Oncogenic Ras inhibits Fas ligand-mediated apoptosis by downregulating the expression of Fas

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Tumor growth is the result of deregulated tissue homeostasis which is maintained through the delicate balance of cell growth and apoptosis. One of the most efficient inducers of apoptosis is the death receptor Fas. We report here that oncogenic Ras (H-Ras) down-regulates Fas expression and renders cells of fibroblastic and epitheloid origin resistant to Fas ligand-induced apoptosis. In Ras-transformed cells, Fas mRNA is absent. Inhibition of DNA methylation restores Fas expression. H-Ras signals via the PI 3-kinase pathway to downregulate Fas, suggesting that the known anti-apoptotic effect of the downstream PKB/Akt kinase may be mediated, at least in part, by the repression of Fas expression. Thus, the oncogenic potential of H-ras may reside on its capacity not only to promote cellular proliferation, but also to simultaneously inhibit Fas-triggered apoptosis.

Keywords: apoptosis/Fas (Apo-1/CD95)/oncogene/tumorigenesis

Introduction

Programmed cell death by apoptosis is essential in regulating tissue development and homeostasis in multicellular organisms (Raff *et al.*, 1993). Apoptosis also serves as a defense mechanism to remove unwanted and potentially dangerous cells, such as virus infected cells and tumor cells. The discovery that tumor-suppressor genes (Levine, 1997) and oncogenes (Cory, 1994; Green *et al.*, 1996) fundamentally regulate apoptosis, thereby promoting neoplastic progression, established the concept that the escape from apoptosis also represents an important step in tumor development (Evan, 1997; Reed, 1997).

Apoptosis is influenced by a wide variety of regulatory stimuli. Among the death receptors (TNF-R1, DR-3/TRAMP, TRAIL-R1 and TRAIL-R2), Fas is one of the most potent inducers of apoptosis (Nagata, 1997). Interaction of Fas ligand (FasL) with its cognate receptor Fas induces receptor trimerization, which in turn results in the recruitment of the adaptor protein FADD and

caspase 8 (FLICE). Subsequent activation of downstream caspases leads to irreversible cell damage (Dixit, 1996; Krammer, 1996; Tschopp *et al.*, 1998).

Several intracellular proteins, e.g. c-FLIPs (Irmeler *et al.*, 1997), c-IAPs (Liston *et al.*, 1996) and Bcl-2 family members (Reed, 1997), are known to interfere with the Fas death signaling cascade thus rendering cells less sensitive to apoptosis (Cory, 1994; Tschopp *et al.*, 1998). Some members of these anti-apoptotic proteins are over-expressed in tumors (Ambrosini *et al.*, 1997; Irmeler *et al.*, 1997), and for Bcl-2 its implication in tumor formation has been clearly demonstrated in human follicular lymphomas (Tsujimoto *et al.*, 1985; Korsmeyer, 1995). Yet another, very efficient way to acquire resistance to FasL-induced apoptosis, is to downregulate death receptor surface expression. Most cells in a variety of tissues, including epithelial cells, constitutively express Fas (Leithauser *et al.*, 1993; Nagata and Golstein, 1995; French *et al.*, 1996). However, neoplastic transformation of these cells often leads to the partial or complete loss of Fas expression. For example, in metastasized colon carcinomas (Moller *et al.*, 1994), testicular tumors (Lebel *et al.*, 1996), melanomas (Hahne *et al.*, 1996) and in hepatomas (Strand *et al.*, 1996), Fas is not expressed.

Many lines of evidence have established that deregulation of growth-promoting proto-oncogenes such as *myc*, *fos* and *ras* are involved in the multistep process of cancer progression. Notably, the small GTP-binding protein Ras is able to transform most immortalized cell lines, and mutations of Ras have been shown to occur in 30% of human tumors (Macara *et al.*, 1996). A number of Ras effector proteins have been identified which bind preferentially to Ras in the GTP-bound state. Raf proteins, which are proto-oncogene-encoded serine/threonine kinases, activate MAP kinases which control the activity of various transcription factors such as c-Myc and c-Jun (Marshall and Wyllie, 1996). Other Ras targets include the GTPase-activating proteins, p120GAP and neurofibrin, the Ral-GDS, a family of exchange factors for the Ras-related Ral family and the phosphoinositide 3-OH kinase (PI 3-kinase), whose activation results in the activation of the serine/threonine kinase B (PKB) or Akt (Marte and Downward, 1997).

Ras not only promotes cell proliferation but also has anti-apoptotic effects. In human colorectal adenomas and carcinomas, activation of the *K-ras* oncogene correlates with decreased apoptosis (Ward *et al.*, 1997). Ras suppresses Myc-induced apoptosis (Kauffmann-Zeh *et al.*, 1997) and protects epithelial cells from apoptosis induced by their detachment from the extracellular matrix (Khwaja *et al.*, 1997). We therefore considered the possibility that *ras*, or another oncogene, may be implicated in the downregulation of Fas expression frequently found during tumor progression. In this study, we provide evidence

Fig. 1. H-ras suppresses Fas expression in mammary epithelial cells and in NIH 3T3 fibroblasts. (A) The mammary epithelial cell line Ep and an H-ras-transfected clone (Ep-Ras) were analyzed for the expression of Fas and Ras by Western blotting, and for surface Fas expression by flow cytometry. The two bands observed for Ras correspond to the farnesylated and the non-modified forms, respectively. (B) Ep clones transfected with an inducible construct to express c-Myc (Ep-Myc, uninduced/OFF; induced/ON) or c-Fos (Ep-Fos, ON and OFF) were analyzed for Fas surface levels by FACS analysis. (C) As (A) but Fas expression was analyzed in NIH 3T3 fibroblasts and an H-ras-transfected NIH 3T3 clone (NIH-Ras)

that mutated Ras (H-ras) effectively inhibits Fas surface expression and that downregulation of Fas occurs at the transcriptional level, most likely through DNA methylation. We conclude that Ras not only promotes cell proliferation by its oncogenic nature, but also mediates this effect by protecting cells from Fas ligand-mediated apoptosis.

Results

H-ras downregulates Fas and renders cells resistant to FasL-mediated apoptosis

In order to test the hypothesis that oncogenes control Fas expression in tumors, we analyzed several established mouse mammary epithelial cell lines (Ep) which constitutively or conditionally express either H-ras, an active form of Ras (V12 Ras, H-Ras), c-Myc or c-Fos (Reichmann *et al.*, 1992; Fialka *et al.*, 1996). Fas was not detected in cells expressing H-Ras by FACS (Figure 1A), whereas in cells expressing ectopic c-Fos and c-Myc, Fas surface expression remained unaltered compared with the Ep cells which do not express the oncogenes (Figure 1B). These results were correlated with Western blot analysis showing the absence of the 45 kDa mouse Fas protein in Ep clones expressing oncogenic Ras (Figure 1A). Fas was undetectable in NIH 3T3 fibroblast clones expressing H-Ras (Figure 1C).

The analysis of a panel of H-ras transfected NIH and Ep clones revealed a strictly inverse correlation between Fas and Ras protein levels (Figure 2): in low H-Ras expressors, the Fas protein was still detectable (Figure 2A), while no Fas at all was found in clones with high H-Ras levels. Moreover, in pools of NIH 3T3 cells infected with an H-Ras-expressing retrovirus, LTR-driven H-Ras expression gradually increased up to day 6, while Fas protein expression decreased correspondingly (Figure 2B). Thus, H-Ras downregulates Fas in a strictly dose-dependent manner.

The absence of surface Fas caused H-Ras expressors to become resistant to FasL-mediated apoptosis (Figure 2C). Only the parental, Fas-expressing lines underwent massive cell death upon addition of recombinant FasL (100 ng/ml), while Ras-expressing Ep or NIH 3T3 clones were resistant to FasL. In contrast, H-Ras expression in Jurkat T cells did not influence Fas expression and these cells were as sensitive to apoptosis as the parental cells (data not shown), indicating that Fas downregulation by Ras is cell type specific.

Downregulation of Fas by H-ras is reversible

Although the previous results showed a clear correlation between Ras expression and the absence of Fas, it remained possible that the lack of Fas and the consequent resistance to apoptosis were the mere result of the procedure used to select H-ras clones (selecting cells with high resistance to apoptosis) and not a direct effect of an activated Ras signaling pathway. In order to exclude this possibility, we analyzed NIH 3T3 cells in which the transient expression of the H-ras oncogene was under the control of an IPTG inducible promoter (NIH^{Rasind}). Similar to the parental line, uninduced NIH^{Rasind} cells showed moderate levels of expression of Fas and no H-Ras (Figure 3A and B). However, when these cells were cultured in the presence of IPTG for 2 days, H-Ras levels were massively increased, and reached highest levels 2 days after IPTG removal. The induction of H-Ras caused morphological changes, typical of *ras*-transformed NIH 3T3 cells (Figure 3C). In parallel with the appearance of Ras, Fas levels diminished (Figure 3A), and no Fas at all was detectable on day 4, at a time when Ras levels reached their maximum. Prolonged culturing of these cells in IPTG-free medium led to a decrease in Ras levels. Fas was concomitantly re-expressed and on day 20 (18 days after IPTG removal) Fas levels were completely restored. Following the kinetics of disappearance and reappearance of Fas, NIH^{Rasind} cells

Fig. 2. Fas expression inversely correlates with levels of H-Ras. (A) Fas and Ras levels of several Ep clones stably transfected with H-ras (Ep-Ras) were analyzed by Western blotting. (B) As (A) but Ras and Fas levels were determined in pools of NIH 3T3 cells transiently infected with an H-ras-expressing retrovirus. Highest Ras levels were detected on day 6 after the infection. (C) The parental Ep, an H-ras transfected clone (Ep-Ras/3), NIH 3T3 fibroblasts and an H-ras transfected clone (NIH-Ras/3) were incubated for 16 h in the presence or the absence of FasL (+/- FasL). The relative percentage of viable cells was determined.

became resistant and again sensitive to FasL-induced apoptosis (Figure 3D).

Ectopic expression of Fas restores FasL sensitivity in H-ras-transformed cells

It was still conceivable that the inactivation of a downstream effector protein and not the absence of Fas was the main cause for the apoptosis-resistant phenotype of *ras*-transformed NIH 3T3 cells. To exclude this possibility, we tested whether the forced expression of Fas would lead to the restoration of FasL sensitivity. The murine Fas cDNA was introduced into H-ras-transformed NIH 3T3 clones. Several clones were selected and analyzed for Fas

expression and sensitivity to FasL. As shown in Figure 4A, NIH-Ras-Fas cells expressed increased levels of Fas protein, as well as cell surface Fas. Consequently, Fas-transfected cells became highly susceptible to FasL-mediated apoptosis (Figure 4B). Collectively, these data provide evidence that the absence of Fas expression and not the inactivation of a protein in the Fas signaling cascade is the main mechanism by which oncogenic Ras inhibits the Fas death signals.

Inhibition of DNA methylation restores Fas expression

Several mechanisms can be envisaged that possibly lead to the observed decrease of Fas protein expression by H-Ras. For example, Ras signals could result in the degradation of the Fas protein by targeted endocytosis of the receptor to lysosomes, as known for the 10.4/14.5 adenoviral proteins (Tollefson *et al.*, 1998). Alternatively, H-Ras signals may lead to the phosphorylation of the cytoplasmic domain of Fas or they may have a direct effect on Fas mRNA stability or inhibit Fas gene transcription. Northern blot analysis supports the latter notion. The Fas mRNA was absent in NIH 3T3-H-ras transfectants (Figure 5), indicating that Fas expression was regulated at the genomic level.

The mechanism by which Ras regulates Fas gene expression was then analyzed. It has been demonstrated previously that the presence of oncogenic H-Ras results in an increase in DNA methyltransferase activity (MacLeod *et al.*, 1995). DNA methyltransferase is responsible for the methylation of cytosine residues located in the dinucleotide sequence CpG. Within the vertebrate genome, 80–90% of the CpGs are methylated and there is a large number of results which document the inverse correlation between DNA methylation and tissue-specific gene expression (Mostoslavsky and Bergman, 1997). Therefore, we analyzed the effect of H-Ras on Fas in cells that had been treated with 5-aza-2'-deoxycytidine, an inhibitor of DNA methylation. When H-Ras was induced in NIH^{Rasind} cells by IPTG, Fas expression was not inhibited in the presence of 5-aza-2'-deoxycytidine (Figure 6) and cells remained sensitive to FasL-mediated apoptosis (data not shown). Moreover, partial Fas re-expression was achieved in stably transfected NIH-Ras clones in the presence of this inhibitor, suggesting that inhibition of Fas gene transcription was caused by DNA methylation.

Activation of the PI 3-kinase pathway downregulates Fas

Finally, we investigated which of the many signaling pathways triggered by Ras was responsible for the inhibition of Fas gene transcription. Ras anti-apoptotic activity in several systems has been proposed to be dependent on its capacity to trigger PI 3-kinase. For instance, *c-myc*-induced apoptosis in fibroblasts is inhibited by active PI 3-kinase or the downstream kinase PKB/Akt (Kauffmann-Zeh *et al.*, 1997). To block this anti-apoptotic pathway, cells were cultured in the presence of the specific PI 3-kinase inhibitor LY294002 (Figure 7A). Even in NIH^{Rasind} cells, which do not express oncogenic Ras, Fas levels were moderately increased, suggesting that some constitutively active PI 3-kinase results in suboptimal Fas expression. When Ras expression was induced by IPTG in NIH^{Rasind}

Fig. 3. Fas downregulation by Ras is reversible. (A) H-Ras was transiently expressed in NIH 3T3 transfected with H-ras under an IPTG-inducible promoter (NIH-Ras^{ind}). NIH-Ras^{ind} cells were cultured for 2 days in the presence of 50 mM IPTG and (A) Fas and (B) Ras protein expression was followed by flow cytometry and by Western blotting, respectively. (C) Upon Ras induction, NIH 3T3 cells acquire the round, transformed phenotype. Day 4 after IPTG addition corresponding to day 2 after IPTG removal is shown (Ras ON), and as control, non-induced NIH cells (Ras OFF). (D) NIH-Ras cells acquire transient resistance to FasL.

cells, downregulation of Fas was not observed in the presence of LY294002, indicating that the Ras-triggered PI 3-kinase pathway contributed considerably to the suppression of Fas.

To corroborate that the PI 3-kinase pathway is implicated in the modulation of Fas expression, we made use of Ras mutants that have been shown to preferentially activate either the Ral.GDS(G37), the Raf (S35) or the PI 3-kinase (C40) pathway (Rodriguez-Viciano *et al.*, 1997). When these various Ras mutants were transduced into NIH cells, Fas was efficiently downregulated by the C40 mutant (PI 3-kinase). A partial downregulation was observed with the S35 mutant that preferentially binds to Raf, while the Ral.GDS binding mutant (G37) had no effect. It is currently not clear whether the effect of the S35 mutant reflects the activation of the Raf/Erk pathway or whether this is due to the fact that this particular Ras mutant also weakly activates the PI 3-kinase pathway (Rodriguez-Viciano *et al.*, 1997).

The importance of the PI 3-kinase pathway in Ras-induced Fas downregulation was demonstrated further by the use of an activated PI 3-kinase mutant that is anchored to the membrane via a CAAX-signal-induced lipid modification. PI 3-kinase consists of two subunits, i.e. the regulatory p85 and the p110 kinase subunits. Translocation of the kinase domain to the membrane spontaneously initiates downstream signaling events, and thus the lipid-modified p110 subunit (p110/CAAX) is constitutively active. Figure 7B shows that the expression of the p110/CAAX protein in NIH cells led to an almost complete disappearance of surface Fas.

Discussion

Our results show that transfection of fibroblast and epithelial cells with a constitutively active Ras mutant downregu-

lates Fas expression to barely detectable levels. A number of independent clones were analyzed and H-Ras levels inversely correlated with surface Fas and susceptibility to FasL-mediated apoptosis. The H-Ras-induced resistance to FasL signaling appeared to be a direct result of the loss of Fas surface expression, since ectopic expression of Fas in H-ras-transfected cells resulted in the restoration of susceptibility. However, we cannot exclude the possibility that upregulated inhibitory proteins (such as FLIP) may have contributed to this effect. H-Ras transfectants were still susceptible to radiation-induced apoptosis (data not shown), which is suppressed by the Bcl-2 inhibitory protein (Strasser *et al.*, 1995), suggesting that Ras does not influence expression levels of members of the Bcl-2 family.

Although there is considerable evidence that activated Ras proteins inhibit apoptosis and promote cell proliferation under many circumstances and in many cell lines, there is also experimental evidence that Ras possesses a pro-apoptotic function (Downward, 1998). For example, Ras-activation induces apoptosis in T lymphocytes (Gulbins *et al.*, 1995; Latinis and Koretzky, 1996). This is consistent with our finding that Ras was unable to modulate Fas levels in Jurkat cells, indicating that Ras signals differ in different cell types.

What is the molecular mechanism whereby oncogenic Ras inhibits Fas expression? H-Ras transfectants do not express Fas mRNA, suggesting that Ras inhibits Fas gene transcription. This is not surprising considering the profound effect of Ras on many transcription factors (although an influence of Ras on Fas mRNA stability can not be formally excluded). The analysis of the 5' regulatory region of the Fas gene revealed consensus sequences for many common transcription factors including AP-1 and NF- κ B, both of which are known to be activated by Ras

Fig. 4. Forced expression of Fas restores sensitivity to FasL. (A) NIH-Ras clones were transfected with a murine Fas expression vector, and a selected clone (NIH-Ras-Fas) was analyzed for Fas expression by Western blot and flow cytometry. (B) Restoration of sensitivity to FasL in NIH-Ras-Fas clones.

(Behrmann *et al.*, 1994; Cheng *et al.*, 1995; Rudert *et al.*, 1995; Downward, 1997). Moreover, a strongly increased frequency of CpG dinucleotides which are known targets of the DNA methyltransferase was noted (Cheng *et al.*, 1995). Within the first 500 nucleotides of the 5' region of Fas, 27 CpG sites were detected. We found that inhibition of DNA methylation by 5-aza-2'-deoxycytidine abolishes the capacity for Ras-mediated downregulation of Fas. Oncogenic Ras has been shown previously to increase the level of DNA methyltransferase (MacLeod *et al.*, 1995). Many of the CpG sites may therefore be methylated in the presence of the *ras* oncogene, leading to the repression of Fas gene transcription. Methylation of CpG dinucleotides as a mechanism of gene inactivation has been found frequently and has been suggested to play an important role in tumorigenesis by inactivation of tumor suppressor genes (e.g. retinoblastoma, p16, CDKN2) (Mostoslavsky and Bergman, 1997). Whether CpG methylation is the mechanism by which Fas gene transcription is regulated remains to be investigated in future studies.

Fig. 5. H-Ras inhibits Fas mRNA expression. NIH-Ras/3 and Ep-Ras/3 clones and the respective parental cell lines were analyzed for Fas mRNA expression by Northern blot analysis. Equal loading is shown by the GAPDH signal intensity.

Fig. 6. Inhibition of DNA methylation blocks Ras-induced downregulation of Fas. NIH 3T3, NIH-Ras and NIH-Ras^{ind} (in the presence or absence of IPTG, Ras OFF; Ras ON) clones were treated with 5-aza-2'-deoxycytidine (azacytidine) for 2 days, and Fas surface expression was analyzed by flow cytometry.

Our results allude to the involvement of the Ras-initiated PI 3-kinase pathway for *fas* gene inactivation. Since this pathway also protects epithelial cells from detachment-induced apoptosis, it is possible that the detachment of cells leads to the upregulation of FasL (which is known to be upregulated under stress conditions (Herr *et al.*, 1997; Faris *et al.*, 1998; Kashibatla *et al.*, 1998), and that subsequent apoptosis is blocked in the presence of oncogenic Ras due to Fas downregulation. This PI 3-kinase-dependent Fas downregulation could also

Fig. 7. Involvement of the PI 3-kinase pathway in Fas downregulation. (A) The PI 3-kinase inhibitor [20 ng/ml LY294002 (LY)] was added to NIH-Ras^{ind} cells cultured in the presence (Ras ON) or absence (Ras OFF) of IPTG over 24 h and Fas surface expression determined by flow cytometry. (B) NIH cells were transduced with Ras mutants V12G37 (activating the Raf.GDS pathway), V12S35 (activating the Raf pathway), V12C40 (activating the PI 3-kinase pathway) and with the constitutively active PI 3-kinase p110 subunit (p110/CAAX). Fas surface expression was determined by FACS 5 days after infection. The Western blot shows equal expression of the Ras mutant proteins.

explain molecularly the observation that c-Myc-induced cell death in fibroblasts is inhibited by oncogenic Ras through the PI 3-kinase pathway (Kauffmann-Zeh *et al.*, 1997), as it has been recently shown that c-Myc-induced cell death is dependent on the FasL/Fas system (Hueber *et al.*, 1997). Blocking the Fas death pathway by Ras would leave the proliferative response of c-Myc intact, while inhibiting apoptosis, predicting that c-Myc and oncogenic Ras co-operate perfectly by combining proliferative, pro-apoptotic signals with the blockade of a major cell death pathway. Several reports are in agreement with this notion. For example, *lpr* mice (lacking functional Fas) crossed with L-*myc* transgenic mice showed an accelerated formation of T- and B-cell lymphoma when compared with normal L-*myc* transgenics (Zornig *et al.*, 1995; Peng *et al.*, 1996). Moreover, transgenic mice expressing

oncogenic *myc* under the MMTV promotor carry tumors with much higher rates of spontaneous apoptosis, as compared with tumors from MMTV-*myc/ras* double transgenic mice (Cardi *et al.*, 1998). Thus, impairment of the Fas apoptotic function, in co-operation with growth-promoting oncogenes, can accelerate tumorigenesis.

Increased levels of Bcl-2 confer on malignant cells resistance to various anticancer drugs and γ -irradiation (Strasser *et al.*, 1995), thus preventing the successful treatment of some types of cancer. The widely used anticancer drugs doxorubicin and methotrexate can also act through the upregulation of FasL expression, leading to apoptosis of Fas-sensitive tumor cells (Friesen *et al.*, 1996). Various Ras-expressing human melanoma and colon carcinoma cell lines have been reported to lack Fas surface expression (Moller *et al.*, 1994; Fenton *et al.*, 1998). Thus, many neoplasms carrying the *ras* oncogene may have a defect in the Fas signaling pathway, and may therefore be resistant to certain chemotherapeutic agents. A further understanding of the anti-apoptotic role of oncogenes in human cancers may therefore lead to improved anticancer treatment strategies.

Materials and methods

Western blot, Northern blot and flow cytometric analysis

The monoclonal mouse anti-p21^{ras} antibody (DAKO, Glostrup, Denmark) and the affinity purified polyclonal antibody AL78 generated against a peptide containing the cytoplasmic domain of murine Fas (amino acids Ser183–Glu306) were used to detect the expression of v-Ha-Ras and Fas respectively by Western blotting. The biotinylated anti-Fas antibody Jo-2 (Pharmingen, San Diego, CA) was used in combination with FITC-conjugated streptavidin for flow cytometric analysis. Cells stained only with FITC-conjugated streptavidin were used as negative controls.

For Northern blot analysis, poly(A)⁺ RNA (1 μ g per lane) was separated on formaldehyde-containing denaturing gels, blotted to Hybond N membranes and probed with ³²P-labeled Fas or GAPDH riboprobes (French *et al.*, 1997).

Cell lines and transfection

Ep-Ras clones were generated by infecting the parental Ep cell line with a retroviral vector containing v-H-*ras* (Reichmann, 1994). Ep-Myc clones were obtained by transfecting c-*myc* into the Ep cell line (Reichmann *et al.*, 1992; Fialka *et al.*, 1996). c-Fos was fused to the estrogen receptor (FosER) and transfected into the Ep cell line. Stable Ep-FosER clones were obtained in which expression of c-Fos can be induced by addition of estrogen (Reichmann *et al.*, 1992; Fialka *et al.*, 1996). NIH-Ras clones were established using the retroviral vector pBabe-Puro containing v-H-*ras* (Pear *et al.*, 1993). NIH-Fas and NIH-Ras-Fas clones were established by transfecting murine Fas cloned into the pCR3 vector (Invitrogen, Carlsbad, CA) into NIH cells or NIH-Ras clones, respectively. In order to produce the inducible NIH-Ras^{ind} clones and Jurkat-Ras^{ind}, v-Ha-*ras* was cloned under the control of an IPTG-inducible promoter (LacSwitch inducible Mammalian expression system, Stratagene, La Jolla, CA), and transfected into NIH or Jurkat cells, respectively. Gene expression was induced by culturing the clones or control cells in 50 mM IPTG for the indicated time. Transient transfections of H-Ras into NIH cells were performed by infecting NIH cells with a retroviral vector containing v-H-*ras* (see above). Cell lysate from cells incubated for various lengths of time after infection were analyzed by Western blot analysis for Fas expression. The Ras mutant constructs were a kind gift of Dr J. Downward, ICRF, London.

Assay for FasL-induced cell death

Exponentially growing cells were detached with 3 mM EDTA/PBS, washed with medium and transferred into 96-well microtiter plates (10⁴ cells per well in 200 μ l of medium). Fas-mediated apoptosis was induced by the addition of 100 ng/ml of recombinant human FasL (Alexis, San Diego, CA), or diluted recombinant protein in the presence of 1 μ g/ml enhancer (Alexis). Cell viability was determined after 16 h. Twenty microliters of a solution containing 2 mg/ml 3-(4,5-dimethylthiazol-2-yl)-

5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt (MTS) reagent (Promega) and 50 µg/ml phenazine methosulfate was added to the cells. Following color development (3 h), absorbance at 490 nm was measured with an ELISA reader. One hundred percent of viable cells correspond to control cells treated for the indicated times with the enhancer only.

PI 3-kinase inhibition and DNA methylation inhibition

When NIH-Ras^{ind} clones were used, Ras expression was induced 2 days prior to the addition of inhibitors by addition of 50 mM IPTG. PI 3-kinase was inhibited by the addition of 20 µM LY294002 (Sigma, St Louis, MO), and subsequent culturing of the cells for 2 days. Inhibition of DNA methylation was achieved by culturing the cells in 10 µM 5-aza-2'-deoxycytidine (Sigma) for up to 36 h. After treatment with or without the indicated inhibitor, cells were analyzed for Fas expression by flow cytometry.

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