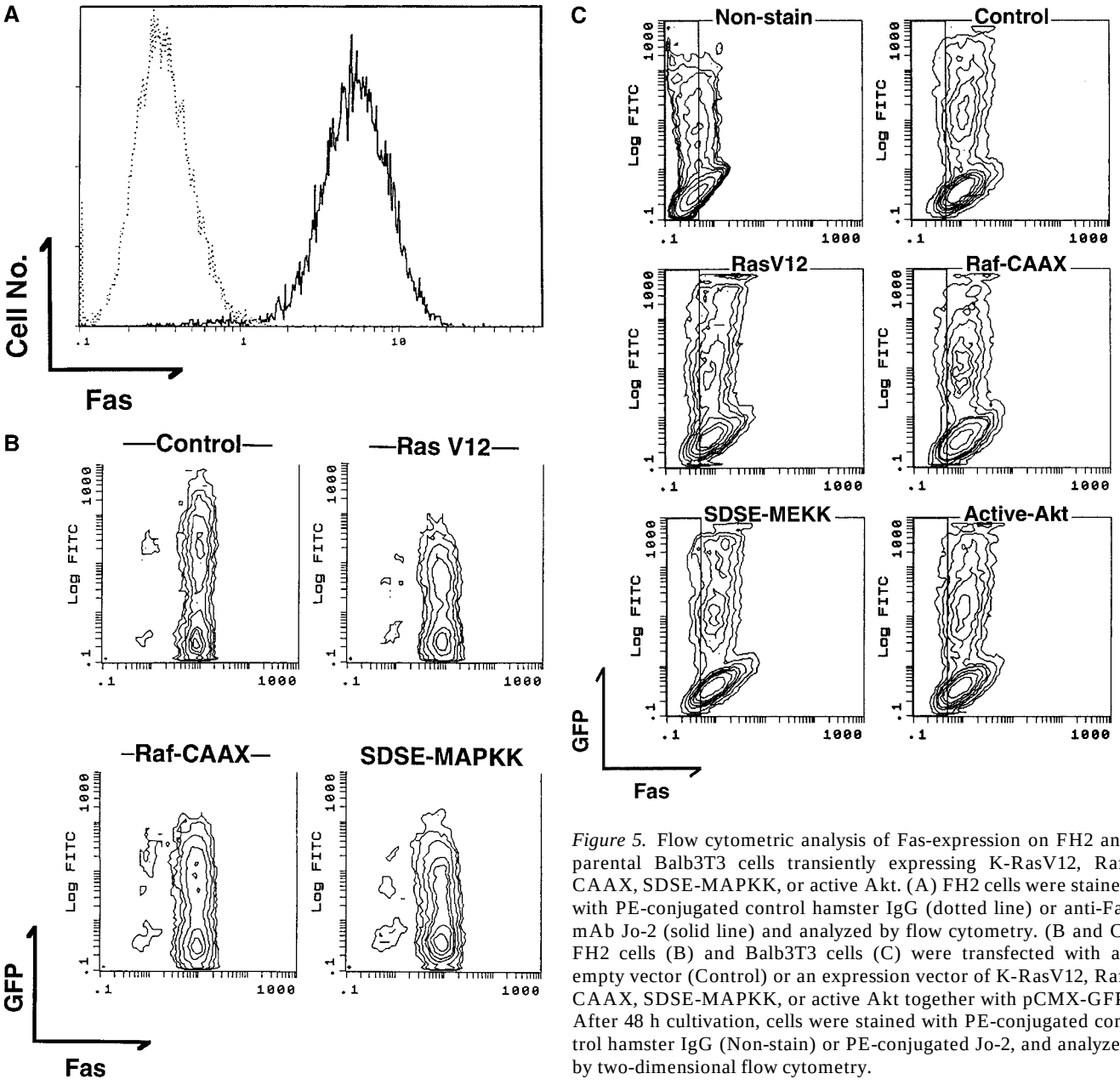

Figure 5. Flow cytometric analysis of Fas-expression on FH2 and parental Balb3T3 cells transiently expressing K-RasV12, Raf-CAAX, SDSE-MAPKK, or active Akt. (A) FH2 cells were stained with PE-conjugated control hamster IgG (dotted line) or anti-Fas mAb Jo-2 (solid line) and analyzed by flow cytometry. (B and C) FH2 cells (B) and Balb3T3 cells (C) were transfected with an empty vector (Control) or an expression vector of K-RasV12, Raf-CAAX, SDSE-MAPKK, or active Akt together with pCMX-GFP. After 48 h cultivation, cells were stained with PE-conjugated control hamster IgG (Non-stain) or PE-conjugated Jo-2, and analyzed by two-dimensional flow cytometry.

Discussion

We report here that transient expression of oncogenic K-Ras inhibits Fas-mediated apoptosis in Fas-transgenic Balb3T3 cells through the activation of the Ras/MAPK pathway. Although the cells transfected with K-RasV12 were strongly resistant to the stimulation of Fas, prolonged stimulation >8 h caused apoptosis in some of these cells (Fig. 2 I). We observed that most of the surviving cells transfected with K-RasV12, even after the prolonged stimulation of Fas, show developed filamentous structures in the cytoplasm, which may indicate strong expression of transfected K-RasV12 (Fig. 2, I and J). These results suggest that strong expression of oncogenic K-Ras can completely prevent untransformed cells from undergoing Fas-mediated apoptosis, and explain how tumor cells escape from immune surveillance by cytotoxic T cells during the multistep progression of oncogenesis, because cytotoxic T cells utilize Fas–Fas ligand system to kill tumor cells (Rouvier et al., 1993; Suda et al., 1993; Kojima et al., 1994).

Among partial loss-of-function mutants of Ras, RasS35 and RasG37 were reported to activate only Raf and Ral-GDS, respectively. Both K-RasS35 and K-RasG37 partially protected FH2 cells from Fas-mediated apoptosis (Fig. 3 B). However, dominant-inhibitory RalN28 could not cancel Ras-dependent protection against Fas-mediated apoptosis (data not shown). Not only dominant-inhibitory Ral but also dominant-inhibitory PI-3 kinase subunit Δp85, which inhibited Ras-dependent activation of PI-3 kinase (Fig. 4 D), could not disrupt the protective activity of K-Ras against Fas-mediated apoptosis (Fig. 4 A). In contrast, MKP-1, a phosphatase specific for activated classical MAPK, could cancel the protective activity of

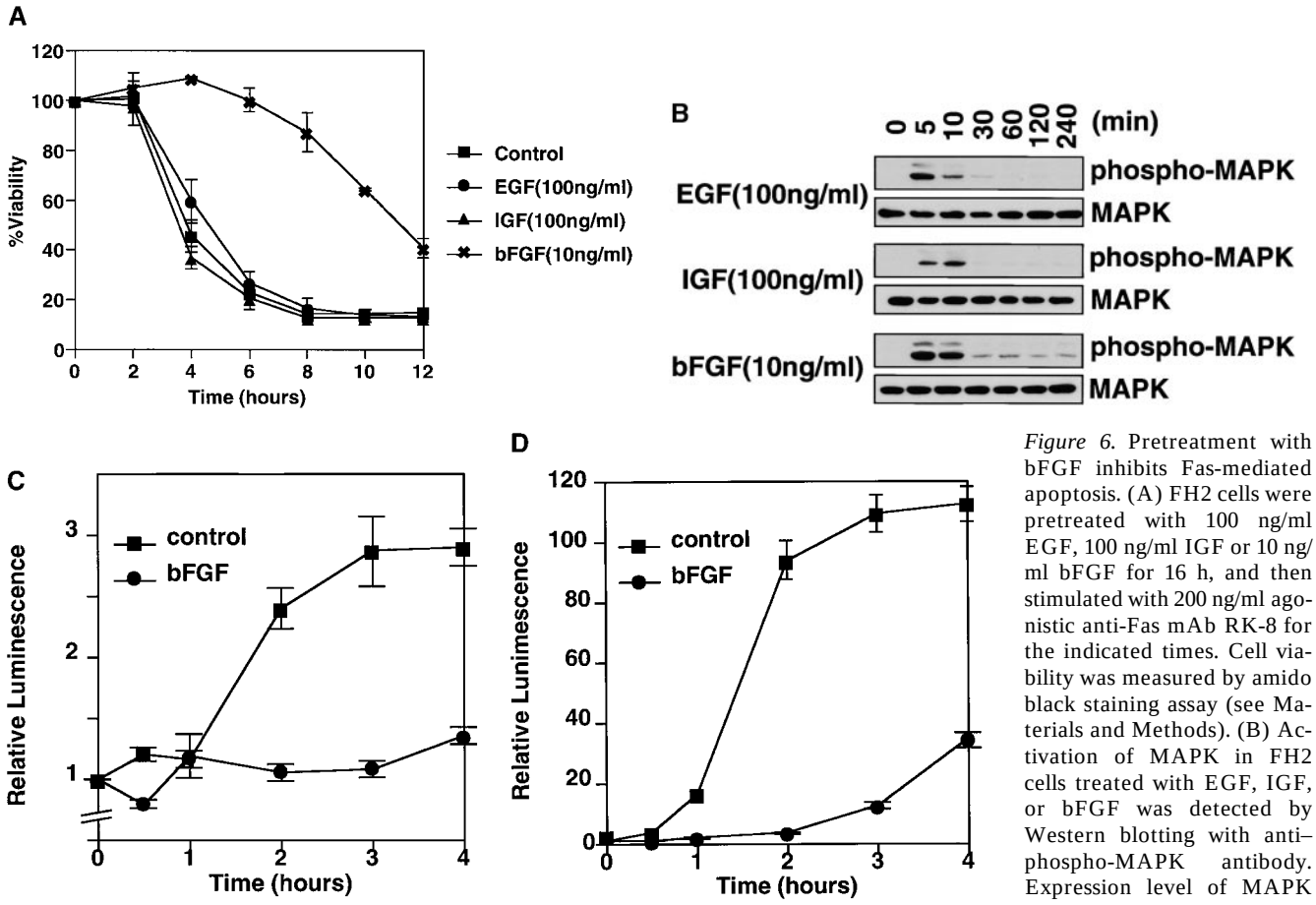

Figure 6. Pretreatment with bFGF inhibits Fas-mediated apoptosis. (A) FH2 cells were pretreated with 100 ng/ml EGF, 100 ng/ml IGF or 10 ng/ml bFGF for 16 h, and then stimulated with 200 ng/ml agonistic anti-Fas mAb RK-8 for the indicated times. Cell viability was measured by amido black staining assay (see Materials and Methods). (B) Activation of MAPK in FH2 cells treated with EGF, IGF, or bFGF was detected by Western blotting with anti–phospho-MAPK antibody. Expression level of MAPK was confirmed by Western blotting with anti-MAPK antibody. (C and D) FH2 cells were pretreated with or without 10 ng/ml bFGF for 16 h and stimulated with anti-Fas mAb RK-8 for the indicated times. Activation of caspases in cell lysate was measured using the fluorescent substrate IETD-MCA and DEVD-MCA for caspase-8/6 (C) and caspase-3/7 (D), respectively.

K-RasV12, Raf-CAAX, and SDSE-MAPKK (Fig. 4 A). Thus, activation of MAPK is essential for K-Ras–dependent protection against Fas-mediated apoptosis in FH2 cells. However, the results indicating that the protective activity of Raf-CAAX and SDSE-MAPKK was slightly lower than that of K-RasV12 (Fig. 4, A and B) suggest that another signaling pathway activated by K-Ras may contribute to Ras-dependent protection against Fas-mediated apoptosis.

We transfected K-RasV12 or K-RasN17 into other Fas-transgenic cells prepared from tumor cell lines such as HeLa and KB cells. Interestingly, Fas-mediated apoptosis in these cells was neither inhibited by transient expression of K-RasV12 nor enhanced by transient expression of K-RasN17 or MKP-1 (data not shown). These cells were relatively resistant to the stimulation with agonistic anti-Fas mAb compared with FH2 cells. These results imply that the protective activity of Ras/Raf/MAPK is specifically observed in untransformed cells or the cells more sensitive to the stimulation of Fas than usual transformed cell lines such as HeLa and KB cells.

We showed here that bFGF treatment desensitized fibroblasts to the stimulation of Fas through activation of the Ras/MAPK pathway, because K-RasN17 and MKP-1 canceled the protective effect of bFGF (Fig. 7 A). However, the protective effect of bFGF was not sustained for a long time (Fig. 6 A), and caspase-3 was gradually activated after a 3-h stimulation of Fas (Fig. 6 D). These results indicate that bFGF can exert its protective ability through the Ras/MAPK pathway, but the activation of endogenous Ras by bFGF may not be sufficient to protect cells against continuous stimulation of Fas. We suppose that a more sustained activation of the Ras/MAPK pathway, such as by high expression of oncogenic K-RasV12 (Fig. 2, I and J), is necessary for complete protection of cells against Fas-mediated apoptosis.

The protective ability of bFGF against Fas-mediated apoptosis was different from that of EGF (Fig. 6 A). Marshall (1995) reviewed that stimulation of pheochromocytoma cell line PC12 with EGF leads to proliferation, whereas stimulation with FGF or NGF leads to outgrowth of neurites and eventual cessation of cell division. For the difference of cellular responses of PC12 cells, the duration of MAPK activation is claimed to be critical. In our experiments, both bFGF and EGF could activate MAPK (Fig. 6 B), but only bFGF inhibited Fas-mediated apoptosis in fibroblasts (Fig. 6 A). The protective effect of bFGF may also result from prolonged activation of MAPK, because

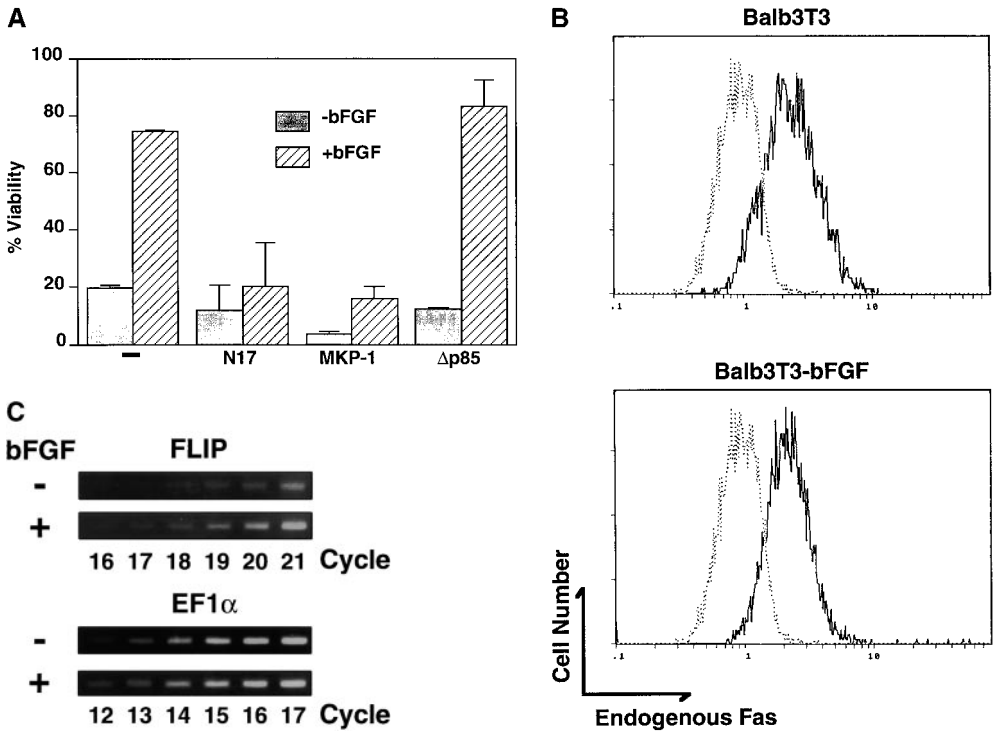

Figure 7. Activation of Ras/MAPK pathway is essential for the protective effect of bFGF on Fas-mediated apoptosis. (A) After transfection of an expression vector of K-RasN17, MKP-1, or Δp85 together with pJ7Ω-LacZ, FH2 cells were cultivated in the presence of 10 ng/ml bFGF for 16 h and then stimulated with 200 ng/ml agonistic anti-Fas mAb for 4 h. The percentages of viable cells were determined as described in Materials and Methods. (B) Parental Balb3T3 cells were cultivated with or without 10 ng/ml bFGF for 16 h, and then stained with PE-conjugated hamster IgG (dotted line) or anti-Fas mAb Jo-2 (solid line). Endogenous expression of Fas of Balb3T3 cells was analyzed by flow cytometry. (C) RT-PCR amplification of c-FLIP and EF1α mRNA was performed with 2 μg total RNA from FH2 cells treated with or without bFGF. After the indicated PCR cycles, amplified products were analyzed.

we observed a more sustained activation of MAPK in FH2 cells treated with bFGF than with EGF (Fig. 6 B). By using the DNA chip technique, Fambrough et al. (1999) reported that the stimulation of fibroblasts with PDGF or FGF induces the expression of a set of genes designated as immediate early genes (IEGs), and EGF induces expression of only a subset of IEGs. We suppose that the difference of the protective effect against Fas-mediated apoptosis between bFGF and EGF might be explained by the difference in the set of IEGs induced by bFGF and EGF.

It was shown that oncogenic H-Ras downregulates the expression of endogenous Fas in fibroblast and epithelial cells through the activation of PI-3 kinase (Peli et al., 1999). We analyzed the expression of Fas on FH2 cells after the transfection with K-RasV12, Raf-CAAX, or SDSE-MAPKK, or the treatment with bFGF. The results did not show the downregulation of the expression level of Fas enforced by β-actin promoter in FH2 cells (Fig. 5 B; data not shown). Thus, the activation of the Ras/MAPK pathway was shown to be able to inhibit Fas-mediated apoptosis even when the expression of Fas was not downregulated. In addition, not only transient expression of K-RasV12, Raf-CAAX, SDSE-MAPKK, or active Akt, but also pretreatment with bFGF did not downregulate the expression of endogenous Fas in parental Balb3T3 cells (Fig. 5 C and Fig. 7 B). Our results indicate that activation of the Ras/MAPK pathway can confer cellular resistance to Fas-mediated apoptosis without affecting the expression of Fas when cells are treated with bFGF. The different observations by others and us (Fenton et al., 1998; Gibson et al., 1999; Peli et al., 1999) might arise from different cells and/or different gene-expression system. In addition, we used K-Ras, whereas the others used H-Ras. A difference of Ras might contribute to the distinct data, because functional differences were reported among Ras homologues (Voice et al., 1999).

In bFGF-treated FH2 cells, we detected about twofold upregulation of c-FLIP transcript by RT-PCR when compared with that in control FH2 cells (Fig. 7 C). However, we could not detect c-FLIP mRNA in FH2 cells treated or untreated with bFGF by Northern hybridization under the condition where mRNA of caspase-8 and EF1α were detected (data not shown). We suppose that bFGF-induced upregulation of c-FLIP mRNA is not sufficient to protect FH2 cells from Fas-mediated apoptosis, because the quantity of c-FLIP mRNA is much lower than that of caspase-8 mRNA in FH2 cells.

Here we clarified that the Ras/MAPK pathway prevents Fas-mediated apoptosis in untransformed fibroblasts, which may contribute to oncogenesis. However, the protective mechanism of the Ras/MAPK pathway remains to be elucidated and must be clarified in the future.

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