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# FasL (CD95L/APO-1L) Resistance of Neurons Mediated by Phosphatidylinositol 3-Kinase–Akt/Protein Kinase B-Dependent Expression of Lifeguard/Neuronal Membrane Protein 35

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**The contribution of Fas (CD95/APO-1) to cell death mechanisms of differentiated neurons is controversially discussed. Rat cerebellar granule neurons (CGNs) express high levels of Fas *in vitro* but are resistant to FasL (CD95L/APO-1L/CD178)-induced apoptosis. We here show that this resistance was mediated by a phosphatidylinositol 3-kinase (PI 3-kinase)–Akt/protein kinase B (PKB)-dependent expression of lifeguard (LFG)/neuronal membrane protein 35. Reduction of endogenous LFG expression by antisense oligonucleotides or small interfering RNA lead to increased sensitivity of CGNs to FasL-induced cell death and caspase-8 cleavage. The inhibition of PI 3-kinase activity sensitized CGNs to FasL-induced caspase-8 and caspase-3 processing and caspase-dependent fodrin cleavage. Pharmacological inhibition of PI 3-kinase, overexpression of the inhibitory protein IκB, or cotransfection of an LFG reporter plasmid with dominant-negative Akt/PKB inhibited LFG reporter activity, whereas overexpression of constitutively active Akt/PKB increased LFG reporter activity. Overexpression of LFG in CGNs interfered with the sensitization to FasL by PI 3-kinase inhibitors. In contrast to CGNs, 12 glioma cell lines, which are sensitive to FasL, did not express LFG. Gene transfer of LFG into these FasL-susceptible glioma cells protected against FasL-induced apoptosis. These results demonstrate that LFG mediated the FasL resistance of CGNs and that, under certain circumstances, e.g., inhibition of the PI 3-kinase–Akt/PKB pathway, CGNs were sensitized to FasL.**

*Key words:* **apoptosis; Fas/CD95; lifeguard; cerebellar granule neurons; PI 3-kinase/Akt; caspase**

## Introduction

Fas (CD95/APO-1) and FasL (CD95L/APO-1L/CD178) are a cytokine receptor/cytokine pair of the tumor necrosis factor/nerve growth factor (TNF/NGF) superfamily. Natural mouse mutants for Fas (*lpr*) and FasL (*gld*) exhibit a syndrome resembling the human autoimmune disease systemic lupus erythematosus, indicating a role for Fas/FasL interactions in the limitation of exogenously triggered immune responses. Fas mutations may cause an autoimmune lymphoproliferative syndrome in humans (Drappa et al., 1996; Clementi et al., 2004; Holzelova et al., 2004). FasL is abundantly expressed in the nervous system (Bechmann et al., 1999), and its presence has been related to the maintenance of immune privilege in the CNS through its ability to trigger the death of invading lymphocytes (French et al., 1996; Saas et al., 1997; Weller et al., 1997; Green and Ferguson, 2001). The cognate death receptor Fas has also been detected in the nervous system. Specific neuronal populations in postnatal mouse brain express Fas mRNA and protein even in nonlesioned animals (Park et al., 1998; Cheema et al., 1999; Felderhoff-Mueser et al., 2000). However, *lpr* and *gld* mice appear to show no obvious neuronal phenotype (Kovac et al., 2002).

Neurons appear to be sensitive to FasL during development and before differentiation (Cheema et al., 1999; Raoul et al., 1999, 2002). However, during *in vitro* maturation and differentiation at

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least, motor neurons become resistant to FasL-induced apoptosis (Raoul et al., 1999). This acquired resistance was attributed to the upregulation of the caspase-8/FLICE (Fas-associated death domain-like IL-1β-converting enzyme) inhibitory protein (FLIP). Under certain pathological conditions, FasL-induced apoptosis has been implicated in cell death of mature neurons. *lpr* and *gld* mice and mice treated with anti-FasL antibodies are protected from focal ischemia *in vivo* (Martin-Villalba et al., 1999, 2001).

We and others reported previously that, like mature motor neurons in culture, differentiated cerebellar granule neurons (CGNs) cultured for 7 d were not sensitive to FasL (Gerhardt et al., 2001; Putcha et al., 2002). This has been questioned by others (Le-Niculescu et al., 1999; Hou et al., 2002). We here wanted to test the mechanisms of this resistance. In addition to FLIP, lifeguard (LFG) is an endogenous inhibitor of Fas-mediated apoptosis in tumor cells (Somia et al., 1999). *LFG* was isolated as a gene from a human lung fibroblast cell line, MRC5, that was not sensitive to FasL. Somia and colleagues showed that LFG bound directly to the Fas receptor but not to Fas adaptor proteins. Its rat homolog was identified as a protein upregulated during the development of spinal motor neurons of the sciatic nerve. Although it is expressed and upregulated during development in the CNS (Somia et al., 1999; Schweitzer et al., 1998, 2002), its antiapoptotic function and mechanisms of its temporal expression profile in the CNS have never been investigated. We here tested the hypothesis that LFG provides protection from neuronal apoptosis and investigated potential mechanisms of the regulation of Fas expression.

## Materials and Methods

*Materials.* Unless otherwise stated, all materials were obtained from Sigma (Deisenhofen, Germany). The antibodies to LFG were described previously (Schweitzer et al., 1998, 2002). They were raised against a synthetic peptide in the N-terminal loop of neuronal membrane protein 35 (NMP35) (SYEEATSGEGLKAGAF). The Fas antibody used for immunocytochemistry (catalog #610197) was obtained from BD Transduction Laboratories (Lexington, KY); the Fas antibody (clone C-20, sc-715) used for immunoprecipitation and the antibody against c-myc (sc-40) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies against the flag epitope (catalog #200472) were from Stratagene (La Jolla, CA), against caspase-8 (SK 441) from SmithKline Beecham Pharmaceuticals (gift from K. K. Kikly, King of Prussia, PA), against c-FLIP (catalog #1159) from ProSci (San Diego, CA), and against fodrin (mab-1622) and neuronal-specific nuclear protein (NeuN) (mab-377) from Chemicon (Temecula, CA).

*Constructs.* The generation of pBCMGS–Apo-1 has been described previously (Weller et al., 1995). pSG5-contitutionally active (gag)-protein kinase B (PKB)/Akt and pSG5-HA-dominant-negative (DN) PKB/Akt (kinase dead 388) plasmids were generous gifts from Dr. Boudewijn Burgering (Utrecht, The Netherlands); pcDNA3-IκB, pFLIP (s), and pFLIP (l) were obtained from Peter Daniel (Berlin, Germany) and Jürg Tschopp (Lausanne, Switzerland). For all other constructs the vector pcDNA 3.1 or a modified 6x-myc-pcDNA 3.1 were used (Invitrogen, Karlsruhe, Germany). In the latter, six repeats of c-myc were cloned into pcDNA 3.1 by using the *Hin*dIII and *Bam*H1 sites of the multiple cloning site. The *LFG* construct was cloned by using cDNA of rat CGNs. The fragment was amplified with the following primers: up, 5′-TTT GAA TTC TCC TCA TTC CCG GTT GGT GC-3′; and down, 5′-TTT GGA TCC TCG AGA GAC GAC ACC ATG AC-3′.

*Generation of pSUPER-LFG (525–545).* The small interfering RNA (siRNA) target sequence was 525–545 (5′-AACCTGATTCTGCTGACCATC-3′). The 64-mer oligos 5′-GATCCCCCCTGATTCTGCTGACCATCTTCAAGAGAGATGGTCAGCAGAATCAGGTTTTTGGAAA-3′ and 5′-TCGATTTCCAAAAACCTGATTCTGCTGACCATCTCTCTTGAAGATGGTCAGCAGAATCAGGGGG-3′ were obtained from MWG Biotech (Ebersberg, Germany), annealed, phosphorylated, and ligated into *Bgl*II and *Sal*I sites of pSUPER (Brummelkamp et al., 2002). The sequence was chosen according to the guidelines published by T. Tuschl (http://www.rockefeller.edu/labheads/tuschl/sirna.html). Integrity of the insert was confirmed by sequencing on an ABI310 capillary sequencer using Big Dye Sequencing Mix (ABI Prism; PerkinElmer Life Sciences, Überlingen, Germany).

*Northern blot analysis.* Total RNA was extracted using the RNeasy RNA purification system (Qiagen, Hilden, Germany). Denatured total RNA (10 μg) was loaded on a 1% agarose gel containing 6.7% formaldehyde. The RNA was separated at 100 V, transferred to a Hybond N+ membrane (Amersham Biosciences, Freiburg, Germany) by capillary blotting, and cross-linked in a UV stratalinker 1800 (Stratagene) at 1200 J. Methylene blue staining was performed as a loading control. The membrane was preincubated for 2 h in Church buffer at 65°C. Full-length rat LFG was labeled using 5 μl (~1.6 MBq) dCTP and the Rediprime II random labeling system (Amersham Biosciences). Filters were hybridized overnight at 65°C in a hybridization oven with a rotisserie device using Church buffer. Binding of radioactive probes was visualized and quantified using a PhosphoImager (FujiBasReader 1500; Fuji, Kangawa, Japan).

*Quantitative reverse transcription-PCR.* Total RNA was extracted with standard protocol using Trizol reagent (Invitrogen, Karlsruhe, Germany). Reverse transcription was performed with iScript cDNA Synthesis kit (Bio-Rad, Munich, Germany). Quantitative analysis of gene expression relative to a cDNA template was performed by reverse transcription (RT)-PCR using the Stratagene Mx3000P thermal cycler. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal standard for antisense experiments (taken from RTPrimerDB, Real Time PCR Primer and Probe Database). The sense primer for *GAPDH* was 5′-ATGATTCTACCCACGGCAAG-3′, and the antisense primer was 5′-CTGGAAGATGGTGATGGGTT-3′. The housekeeping gene *actin* was used as an internal standard for determination of *LFG* in cerebellum. The actin sense primer was 5′-ATTGCCGACAGGATGCAGAA-3′, and the antisense primer was 5′-GCTGATCCACATCTGCTGGAA-3′. LFG and Fas primers were created using Primer Express software (Applied Biosystems, Foster City, CA): LFG, sense primer, 5′- CATCCTCCTGCCCTTCCAAT-3′; antisense primer, 5′- CACACCCGCTCCTAGCACA-3′; Fas receptor, sense primer, 5′- CCCAGAATACCAAGTGCAGGTG-3′; antisense primer, 5′-TGTGCAAGGCTCAAGGATGTC-3′. The relative RNA expression levels were calculated using the ΔCT (difference of cycle thresholds) method, and the average results from three samples for each condition are shown.

*Cell culture and survival assays.* CGNs were obtained from 7-d-old Sprague Dawley rats and cultured as described previously (Schulz et al., 1996). All experiments with CGNs were performed on day *in vitro* (DIV) 8. The viability of CGNs was assessed by fluorescein diacetate (FDA) staining (Gerhardt et al., 2001). HEK-293, PC12, N2A murine neuroblastoma, and LN-18 human glioma cells were cultured in DMEM supplemented with 10% FCS and penicillin (100 U/ml)/streptomycin (100 μg/ml). All cell lines were seeded at $6 \times 10^5$ cells/cm$^2$. To ectopically express LFG in LN-18 cells, the cells were transfected using Effectene transfection reagent (Qiagen), followed by selection with 100 μg/ml G418. Stable expression of LFG was assessed by immunocytochemistry. The survival of LN-18 cells was measured by crystal violet staining (Weller et al., 1994). In brief, supernatant was removed, and the cells were incubated for 10 min in 2% of crystal violet dissolved in 20% methanol. The plates were washed in running tap water and air dried for 24 h. After addition of 0.1 M sodium citrate buffer, OD values were read at 550 nm on a Dynatech (Denkendorf, Germany) plate photometer.

*Production of FasL.* FasL was obtained from FasL-transfected murine N2A neuroblastoma cells, which secrete high amounts of soluble FasL into the supernatant (Rensing-Ehl et al., 1995; Schneider et al., 1998). One unit is defined as the activity of FasL required to kill 50% of LN-18 glioma cells in 100 μl volume 96-well assays.

*LFG antisense studies.* An LFG antisense phosphothioate oligonucleotide complementary to the region around the initiation codon (5′-GAG CTT TCC CCG GGT CAT GG-3′) of LFG was purchased from MWG

Biotech. A scrambled oligonucleotide composed of the same nucleotides (5′-CGT ATT GCC ATG CGG CGT CTC-3′) was used as a control. The sequence is not complementary to any eukaryote mRNA as controlled with the National Center for Biotechnology Information basic local alignment search tool Blast (http://www.ncbi.nlm.nih.gov/BLAST). Both oligonucleotides contained three phosphothioate linkages at the 3′ and the 5′ ends. The 5′ ends of both oligonucleotides were conjugated to fluorescein to monitor the transfection efficacy.

*Western blot and coimmunoprecipitation.* Western blotting was performed according to standard procedures (Gerhardt et al., 2001). For immunoprecipitation, HEK-293 cells were seeded in 10 $cm^2$ dishes. On the next day, the cells were transfected with 2 $\mu$g of plasmid using Effectene transfection reagent (Qiagen). The cells were collected 48 h later and lysed for 45 min on ice (140 mM NaCl, 20 mM Tris HCl, 1 mM $CaCl_2$, and 0.5% NP-40). After spinning down the pellet (13,000 $min^{-1}$, 4°C, 10 min), 250 $\mu$l of the supernatant were incubated for 2 h on ice with 1.5 $\mu$g of Fas antibody (C-20; Santa Cruz Biotechnology). Agarose-coupled A/G protein (Santa Cruz Biotechnology) was added, and, 1 h later, the pellet was washed three times with PBS and protease inhibitors. All samples (30 $\mu$g) were denaturated using 1% SDS/1% mercaptoethanol and used for Western blotting.

*Immunocytochemistry.* Cells were seeded on coverslips and transfected with $CaCl_2$ on DIV 6 as described previously (Kohrmann et al., 1999). After rinsing with PBS, cells were fixed with 4% paraformaldehyde for 5 min, washed, blocked with normal goat serum (10% in PBS with 1% Triton X-100), and incubated overnight with antibodies against Fas (1:1000) or LFG (1:500) in PBS containing 1% serum and 0.1% Triton X-100. After washing with PBS, the sections were incubated at room temperature for 2 h with carbocyanine 2- and/or 3-labeled secondary antibodies (Biotrend, Cologne, Germany). Cells were analyzed by confocal laser scanning microscopy (LSM 510; Zeiss, Jena, Germany).

*Transfection methods and flow cytometry.* CGNs were transfected using $CaCl_2$ or the GeneGun as described previously (Kohrmann et al., 1999; Gleichmann et al., 2002) as indicated. The oligonucleotides were delivered into CGNs using Effectene transfection reagent (Qiagen). Tumor cells were transfected using FuGene (Roche, Mannheim, Germany).

For flow cytometry, LN-18 cells were seeded in 24-well plates. On the next day, they were transfected with 0.3 $\mu$g of DNA per well [0.1 $\mu$g of green fluorescent protein (GFP) and 0.2 $\mu$g of the plasmid of interest or the control plasmid] using Effectene. After 24 h, 50 U/ml FasL was added, and, 18 h later, both adherent and floating cells were collected. The cells were once washed with PBS, fixed with 4% formaldehyde for 5 min, and washed with PBS and PBS containing 0.1% Triton X-100. The cells were then incubated for 30 min in PBS containing 1% propidium iodide and 1% RNase A and analyzed on a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ).

*Reporter gene assays.* The predicted promoter fragment of *LFG* (www.genomatix.com) was amplified from rat genomic DNA using the following primers: up, 5′-GTCCGGGAACCCTGATAGA-3′; and down, 5′-GAGTCAGGAGAGGATGCT-3′. The resulting fragment was further amplified in a nested PCR with the primer pair 5′-TTTCTCGAGCTCAGTCAATGG-3′ (up) and 5′-TCTAAGCTTATAGCAGGGAAG-3′ (down) and cloned into the *Xho*I and *Hind*III sites of pGL3-basic (Promega, Mannheim, Germany). For the assay, PC12 cells were seeded into 96-well plates ($2 \times 10^4$ cells per well) and left to adhere overnight, before they were cotransfected with 0.08 $\mu$g of LFG-Luc reporter gene plasmid, 0.02 $\mu$g of the respective activator plasmid (or irrelevant DNA), and 0.02 $\mu$g of pRL-cytomegalovirus (CMV) (Promega), using FuGene 6 (Roche). At 32 h after transfection, the cells were treated as indicated. Another 16 h later, Reporter Lysis Buffer (Promega) was added, and cell lysates were transferred to a LumiNunc plate (Nunc, Roskilde, Denmark). The respective activities of firefly and renilla reniformis luciferase were determined sequentially in a LumimatPlus (Berthold, Pforzheim, Germany) using a noncommercial dual luciferase assay (Dyer et al., 2000). Luciferin, coenzyme A, and coelenterazine were all from PJK (Kleinblittersdorf, Germany). Background was subtracted from all values, and the counts obtained from the measurement of firefly luciferase were normalized with respect to pRL-CMV.

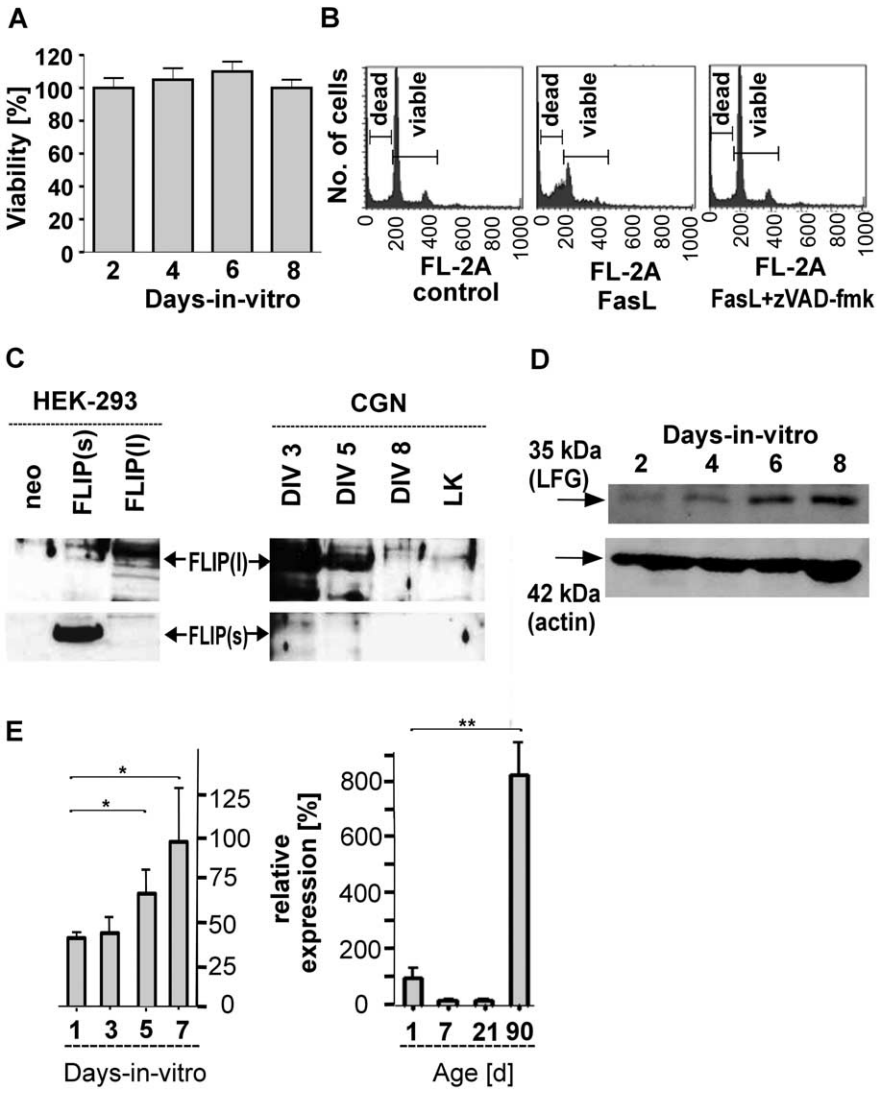



**Figure 1.** Resistance of CGNs against FasL-induced apoptosis and expression of LFG. ***A***, The resistance of CGNs cultured in medium containing 10% FCS to 500 U/ml FasL did not change in the course of *in vitro* maturation. CGNs were resistant to 500 U/ml FasL at all time points ($n = 3$ per time point). ***B***, To test the cross-reactivity of human FasL used in the experiments with rat Fas, rat lymphocytes isolated from spleens of 8-d-old rats were coincubated with FasL. Cell death was detected as the sub-$G_0/G_1$ peak by propidium iodide staining and flow cytometry at 16 h. ***C***, ***D***, The temporal profile of FLIP (long), FLIP (short), and LFG expression in CGNs was studied by Western blot analysis. Lysates from CGNs were prepared at the time points indicated, and 30 $\mu$g of protein was used per lane. As positive control lysates of HEK-293, cells were transfected with FLIP (long) and FLIP (short) using Effectene. FLIP was expressed in CGNs until DIV 5 but was no longer detected at DIV 8. In contrast, expression of LFG was upregulated from DIV 2 to DIV 8. ***E***, LFG mRNA expression in CGNs *in vitro* (left) and in the cerebellum *in vivo* (right) during maturation. LFG mRNA expression was assessed using RT-PCR and compared with GAPDH expression (*in vitro*) or actin expression (*in vivo*) shown as relative data ($n = 3$ per time point; $^*p < 0.05$, $^{**}p < 0.001$, ANOVA followed by Scheffe's *post hoc* test.).

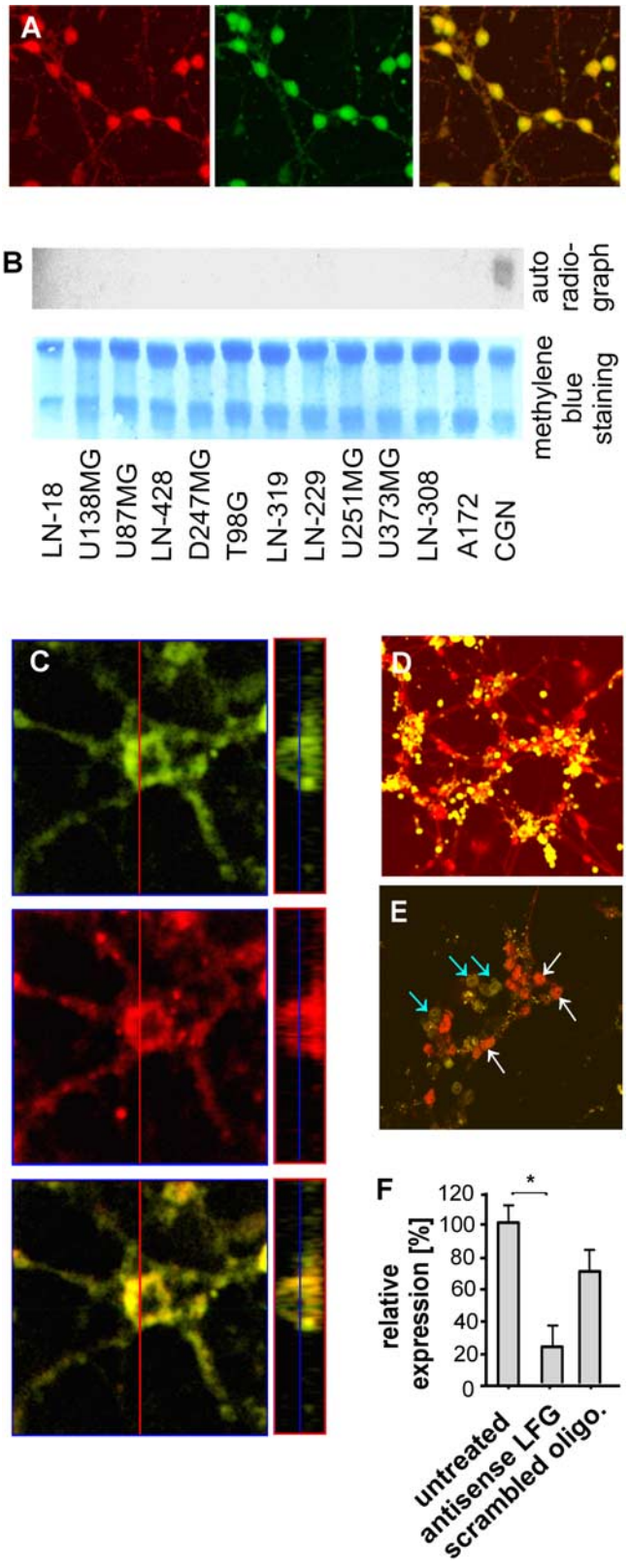



**Figure 2.** Colocalization of LFG and Fas in CGNs. ***A***, CGNs were stained for LFG (green) and NeuN (red). All LFG-positive cells showed colocalization with NeuN (yellow). ***B***, In contrast to all glioma cell lines tested, CGNs expressed LFG mRNA, as shown by Northern blot analysis. Methylene blue staining was performed as a loading control. ***C***, Confocal image of Fas (red)-labeled and LFG (green)-labeled CGNs viewed in three dimensions after orthogonal reconstruction of z-scans with a thickness of 0.77 $\mu$m. There is colocalization of both stainings in neurites, cytoplasm, and the cell membrane. ***D***, ***E***, Fluorescein-labeled antisense oligonucleotides (green) efficiently transfected CGN. Antisense oligonucleotides to LFG reduced the LFG levels in CGN. After transfection, the uptake of oligonucleotides was reflected by green fluorescence. Cells were fixed and stained for LFG (red). Those neurons with a strong uptake of fluorescein-labeled oligonucleotides showed no or weak LFG immunostaining (blue arrows), whereas CGNs with a limited uptake were still positive for LFG (white arrows). ***F***, LFG mRNA expression in CGNs, which were not transfected or transfected with antisense or scrambled oligonucleotides (oligo) for 18 h. LFG mRNA expression was assessed using RT-PCR and compared with actin expression shown as relative data ($n = 3$ per time point; $^*p < 0.05$).

*Statistical analysis.* Data are expressed as mean ± SEM. Statistical analysis was assessed by one-way ANOVA, followed by Scheffe's *post hoc* test. If not otherwise stated, all experiments reported were done with $n = 3$ per group or time point and represent one of at least three independent replications.

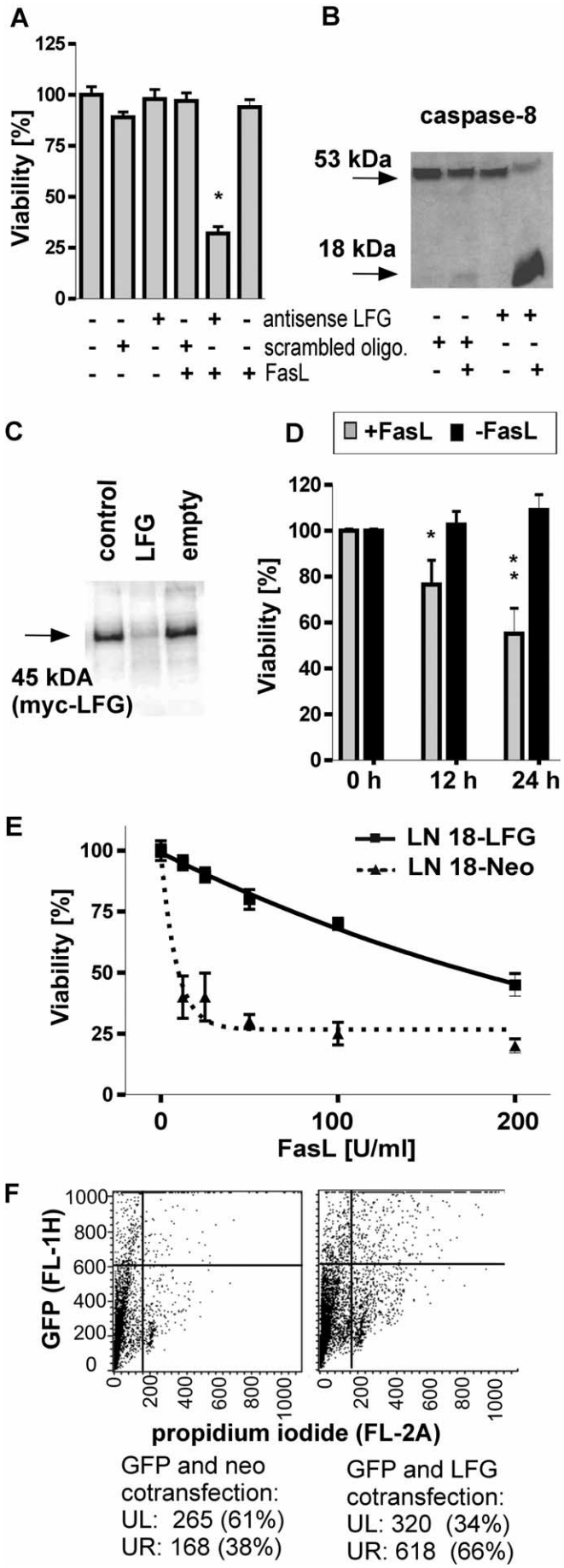
## Results

### Differentiated CGNs are resistant to FasL-induced apoptosis

We reported previously that potassium withdrawal-induced neuronal death is not altered in *lpr* and *gld* mice or by pharmacological inhibition of caspase-8 and that CGNs are resistant to exogenous FasL (Gerhardt et al., 2001). CGNs express Fas (Le-Niculescu et al., 1999; Gerhardt et al., 2001; Hou et al., 2002), but the exposure of CGNs to up to 500 U/ml FasL did not induce cell death (data not shown). To investigate whether the inhibition of the events leading to cell death in CGNs occurred upstream or downstream from the activation of caspases, we studied the processing of fodrin (also called spectrin), a specific substrate of caspase-3 (Jänicke et al., 1998). We did not detect any fodrin cleavage after the addition of up to 500 U/ml FasL for 24 h (data not shown). Because the level of Fas expression as well as of inhibitory molecules varies during neuronal maturation (Cheema et al., 1999), we investigated whether the sensitivity of CGNs changed during their *in vitro* maturation. No alteration was found from DIV 2 to DIV 8 (Fig. 1*A*). As a positive control for the effects of FasL on rat cells, we ascertained that lymphocytes from rat spleen undergo zVAD-fmk (*N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone)-sensitive apoptosis in response to treatment with 50–500 U/ml FasL (Fig. 1*B*).

### Endogenous LFG protects CGNs against FasL

To further investigate the mechanisms of resistance to FasL-mediated cell death in CGNs, we studied proteins that are known to confer resistance to FasL. The resistance of differentiated motor neurons to FasL is linked to the expression of FLIP (Raoul et al., 1999). The long isoform of FLIP was expressed in CGNs until DIV 5 but became undetectable at DIV 8 (Fig. 1*C*). Because the resistance of CGNs to FasL was unaltered during this period, we expected another protein to mediate resistance to FasL. A candidate protein was LFG, which inhibits the Fas-dependent killing pathway via an unknown mechanism (Somia et al., 1999). Its rat homolog, NMP35, is abundantly expressed in the CNS (Schweitzer et al., 1998) but has not yet been linked to neuronal apoptosis. In contrast to FLIP, protein expression of LFG was upregulated during the maturation of CGNs from DIV 2 to DIV 8 (Fig. 1*D*,*E*). This corresponds to a gradual increase in LFG protein expression in brain lysates taken from postnatal day 7 (P7), P14, P21, and P60 (Schweitzer et al., 1998). Using quantitative PCR, we found that this increased expression was caused by a transcriptional upregulation in CGNs *in vitro* and in the cerebellum *in vivo* (Fig. 1*E*). In contrast, Fas receptor expression remained unchanged during *in vitro* maturation (data not shown). Using an antibody (Schweitzer et al., 1998, 2002) against LFG, we detected LFG expression in all cultured CGNs (Fig. 2*A*)




but no colocalization with GFAP (data not shown). CGNs expressed LFG mRNA but none of the 12 glioma cell lines examined (Fig. 2*B*). We next investigated whether LFG interacted with components of the Fas signaling pathway. LFG and Fas exhibited similar expression patterns and colocalized when double stained with specific antibodies (Fig. 2*C*). We found intense staining in the cytosol, cellular membranes, and neurites. Furthermore, we confirmed the previous demonstration (Somia et al., 1999) that Fas and LFG interacted physically using ectopic expression of both proteins in HEK-293 cells and subsequent coimmunoprecipitation (data not shown).

To assess whether LFG was responsible for the resistance of CGNs to FasL, we transfected CGNs transiently with LFG antisense oligonucleotides. To monitor the transfection efficacy, the oligonucleotides were conjugated to fluorescein (Fig. 2*D*). Approximately 70% of the neurons were transfected with LFG antisense oligonucleotides. Transfected neurons showed decreased LFG staining compared with untransfected neurons (Fig. 2*E*). The antisense oligonucleotide-mediated downregulation of LFG was confirmed by quantitative RT-PCR (Fig. 2*F*). In contrast to the antisense oligonucleotides, scrambled oligonucleotides were not effective. Cells transiently transfected with LFG antisense oligonucleotides acquired sensitivity to FasL-induced cell death and caspase-8 cleavage (Fig. 3*A*,*B*). In contrast, CGNs transfected with scrambled oligonucleotides were still resistant to FasL (Fig. 3*D*). As a second method to decrease LFG expression, we used two different siRNA expressed in pSUPER-LFG (Brummelkamp et al., 2002) and directed against LFG, of which only one was successful (successful sequence is given in Materials and Methods). To confirm that the siRNA reduced LFG expression, myc-LFG and pSUPER-LFG were cotransfected into HEK-293 cells. The empty vector and a scrambled siRNA sequence were used as a negative control. Only pSUPER-LFG decreased the expression of myc-tagged LFG in HEK-293 cells (Fig. 3*C*). The siRNA-mediated reduction of LFG expression led to an increase of FasL sensitivity in CGNs (Fig. 3*D*). To confirm that LFG confers resistance to Fas-mediated apoptosis, we stably transfected the FasL-susceptible glioma cell line LN-18 with LFG. Approximately 80% of the cells stably ectopically expressed LFG. Expression of LFG was confirmed by immunocytochemistry (data not shown). LFG-transfected glioma cells were protected from FasL-induced cell death (Fig. 3*E*).

There has been a debate about whether or not potassium withdrawal-induced apoptosis is mediated by Fas. Although two

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**Figure 3.** LFG in FasL-induced apoptosis. ***A***, ***B***, Incubation with antisense LFG (0.5 pM) or FasL (500 U/ml) for 24 h alone did not alter cell survival, whereas cotreatment resulted in cell death and caspase-8 cleavage. Exposure to scrambled oligonucleotide (oligo) alone or in combination with FasL had no such effect. Cell viability was assessed by FDA staining ($n = 3$ per condition; $^*p < 0.001$, ANOVA followed by Scheffe's *post hoc* test). ***C***, To analyze the efficacy of the pSUPER-LFG siRNA vector, HEK-293 cells were cotransfected with pcDNA3-LFG and pSUPER-LFG, pSUPER control, or the empty vector using FuGene. Cells were lysed at 48 h after transfection. ***D***, The siRNA expressed in pSUPER-LFG sensitized CGNs to FasL-induced cell death in transfected cells. $CaCl_2$ was used for transfection. Four hours after transient cotransfection of CGNs with GFP and pSUPER (ratio of 1:2), 450 cells were evaluated per condition (−FasL, +FasL). Another 12 and 24 h later, the survival of the transfected CGNs was assessed by counting again the same visual fields. The viability compared with untreated conditions is given [$n = 3$ per condition; $^*p < 0.05$, $^{**}p < 0.01$ compared with untreated (0 h) condition]. ***E***, Stable overexpression of LFG protected LN-18 glioma cells from FasL-induced death. Viability was assessed by crystal violet staining ($n = 3$ per time point and condition). ***F***, LN-18 glioma cells were protected from FasL-induced cell death when transiently cotransfected with GFP and LFG (GFP/LFG of 2:1). Cell death was assessed 18 h after addition of 50 U/ml FasL by propidium iodide staining, followed by flow cytometric analysis of DNA content in GFP-positive cells.

groups reported that neutralizing anti-FasL antibodies protected from potassium withdrawal-induced apoptosis (Le-Niculescu et al., 1999; Castiglione et al., 2004), we and others did not find a role for Fas in this system (Gerhardt et al., 2001; Putcha et al., 2002). In agreement with our previous results, we did not find a protection from potassium withdrawal-induced apoptosis after forced expression of LFG in CGNs (data not shown).

To show that LFG prevents DNA loss as well as cell death, we cotransfected LN-18 glioma cells with GFP and LFG. After incubation with 50 U/ml FasL for 18 h, we determined DNA content by propidium iodide staining and subsequent flow cytometry (Fig. 3*F*). Whereas 61% of the GFP control cells exhibited reduced DNA content, this was true for only 34% of the cells cotransfected with GFP and LFG.

### FasL resistance of CGNs is mediated by phosphatidylinositol 3-kinase-dependent expression of LFG

The IGF-1/phosphatidylinositol 3-kinase (PI 3-kinase)-mediated pathway signals survival in paradigms of neuronal death, e.g., in potassium withdrawal-induced apoptosis of CGNs (Miller et al., 1997; D'Mello et al., 1998; Gleichmann et al., 2000). CGNs cultured in medium containing 10% FCS did not die when exposed to the PI 3-kinase inhibitors wortmannin or LY294002 [2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one]. In contrast, treatment of CGNs with PI 3-kinase inhibitors in combination with FasL concentration dependently induced cell death (Fig. 4*A*). The combination of the PI 3-kinase inhibitor wortmannin and FasL induced caspase-dependent fodrin cleavage, whereas either treatment alone was ineffective (Fig. 4*B*). Activation of caspase-8 and caspase-3 was observed after coincubation of CGNs with wortmannin and FasL for 6 h (Fig. 4*C*).

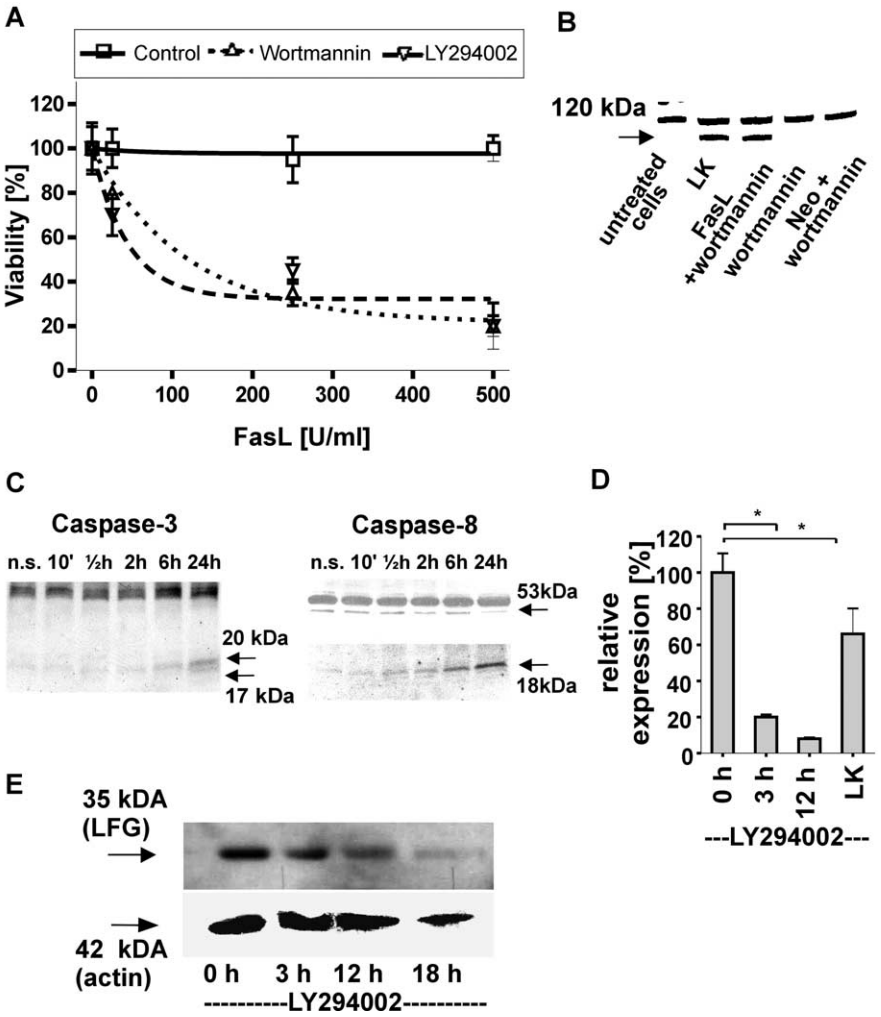


**Figure 4.** Inhibition of PI 3-kinase establishes sensitivity of CGNs to FasL. ***A–C***, CGNS treated with wortmannin (0.2 $\mu$M), LY294002 (10 $\mu$M), or FasL (25–500 U/ml) alone for 24 h did not undergo apoptosis. Only the combination of wortmannin (0.2 $\mu$M) and FasL (500 U/ml) caused a significant decrease in cell viability as well as fodrin cleavage at 24 h and caspase-8 and caspase-3 cleavage at the time points indicated. For Western blot analysis, 30 $\mu$g of protein per lane was used ($n = 3$ per condition). ***D***, ***E***, CGNs treated with LY294002 (10 $\mu$M) downregulated LFG mRNA and protein expression. mRNA content was assessed using real-time-PCR ($n = 2$ per time point). LFG mRNA bands were normalized to 18 S mRNA expression and shown as relative data (*$p < 0.05$; ANOVA followed by Scheffe's *post hoc* test). Protein levels were assessed by Western blot analysis. LK, Low potassium.

We then asked whether the increased sensitivity of CGNs treated with PI 3-kinase inhibitors resulted from decreased expression of LFG. Treatment with the PI 3-kinase inhibitor LY294002 induced a time-dependent decrease of LFG mRNA (Fig. 4*D*) and protein (Fig. 4*E*) expression. After 12 h, mRNA expression was reduced to <10% compared with untreated cells (Fig. 4*D*).

Because FasL-induced cell death in motor neurons is mediated by a DAXX–ASK1–p38 pathway (Raoul et al., 2002), we questioned whether or not the classical caspase-8-dependent pathway is relevant in CGNs. The activation of caspase-8 is a key event because its inhibition by 100 $\mu$M of a specific inhibitor of caspase-8, IETD-fmk [Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethyl ketone], almost completely prevented apoptosis and fodrin cleavage in CGNs treated with FasL and wortmannin (Fig. 5*A*,*B*). In contrast, IETD-fmk did not block potassium withdrawal-induced apoptosis (Fig. 5*A*), which is mediated via the endogenous, mitochondrial pathway involving the translocation of cytochrome *c* to the cytosol and the sequential activation of caspase-9 and caspase-3 (Gerhardt et al., 2001). To further confirm the interaction between the PI 3-kinase pathway and Fas, we treated CGNs from *lpr* mice with the activating Fas antibody Jo2 and wortmannin or LY294002, respectively (Fig. 5*C*). *lpr* mice lack a functional Fas receptor. Whereas CGNs from wild-type controls died when exposed to wortmannin and FasL or Jo2, CGNs from *lpr* mice were resistant (Fig. 5*C*).

To confirm that the increased sensitivity of CGNs to FasL-induced apoptosis after PI 3-kinase inhibition is caused by decreased LFG expression, we tested whether transient overexpression of LFG in this paradigm is protective. In contrast to control transfection with GFP, LFG overexpression provided almost complete protection against FasL-induced apoptosis after PI 3-kinase inhibition (Fig. 5*D*).

### The LFG promoter is regulated by PI 3-kinase activity

To further investigate the regulation of LFG transcription, we cloned the rat LFG promoter into the reporter plasmid pGL3-basic (Promega). Because of the poor transfection rates obtained

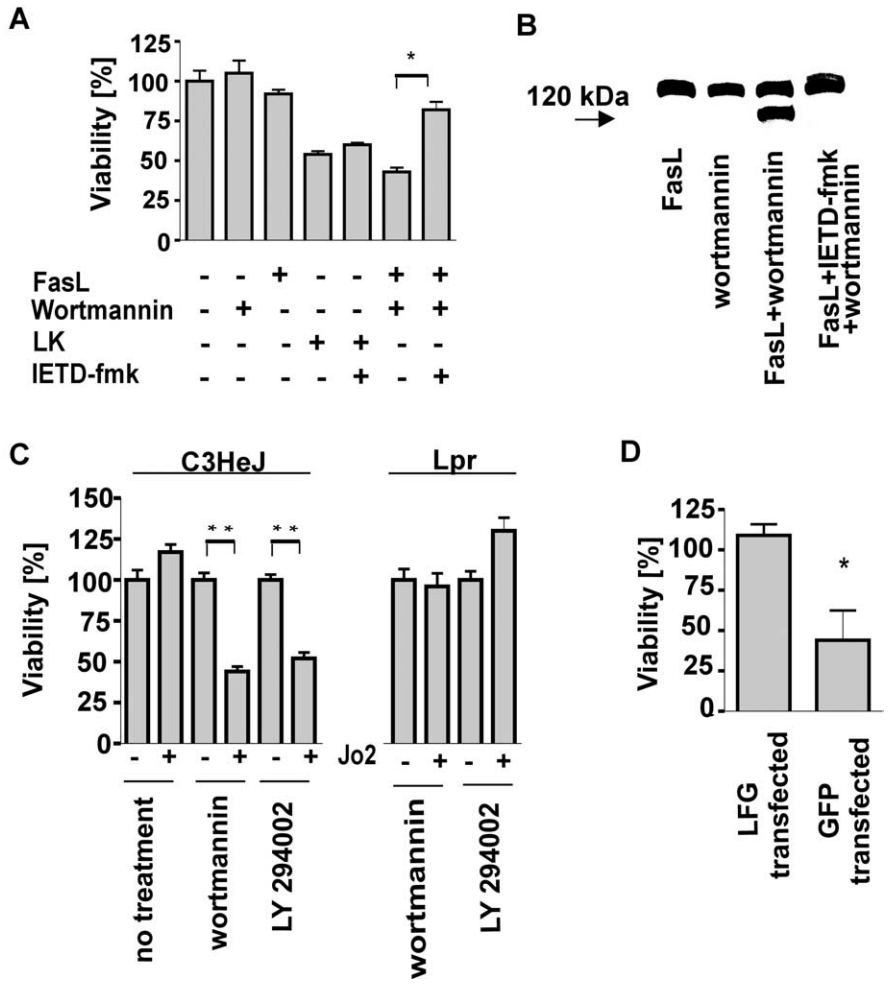


**Figure 5.** Activation of caspase-8 in FasL induced apoptosis of CGNs. ***A***, ***B***, The specific caspase-8 inhibitor IETD-fmk (100 $\mu$M) prevented cell death and fodrin cleavage induced by the combination of FasL (500 U/ml) and wortmannin (0.2 $\mu$M). Cell viability of CGNs was measured by FDA staining after 24 h of treatment with substances as indicated ($n = 3$ per condition; $^*p < 0.05$, ANOVA followed by Scheffe's *post hoc* test). LK, Low potassium. ***C***, CGNs from *lpr* mice were protected against cell death induced by the combination of the Fas agonistic antibody Jo2 (20 $\mu$g/ml) with wortmannin (0.2 $\mu$M) or LY294002 (10 $\mu$M), whereas CGNs obtained from strain-specific controls underwent apoptosis ($n = 3$ per condition; $^{**}p < 0.01$, ANOVA followed by Scheffe's *post hoc* test). ***D***, CGNs were cotransfected with GFP and pcDNA3-LFG or an empty vector (ratio 2:1) using the GeneGun. With this method ~2–5% of cells were transfected. Approximately 300 GFP-positive cells in two wells per condition were evaluated per condition (LFG vs empty vector). CGNs were then treated with the combination of 500 U/ml FasL and 0.2 $\mu$M wortmannin. Another 24 h later, the survival of the transfected CGNs was assessed by counting again the same visual fields. The viability compared with pretreatment conditions is given ($^*p < 0.05$; ANOVA followed by Scheffe's *post hoc* test).

with CGNs, we chose the rat pheochromocytoma cell line PC12 for the reporter gene experiments. PC12 cells endogenously express LFG (data not shown). Accordingly, there was a marked activation of the LFG reporter plasmid (LFG-Luc) in PC12 cells without stimulation (Fig. 6*A*). Treatment with NGF, an activator of PI 3-kinase, further increased the LFG-Luc signal. After incubation with the PI 3-kinase inhibitor LY294002, the activity of the LFG promoter was reduced close to background levels, suggesting that a PI 3-kinase-dependent signaling pathway is the main activator of LFG transcription in neuronal cells. This suppression of LFG activation was not reversed by the addition of NGF (Fig. 6*A*).

The activation of Akt/PKB by PI 3-kinase is essential for neuronal survival (Burgering and Coffer, 1995; Crowder and Freeman, 1998; Chan et al., 1999; Datta et al., 1999). We therefore asked whether Akt/PKB is involved in the regulation of LFG transcription. Cotransfection of the LFG reporter plasmid with DN-Akt/PKB but not with an empty plasmid blocked LFG reporter activity (Fig. 6*B*). Conversely, overexpression of gag-Akt/PKB (Bellacosa et al., 1991) was followed by an increase in LFG reporter activity. Furthermore, there was no reduction of LFG-Luc activation after addition of LY294002 if cells had been transfected with gag-Akt/PKB. Together, these results show an essential role of the PI 3-kinase–Akt/PKB signaling pathway in the regulation of LFG transcription.

Akt/PKB activates various transcription factors, including forkhead transcription factors and nuclear factor $\kappa$B (NF$\kappa$B). The rat LFG promoter cloned into LFG-Luc contained a consensus site for NF$\kappa$B. If NF$\kappa$B was of importance for the transcription of LFG, the inhibitory protein I$\kappa$B should suppress the LFG-Luc activation. Cotransfection of PC12 cells with I$\kappa$B and LFG-Luc led to a significant reduction of the LFG reporter activation compared with cells transfected with a control plasmid. Still, there was a marked activity left, suggesting that there is more than one transcription factor involved in the regulation of LFG (Fig. 6*C*).

## Discussion

Fas is a member of the TNF/NGF receptor family that triggers apoptosis. CGNs express Fas but are resistant to exogenous FasL or agonistic Fas antibodies (Gerhardt et al., 2001; Putcha et al., 2002), although this has been disputed by others (Le-Niculescu et al., 1999; Hou et al., 2002). The expression of c-FLIP appears to mediate this resistance in lymphocytes, endothelial cells, and differentiated spinal motor neurons (Irmler et al., 1997; Thome et al., 1997; Raoul et al., 1999; Scaffidi et al., 1999; Yeh et al., 2000; Panka and Mier, 2003). At DIV 8, this resistance appears to be independent of FLIP and, rather, attributable to LFG expression (Fig. 1*C–E*). Using a genetic screen, LFG was identified as protecting cells uniquely from FasL but not from TNF-$\alpha$ (Somia et al., 1999). We here show that CGNs, but not glial cells, expressed LFG. LFG colocalized (Fig. 2*C*) and physically interacted (Somia et al., 1999, and data not shown) with Fas. Furthermore, inhibition of LFG expression by treatment with antisense oligonucleotides or siRNA rendered CGNs susceptible to FasL (Fig. 3*A*,*D*). In contrast, expression of LFG in the FasL-susceptible glioma cell line LN-18 provided protection from FasL-induced cell death, proving that indeed LFG inhibits apoptosis mediated by Fas signaling (Fig. 3*E*,*F*).

The IGF-1–PI 3-kinase–Akt/PKB kinase-mediated pathway signals survival in paradigms of neuronal survival, e.g., in potassium withdrawal-induced apoptosis of CGNs (Miller et al., 1997; D'Mello et al., 1998; Gleichmann et al., 2000; Subramaniam et al., 2003). PI 3-kinase-generated phosphoinositides are important second messengers in neurons that are activated by various

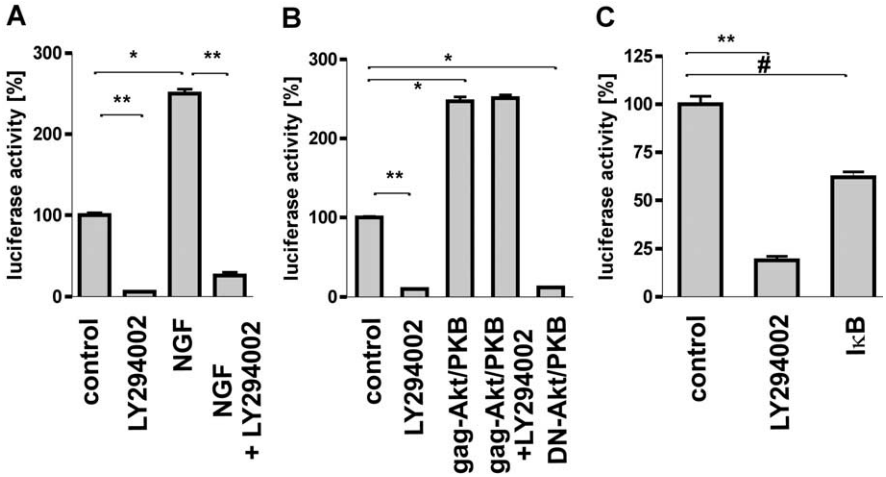


**Figure 6.** LFG promoter activity regulated by Akt/PKB and NFκB in PC12 cells. ***A***, PC12 cells were cotransfected (ratio of 4:1) with LFG-Luc and pCMV-RL and incubated as indicated (100 ng/ml NGF, 10 $\mu$M LY294002). Reporter gene activities were assessed after 16 h ($n = 5$; $^{\#}p < 0.05$, $^{*}p < 0.01$, $^{**}p < 0.001$, ANOVA followed by Scheffe's *post hoc* test). ***B***, ***C***, Cells were cotransfected with LFG-Luc, pCMV-RL, and the vectors given (ratio 4:1:1). Reporter gene activities were measured at 48 h after transfection and 16 h after treatment.

growth factors. They activate Akt/PKB, a kinase that interferes with the apoptotic cascades by phosphorylation of proteins such as Bax or caspase-9. Inhibition of Akt/PKB activation induces Fas-dependent apoptosis in endothelial cells, likely mediated by a downregulation of FLIP (Panka et al., 2001; Panka and Mier, 2003). Coexpression of Fas with constitutively active PI 3-kinase in COS7 cells resulted in protection from FasL-induced death (Hausler et al., 1998). An interaction between PI 3-kinase–Akt/PKB signaling and Fas has been described previously in T-cells in which PI 3-kinase inhibited the formation of Fas aggregates by mediating changes in the actin cytoskeleton (Varadhachary et al., 1999, 2001).

In our study, Fas-mediated apoptosis of CGNs was regulated by PI 3-kinase–Akt/PKB-dependent expression of LFG. Inhibition of PI 3-kinase activity with specific inhibitors was followed by a massive reduction of LFG mRNA and protein expression (Fig. 4*D*,*E*). This downregulation caused increased sensitivity to FasL-induced apoptosis (Fig. 4*A–C*). In other cell types, including T lymphocytes, endothelial cells, and different tumor cell lines, PI 3-kinase–Akt/PKB inhibition sensitizes to FasL toxicity (Hausler et al., 1998; Varadhachary et al., 1999, 2001; Panka et al., 2001; Suhara et al., 2001; Ivanov et al., 2002; Jones et al., 2002; Panka and Mier, 2003). As potential mechanisms an upregulation of FLIP (Panka et al., 2001; Panka and Mier, 2003), changes in the actin cytoskeleton (Varadhachary et al., 1999, 2001), Fas (Ivanov et al., 2002), or FasL expression (Suhara et al., 2001) or inhibition of procaspase-8 translocation to the death-inducing signaling complex (Jones et al., 2002) have been discussed. In CGNs, it is unlikely that mechanisms other than LFG are involved in this increased sensitivity to FasL, because overexpression of LFG completely protected CGNs against FasL toxicity after inhibition of PI 3-kinase activity (Fig. 5*D*).

In contrast to some reports (Datta et al., 1997; Miller et al., 1997; Shimoke et al., 1999) but in line with previous reports (Gleichmann et al., 2000; Subramaniam et al., 2003), we did not observe death in CGNs after the exposure to wortmannin or LY294002 as long as CGNs were not withdrawn from potassium. This might reflect differences in culture conditions.

To further investigate the intracellular signaling cascade regulating LFG expression, we cloned its promoter sequence into a reporter plasmid. Using this tool, we were able to investigate potential regulatory proteins involved in the regulation of LFG expression. The kinase Akt/PKB turned out to be a crucial member of the signaling cascade. Overexpression of DN-Akt/PKB resulted in a complete suppression of LFG-Luc activity (Fig. 6*B*). Furthermore, the transient overexpression of constitutive active Akt/PKB overcame the suppression of LFG transcription by PI 3-kinase inhibitors.

Akt/PKB activates several transcription factors, among them NFκB. Inhibition of NFκB by transient overexpression of IκB, an endogenous inhibitor of NFκB, was followed by a marked reduction of LFG-Luc activity, indicating that NFκB is involved in the transcriptional regulation of LFG expression.

We thus show here for the first time that expression of LFG is mediated by the PI 3-kinase–Akt/PKB pathway. Activators of PI 3-kinase, e.g., IGF-1 and several neurotrophins, including NGF, neurotrophin-3, and neurotrophin-4, provide neuroprotection in several paradigms of neuronal death (Zheng et al., 2002). Activation of the PI 3-kinase–Akt/PKB pathway protects cells through different pathways, e.g., phosphorylation of caspase-9, Bad, or forkhead family transcription factors (Datta et al., 1999). These molecular modifications all affect the endogenous, mitochondrial death pathway. In addition, in CGNs, Brunet et al. (1999) identified a forkhead transcription factor (FKHLR1) to be negatively regulated by Akt/PKB activation, to be retained in the cytoplasm by 14-3-3, and that FasL is a major mediator of this transcription factor (Brunet et al., 1999). The transcription of LFG can be added here as an important downstream target of Akt/PKB. By identifying LFG transcription as another target of Akt/PKB, this adds to the mechanisms of how neurons are protected from FasL toxicity.

In experimental conditions of epilepsy (Henshall et al., 2002), progressive motor neuropathy (Wagey et al., 2001), and global brain ischemia (Noshita et al., 2001), PI 3-kinase and/or Akt activities are differentially regulated, and inhibition of PI 3-kinase further exacerbates neuronal death. There is increasing interest in the role and regulation of Fas in neurons of the CNS, because its inactivation in *lpr* mice or with antagonistic antibodies confers protection in animal models of stroke and multiple sclerosis (Martin-Villalba et al., 1999, 2001; Waldner et al., 1997). As a consequence, activation of Fas must be considered as a key event for the induction of neuronal apoptosis in these diseases. In contrast to these results, healthy neurons do not appear to be vulnerable to FasL-mediated toxicity because neurons express LFG throughout the brain. At the moment, it remains a challenging hypothesis whether, in disease conditions such as ischemia, Parkinson's disease, or multiple sclerosis, the expression of LFG is downregulated, resulting in a sensitization of the Fas/FasL cell death pathway. Activation of the PI 3-kinase–LFG pathway might become a promising target for the acute treatment of neurological disorders.

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