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Signaling through the ARK tyrosine kinase receptor protects from apoptosis in the absence of growth stimulation

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ARK (AXL) is the prototype of a distinctive family of receptor tyrosine kinases which contain in their extracellular domains features reminiscent of cell adhesion molecules. ARK is capable of homophilic binding, which results in a degree of receptor activation, but can also be activated by a heterophilic ligand, Gas6, a member of the family of vitamin K dependent proteins that is preferentially expressed in quiescent cells. Since a number of tissues and cell lines express both ARK and Gas6, we studied the effect of endogenous and exogenous Gas6 on the phenotype of ARK expressing cells. Here we show that constitutive expression of Gas6 in an NIH3T3 cell line that does not spontaneously express this protein does not result in cell transformation or uncontrolled growth, but protects from apoptosis induced by serum deprivation. Recombinant exogenous Gas6 was also capable of protecting cells from apoptosis at concentrations that did not result in significant induction of DNA synthesis. Activation of ARK phosphorylation and a weak but significant induction of MAP kinase activity accompanied the increased survival of cells treated with Gas6. The antiapoptotic effect of ARK signaling was confirmed by studies using fibroblasts from ARK knock-out mice, that showed that the absence of ARK resulted in higher levels of serum deprivation-induced apoptosis, that could not be rescued by the addition of Gas6. Interestingly ARK signaling protects from apoptosis induced by serum deprivation, *myc* overexpression, or by TNFα but not from u.v. irradiation or Staurosporine. These results suggest that a major function of Gas6–ARK signaling is that of increasing cell survival under conditions which do not allow cell proliferation.

Keywords: programmed cell death; gene knock-out; TWF; c-*myc*

Introduction

Apoptosis or programmed cell death (PCD), is a physiological process essential to the normal development and homeostasis of multicellular organisms (White, 1996; Raff, 1992; Weil *et al*., 1996). Cells undergoing apoptosis are characterized by specific morphological changes including DNA degradation, chromatin condensation and nuclear fragmentation (Wyllie, 1980; Wertz and Hanley, 1996). At the biochemical level apoptosis is regulated by a cascade of positive or negative signals which are controlled by the *bcl*-2 family of proteins, and result in the activation of the interleukin-1β-converting enzyme (ICE) family of cysteine proteases (Reed *et al*., 1996; Nagata, 1997) recently named caspases (Alnemri *et al*., 1996). It has been shown that the machinery triggering PCD can be activated in any point of the cell cycle (Evan *et al*., 1995) and indeed apoptosis can be induced by lack of growth factors as well as by specific 'killer molecules' such as TNFα or FAS-ligand (Chinnaiyan *et al*., 1996). While TNFα and the FAS-ligand are known to function through a cascade of signals triggered by transmembrane receptors containing the so called 'death domain' (Kitson *et al*., 1996; Fraser and Evan, 1996), little is known about the molecular events involved when apoptosis is induced by growth factor and cytokine deprivation.

Deregulated expression of genes which are important for the cells to progress through specific points of the cell cycle can also cause apoptosis. At the level of transcriptional regulators an important role is played by the cellular proto-oncogene c-*myc* and the tumor suppressor gene p53. p53 expression is necessary for and can induce apoptosis (Levine, 1997) and deregulated expression of c-*myc* in serum deprived fibroblasts causes massive apoptotic cell death (Evan *et al*., 1992).

Protection from apoptosis by cytokines and growth factors has generally been found associated with induction of DNA synthesis. Recent studies however, have shown that these two phenomena can be dissociated by the use of specific kinase inhibitors or receptor mutants, indicating distinct signaling pathways for induction of proliferation or prevention of apoptosis (Kinoshita *et al*., 1995; O'Connor *et al*., 1997). Similarly, *myc*-induced apoptosis can be rescued by several cytokines but protection does not always correlate with the cytokine's ability to promote cell growth (Harrington *et al*., 1994). Signaling through tyrosine kinases receptors can promote cell survival by either modulating the activity of antiapoptotic molecules of the *bcl*-2 family (Carson *et al*., 1994) or triggering specific pathways such as the *ras* pathway leading to mitogenic-activated protein kinase (MAPK). In addition, recently a pathway that includes the lipid-kinase PI3K, has been shown to play an important role in the mechanism by which receptor tyrosine kinases can promote cell survival (Yao and Cooper, 1995; Hemmings, 1997).

The ARK/AXL tyrosine kinase (Rescigno *et al*., 1991; Janssen *et al*., 1991; O'Bryan *et al*., 1991) is the prototype of a family of transmembrane receptors which includes *Tyro*3/*rse*/*sky*/*brt* (Lai *et al*., 1994; Mark *et al*., 1994; Ohashi *et al*., 1994; Fujimoto and

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Yamamoto, 1994) and c-*mer*/*nyk*/*eyk* (Graham *et al*., 1995; Ling and Kung, 1995; Jia and Hanafusa, 1994). Members of this family display IgG-like and fibronectin type III domains in their extracellular domain, features reminiscent of cell adhesion molecules; indeed, we were able to demonstrate that the ARK receptor can behave like a cell adhesion molecule by inducing cell aggregation through a homophelic mechanism (Bellosta *et al*., 1995). Recently Gas6 was identified as a heterophilic ligand for ARK and *Tyro*3 receptors and c-*mer*, while Protein S was identified as the ligand for the *Tyro*3 receptor (Nagata *et al*., 1996; Mark *et al*., 1996; Stitt *et al*., 1995; Varnum *et al*., 1995). Both proteins belong to the class of vitamin K dependent proteins but while Protein S is involved in the anticoagulation cascade (Dahlback, 1991), Gas6 was originally isolated as a Growth Arrest Specific gene from quiescent fibroblasts (Manfioletti *et al*., 1993). Because Gas6 production is up-regulated during quiescence, we considered the possibility that the major effect of ARK signaling could be that of promoting cell survival under conditions which do not allow cell proliferation.

The results presented here show that constitutive activation of the ARK receptor by Gas6 protects serum depleted NIH3T3 fibroblasts from apoptosis without inducing cell growth and stimulation of DNA synthesis. Treatment with recombinant exogenous Gas6 was also capable of preventing apoptosis at concentrations that did not result in induction of DNA synthesis. These results were confirmed using mouse embryonic fibroblasts from ARK knock-out mice which showed that the absence of ARK results in hypersensitivity to induction of apoptosis. Interestingly ARK signaling protects from apoptosis induced by growth factor deprivation, c-*myc* overexpression, or by TNFα, but not from apoptosis induced by u.v. irradiation or Staurosporine. Thus, although the precise biological functions of this receptor remain to be elucidated, ARK signaling appears to play an important role in protecting cells from premature death under conditions of growth factor deprivation, a function which could be important in tissue homeostasis and is consistent with the up-regulation of Gas6 expression following serum deprivation.

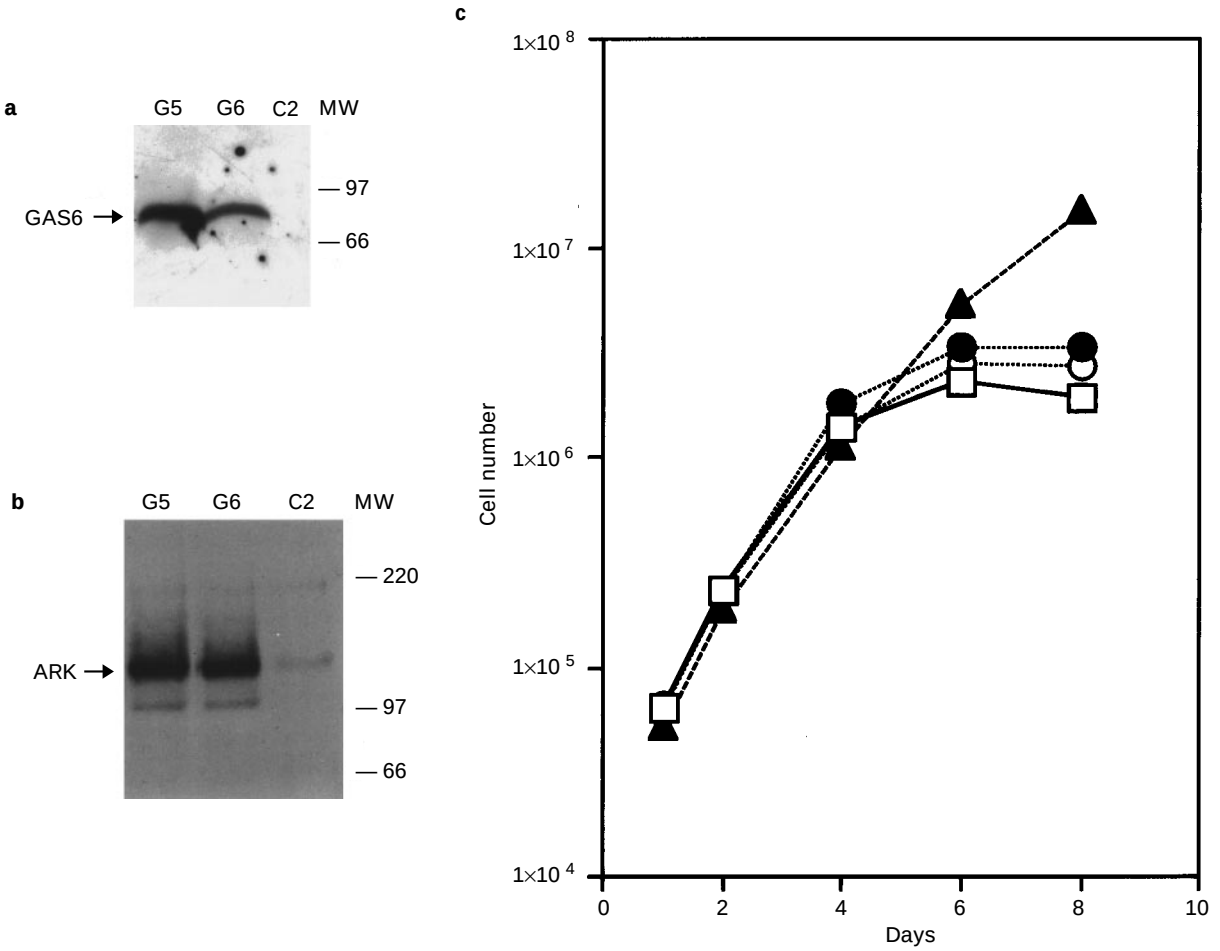

Figure 1 Expression of Gas6 does not affect proliferation of transfected NIH3T3 cells. (**a**) Supernatants of cultures of NIH3T3 cells stably transfected with Gas6 cDNA (G5 and G6), or control cells (C2), were run on a 8% SDS–PAGE and Western blotting analysis was performed using anti-Gas6 polyclonal antiserum. Molecular weight markers (MW) are indicated to the left. (**b**) Cells were incubated in DMEM + 0.5% serum for 24 h then lysed and immunoprecipitated with anti-ARK serum. Immunoprecipitates were run on 6% SDS–PAGE and Western blotted using anti-phosphotyrosine monoclonal antibodies. (**c**) C2 cells, (□), the Gas6 expressing clones G5 and G6 (● and ○) or 8bG cells, expressing FGF-4 (▲) were grown in 10% serum. Cells were counted at the indicated times using a Coulter Counter. Medium was changed every 2 days

Results

Constitutive expression of Gas6 in NIH3T3 prevents cells from undergoing apoptosis without inducing cell proliferation

Because Gas6, the heterophilic ligand for the ARK receptor, is up-regulated during growth arrest in fibroblasts (Manfioletti *et al*., 1993), we wanted to determine whether its constitutive expression could modify the cell response to conditions causing quiescence. We have addressed this question by transfecting a murine Gas6 cDNA into our line of NIH3T3 cells, which do not express detectable levels of this protein but express ARK (Costa *et al*., 1996) and asking whether constitutive expression and release of Gas6 in the supernatant affected cell growth or survival. Since Gas6 is a vitamin K dependent protein, all experiments were performed in the presence of the vitamin K analog, sodium-menadione bisulfite. As shown in Figure 1a a band of 90 kDa is present only in the supernatant of Gas6 stably transfected cells (G5 and G6) while control C2 cells (transfected with the vector alone) do not express detectable levels of this protein. Figure 1b shows that the Gas6 expressing clones, G5 and G6, have a high level of constitutive ARK phosphorylation, consistent with the idea that ARK signaling is activated in these cells. Expression of Gas6 in NIH3T3 cells however did not affect the growth rate in medium containing 10% serum and Gas6 expressing cells, as well as control cells, stopped growing after reaching confluence (Figure 1c). In contrast, as previously shown (Talarico and Basilico, 1991), NIH3T3 cells constitutively expressing FGF-4 showed loss of contact-inhibition and proliferated to very high densities. Furthermore, while FGF-4 expressing cells grew in soft-agar and in serum-free medium, cells expressing Gas6 did not show such transformed properties (data not shown). However, Gas6 expression was not entirely without effect. Examination of several clones of Gas6 expressing cells showed that cells producing Gas6 had a flatter, more contact-inhibited morphology at confluence compared to control cells (Figure 2). These results indicate that constitutive expression of Gas6 in 3T3 cells did not induce autocrine growth or a transformed phenotype, but Gas6 expressing clones appeared 'healthier' at quiescence than their parental NIH3T3 cells.

To determine if ARK activation could produce signals important for cell survival, and whether Gas6 played a role in preventing apoptosis following growth factor deprivation, we induced apoptosis in 3T3 cells by lowering the serum concentration from 10 to 0.1%, and analysed the morphology of the nuclei stained with the Hoechst 33342 dye. Figure 3 shows micrographs of nuclei from control NIH3T3 (a) or Gas6 expressing cells (b) after 24 h of serum deprivation. While control cells showed typical chromatin condensation and apoptotic nuclei, constitutive expression of Gas6 appeared to induce protection from apoptosis. Analysis of the soluble cytoplasmic DNA (Hockenbery *et al*., 1996) from these cells is shown in Figure 3c. Agarose gel electrophoresis of DNA isolated from control cells (C2) displayed chromatin cleavage and the typical ladder like pattern characteristic of cells undergoing programmed cell death. By contrast DNA cleavage was much less pronounced for cells producing Gas6 (G5 and G6).

Many cytokines or growth factors can prevent cells from undergoing apoptosis by stimulating cellular DNA synthesis and proliferation. It was therefore important to determine the rate of DNA synthesis in serum-starved Gas6 expressing cells. Cells were plated in medium containing 10% serum. After 24 h the medium was changed to 0.1% serum, and the cultures were labeled with BrdU as indicated in Materials and methods. Cells were fixed at the indicated times and nuclei were scored for chromatin condensation, nuclear fragmentation and for BrdU incorporation, to determine the frequency of cells undergoing apoptosis and of cells synthesizing DNA. As shown in Figure 4 Gas6 expressing clones (G5 and G6) showed a lower frequency of apoptotic cells than control cells (C2) and this phenomenon was not accompanied by sustained DNA synthesis. In contrast 8bG cells, which express FGF-4, were also protected from cell death, but protection in this case was accompanied by a high rate of BrdU incorporation. We can conclude that constitutive expression of Gas6 in 3T3 cells prevents cells from entering the apoptotic program without

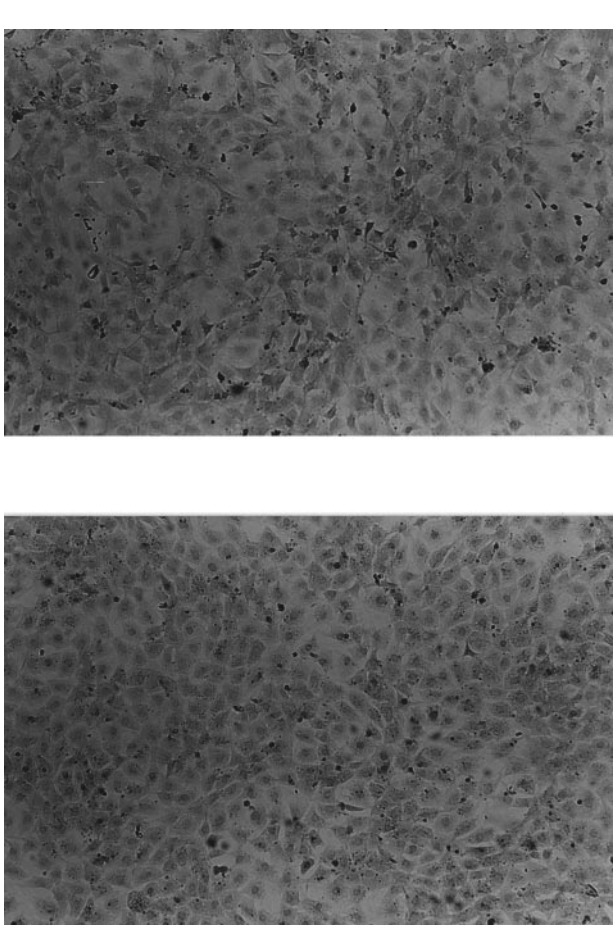

Figure 2 Morphology of NIH3T3 cells expressing Gas6 at confluence. Micrographs of control NIH3T3 cells (**a**) or cells expressing Gas6 (**b**) 24 h after reaching confluence in DMEM with 10% serum containing 4 μM of sodium menadione bisulfite. Cells were fixed and stained with Crystal Violet and photographed using a Zeiss microscope (10×)

inducing cell proliferation; these data therefore support the idea that Gas6 may act as a survival factor in fibroblasts.

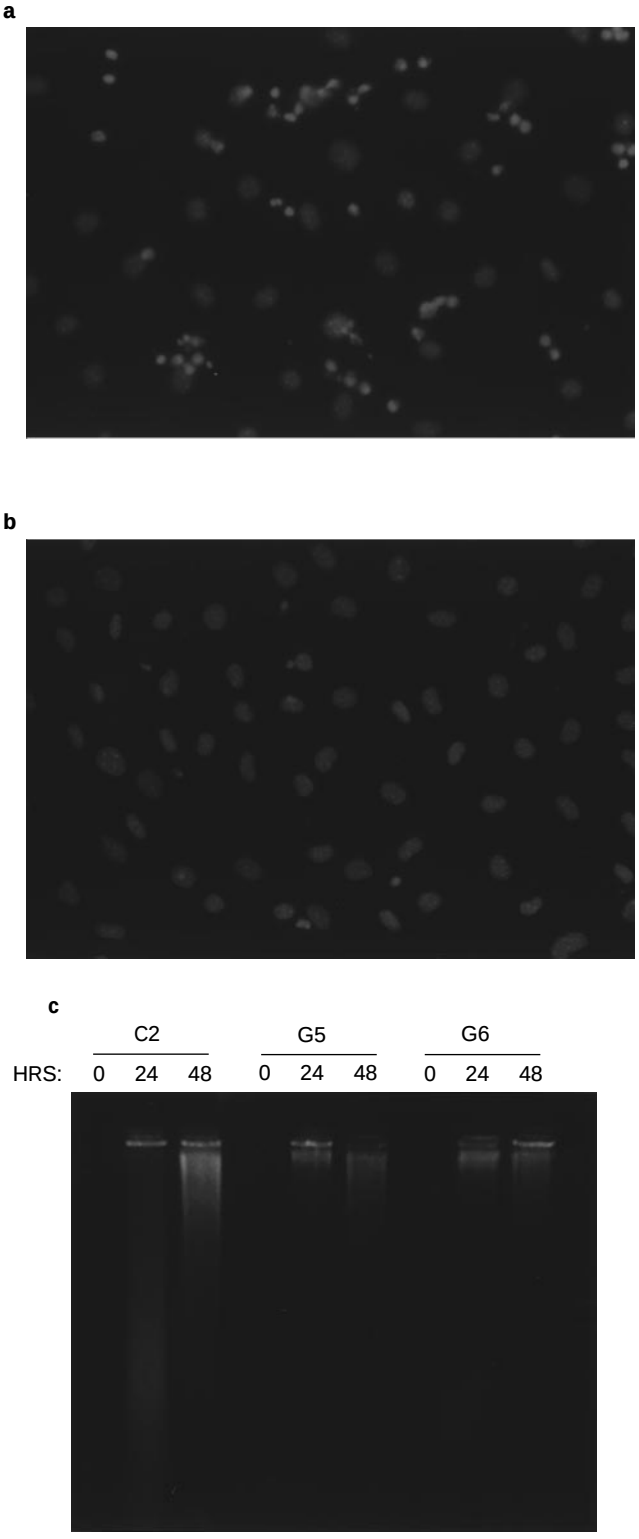

Figure 3 Expression of Gas6 protects NIH3T3 cells from apoptosis. Control C2 cells (**a**) or G5 cells, expressing Gas6 (**b**) were incubated in medium containing 0.1% serum. After 48 h the cells were fixed and nuclei were stained using the Hoechst 33342 dye. Apoptotic nuclei are brighter and smaller than normal nuclei as a result of chromatin condensation (**c**) Cytosolic DNA from NIH3T3 control cells (C2) or Gas6 expressing clones (G5 and G6) in 0.1% serum, was extracted at the indicated times and run on a 1.5% agarose gel. DNA laddering was stained using propidium iodide

Exogenous Gas6 acts as a survival factor by preventing apoptosis induced by serum withdrawal

In order to determine whether the protection from apoptosis observed in the Gas6 expressing clones was mediated specifically by Gas6, we tested directly the effect of Gas6 addition to NIH3T3 cells. Recombinant murine Gas6 (purified from the supernatant of stably transfected CHO cells) was tested for its ability to induce phosphorylation of the ARK tyrosine kinase in serum-starved NIH3T3 cells. Cell lysates were immuno-precipitated using antibodies against the ARK

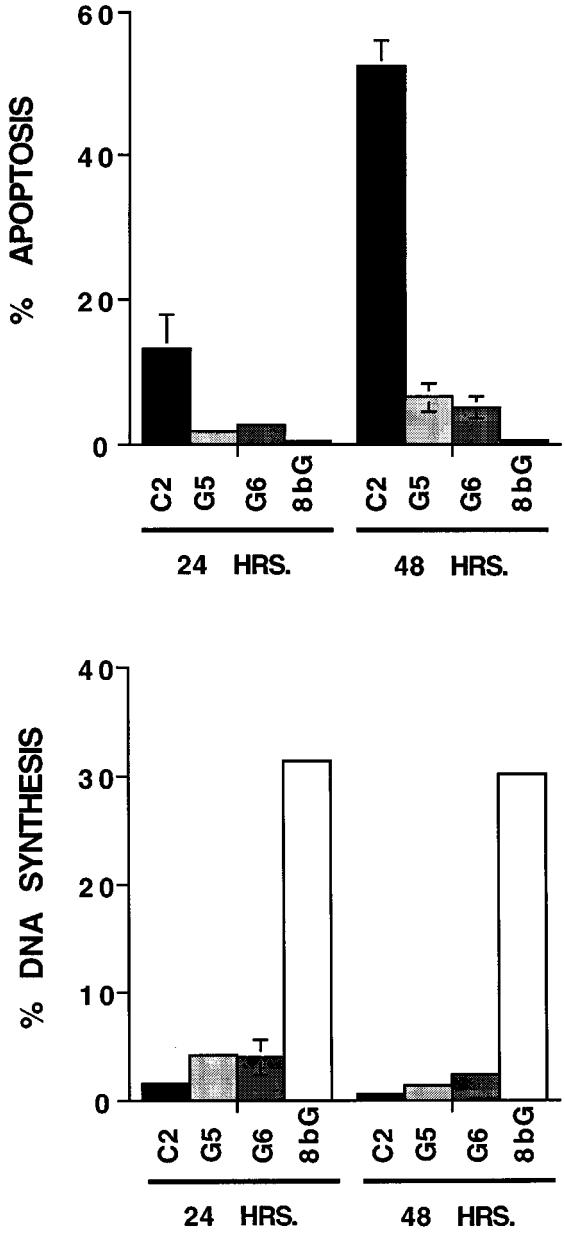

Figure 4 Gas6 expression protects from apoptosis in the absence of DNA synthesis. 3T3 control cells (C2), Gas6 expressing clones (G5 and G6) or FGF-4 expressing cells (8bG), were incubated in medium containing 0.1% serum for the indicated times. BrdU was added for 1 h to the medium and nuclei were scored for BrdU incorporation (DNA synthesis) or apoptosis by Hoechst staining as described in Materials and methods. The data are expressed as percentage of nuclei in apoptosis (upper panel) or the percentage of nuclei in DNA synthesis (lower panel). Data represent the mean and s.d. from duplicate samples

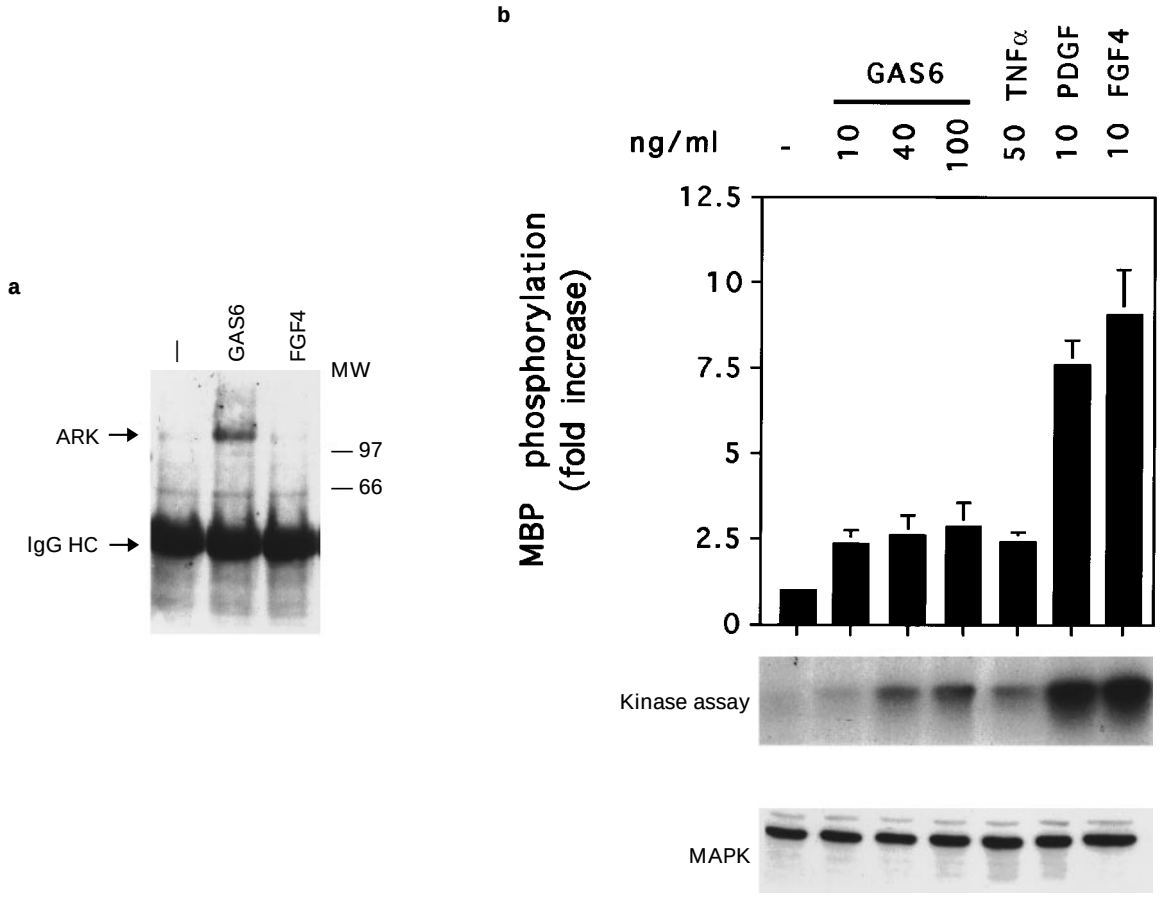

Figure 5 Murine recombinant Gas6 activates the ARK receptor and MAP-Kinase activity. (**a**) Lysates from serum-starved NIH3T3 cells, untreated (−) or stimulated with purified recombinant Gas6 or FGF-4 (100 ng/ml for 10 min), were immunoprecipitated with anti-ARK antiserum. Immunoprecipitates were run on an 8% SDS–PAGE and Western blotting analysis was performed using anti-phosphotyrosine antiserum. Molecular weight markers are indicated on the right. (**b**) *In vitro* kinase assays of MAPK immunoprecipitated from lysates of NIH3T3 cells. Serum-starved 3T3 cells were treated with the indicated concentrations of growth factors for 10 min. Lysates were immunoprecipitated using anti-ERK1 antibodies and *in vitro* kinase assays were performed using MBP as a substrate. MAPK activity was determined as described in Materials and methods and normalized to the activity of untreated cells. The data presented are the average of two separated experiments. The lower part of the panel shows an *in vitro* kinase assay of MBP phosphorylation, and Western blot analysis of MAP-Kinase expression on total cell lysates

receptor and Western blot analysis was performed using anti phosphotyrosine antiserum. As shown in Figure 5a addition of exogenous Gas6 to serum-starved 3T3 cells induces ARK phosphorylation, indicating that the purified recombinant Gas6 can activate the receptor.

We next analysed whether exogenous Gas6 exerted the same survival effect observed in Gas6 expressing clones. NIH3T3 cells were seeded in 10% serum for 24 h and then shifted to medium containing 0.1% serum and the indicated concentrations of Gas6 or FGF-4. Cells were labeled with BrdU, fixed at the indicated times and analysed for apoptosis or for BrdU incorporation, as described above. Figure 6 shows that addition of recombinant murine Gas6 also prevented NIH3T3 cells from undergoing apoptosis. This effect was dose dependent and was not accompanied by significant BrdU incorporation, indicating that Gas6 does not induce the cells to re-enter the cell cycle. In contrast, FGF-4 or serum also prevented cells from undergoing apoptosis, but in this case protection from apoptosis was always accompanied by stimulation of DNA synthesis. Thus protection from cell death by Gas6 can be separated from DNA synthesis and cell growth.

It is important to note that we and others (Goruppi *et al*., 1996; Costa *et al*., 1996) had previously shown that Gas6 could exert a moderate mitogenic effect on NIH3T3 cells. Such an effect however could only be detected at very high (approximately 400 ng/ml) ligand concentrations. Figure 6 shows that maximum Gas6 protection from apoptosis requires approximately a tenfold lower ligand concentration.

Many tyrosine kinases and cytokine receptors have been shown to activate serine/threonine kinases which are part of the family of mitogen-activated protein kinases (MAPKs). It is known that activation of the MAP-kinase pathways in 3T3 cells is necessary and sufficient for cell proliferation (Marshall, 1995). As mentioned above, previous studies have indicated that high concentrations of Gas6 can induce a low level of MAP-Kinase activation as well as a weak mitogenic signal in 3T3 cells (Goruppi *et al*., 1996; Costa *et al*., 1996; Fridell *et al*., 1996). We analysed the level of activation of MAP-kinase under conditions in which recombinant murine Gas6 was protecting cells from apoptosis and was not exerting a mitogenic effect. NIH3T3 cells were serum-starved and treated with recombinant murine Gas6 (from 10–100 ng/ml). Lysates were immunoprecipitated with antibodies

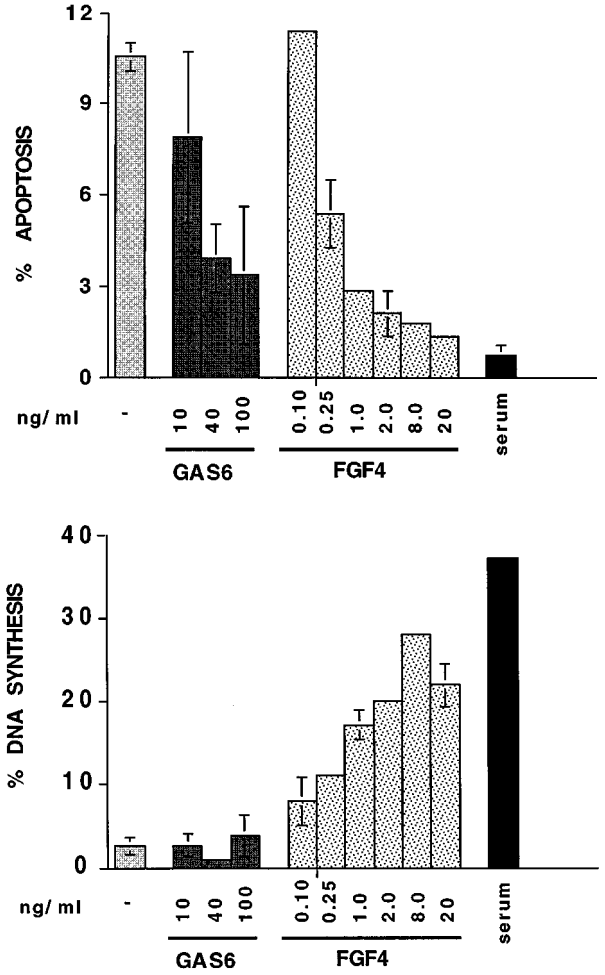

Figure 6 Recombinant murine Gas6 protects cells from apoptosis without inducing DNA synthesis. NIH3T3 cells were treated with medium containing 0.1% serum and the indicated concentration of recombinant Gas6 or FGF-4. Twenty-four hours later BrdU was added for 1 h and nuclei were scored for DNA synthesis (BrdU incorporation) or apoptosis as described in Materials and methods. The data are expressed as percentage of nuclei in apoptosis (upper panel) or the percentage of nuclei in DNA synthesis (lower panel). Data represent the mean and s.d. from duplicate samples

against ERK1 and the level of ERK activity analysed by *in vitro* kinases assay using MBP as a substrate. As shown in Figure 5b, Gas6 is capable of inducing a weak activation of MAP-Kinase, about 2–3 times higher than the background. This level has not increased using higher doses of Gas6 (data not shown). In contrast, growth factors such as FGF-4 or PDGF induced a level of MAPK activation about 8–9 times above the background.

Thus Gas6-induced ARK signaling results in a weak but significant activation of MAP-kinase activity, and protection from apoptosis induced by serum deprivation without a concomitant stimulation of DNA synthesis. To confirm that ARK signaling was critical to the antiapoptotic affect, we examined the response to serum deprivation of a clone of NIH3T3 cells which expresses a truncated form of ARK (Costa *et al*., 1996). This truncated receptor, lacking the entire tyrosine kinase domain, inhibits receptor phosphorylation in response to Gas6 (data not shown) and would therefore, be expected to exert a dominant negative effect. Upon serum starvation these cells underwent apoptosis, that was not affected by the addition of Gas6 to the culture medium (data not shown).

Embryonic fibroblasts from ARK knock-out mice are highly susceptible to apoptosis induced by serum deprivation

We were interested in determining whether loss of ARK function resulted in increased susceptibility to apoptosis. Fifteen days embryos generated by crossing two heterozygous ARK+/− mice, were sacrificed and used to prepare individual primary cultures of mouse embryonic fibroblasts (MEF) from each embryo. PCR analysis was perfomed to analyse the genotype of the primary cell lines. RNA was extracted from ARK−/− and ARK+/+ fibroblasts and tested for expression of Gas6 and ARK mRNAs. Northern blot analysis showed that Gas6 was expressed at a similar level in ARK knock-out or wild-type cells, whereas ARK mRNA was present only in the ARK+/+ cells and absent from the ARK−/− MEFs (Figure 7a). We compared the ability of serum withdrawal to induce apoptosis in ARK+/+ and ARK−/− MEFs. Cells were plated in DMEM containing 10% serum and after 48 h the medium was shifted to either DMEM or DMEM containing 100 ng/ml of purified Gas6, or 100 ng/ml of FGF-4. Cells were fixed at different times and apoptosis visualized by analysis of nuclear morphology upon Hoechst 44432 staining. Figure 7b shows that MEF derived from ARK null embryos, were more sensitive than MEF derived from the wild-type embryos to undergo apoptosis following serum withdrawal. At 20 h the frequency of apoptotic cells in the ARK−/− fibroblasts was about three times higher than in fibroblasts expressing ARK. This difference was also apparent at 40 h when apoptosis was observed not only in the ARK−/− MEF, but also in the ARK+/+ MEF. Addition of exogenous Gas6 further emphasized these differences as it significantly reduced the formation of apoptotic nuclei in the case of wild-type cells, but had no effect on ARK−/− cells. These data indicate not only that MEF lacking the ARK receptor are less protected from apoptosis than wild-type cells, but also that the amount of Gas6 produced by these cells is not sufficient for full protection under these experimental conditions.

These experiments strengthen the conclusion that ARK signaling protects cells from apoptosis induced by serum withdrawal. Also in this case, this effect did not require stimulation of DNA synthesis because experiments of BrdU incorporation showed that serum starvation induced growth arrest in MEF from ARK+/+ as well as in cells from the ARK knock-out mice (data not shown).

*Gas6 prevents apoptosis induced by TNFα and c-*myc *but not apoptosis induced by u.v. irradiation or Staurosporine*

In order to understand the possible role of ARK in the protection from Programmed Cell Death, we investigated whether ARK activation could prevent apoptosis

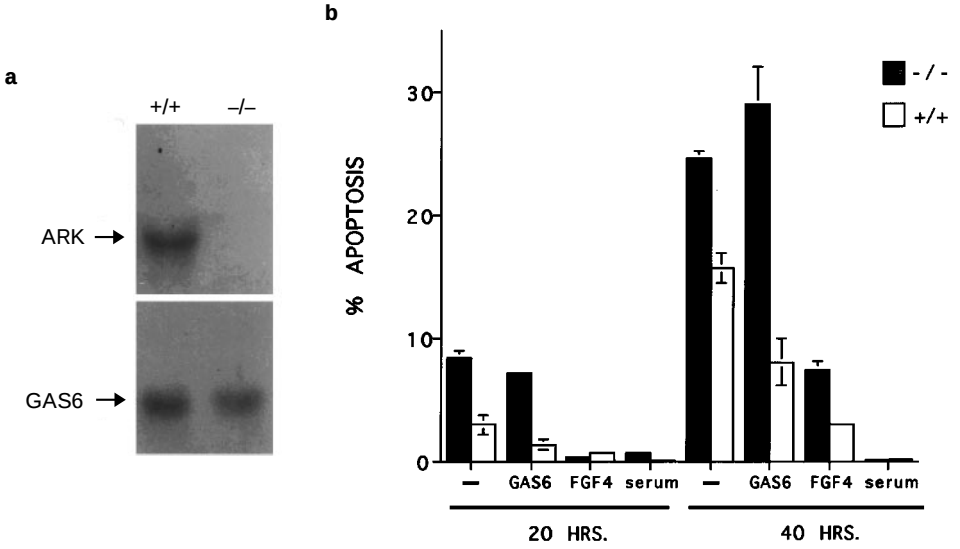

Figure 7 Induction of apoptosis in embryo fibroblasts from wild-type and ARK knock-out mice. (**a**) Shows Northern blot analysis of ARK and Gas6 mRNA expression from wild-type (+/+) or ARK knock-out (−/−) MEF. (**b**) MEF were incubated in serum-free medium containing 100 ng/ml of Gas6 or FGF-4. Cells were fixed at the indicated times and nuclei scored for apoptosis as described in Materials and methods. Data represent the mean and s.d. from duplicate samples

induced by TNFα, c-*myc* overexpression, Staurosporine, or u.v. irradiation.

Activation of the TNF-R1 by TNFα results in formation of a complex between the 'death domain' of this receptor and the TRADD protein which eventually results in the induction of apoptotic ICE proteases (Chinnaiyan *et al*., 1996). In order to analyse if ARK activation could protect from apoptosis induced by TNFα, NIH3T3 cells were seeded in 10% serum for 24 h and treated with 5 ng/ml of TNFα in the absence or presence of different concentrations of purified recombinant Gas6. Cells were fixed for different times and nuclei analysed for apoptosis as described above. Addition of Gas6 to HIH3T3 cells could prevent apoptosis induced by TNFα, indicating that ARK activation may interfere with the apoptotic program activated by TNFα (Figure 8a).

We next used a strain of Rat-1 cells in which activation of a c-*myc*-ER chimera can be induced by Tamoxifen to study whether ARK signaling could inhibit *myc*-induced apoptosis (Harrington *et al*., 1994). We previously showed that Rat-1 cells express ARK (Costa *et al*., 1996). Cells were exposed to 200 nM 4-hydroxy tamoxifen in medium containing 0.5% serum and different concentration of recombinant Gas6 and the frequency of apoptotic cells measured 15 h later. Figure 8b shows that also in this case addition of Gas6 to the culture medium resulted in a significant inhibition of apoptosis.

We also examined whether ARK activation could inhibit programmed cell death induced by Staurosporine (STS) or by u.v. irradiation (Lu and Lane, 1993). Staurosporine is a bacterial alkaloid acting as a potent inhibitor of protein kinases, with a broad spectrum of activity (Jacobson *et al*., 1996). Cells were seeded in 10% serum and treated with 0.25 μM of STS for different times in the presence of Gas6. As shown in Figure 8c treatment with STS leads to rapid apoptosis which is not rescued by Gas6 treatment.

To determine if ARK activation could rescue cells from apoptosis induced by u.v. irradiation, NIH3T3 cells were irradiated with 50 J/m^2 or 65 J/m^2, as described in Materials and methods. Cells were fixed at different time-points and nuclei scored for apoptosis or for incorporation of BrdU. Figure 8d shows that NIH3T3 cells irradiated with 50 J/m^2 underwent apoptosis and this effect could not be antagonized by addition of exogenous Gas6. Gas6 also had no effect on DNA synthesis, that declined to very low levels 24 h after irradiation and resumed eventually 48 h later. Similar results were obtained with 3T3 cells irradiated with 65 J/m^2 (data not shown).

Discussion

The results presented in this report indicate that signaling through the receptor tyrosine kinase ARK (AXL) induced by its Gas6 ligand does not induce significant cell proliferation in fibroblasts, but protects them from apoptosis induced by serum deprivation as well as by TNFα and c-*myc*. These results are important with regard to several issues which have been raised about ARK/AXL physiological and pathological function.

The human homolog of ARK, AXL was originally isolated as an oncogene, but its oncogenic activity is, at best, weak and transformation could only be demonstrated in cells expressing very high levels of AXL receptor (O'Bryan *et al*., 1991). Similarly, a retrovirus encoding a gag-AXL fusion protein was only weakly transforming in NIH3T3 cells (Zhang *et al*., 1996). It is not known whether Gas6, the ARK heterophilic ligand, was expressed in the transformants, but a number of RTK have been shown to cause transformation when overexpressed, likely because high receptor surface density induces receptor dimerization and activation, a process which in this case could be also facilitated by ARK's ability to undergo homophilic binding (Bellosta *et al*., 1995). On the other hand, when we expressed constitutively Gas6 in ARK

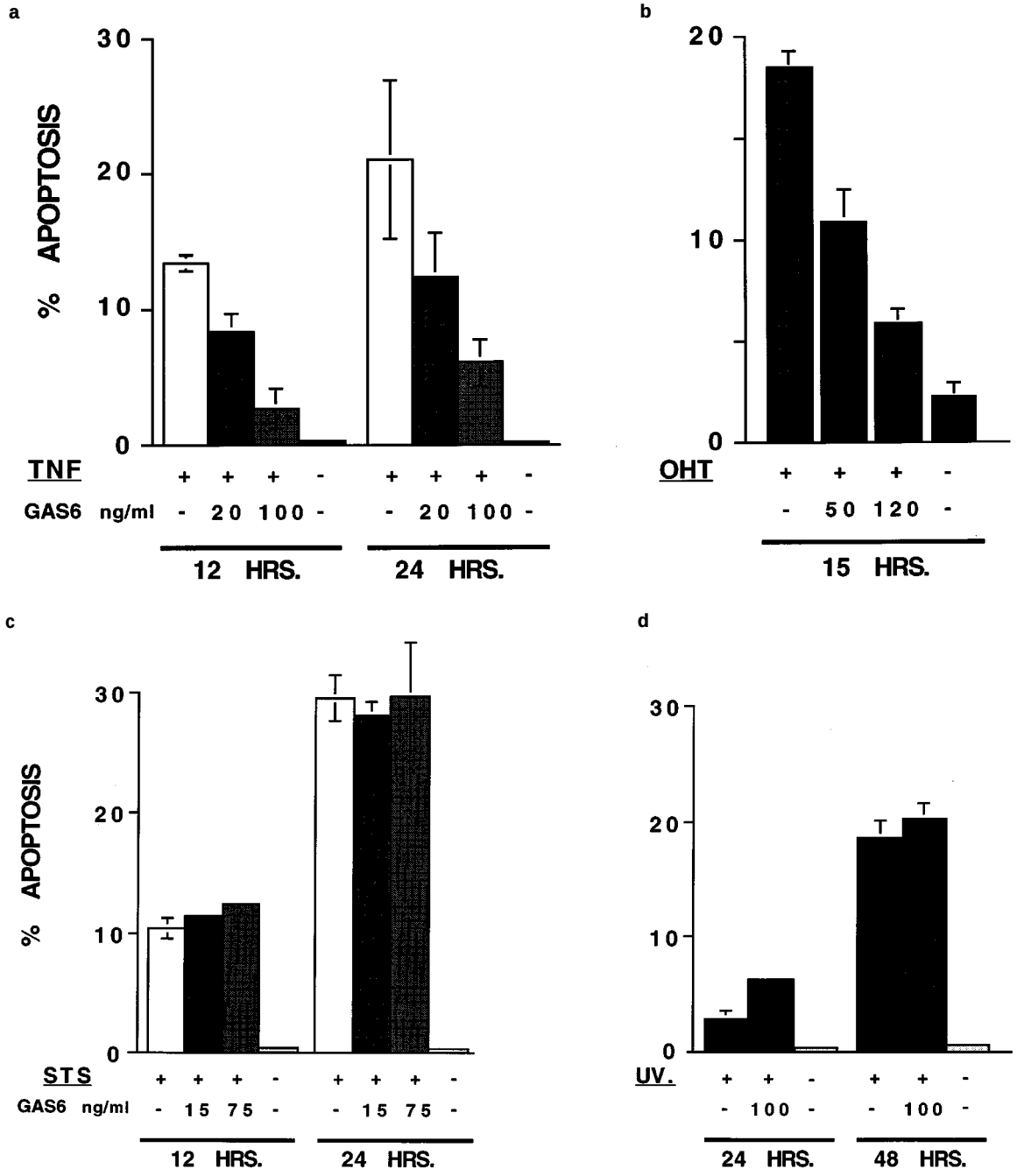

Figure 8 Gas6-ARK interaction can protect from TNFα and c-*myc* induced apoptosis but not from Staurosporine or u.v. NIH3T3 cells were seeded in medium containing 10% serum and induced to undergo apoptosis using 5 μg/ml of TNFα (**a**) 0.25 μM Staurosporine (**c**) or irradiated with 50 J/m^2 (**d**). *myc*-induced apoptosis (**b**), was analysed in Rat1-*myc*ER cells where *myc* activation was induced by addition of 200 nM 4-hydroxy tamoxifen (OHT) in medium containing 0.5% serum. Recombinant murine Gas6 was added to the medium at the indicated concentrations as described in Materials and methods. Cells were fixed at the indicated times and apoptosis was analysed by scoring the nuclei stained with the Hoechst dye. Data represent the mean and s.d. from duplicate samples

expressing NIH3T3 cells, we could not detect evidence of morphological transformation, ability to grow in low serum or at high densities, although we could show that ARK was activated in these cells. This observation contrasts with that observed with a growth factor, such as FGF-4, where ectopic expression in cells expressing FGF receptors results in autocrine growth and transformation. The only clear effect of Gas6 expression we could detect was the protection from apoptosis induced by serum deprivation. Prevention of apoptosis could result in an oncogenic signal under specific circumstances, as illustrated by the original identification of Bcl-2, which powerfully prevents apoptosis, as an oncogene (Reed *et al*., 1996). We would like therefore to suggest that the weak oncogenic ability of ARK/AXL may derive both from exaggerated signaling, resulting in a weak mitogenic signal, and prevention of apoptosis.

We and others had previously shown that high doses of Gas6 could have a modest mitogenic effect on ARK expressing cells (Costa *et al*., 1996; Goruppi *et al*., 1996). The doses of Gas6 necessary to induce cell proliferation are however very high, (200–400 ng/ml), and although it is not known what concentrations

Gas6 may reach *in vivo*, it should be noted that most *bona fide* growth factors act at concentrations on the other order of 1 ng/ml. In contrast, we could detect an antiapoptotic effect of Gas6 at concentrations ten times lower than those needed to stimulate mitogenesis, and much more likely to approximate a physiological level of expression. At these concentrations, no significant stimulation of DNA synthesis was detected. It is therefore possible that the true role of ARK signaling consists of promoting cell survival, and that the mitogenic effect is a tissue culture artifact caused by an unphysiological level of receptor stimulation.

Goruppi *et al*. (1996) have also noted that Gas6 can protect cells from apoptosis induced by growth factor deprivation. These authors however used high Gas6 concentrations, which can produce a mitogenic effect. Nevertheless, they also concluded that Gas6 had survival activity in serum-starved NIH3T3 cells and that this effect did not require induction of cell proliferation.

Fridell *et al*. (1996) have obtained very interesting results in 32D cells by comparing the effect of AXL activation by Gas6 with that of EGF stimulation of an EGFR-AXL chimera (EGFR extracellular domain and transmembrane region fused to AXL tyrosine kinase domain). While EGF could stimulate the proliferation of 32D cells expressing the chimeric receptor, Gas6 did not induce proliferation in AXL expressing cells, although it clearly activated AXL phosphorylation. The results with the chimeric receptor clearly showed that the ARK kinase domain is capable of activating a mitogenic pathway, but that the Gas6-AXL interaction does not result in such activation. Although it is possible to hypothesize that a weak activation of the RAS/MAPK pathway (such as the one we observed) could result in protection from apoptosis without induction of DNA synthesis, this hypothesis does not conform with the results obtained with the IL-3 and IGF-1 receptors, which indicate distinct pathway for apoptosis protection and mitogenicity (Kinoshita *et al*., 1995; O'Connor *et al*., 1997). Thus we suggest that ARK normally activates a signaling pathway which is involved in promoting cell survival. Recently it has been shown that a critical role in protection from apoptosis is played by the lipid kinase P-I3-K, and its downstream effector Akt/PkB (Hemmings, 1997). c-*myc*-induced apoptosis can also be suppressed by *ras* signaling through PI3K and PkB (Kauffmann-Zeh *et al*., 1997). Considering that Gas6-ARK interaction can prevent apoptosis induced by c-*myc* and that ARK can bind and activate PI3K, (our unpublished results) activation of this enzyme could play an important role in the mechanism by which ARK activation protects cells from apoptosis.

The differential effect of Gas6 on the apoptotic process induced by different conditions suggests that ARK signaling does not prevent apoptosis by inducing broadly antiapoptotic molecules, such as *bcl*-2, or by inhibiting the activity of ICE-like proteases. Indeed we could not find significant induction of *bcl*-2 following GAS-6 treatment of NIH3T3 cells (data not shown). The inability of GAS-6 to inhibit Staurosporine-induced apoptosis could result from the inhibition of protein kinases activity produced by this drug, which could reduce ARK autophosphorylation and activation. On the other hand, the lack of effect on u.v.-induced apoptosis, which is known to require p53 function, suggests that ARK signaling activates pathways that do not interfere with the processes induced by high levels of p53 expression. Harrington *et al*. (1994) have shown that apoptosis induced by c-*myc* in fibroblasts is inhibited by some but not other cytokines, irrespective of their ability to promote cell proliferation. These authors have suggested that apoptosis induced by elevated levels of c-*myc* expression results from an intrinsic ability of c-*myc* to activate an apoptotic program, which is counteracted only by specific cytokines, possibly depending on their ability to activate PI-3 kinase. In this context, since ARK inhibits c-*myc* induced apoptosis, it could be thought that ARK signaling can activate signal transduction pathways which are shared with other growth factors which can protect from *myc*-induced apoptosis (i.e. PDGF and IGF-I), while only weakly capable of activating mitogenic pathways.

The experiments performed with embryonic fibroblasts from ARK knock-out mice also point to a role of ARK signaling in protecting cells from apoptosis, although clearly ARK is unlikely to be the only antiapoptotic signaling molecule in these cells. It is interesting to note that Gas6 addition had a further protection effect on wild-type cells, while it did not on ARK$-/-$ cells. Since these cells also express tyro3, another member of the ARK family to which Gas6 has been reported to bind (Mark *et al*., 1996), these results suggest that either tyro3 activation does not protect from apoptosis, or that Gas6 does not activate tyro3 appreciably.

In conclusion, our results indicate that the activation of the ARK receptor by its heterophilic ligand Gas6, does not generally result in fibroblast proliferation but has a significant antiapoptotic effect in cells deprived of serum or treated with TNFα. This observation provides a plausible explanation for the finding that Gas6 expression is up-regulated in quiescent cells. The investigation of the specific signals which mediate ARK's antiapoptotic effect is in progress and should reveal why specific apoptosis signals are counteracted and others are not.

Materials and methods

Generation of Gas6 producing clones

NIH3T3 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% calf serum. The mouse Gas6 cDNA was cloned from liver cDNA library (Stratagene) and a fragment of 2.5 kb, corresponding to the full-length Gas6 cDNA was sub-cloned into the *Eco*RI sites of the PLXSN vector under the control of the mouse Moloney leukemia virus LTR. The PLXSN vector also carries the neomycin resistance gene under the SV40 promoter. Co-transfection of the PLXSN-Gas6 construct with an ecotropic packaging construct allowed us to produce a replication-defective retrovirus (Muller *et al*., 1991). 293 cells were used as recipient cells and transfected by the calcium phosphate method. Supernatants were collected after 48 h of transfection and used to infect NIH3T3 cells. Clones were selected using 400 μg/ml of G418 and the culture medium was used to determine the

expression of Gas6 in each clone using anti-Gas6 antiserum (Costa *et al*., 1996) in Western blot analysis.

Production of recombinant murine Gas6

CHO-DG44 cells, which are dihydrofolate reductase-negative (DHFR$^-$), were maintained in DMEM containing 10% fetal calf serum (FCS), 0.1 mM hypoxanthine, 0.02 mM thymidine and 10 mM proline. Transfection of the 2.5 kb murine Gas6 cDNA, cloned into the P91023 (B) vector (Wong *et al*., 1985) was performed using 5 μg of plasmid using the calcium phosphate procedure. Positive clones were selected in medium containing dialyzed FCS and lacking hypoxanthine and thymidine. Expression of Gas6 was determined by Western blotting analysis using anti-Gas6 antiserum. CHO-Gas6 clones were grown in DMEM supplemented with 10% FCS and proline. Recombinant Gas6 was obtained from cells growing in serum-free DMEM containing 5 μg/ml of insulin, 5 μg/ml of transferrin, 10 mM of proline and 4 μM of menadione sodium bisulfate (Sigma). Supernatant was collected every 24 h for 3 days and recombinant Gas6 was purified using an hydroxiapatite column (Varnum *et al*., 1995). Bound material was eluted using a gradient of Na Phosphate buffer from 100–400 mM, pH 7.5, and the presence of Gas6 in the fractions was detected by Western blot using anti-Gas6 antibodies. The concentration of active protein was determined by binding to the murine AXL/ARK-Fc fusion protein (Stitt *et al*., 1995) using the BIACORE and by the ability to phosphorylate the ARK receptor.

DNA synthesis and apoptosis assay

4×10^4 cells were seeded on coverslips on 24 wells plates in DMEM/10% calf serum. After 24 h cells were incubated with 4 μg/ml Bromodeoxyuridine (BrdU) for 1 h at 37°C and fixed with a solution of 70% ethanol/20 mM glycine pH 2.0 at −20°C. Analysis of BrdU incorporation was performed using the BrdU fluorescein-labeling and detection kit I (Boehringer). Nuclei were stained with 1 μg/ml of Hoechst 33342 dye in PBS for 10 min and coverslips were mounted using elvanol. The frequency of S phase cells was calculated as the ratio between nuclei positive for BrdU incorporation and total nuclei (Hoechst). When apoptosis only was analysed cells were fixed with a solution of 3.7% of paraformaldehyde for 30 min at room temperature and permeabilized with 0.5% TritonX-100.

TUNEL assay was performed by using the *in situ* cell death detection kit (Boehringer). All the experiments were analysed using a Zeiss Axiophot II microscope using FITC or Hoechst filters.

DNA fragmentation

Cytosolic DNA was isolated for fragmentation analysis by lysing 5×10^6 cells in buffer containing 50 mM HEPES (pH 7.4) and 0.5% Triton X-100. The nuclei were centrifuged and the cytosolic fraction was treated with RNase (1 μg/ml) for 1 h at 37°C. DNA was phenol extracted, precipitated by the addition of 0.1 volume of 3.3 M sodium acetate pH 5.2 and two volumes of ethanol and visualized by electrophoresis in 1.5% agarose gel. Gels were stained with ethidium bromide.

Northern blotting

Mouse embryonic fibroblasts were grown in DMEM containing 10% calf serum. Total RNA was extracted as described (Costa *et al*., 1996) and 15 μg of total RNA were run in a 1% agarose gel containing 6.5% of formaldehyde. A 1.6 kb fragment encoding the extracellular and transmembrane domain of the ARK receptor was used as a probe for detecting the ARK mRNA; while the 1.2 kb *Eco*RI fragment, which contains the 3′ UTR of the murine Gas6, was used to detect the Gas6 mRNA (Manfioletti *et al*., 1993).

Receptor phosphorylation and in vitro *kinase assay*

After starvation for 24 h in 0.5% calf serum, untreated cells, or cells treated with Gas6 (100 ng/ml) or FGF-4 (100 ng/ml) for 10 min at 37°C were lysed in 20 mM Na Phosphate buffer containing 1 mM EDTA, 1% Triton X-100, 1 mM of PMSF, 1 mM aprotinin, 1 mM leupeptin and 1 mM Na Orthovanadate and lysates were immunoprecipitated using anti-ARK antiserum #318 (Bellosta *et al*., 1995). Samples were separated on 8% SDS–PAGE and Western blot analysis was performed using anti-phosphotyrosine antiserum #72 (Bellosta *et al*., 1995), or a monoclonal antibody (4G10, Oncogene Science). Positive bands were visualized using the ECL kit (Amersham). For analysis of MAP-Kinase activation NIH3T3 cells were starved as described above and treated with different concentration of growth factors or cytokines. After 10 min cells were lysed and MAP-kinase was immunoprecipitated using anti ERK1 antiserum (Santa Cruz). Immunoprecipitate were subjected to an *in vitro* kinase assay using a buffer containing 50 mM Tris pH 7.5 and 10 mM $MgCl_2$ for 20 min at 30°C using 5 μCi of $\gamma[^{32}P]$-ATP and 25 μg of Mielin Basic Protein (MBP) as a substrate; reaction was stopped by adding $2 \times$ Laemmli sample buffer and immunoprecipitates were run in a 15% SDS–PAGE. Gels were fixed in a solution of 30% acetic acid and 10% methanol in the presence of Comassie Blue dye. Gels were dried and exposed to a X-ray film for 15 min $\gamma[^{32}P]$-ATP incorporated in MBP was calculated by cutting the band from the gel and radioactivity detected using a β scintillation counter (Beckman).

*Induction of apoptosis by serum deprivation, c-*myc *activation, TNF*α *or Staurosporine*

4×10^4 cells were seeded in 10% calf serum in 24 wells plates. Apoptosis by serum starvation was induced, in NIH3T3 by shifting the concentration of serum from 10–0.1%. Apoptosis in MEF was induced by incubation in serum free medium. *myc*-induced apoptosis was analysed in Rat1-MycER cells where *myc* was induced by addition of 200 nM 4-hydroxy tamoxifen (OHT) (Harrington *et al*., 1994). Apoptosis induced by TNFα and Staurosporine was performed using DMEM containing 10% calf serum and murine recombinant TNFα (from Paul Schwenger – NYU) at the concentration of 5 μg/ml, or Staurosporine at the concentration of 0.25 μM (Sigma). Cells were fixed at different times with 3.7% paraformaldehyde and nuclei were stained using the Hoechst dye as described above.

For u.v. irradiation medium was removed and cells irradiated with 50 J/m^2 or 65 J/m^2 using a u.v.-crosslinker (Stratagene). After addition of DMEM containing 10% calf serum cells were labeled for 1 h with 4 μg/ml BrdU. Analysis of apoptotic nuclei and DNA synthesis was performed by staining the nuclei either with the Hoechst dye or for BdrU incorporation as described above.

Preparation of mouse embryonic fibroblasts from ARK knock-out mice

The generation of the ARK knock-out mice will be described in detail elsewhere (Zhang and Goff, in preparation). Briefly, a genomic DNA clone containing 11 kb of the *ark* locus was used to generate a targeting construct; all of exon 9 and small portions of the flanking intronic sequences were deleted and replaced with a PGK-

neo cassette. The CB 1–4 embryonic stem cell line was transformed by electroporation and targeted clones were identified by Southern blot. ES cells were injected into C571/6J blastocysts and chimeric mice were identified and mated to obtain germline transmission. The heterozygous mutant animals were intercrossed to obtain homozygotes. Examination of RNA from embryo fibroblasts by Northern blot and of protein in brain extracts by Western blot yielded no detectable signals from homozygous animals, suggesting that the mutant allele did not produce stable transcripts. Embryos at 15 days p.c. generated by crossing two heterozygous ARK+/− mice, were sacrificed and used to prepare individual primary cultures of mouse embryonic fibroblasts (MEF). Each embryo was minced in PBS and transferred to a solution of 0.25% of trypsin. After 20 min of gentle stirring, the supernatant was filtered and diluted with DMEM containing 20% calf serum. The incubation with trypsin was repeated three times. Cells were centrifuged and plated in DMEM containing 20% calf serum at the concentration of 1×10^7 cells/plate.

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