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

Hirotaka Kazama and Shin Yonehara

Institute for Virus Research, Kyoto University, Kyoto 606-8507, Japan

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 as mutants revealed that oncogenic mutant K-Ras (RasV12) strongly inhibited, whereas dominant-inhibitory mutant K-Ras (RasN17) enhanced, Fas-mediated apoptosis by inhibiting Fas-activated stimulation 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 MAPK (SD SE-MAPK) 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 SD SE-MAPK. 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. 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; Y in et al., 1997).

A apoptosis is also induced by the stimulation 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 (Y onehara et al., 1989; I toh 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., 1995).
Some types of apoptosis are known to be inhibited by the activation of A kt/potential kinase (PKB) or mitogen-activated protein kinase (MAPK)/extracellular signal regulatory kinase (ERK). A cation of A kt/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). A k/PKB also inhibits the apoptosis induced by activation of M yc in the absence of serum in fibroblasts (K aufmann-Zeh et al., 1997; K ennedy et al., 1997) and by detachment of epithelial cells from the extracellular matrix (K hwa et al., 1997). M APK was reported to inhibit the apoptosis induced by withdrawal of NGF in neurons (X ia et al., 1995) and by the expression of H id in Drosophila (B ergmann et al., 1998; K urada and W hite, 1998). In case of the death receptors, Fas-mediated apoptosis was reported to be inhibited by a cellular gene, c-FL I C-inhibitory protein (FLIP) (I rmler et al., 1997), the expression of which was suggested to be upregulated by activated M APK in T lymphocytes (Y eh et al., 1998). However, it was also reported that activated T cells in early phase are resistant to Fas stimulation independently of c-FL I P.

The key regulator upstream of both A kt/PKB and M APK is a small G protein Ras, known as an oncogene product. GTP-bound active R as recruits its effector molecules, including R af and phosphatidylinositol 3 (PI-3) kinase, under the plasma membrane and then activates the R af/M APK pathway and the PI-3 kinase/A kt pathway, respectively. Here, we report that c-K-R as suppresses Fas-mediated apoptosis, and oncogenic R as strongly protects cells against Fas-mediated apoptosis through the activation of the M APK 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 R as/M APK pathway. Although it was recently reported that oncogenic R as downregulates the expression of Fas through activation of the PI-3 kinase/A kt pathway (Peli et al., 1999), the M APK pathway inhibited Fas-mediated apoptosis without affecting the expression level of Fas. Our results indicate that the activation of M APK 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. Nagaoka (K yoto U niversity, K yoto, J apan). The cells were maintained in DM E supplemented with 10% F BS 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 (G unning 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 DM E with 10% FBS containing 200 μg/ml of hygromycin B (Sigma Chemical Co.). Stably Fas-expressing Balb3T3, designated F H 2, was cloned based on the high-level expression of Fas analyzed by flow cytometry after the staining with FITC-conjugated anti-Fas antibody RM F-6 (Nishimura et al., 1995), or phycoerythrin (PE)-conjugated anti-Fas antibody Jo-2 (PharM gen).

cDNA Library and Plasmid Constructs
cDNA was prepared by using time saver cDNA synthesis kit (A mersh Pharmacia Biotech) from polya  RNA of Balb3T3 cells purified by oligo-dT column (A mersh Pharmacia Biotech), and then subcloned into pM E 18S expression vector (Sakamaki et al., 1992). Various mutants of mouse R as, K -R asV 12, K -R asN 17, K -R asS 35, K -R asG 37, and K -R asA 40 (K aufmann-Zeh et al., 1997), were prepared from c-K -R as by using Quick-Change site-directed mutagenesis kit (Stratagene). pJ 1-lacZ (M orgenstern and L and, 1990) was used for the expression of β-galactosidase. Flag-tagged mammalian STE-20-like protein kinase (M ST1) (Lee et al., 1998) was mutagenized to be kinase-defective (M ST1-K D) by replacement of lysine 59 with arginine (K 59R). The proper construction of all the mutants was confirmed by DNA sequencing. cDNA encoding constitutively active R af (R af-C A A X), ΔR 85, A kt, constitutively active A kt (H A-m a34-129 A kt), and R aln 26 were kind gifts from J. F. H ancock (U niversity of Queensland M edical School, Brisbane, A ustralia), W. O gawa (K obe U niversity, K obe, J apan), U. K ikkawa (K obe U niversity), R. R oth (Stanford U niversity, Stanford, C AL), and L. A. F eig (U niversity of M edford, M edford, M A), respectively. cDNA s for constitutively active M APK K (SDS-M APK K ) were provided by E. N ishida (K yoto U niversity), and an expression vector for green fluorescence protein (GFP) was from K. U mesono (K yoto U niversity).

Antibodies and Reagents

A gonic anti-mouse Fas mAb R K-8 (Nishimura et al., 1995) was provided by M edical and B iological Laboratories (Nagoya, J apan). mAbs against R as (clone 1B 8) and phospho-p44/42 M APK (E 10) were purchased from Transduction Laboratories and New England Biolabs, respectively. Polyclonal antibody against phospho-A kt was purchased from New En gland B iolabs. EGF purified from mouse submaxillary glands was from Sigma Chemical Co. Recombinant human IGF-1 and recombinant human bFGF were from G IBCO B RL. Fluorescent substrates acetyl-A sπ-Glu-Val-A sπ-(4-methyl-coumaryl-7-amide) (A c-DEVD-M C A) and acetyl-Ile-Glu-Thy-A sπ-(4-methyl-coumaryl-7-amide) (A c-IETD-M C A) for caspase-3 and caspase-8/6, respectively, were purchased from Pep tide I nstitute. For staining β-galactosidase-positive cells, 5-bromo-4-chloro-3-indolyl-b-o-(--)-galactopyranoside (X -GaI) was purchased from Wako.

Expression Cloning

Subconfluent F H 2 cells in five 10-cm dishes were transfected with pM E 18S 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 R K-8 for 4–6 h. A poptotic cells were removed by washing with PBS three times. The surviving cells that adhered to the dishes were collected and episcopal plasmids in the collected cells were recovered according to the method described by Itoh et al. (1991). The recovered plasmids were amplified in E lectroMAX DH 10B cells (G IB CO B RL) and transfected into F H 2 cells. This cycle was repeated six times with the amplified plasmids obtained in the previous cycle.

Transient Transfection of Expression Vectors

For transient transfection, F H 2 cells were seeded at 1 × 10 6 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) using Lipofectamine plus (G IBCO B RL) according to the manufacturer’s protocol.

Assay of Fas-mediated Apoptosis in the Transfected Cells

F H 2 cells transfected with expression vectors (0.4 μg/each vector) and 0.4...
Fig. 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 pJ71-LacZ. Cells were treated with indicated concentrations of agonistic anti-Fas mAb R K-8 for 8 h. The percentages of viable cells after Fas stimulation were determined as described in Materials and Methods.

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 b-galactosidase, constitutively active Ras (K-RasV12), or dominant-inhibitory Ras (K-RasN17). We counted the b-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 indicate that the overexpression of c-K-Ras decreases the sensitivity of fibroblasts to Fas-mediated apoptosis.

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 D Nase (ICAD), poly (ADP-ribose) polymerase (P ARP), and protein ki-
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, or 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...
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 SD-SEMA PKK 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 SD-SEMA PKK 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 SD-SEMA PKK 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 SD-SEMA PKK. In addition, the expression level of endogenous Fas in parental BAlb3T3 cells was also unaffected by overexpressed K-RasV12, Raf-CAAX, or SD-SEMA PKK (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 RK-8. A 4-h 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 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.
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, MAPK-1, or Δp85, and then treated with bFGF followed by the stimulation with anti-Fas mAb. Fig. 7 A shows that K-RasN17 and MAPK-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-FIP, which can inhibit Fas-induced apoptosis (Irmler et al., 1997), is upregulated by MAPK activation in lymphocytes (Yeh et al., 1998). We analyzed c-FIP expression in FH2 cells pretreated with or without bFGF by Northern hybridization. However, we could not detect specific expression of c-FIP mRNA by Northern hybridization, which indicates that the expression level of c-FIP mRNA in FH2 cells treated with or without bFGF is low. We carried out RT-PCR with c-FIP-specific primers and detected the expression of c-FIP mRNA (Fig. 7 C). The PCR product of c-FIP from the bFGF-treated cells was detected about twice, but the expression level of c-FIP was low.
**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 (Rouviere 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 Raf 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...
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 R K-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.

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
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 PI3 kinase (Peli et al., 1999). We analyzed the expression of Fas on FH2 cells after the transfection with K-RasN17, 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-RasN17, 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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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, M KP-1, or Δp85 together with pJ70-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 J0-2 (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. A faster the indicated PCR cycles, amplified products were analyzed.
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