Lifeguard Inhibits Fas Ligand-mediated Endoplasmic Reticulum-Calcium Release Mandatory for Apoptosis in Type II Apoptotic Cells*

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Death receptors are members of the tumor necrosis factor receptor superfamily involved in the extrinsic apoptotic pathway. Lifeguard (LFG) is a death receptor antagonist mainly expressed in the nervous system that specifically blocks Fas ligand (FasL)-induced apoptosis. To investigate its mechanism of action, we studied its subcellular localization and its interaction with members of the Bcl-2 family proteins. We performed an analysis of LFG subcellular localization in murine cortical neurons and found that LFG localizes mainly to the ER and Golgi. We confirmed these results with subcellular fractionation experiments. Moreover, we show by co-immunoprecipitation experiments that LFG interacts with Bcl-XL and Bcl-2, but not with Bax or Bak, and this interaction likely occurs in the endoplasmic reticulum. We further investigated the relationship between LFG and Bcl-XL in the inhibition of apoptosis and found that LFG protects only type II apoptotic cells from FasL-induced death in a Bcl-XL dependent manner. The observation that LFG itself is not located in mitochondria raises the question as to whether LFG in the ER participates in FasL-induced death. Indeed, we investigated the degree of calcium mobilization after FasL stimulation and found that LFG inhibits calcium release from the ER, a process that correlates with LFG blockage of cytochrome c release to the cytosol and caspase activation. On the basis of our observations, we propose that there is a required step in the induction of type II apoptotic cell death that involves calcium mobilization from the ER and that this step is modulated by LFG.

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34 The abbreviations used are: DR, death receptor; C-ter, C-terminal; DISC, death-inducing signaling complex; ER, endoplasmic reticulum; FasL, Fas ligand; FLIP, Flice inhibitory protein; LFG, lifeguard; N-ter, N-terminal; TMBIM, transmembrane Bax inhibitory motif; ANOVA, analysis of variance.
LFG Impairs Ca\textsuperscript{2+} Mobilization after FasL Treatment

Here we performed a study to elucidate the detailed subcellular distribution of LFG. We demonstrate that it localizes mainly at the ER membrane of neurons, whereas no co-localization with mitochondria markers was found. We also studied the relation of LFG with Bcl-2 family members, showing that it interacts with Bcl-X\textsubscript{L} and Bcl-2 but not with Bax or Bak. In addition, we also demonstrate that LFG overexpression protects type II apoptotic cells, but not type I, from FasL-induced apoptosis and that this protection depends on the endogenous expression of Bcl-X\textsubscript{L}. Additionally, LFG was found to modulate calcium release from the ER after FasL treatment. Our results suggest a novel function of LFG in the ER relevant to Fas-mediated cell death.

**Experimental Procedures**

**Reagents**—Recombinant human FasL expressing plasmid was a generous gift from Pascal Schneider (23). Fluorogenic caspase substrate Ac-DEVD-afc was purchased from Calbiochem/Merck Biosciences. Unless otherwise specified, all biochemicals required. Oligonucleotides were obtained from Sigma-Aldrich.

**Plasmids**—LFG, LFG-FLAG, 3\times HA-Bcl-X\textsubscript{L}, Bcl-2, 3\times HA-Bax, 3\times HA-Bad, and 3\times HA-FLIP were expressed under the control of a cytomegalovirus constitutive promoter in the pcDNA3 expression vector (Invitrogen). LFG, Bcl-X\textsubscript{L}, and FLIP cDNAs were also subcloned into the pEIGW vector, giving rise to the lentiviral LFG-, Bcl-X\textsubscript{L}-, and FLIP-overexpressing plasmids. For RNAi experiments, constructs were generated in pSUPER.retro.puro (OligoEngine) using specific oligonucleotides targeting Bcl-X\textsubscript{L} and LFG sequences as follows: shBcl-X\textsubscript{L}, GATTGCAAGTTGGATGCG; and shLFG, GGAGAGAAGGATGCT. The control scrambled sequence was GGTCCCTTTCCTCTGTAGT. Adaptors to clone the oligonucleotides into the BglII/HindIII sites of pSUPy.retro.puro were added as required. Oligonucleotides were obtained from Sigma-Aldrich. Lentiviral constructs were generated by digesting pSUPER-sh with EcoRI and ClaI to replace the H1 promoter with the H1-shRNA cassette in pLVTHM. Truncated forms of LFG were amplified from LFG-FLAG-pcDNA3 plasmid using the following primers: Fw-LFG-N-ter, CCAAAAGCTTTAGCCCAAGGAAAGCT-CCTCTGTGG; and Rv-LFG-N-ter, TTGCGGCCGCAAGAGGACGC-GACGCGAATTTTCTGCTAT. For the C-ter-LFG construct, amplified cDNA was digested with HindIII/NotI and inserted into the pcDNA3 plasmid. For the N-ter-LFG construct, amplified cDNA was digested with EcoRI/NotI and inserted into the pcDNA3 plasmid.

**Lentiviral Production**—HEK293T cells were seeded at a density of 1 \times 10^5 cells/cm\textsuperscript{2}. Cell culture plates were kept at 37 °C in a humidified incubator with 5% CO\textsubscript{2}/95% air.

**Cell Culture**—HEK293T, HEK293, and SK-N-AS cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 20 units/ml penicillin, and 20 \mu g/ml streptomycin. Cell culture plates were kept at 37 °C in a humidified incubator with 5% CO\textsubscript{2}, 95% air.

**Western Blot**—Cells were harvested and rinsed once with ice-cold 1× PBS, pH 7.2, and lysed in immunoprecipitation lysis buffer (20 mM Tris, pH 7.4, 140 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM EGTA, and 1% Triton X-100) supplemented with 1× EDTA-free Complete protease inhibitor mixture (Roche). They were then centrifuged at 16,000 \times g for 4 °C for 30 min, and the supernatants were collected. Protein concentration was quantified by a modified Lowry assay (DC protein assay; Bio-Rad). The cell lysates obtained were resolved by SDS-PAGE and transferred onto PVDF Immobilon-P membranes (Millipore). After blocking with 1× TBS, 0.1% Tween 20 containing 5% nonfat dry milk for 1 h at room temperature, membranes were probed with the appropriate primary antibodies. They were then incubated for 1 h with the appropriate specific peroxidase-conjugated secondary antibody. Membranes were developed using the EZ-ECL chemiluminescence detection kit (Biological Industries).

The following primary antibodies were used: anti-FLAG (1:2000; Sigma), anti-LFG (1:200; Santa Cruz), anti-HA (1:2000; Sigma), anti-Bcl-2 (1:1000; BD Biosciences), anti-Bcl-X\textsubscript{L} (1:1000; Sigma), anti-GlutR2 (1:500; Millipore), anti-Rab5 (1:1000; Cell Signaling), anti-calnexin (1:1000; Cell Signaling), anti-histone H3 (1:1000; Cell Signaling), anti-GAPDH (1:500; Santa Cruz), anti-cytochrome c (1:1000; BD Biosciences), and anti-caspase-8 (1:1000; Cell Signaling). Naphtol
Blue staining of the PVDF membrane verified equal loading between lanes.

**Immunoprecipitation**—Samples were lysed in immunoprecipitation lysis buffer as described above, and lysates were quantified by a modified Lowry assay (DC protein assay; Bio-Rad). 1 mg of total protein was adjusted with immunoprecipitation buffer to achieve a concentration of 1 mg/ml. A total of 30 μl of anti-FLAG M2-agarose-coupled antibody was added to each sample and incubated overnight at 4 °C in an orbital shaker. Beads were then washed twice with immunoprecipitation buffer and three more times with 1× TBS. They were then eluted for 30 min at 4 °C with 100 μl of immunoprecipitation lysis buffer containing 10 mM HEPES/NaOH, pH 7.4, 300 mM NaCl, 10 mM DTT, 5 mM EDTA, 1% Triton X-100, 0.1% CHAPS, and 1× EDTA-free Complete protease inhibitor mixture (Roche). The lysates were cleared by centrifugation at 16,000 × g at 4 °C for 10 min, and supernatants were quantified by the Bradford method (Bio-Rad). Assays were performed in triplicate using 25 μg of protein in the same specific lysis buffer supplemented with 50 μM of the fluorogenic substrate Ac-DEVD-afc. After incubation for 1 h at 37 °C, plates were read in a fluorimeter using a 360-nm (40 nm bandwidth) excitation filter and a 530-nm (25 nm bandwidth) emission filter.

**Cell Viability Assays**—For apoptotic nuclear morphology, the cells were seeded in 24-well plates and treated as indicated. They were then fixed with 2% p-formaldehyde and stained with 0.05 μg/ml of Hoechst 33258 for 30 min at room temperature in a buffer composed of 2% p-formaldehyde and 1% Triton X-100. Condensed or fragmented nuclei were counted as dead cells, as described previously (25). The determination of cell death by chromatin condensation was performed in blind testing, counting at least 1000 cells for each data point, and was repeated at least three times in independent experiments.

**Immunofluorescence**—Embryonic day 16 cortical neurons at 1 day in vitro were transduced or not with LFG-FLAG-overexpressing lentiviral particles. Seventy-two hours later they were rinsed with PBS at room temperature and fixed in 4% p-formaldehyde/PBS for 30 min at room temperature. Next, they were washed twice with PBS and subsequently permeabilized and blocked with 5% bovine serum albumin and 0.1% Triton X-100 in PBS for 90 min at room temperature. The cells were incubated overnight with the indicated antibodies, rinsed three times with PBS, and incubated with secondary antibodies as indicated for 1 h at room temperature protected from light. Confocal micrographs were obtained using a FluoView1000 spectral confocal microscope.

The following primary antibodies were used: anti-FLAG (1:2000; Sigma), anti-LFG (1:100, Anaspec), anti-calnexin (1:50; Cell Signaling), anti-Rab11 (1:100; Cell Signaling), anti-GM130 (1:25; BD Biosciences), anti-EAA1 (1:200; BD Biosciences), anti-Rab11 (1:50; BD Biosciences), anti-Rab7 (1:250; Sigma), anti-TGN38 (1:200, Affinity Bioreagents), anti-Bcl-XL (1:250; Sigma), Mitotracker (Life Technologies), and Lysotracker (Life Technologies). Alexa Fluor 488, 568, and 594 were used as secondary antibodies at a dilution of 1:300.

**Image Analysis**—Co-localization was quantified in 10–15 randomly chosen cells from each sample using ImageJ (JaCoP) software to calculate Manders’ co-localization coefficients (M1 and M2) and Pearson’s correlation coefficient (R) (26). Threshold values were determined using the method described by Costes (27), incorporated in the JaCoP software. Only pixels whose red and green intensity values were both above their respective thresholds were considered to be pixels with co-localized probes. M1 and M2, corresponding to channel 1 (LFG) or channel 2 (other markers), were then calculated as the fractions of total fluorescence in the region of interest that occurred in these “co-local” pixels (with a higher value indicating more co-localization). The M1 values shown represent mean percentages of co-localization.
Cytosolic Calcium Measurements—Cells were incubated in prewarmed control buffer (140 mM NaCl, 3.6 mM KCl, 1.2 mM MgSO₄, 1 mM CaCl₂, 20 mM HEPES sodium salt, and 5 mM glucose, pH 7.4) in the presence of 5 μM fluo-4 acetoxymethyl ester (F-14201; Molecular Probes) for 30 min at 37 °C, protected from light. Buffer with the dye was washed, and fresh prewarmed control buffer was added.

The cells were excited at 488 nm using an argon/krypton laser confocal system (Yokogawa CSU10, Nipkow spinning disk) set on an Olympus IX70 (VoxCell Scan; Visitech) with a 60×/1.100 oil immersion objective lens. Emission was captured by a digital CCD camera (ORCA, Hamamatsu, Japan) and analyzed using VoxCell Scan Software (Visitech).

After measurement, each cell in the captured fields was analyzed separately. Cells with a fold-induction of calcium release ≥ 1.2 were considered for further analysis. The cells were normalized at the time point they began to react after Fc-FasL treatment.

Statistical Analysis—All the experiments were repeated at least three times. The values are expressed as means ± S.D.

Results

LFG Localizes Mainly at the ER and Golgi Membranes—LFG has been reported to localize at the plasma membrane (18) and trans-Golgi network membrane (19); however, a more detailed study of its distribution inside the cell is needed. To determine LFG neuronal subcellular localization, we examined its co-localization with markers of cellular organelles and Manders’ co-localization coefficients (M1) and Pearson correlation coefficients (Rr) were obtained for each marker (Fig. 1, A and B). LFG co-localized with the ER marker calnexin (M1: 34.6 ± 3.6; Rr: 0.562 ± 0.032), the Golgi marker GM130 (M1: 22.8 ± 1.0; Rr: 0.582 ± 0.04), and the early endosome marker Rab5 (M1: 28.9 ± 1.9; Rr: 0.405 ± 0.039). Moreover, LFG presented a finely punctate pattern with some aggregation in the perinuclear region, which suggests an endoplasmic and vesicular localization. In contrast, no significant co-localization was observed with Mitotracker (M1: 4.5 ± 0.3; Rr: 0.105 ± 0.023), a mitochondrial marker, or Hoechst staining (M1: 8.6 ± 2.4; Rr: 0.057 ± 0.038), a nuclear marker (Fig. 1B). Thus, these observations suggest that LFG localizes at ER.

LFG Is Localized along the Endocytic Pathway—Because the localization of LFG appears to be vesicular, we sought to study the localization of LFG along the endocytic pathway in greater depth. LFG co-localization with markers of proteins of early, late, and recycling endosomes, lysosomes, as well as markers of the ER and trans-Golgi network, was analyzed (Fig. 2A). LFG preferentially co-localized with BiP (M1: 77.1 ± 7.7; Rr: 0.715 ± 0.028), an ER marker, as values for coefficients of LFG with these organelles are significantly higher than for endosome markers, EEA1 (M1: 45.8 ± 2.6; Rr: 0.53 ± 0.015), Rab7 (M1: 54.1 ± 2.9; Rr: 0.702 ± 0.032), Rab11 (M1: 56.7 ± 4.8; Rr: 0.666 ± 0.086), Lysotraker (M1: 56.4 ± 3.8; Rr: 0.537 ± 0.031),

FIGURE 1. LFG subcellular localization. A, representative confocal images of cortical neurons. Cortical neurons were fixed, permeabilized, and immunostained with anti-LFG (green), Mitotracker (mitochondria marker), Hoechst (nucleus marker), anti-calnexin (ER marker), anti-Rab5 (early endosome marker), and anti-GM130 (Golgi marker) (red). The third column shows a merge of the green and red channels. Scale bar, 10 μm. B, Manders’ co-localization coefficients for LFG are shown as percentage of LFG co-localization with each marker (one-way ANOVA test). ***, p < 0.001; **, p < 0.005; *, p < 0.05). The values of all Manders’ co-localization coefficients and Pearson correlation coefficients for each condition are detailed under “Results.”
and TGN38 (M1: 65.9 ± 8.2; R: 0.754 ± 0.059) (Fig. 2B). Altogether, these results indicate that most cellular LFG in neurons is located at the ER and, to a lesser extent, at the Golgi and vesicular membranes.

**LFG Interacts with Bcl-XL**—LFG is a member of the TMBIM family of proteins (28). Some of these members have the capacity to modulate the activity of Bcl-2 family members (29), such as Bcl-XL and Bcl-2, which are found in mitochondria but also in the ER. We hypothesized that LFG also modulates the activity of Bcl-2 family members in a similar manner. Therefore, we sought to determine LFG interaction with Bcl-2 family proteins. In this regard, we observed that LFG co-immunoprecipitated with Bcl-XL and Bcl-2 but not with Bax or Bak (Fig. 3A).

Because Bcl-XL and LFG are highly expressed in the CNS (30, 31), we focused our attention on the LFG-Bcl-XL relationship. To further characterize this interaction, we constructed truncated forms of LFG (Fig. 3B). The first 101 amino acids of the LFG sequence, corresponding to the N-ter unfolded domain, were subcloned and fused to a 3×FLAG tag in a pcDNA3 expression vector to form the 3×FLAG-C-LFG construct. The rest of the sequence, corresponding to the transmembrane domain with the C-ter tail, was subcloned along with a FLAG tag and a methionine residue at the start to initiate the open reading frame, forming the ΔN-LFG-FLAG vector. These constructs and LFG-FLAG were overexpressed, along with 3×HA-Bcl-XL. FLAG immunoprecipitation and HA detection revealed that LFG interacts with Bcl-XL through its C-ter region (Fig. 3C). Endogenous co-localization of LFG with Bcl-XL in cortical neurons was also studied by immunofluorescence. We observed LFG co-localization with Bcl-XL (Fig. 3D; M1: 47.6 ± 2.9; R: 0.558 ± 0.048), located at the cytosol excluding the nucleus, with a stronger staining in the perinuclear region, thereby suggesting ER co-localization. To further confirm the observation that LFG interacts with Bcl-XL and to rule out possible bias in overexpression experiments, we performed an immunoprecipitation of endogenous Bcl-XL in lysate from adult mice cerebellum, in which both proteins are highly expressed. We observed that LFG is co-immunoprecipitated with Bcl-XL (Fig. 3E). To study in more detail the localization of LFG-Bcl-XL interaction, we performed a subfractionation assay in lysate from adult mice brain. LFG is detected in the heavy and light membrane fractions (Fig. 3F), further confirming our results about LFG localization in the ER and endocytic vesicles membranes. Moreover, Bcl-XL is also detected in the heavy and light membranes fraction. Thus, all these data indicate that LFG interacts with Bcl-XL, and this interaction is likely to occur in the ER membrane.

Although LFG is not localized at the mitochondria, it protects type II cells from FasL-induced cell death—LFG prevents FasL-induced cell death in neurons and neuronal-like cells among others (16, 20), all of them classified as type II apoptotic cells. Nonetheless, there is no direct evidence that LFG protects...
LFG Impairs Ca\(^{2+}\) Mobilization after FasL Treatment

A

+LFG-FLAG

| Treatment    | HA   | Bcl-2 | FLAG |
|--------------|------|-------|------|
| 3xHA-Bcl-xL  |      |       |      |
| 3xHA-Bax     |      |       |      |
| Bcl-2        |      |       |      |
| 3xHA-Bax     |      |       |      |
| Bcl-2        |      |       |      |
| 3xHA-Bax     |      |       |      |

IP FLAG | INPUT

M\(_f\) (K)

11
26
37

B

\(\Delta N\)-LFG-FLAG (~28 kDa)

3xFLAG-ΔC-LFG (~16 kDa)

LFG-FLAG (~36 kDa)

C

+3xHA-Bcl-xL

| Treatment    | HA   | FLAG |
|--------------|------|------|
| LFG-FLAG     |      |      |
| \(\Delta N\)-LFG-FLAG |      |      |
| 3xFLAG-ΔC-LFG |      |      |
| LFG-FLAG     |      |      |
| \(\Delta N\)-LFG-FLAG |      |      |
| 3xFLAG-ΔC-LFG |      |      |

IP FLAG | INPUT

M\(_f\) (K)

35
36
28
16

D

Hoechst | LFG

Bcl-xL | Merge

E

IP

| Treatment | M\(_f\) (K) |
|-----------|------------|
| control IgG |           |
| Bcl-xL     |           |

F

| Protein    | N | HM | LM | Mi | Cy | M\(_f\) (K) |
|------------|---|----|----|----|----|-------------|
| LFG        |   |    |    |    |    | 35          |
| Bcl-XL     |   |    |    |    |    | 28          |
| Rab5       |   |    |    |    |    | 24          |
| Calnexin   |   |    |    |    |    | 20          |
| CytC       |   |    |    |    |    | 14          |
| GluR2      |   |    |    |    |    | 100         |
| H3         |   |    |    |    |    | 15          |
| GADPH      |   |    |    |    |    | 37          |
type I cells against FasL-induced apoptosis. To address this question, we performed a comparative study of LFG inhibition of FasL-induced apoptosis in type I and neuronal-like type II apoptotic cells. To this aim, we overexpressed this protein in parallel with other anti-apoptotic proteins (Bcl-XL and FLIP) in HEK293 and SK-N-AS cells as models of type I and type II apoptotic cells, respectively. SK-N-AS cells are neuroblastoma-derived cells that are sensitive to Fas-L-induced apoptosis and behave as type II apoptotic cells as expected by their neuronal origin. Bcl-XL inhibits FasL-induced apoptosis in type II cells but not in type I FLIP, which competes with caspase-8 for recruitment to the DISC after DR stimulation (32), protects against DR-induced apoptosis in type I and II cells.

Our results show that FasL-induced cell death in SK-N-AS cells, as measured by apoptotic nuclei counting, was inhibited by Bcl-XL and LFG overexpression at 24 and 48 h after treatment (Fig. 4C and data not shown). Such inhibition was not observed in HEK293 cells, where only FLIP overexpression prevented apoptosis (Fig. 4D). Moreover, LFG overexpression, as well as that of Bcl-XL, decreased caspase-3 activation in SK-N-AS (Fig. 4A) cells but not in HEK293 cells (Fig. 4B). The efficiency of overexpression of the DR antagonists was assessed by Western blot (Fig. 4G). Altogether, these data demonstrate that LFG overexpression protects type II apoptotic cells, but not type I apoptotic cells, against Fas-induced cell death.

Requirement of Endogenous Bcl-XL for LFG Anti-apoptotic Effects—Our findings that LFG protects type II apoptotic cells from Fas-induced cell death and that it interacts with Bcl-XL suggested that one of these molecules might depend on the other to exert its anti-apoptotic effects. To study this hypothesis, we used SK-N-AS cells, which express both LFG and Bcl-XL endogenously.

Cells were transduced with lentiviruses carrying LFG, Bcl-XL, or Scrambled shRNA, and after 3 days of transduction they were infected again with overexpression lentiviral particles for LFG or Bcl-XL for 3 additional days. Knockdown efficiency was determined by Western blot (Fig. 5C). LFG and Bcl-XL overexpression reduced caspase activity and the percentage of apoptotic nuclei after Fas-L treatment (Fig. 5, A and B), in concordance with previous results. However, when Bcl-XL was down-regulated, LFG overexpression was unable to protect SK-N-AS cells. This was not the case in the opposite situation; thus Bcl-XL overexpression maintained its anti-apoptotic activity despite endogenous LFG down-regulation (Fig. 5, A and B). On the basis of these observations, we conclude that endogenous levels of Bcl-XL are required for LFG anti-apoptotic action, whereas protection from FasL-induced cell death by Bcl-XL overexpression does not require LFG.

LFG Overexpression Down-regulates Calcium Release from the ER after FasL Stimulation—Calcium transfer from the ER to mitochondria is a crucial step in various forms of cell death (33, 34), including FasL-induced apoptosis, as demonstrated in experiments in Jurkat cells (35). Bcl-XL exerts anti-apoptotic effects in the ER by blocking calcium release (36, 37). In the same way, BI-1, also known as TMBIM5, modulates calcium release from the ER (29) and has an anti-apoptotic role. Because LFG is also a member of the TMBIM family, and we have demonstrated that it exerts its anti-apoptotic effects in a Bcl-XL-dependent manner, we hypothesized that it may be involved in the regulation of calcium release from the ER after Fas engagement.

After FasL treatment, SK-N-AS cells released calcium from the ER (Fig. 6A), as demonstrated by the lack of calcium release after depletion of the ER calcium content by pretreatment with the sarcoplasmic/endoplasmic reticulum calcium-ATPase inhibitor thapsigargin (Fig. 6A). This is the first report involving calcium mobilization from the ER after FasL stimulation in cells from neuronal lineage. In LFG-overexpressing SK-N-AS cells, calcium release from the ER was significantly decreased (Fig. 6, A and D), and as expected and previously described (36–38), Bcl-XL overexpression dramatically abrogated calcium release (Fig. 6, A and D). On the other hand, LFG or Bcl-XL down-regulation did not significantly alter calcium release from the ER (Fig. 6B); nevertheless, these cells showed a tendency to reach higher rates of calcium release than the scrambled control. Strikingly, Bcl-XL overexpression in shLFG-transduced cells was unable to inhibit calcium release from the ER (Fig. 6C). This indicates that Bcl-XL effects on the ER are dependent on LFG expression, and because these cells are resistant to FasL-induced apoptosis (Fig. 5, A and B), it suggests that the Bcl-XL anti-apoptotic effect in the mitochondria is enough to protect from FasL-induced apoptosis. LFG overexpression in shBcl-XL-transduced cells was unable to prevent calcium release from the ER (Fig. 6C), in accordance with previous results that showed a requirement of Bcl-XL for LFG anti-apoptotic effects. To rule out that the observed effects are not due to changes in ER luminal calcium concentration, we compared the baseline levels of each condition. Baseline levels of intracellular calcium fluorescence were similar in all groups of treatment (data not shown). Our data are in agreement with previously reported studies that point out Bcl-XL regulation of ER calcium efflux, rather than decreasing intraluminal calcium content, as the mechanism involved in its anti-apoptotic role in the ER (37, 38).
LFG Impairs Ca\(^{2+}\) Mobilization after FasL Treatment

**A**

![Graph A]

**B**

![Graph B]

**C**

![Graph C]

**D**

![Graph D]

**E**

![Image E]

**F**

![Image F]

**G**

![Image G]
In summary, LFG and Bcl-X₁ overexpressing cells showed a marked reduction in calcium release after FasL treatment when compared with sh scrambled-transduced cells (Fig. 6A), whereas down-regulation of LFG or Bcl-X₁ did not induce a significant increase (Fig. 6B). Overexpression of LFG in shBcl-X₁-transduced cells showed no effect, in the same way as Bcl-X₁ overexpression in shLFG-transduced cells (Fig. 6C). Taken together, these results suggest that LFG anti-apoptotic effects are mediated by inhibition of calcium release from the ER and that Bcl-X₁ is essential for this step. Notably, Bcl-X₁ effects on calcium release also appear to be dependent on LFG expression.

FasL-induced Calcium Release from the ER Is Mediated by Caspase-8 Activation in Neuron-like Cells—It has been reported that ER calcium release is mediated by caspase-8 activation and the cleavage of its substrate Bap31 (35, 39). We decided to explore this pathway in our neuronal-like model SK-NA-S cells. Effectively, we observed that ER calcium release after FasL treatment was dependent on caspase-8 activation, because no calcium release from the ER was detected after FasL stimulation when cells were pretreated with the caspase-8 inhibitor IETD (Fig. 7A). We also observed the cleavage of procaspase-8 in our conditions after FasL treatment (Fig. 7B). More importantly, we were able to detect the cleavage of Bap31 in its p20 fragment, which in turn participates in the cytochrome c release from the mitochondria by enhancing calcium signals (40) (Fig. 7C). The observed cleavage was caspase-8-mediated, because its inhibition by IETD or the competitive antagonist, FLIP, resulted in no cleavage of Bap31. These data indicate that in neuronal-like cells, caspase-8 activation after FasL stimulation is necessary for ER calcium release.

LFG Overexpression Inhibits Cytochrome c Release from the Mitochondria after FasL Stimulation—Because calcium mobilization from the ER to mitochondria has been described as an important step in the inducement of mitochondria permeabilization (35), we sought to study the effects of LFG in this process. After 6 h of FasL treatment, cytochrome c could be detected in the cytosolic fraction of sh scrambled-transduced cells (Fig. 7D). As has already been described, Bcl-X₁ overexpression inhibited mitochondria permeabilization, as it does FLIP. According to the results for calcium release from the ER, LFG-transduced cells showed a marked reduction of cytochrome c release to the cytosol after FasL stimulation, suggesting that LFG reduction on ER calcium release may be the mechanism through which LFG performs its anti-apoptotic effect.

On the other hand, down-regulation of Bcl-X₁ showed no difference in the amount of cytochrome c release compared with sh scrambled control cells (Fig. 7D), and LFG overexpression in shBcl-X₁-transduced cells was unable to inhibit mitochondrial permeabilization, further confirming the dependence of LFG effects on Bcl-X₁ endogenous expression. In shLFG-transduced cells, cytochrome c was detected in the cytosol as in sh scrambled-transduced cells, and Bcl-X₁ overexpression in these cells blocked cytochrome c release, suggesting that Bcl-X₁ inhibitory function on mitochondria permeabilization is able to protect from cell death despite the calcium release from the ER. In summary, LFG overexpression reduces cytochrome c release from the mitochondria. However, LFG failed to inhibit this processes when Bcl-X₁ was down-regulated, highlighting the importance of the association between both proteins on LFG anti-apoptotic function.

Discussion

The protein LFG was discovered during screening for genes that confer resistance to Fas-induced cell death (16). Its predicted structure contains seven hypothetical transmembrane domains and one unfolded region at the N-ter of the protein. However, a functional role of the structural domains of LFG has not yet been described. To date, the mechanism by which LFG protects against Fas-mediated apoptosis is still unknown. It has been proposed that this protein acts upstream of caspase-8 activation because it is able to inhibit the activity of this caspase. The finding that LFG interacts with Fas receptor in the lipid raft has revealed this union as the most plausible mechanism of protection (17). However, no differences in the formation of DISC or in the agonist binding to the receptor have been found when LFG is overexpressed (20).

Studies addressing LFG localization show that it is expressed in postsynaptic sites and dendrites (18). However, a clear punctate pattern not only in dendrites but also in the cell body is detectable when using LFG antibodies for immunofluorescence. We observed that LFG is not localized at the mitochondria. Instead, we demonstrate that LFG localizes mainly in the ER and Golgi apparatus. In agreement with this finding, a study addressing other TMBIM family members reported that LFG also localizes at the membranes of the trans-Golgi complex (19, 28).

LFG is highly homologous to BI-1 (15). BI-1 blocks the intrinsnic pathway that protects cells from DNA-damaging agents and ER stress inducers (41, 42). It interacts with Bcl-X₁ in the ER and regulates calcium signaling (29). Fas-mediated apoptosis shares some features with intrinsic death pathways, such as ER stress (43, 44), and promotes a rise in intracellular calcium (45, 46), which mediates some apoptotic features attributed to Fasl-induced cell death (35). The cross-talk between the ER and mitochondria and the calcium efflux between them are

FIGURE 4. LFG protects type II but not type I cells against FasL-induced cell death. A, SK-N-AS cells were transduced with empty plasmid, LFG, Bcl-X₁, or FLIP-overexpressing lentiviral particles for 3 days. Cells were treated with Fc-FasL (100 ng/ml) or left untreated (UT) for 6 h. DEVDasa activity was assessed (one-way ANOVA test). ***p < 0.001; **p < 0.005; *p < 0.05. B, HEK293 cells were transduced with pcDNA3-, LFG, 3×HA-Bcl-X₁, and 3×HA-FLIP-overexpressing plasmids for 48 h and treated with Fc-FasL (100 ng/ml) or left untreated for 6 h. DEVDasa activity was assessed (one-way ANOVA test). ***p < 0.001. C, SK-N-AS cells were transduced as in A. The cells were treated with Fc-FasL (100 ng/ml) or left untreated for 24 h. The percentage of apoptosis was assessed by counting cells with apoptotic nuclei (one-way ANOVA test). ***, p < 0.001; *p < 0.05. D, HEK293 cells were transfected as in B. The cells were treated with Fc-FasL (100 ng/ml) or left untreated for 10 h. Percentage of apoptosis was assessed by counting cells with apoptotic nuclei (one-way ANOVA test). ***, p < 0.001; *, p < 0.05. E, representative images of SK-N-AS cells with Hoechst staining, transduced as in A, and treated with Fc-FasL (100 ng/ml) or left untreated for 24 h. Scale bar, 10 μm. F, representative images of HEK293 cells with Hoechst staining, transfected as in B, and treated with Fc-FasL (100 ng/ml) or left untreated for 24 h. Scale bar, 10 μm. G, immunoblot analysis was performed to assess LFG, Bcl-X₁, and FLIP overexpression in HEK293 cells. *, short exposure; **, long exposure. Naphtol Blue staining was performed to confirm equal loading.
FIGURE 5. Endogenous Bcl-X\textsubscript{L} is essential for LFG protection against FasL-induced cell death. A, SK-N-AS cells were transduced with lentiviral particles carrying shRNA against LFG, Bcl-X\textsubscript{L}, or scrambled (Scr) for 3 days and then transduced with LFG- or Bcl-X\textsubscript{L}-overexpressing lentiviral particles for 3 additional days as indicated. The cells were treated with Fc-FasL (100 ng/ml) or left untreated (UT) for 6 h. DEVDase activity was assessed (one-way ANOVA test). ***, \(p = 0.001\). B, SK-N-AS cells were transduced as in A. The cells were treated with Fc-FasL (100 ng/ml) or left untreated for 24 h. The percentage of apoptosis was assessed by counting cells with apoptotic nuclei (one-way ANOVA test). ***, \(p = 0.001\); *, \(p = 0.05\). C, Immunoblot analysis was performed to assess LFG and Bcl-X\textsubscript{L} down-regulation in SK-N-AS cells. Naphtol Blue staining was performed to confirm equal loading.
important steps in Fas apoptotic signaling, at least in the immune system (47). Nonetheless, nothing is known in other systems. Thus, we propose that LFG exerts its anti-apoptotic effects by modulating mitochondria permeabilization via an ER-dependent mechanism in the nervous system. LFG inhibits calcium release from the ER after FasL treatment. Accordingly, a marked reduction in cytochrome c release, and caspase-8 activation is observed in LFG-transduced cells. However, we observed that LFG effects are Bcl-XL-dependent. Bcl-XL overexpression also inhibits calcium release from the ER, and cytochrome c release. When LFG is down-regulated, Bcl-XL is still able to inhibit mitochondria permeabilization and caspases activation; nevertheless, calcium release from the ER remains unaffected.

On the basis of these observations, we suggest that LFG may play a role in the regulation of calcium release from the ER after an apoptotic stimulus. Blockade of the calcium efflux from ER to the mitochondria would be a limiting step to inhibit the cytochrome c release and subsequent activation of caspase-3, thereby preventing apoptosis (Fig. 8).

Taking into the account that LFG did not influence ligand binding to Fas-receptor or DISC formation (16), it was suggested that LFG might act at the level of caspase-8 activation. Nevertheless, we report data that contradicts this view. First, we report that LFG is only able to protect type II apoptotic cells, whereas it fails to inhibit apoptosis in type I apoptotic cells. If LFG was able to inhibit caspase-8 activation directly, it should be able to inhibit type-I Fas-induced apoptosis, in the same way as other caspase-8 inhibitors, such as FLIP. In addition, LFG lose its protective effects when Bcl-XL is down-regulated. Because our data suggest that the interaction between both proteins is essential for LFG function, is highly unlikely that LFG may exert its main effect at the receptor level, where Bcl-XL is not localized. If LFG effects were located at the DISC level, effects on blockade of calcium release from the ER and prevention of cytochrome c release from mitochondria should be observed despite Bcl-XL down-regulation.

Bcl-XL plays a central role in the regulation of FasL-induced apoptosis in type II cells. It is located at both the mitochondria and the ER (48, 49), and its anti-apoptotic function through
**FIGURE 7.** LFG overexpression inhibits cytochrome c release from the mitochondria after Fas stimulation. A, SK-N-AS cells were treated with IETD (20 μM) for 30 min prior to Fc-FasL (200 ng/ml) treatment. Intracellular calcium mobilization was assessed. B and C, SK-N-AS cells were transduced FLIP-overexpressing lentiviral particles for 3 days or treated with IETD (20 μM) as indicated. The cells were treated with Fc-FasL (100 ng/ml) or left untreated (UT) for 6 h, and then Western blot was used to assess the cleavage of procaspase-8 (B) or Bap31 (C). Tubulin was used as loading control. D, SK-N-AS cells were transduced with lentiviral particles carrying shRNA against LFG, Bcl-XL, or scrambled (Scr) for 3 days and then transduced with LFG- or Bcl-XL-overexpressing lentiviral particles for 3 additional days as indicated. Cells were treated with Fc-FasL (100 ng/ml) or left untreated for 4 h. Cytochrome c (Cyt C) release to the cytosol was assessed by Western blot. Naphtol Blue staining was used as a loading control.

**FIGURE 8.** Schematic representation of Fas apoptotic signaling pathway and the hypothetic steps inhibited by LFG. After Fas stimulation, caspase-8 cleaves Bap31 and tBid. p20 fragment induces Ca^{2+} efflux from the ER to the mitochondria, which in conjunction with tBid induces mitochondrial permeabilization and cytochrome c release. Although Bcl-X_L is able to inhibit ER calcium release and mitochondria permeabilization directly, LFG is only able to block the ER step.
both organelles is well documented. Strikingly, Bcl-XL effects on ER calcium signaling appear to be dependent on LFG endogenous expression.

In summary, our results reveal a hitherto undescribed step in the extrinsic apoptotic signaling pathway in cells from neuronal lineage. Calcium mobilization from the ER has been shown to be relevant in FasL apoptotic signaling. We demonstrate that LFG modulates calcium release from the ER after FasL stimulation and inhibits FasL-induced apoptosis in neuron-like cells that are type II apoptotic cells regarding the way they subside apoptosis. On the basis of our observations, we propose that LFG protects against FasL-induced apoptosis by modulating calcium release from the ER.

Author Contributions—J. X. C., B. B.-Z., S. R., and J. U. designed the project. D. G.-D., J. U., M. R. M., J. C., and C. F.-S. co-designed and performed laboratory work and analyzed the results of calcium release. J. U., J. C. A., E. C., K. M. O. G., L. P.-F., R. S. M., N. L.-C., and S. R. performed laboratory work and collected the data. J. X. C., B. B.-Z., and J. U. wrote the manuscript. The final manuscript was read and approved by all signing authors.

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