Identification, expression and functional characterization of the GRAL gene

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Abstract

The glial cell line-derived neurotrophic factor (GDNF) family is a group of neurotrophic factors with diverse biological functions. Members of the GDNF family exert their functions by interacting with a specific GDNF family receptor α (GFRα) and activation of the cRET. Here we report the identification and characterization of GDNF receptor-alpha-like (GRAL) gene. Sequence analysis indicated that GRAL is a distant homolog of the GFRα family, with 30% of its amino acid sequence identical to that of GFRα-3. There are two splice variants of GRAL: the full-length form (GRAL-A) represented by a 2080 bp mRNA and a short form (GRAL-B) represented by a 1833 bp mRNA. In adult mouse, GRAL transcripts have been found primarily in the CNS. In the developing mouse brain, the mRNA level of GRAL in the cerebrocortex and hippocampus reached a maximum at birth and declined afterwards. GRAL-A protein was localized predominantly in the plasma membrane. Overexpression of GRAL-A protected PC12 cells and cultured hippocampal neurons from serum starvation-induced cell apoptosis. The neuroprotective effect of GRAL was associated with marked inhibition of the Jun-N-terminal kinase signaling pathway. Our results suggest that GRAL belongs to a superfamily of GFRα and might take part in neuroprotection and brain development.

Keywords: apoptosis, glial cell-line-derived neurotrophic factor α receptor-like, hippocampus, PC12.


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Abbreviations used: BAC, bacterial artificial chromosome; BDNF, brain-derived neurotropic factor; DMEM, Dulbecco’s modified Eagle’s medium; ERK, extracellular-signal-regulated kinase; FACS, fluorescence-activated cell sorting; GDNF, glial cell line-derived neurotropic factor; GFRα, family receptor α; GpDlns, glycosylphosphatidylinositol anchor; GRAL, GDNF receptor alpha-like gene; JNK, Jun-N-terminal kinase; PBS, phosphate-buffered saline; MAPK, mitogen-activated protein kinase; NGF, nerve growth factor; ORF, open reading frame; PtdIns-3K, phosphatidylinositol-3 kinase; RACE, rapid amplification of cDNA ends; SDS, sodium dodecyl sulfate.
action of neurotrophic factors and of other neuronal survival factors is crucial for understanding the development and maintenance of the nervous system and for finding efficient approaches for repairing injured or diseased brain.

The GDNF family is one of the best characterized families of neurotrophic factors, which support the survival of a variety of target neurons such as nigral dopaminergic neurons, spinal motor neurons and a subpopulation of peripheral ganglia neurons (for a review, see Airaksinen et al. 1999). Four ligands are identified in this family: GDNF (Lin et al. 1993), neurturin (Kotzbauer et al. 1996), persephin (Milbrandt et al. 1998), and artemin (Baloh et al. 1998b). All four trophic factors require a heterodimeric receptor complex in order to carry out downstream intracellular signal cascades. The receptor complex is comprised of two molecules, a common transmembrane receptor tyrosine kinase cRet and an accessory glycosylphosphatidylinositol (GPItdIns)-anchored membrane protein GDNF family receptor a (GFRα). Four GFRαs have been described thus far, namely, GFRα-1 (Cacalano et al. 1998), GFRα-2 (Baloh et al. 1997), GFRα-3 (Baloh et al. 1998a) and GFRα-4 (Enokido et al. 1998). Each of the four trophic factors binds to one of the four GFRα proteins and then activates cRet to transduce a signal intracellularly. Although each ligand has its preferred GFRα receptor proteins, cross-talk between the ligands and GFRα receptors has been described both in vitro and in vivo (Airaksinen et al. 1999).

The complex interaction networks of ligands and receptors of the GDNF family prompted us to search for as yet unidentified members of this family. Here we describe the cloning and characterization of a novel gene, designated GDNF receptor-alpha-like (GRAL), that shares sequence similarity with known GFRα proteins. Surprisingly, functional assays of the newly identified GRAL protein indicate that, rather than being a novel GFRα receptor, the protein can directly exert protective effects on neuronal cells differently from a typical GFRα receptor and thus representing a novel neuroprotective molecule.

**Experimental procedures**

**Database homology searching and computational sequence analysis**

Similarity searching was carried out on the GenBank mouse genome draft sequence database (http://www.ncbi.nlm.nih.gov/) using BLAST (Altschul et al. 1990). Gene prediction analysis on the genomic sequences was performed using GENSCAN (Burge and Karlin 1997) at BIOSINO (http://www.biosino.org/). Multiple alignments of mouse GFRα protein sequences were performed using the CLUSTAL W multiple alignment program, version 1.8 (Thompson et al. 1994). Prediction of signal peptide cleavage was done using SIGNALP (http://www.cbs.dtu.dk/services/SignalP-2.0/) (Nielsen et al. 1997). Phylogenetic tree analysis was carried with TREEVIEW (Page 1996). Prediction of N-glycosylation sites was performed using the NetNGlyc server (http://www.cbs.dtu.dk/services/NetNGlyc/) (Gupta et al. 2004). The program CONSITE was used to predict transcription factor binding sites shared by orthologous pairs of genomic sequences (http://mordor.cgb.ki.se/cgi-bin/CONSITE/consite/).

**Cloning of full-length cDNA encoding mouse GRAL**

The mRNA pool of adult mouse brain (Clontech BD Biosciences, Palo Alto, CA, USA) was reverse transcribed using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). 3' and 5'-rapid amplification of cDNA ends (RACE) reactions were performed with SMART RACE cDNA synthesis kit (Clontech BD Biosciences), according to the manufacturer’s instructions. The 3'-RACE gene specific primer used was 5'-CAC-TGCCAAGCAGCCATACGGTTC-3', and 5'-RACE gene specific primer was 5'-CATGGCTTGCTGTGAAGGTTC3'TTTTG-3'. Primers used to amplify the full-length GRAL were 5'-CTGG-TAGAGGACACGTGACA-3' and 5'-CATCATGATACATTGAG-TGATCATT-3'. Advantage 2 PCR system (Clontech BD Biosciences) was used to amplify the gene of interest. The resulting amplification products were cloned into the pGEM®-T Easy vector (Promega, Madison, WI, USA) and sequenced using ABI automated sequencer (Perkin–Elmer, Boston, MA, USA).

**RNA isolation and semi-quantitative RT-PCR**

Adult mice were killed by neck dislocation and various tissues of interest were removed quickly. Total RNA was extracted using TRIzol reagent (Invitrogen). Single-strand cDNA was synthesized from 1 μg total RNA using Superscript II Reverse Transcriptase (Invitrogen). Primers used to amplify a region of interest from mouse GRAL were: forward primer 5'-CCAGGGAGACCATCTTGAGTGTC-3', reverse primer 5'-TCACACAGACATTGTTGG-3'. Negative control templates for each set of PCR included water and total RNA from all samples to show that no DNA contaminants were introduced during RNA preparation. Amplification was performed using ExTaq system (Takara, Dalian, China). A series of preliminary PCR experiments was performed to determine the appropriate cycling numbers for semi-quantitative amplification. The concentration of template cDNA was adjusted using β-actin as the internal control. Briefly, after an initial denaturing step at 95°C for 3 min, the reaction mix was subjected to the following PCR conditions: 15 s at 94°C, 30 s at 65°C, and 2 min at 72°C (29 cycles for GRAL and 24 cycles for β-actin). The products were separated in 1.5% agarose gel and stained with ethidium bromide. All PCR products obtained were sequenced using an automated PCR sequencer (ABI Prism System, Perkin–Elmer).

**Northern blot analysis**

Total RNA was isolated from various tissues of newborn mice. mRNA was purified from total RNA using oligotex mRNA purification kit (Qiagen GmbH, Hilden, Germany). Ten micrograms of mRNA per lane were size-fractionated by 1.0% formalin-denatured agarose gel electrophoresis and transferred to a nylon membrane (Amersham Pharmacia Biotechnology, Uppsala, Sweden). cDNA probes specific were 32P-labeled using a random primer labeling kit (Promega). After hybridization at 42°C overnight, the blots were subjected to washing with ascending stringency. The blots were exposed to X-ray film for 1 week (GRAL) or 24 h (GAPDH).
Culture of PC12 cells and primary culture of hippocampal neurons

PC12 cells (ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 6% heat-inactivated horse serum and 10% fetal bovine serum (Gibco Life Technologies). Primary hippocampal cultures were established from newborn female Sprague–Dawley rats as previously described (Brocco et al. 2003) with a few modifications. Briefly, hippocampal tissue was treated with 0.025% trypsin in D-Hanks's solution for 15 min at room temperature. Cell suspension was prepared by pipetting and then seeded at a density of 3 × 10^5 cells/cm² on plates pre-coated with 10 μg/mL poly-I-lysine (Sigma, St. Louis, MO, USA), in DMEM/Ham’s F12 (1 : 1) supplemented with 5% fetal bovine serum and 5% horse serum (Gibco and Invitrogen). After 1 day in vitro (DIV) cytosine arabinoside (Ara-C, Sigma) was added into the cultures at a final concentration of 6 μM to inhibit glial cell proliferation. Under these conditions, > 90% of cells in culture were neuronal cells. Cells were incubated at 37°C in a 95% air/5% CO₂ humidified atmosphere.

Expression constructs and transient transfection of cultured cells

The pcDNA3.0-GRAL-A expression construct was generated by subcloning the mouse GRAL-A coding sequence into the mammalian expression vector pcDNA3.0 (Invitrogen). This construct had a FLAG tag (DYKDDDDK) inserted (pFLAG-GRAL-A) by recombinant PCR between the predicted signal sequence and the mature coding sequence (positions 28/29 of the amino acid sequence of GRAL-A protein). An EGFP fusion construct was also generated (pGRAL-A-EGFP) by subcloning the mouse GRAL-A coding sequence into the mammalian expression vector pEGFP—N1 (Clontech).

PC12 cells were transiently transfected with the expression constructs described above using Lipofectamin 2000 (Invitrogen). Hippocampal neurons were transfected at 1 DIV with pEGFP—N1 (Clontech) in combination with either pcDNA3.0- or pFLAG—GRAL-A in a ratio of 1 : 3 using Lipofectamine 2000 (Invitrogen). The DNA–liposome complex was removed from the hippocampal cell culture at 4 h following transfection and the medium was replaced with Ara-C-containing medium. Forty-eight hours after transfection, the cultured neurons were switched to serum-free medium and maintained for another 48 h. At 5 DIV, various cells were fixed with 4% paraformaldehyde and subjected to various analyses as described below.

Generation of GRAL stable-transfected cell line

Transfected PC12 cell clones that stably express GRAL-A or empty vector were generated by methods described previously (Hu et al. 2003). Briefly, pFLAG–GRAL-A or empty pFLAG vector cDNA were separately transfected into PC12 cells followed by antibiotic selection in culture media containing 800 μg/mL G418 (Sigma) for 2 weeks. Resulted cell clones that stably express GRAL-A were identified by RT-PCR, western blot or immunoprecipitation analysis.

PC12 cell survival assay

Cells were serum-deprived by three washes of phosphate-buffered saline (PBS) and resuspended in DMEM. The suspended cells were plated at a concentration of 1 × 10^5 cells/well on 24-well plates and treated with the indicated reagent(s). For clonal transfectants, the cells were stained with 10 μg/mL fluorescein diacetate (C2,H11,O4, Sigma), which rendered viable cells bright green under epifluorescence using an Olympus BX51 microscope equipped with a SPOT digital camera (Diagnostic Inc). The number of surviving cells in the cultures transfected with either empty vector or pFLAG–GRAL-A was counted in five randomly chosen fields (×200 magnification). The results were expressed as a percentage of live cells counted in paired untreated cultures. For non-clonal transfectants, cell viability in suspensions of dissociated cells was determined by the ability of viable cells to exclude the Trypan Blue dye. Cell counting was carried out by a blinded observer.

Detection of apoptosis

Apoptosis studies were performed using three methods. First, fluorescence-activated cell sorting (FACS) was used to quantitate apoptotic cells. Briefly, 10⁶ cells were collected and fixed with citrate buffer 24 h after serum starvation, followed by staining with 50 μg/mL propidium iodide. Stained cells were analyzed in a fluorescence cell sorter (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ, USA) excited at 488 nm. The percentage of apoptotic cells in each transfected population was calculated using CELLQUEST software. Second, Hoechst staining was used to view apoptotic cells with condensed/fragmented nuclei. Cells were fixed with 4% paraformaldehyde and stained with Hoechst 33342 (1 μg/mL) for 30 min at room temperature. Cells with condensed/fragmented nuclei were counted in fluorescent photomicrographs in triplicate experiments and the percentage of apoptotic cells in the whole cell population was calculated. Cell counting was performed by a blinded observer. Third, to further confirm apoptosis, DNA fragmentation assay was carried out as previously described with modifications (Yamanoshita et al. 2000). Briefly, 24 h after serum deprivation, PC12 cells were lysed with 10 mM Tris–HCl buffer, pH 7.5, containing 150 mM NaCl, 10 mM EDTA, 0.5% sodium dodecyl sulfate (SDS). The lysate was incubated with 0.1 mg/mL RNase at 37°C for 1 h, followed by phenol/chloroform extraction and ethyl alcohol precipitation. DNA ladders were visualized on a 2% agarose.

In vitro hippocampal neuron survival assay

Postnatal hippocampal neurons were plated at a density of 3 × 10⁵ cells/cm² followed by cotransfection with either pEGFP—N1/pFLAG–GRAL-A or empty vectors pEGFP—N1/pcDNA3.0. Two days later, the cultures were switched to serum-free condition medium. Forty-eight hours following induction of apoptosis, the number of cells with characteristics of apoptosis, such as neurite retraction and nuclear condensation or fragmentation (revealed by Hochest 33342 staining), in corresponding cultures were counted in eight randomly chosen fields (×200 magnification). The results were expressed as a percentage of EGFP-positive cells with characteristics of apoptosis counted against entire number of transfected neurons in the cultures.

Immunocytochemistry and fluorescent microscopy

Forty-eight hours following transfection, cells were fixed with 4% paraformaldehyde for 30 min at room temperature, washed with PBS, blocked with 4% goat serum in PBS with or without 0.5% Triton X-100 for 1 h at room temperature. The cells were incubated...
with 5 μg/mL monoclonal anti-(FLAG M2) IgG (Sigma) overnight at 4°C. Immunoreactivity was detected using a fluorescein-conjugated anti-mouse whole serum (dilution 1 : 400, Sigma). Fluorescence was visualized with a confocal laser scanning microscope (TCS SP2, Leica, Bensheim, Germany). To visualize plasma membrane, freshly prepared the lipophilic tracer Dil (2 μg/mL, Molecular Probes, Inc., Orlando, FL, USA) in PBS was added to the monolayer cells. Cells were then incubated for 2 min at room temperature, and then for an additional 10 min at 4°C to stop further staining.

Western blotting and immunoprecipitation

The following primary antibodies were used in these analyses: anti-GFP (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-(FLAG M2) (Sigma), anti-(phospho-JNK) (Cell Signaling Technology Inc., Beverly, MA, USA), anti-JNK1 (Santa Cruz Biotechnology), anti-(phospho-ERK), anti-ERK (Cells Signaling Technology Inc.), and anti-(β-tubulin) (Sigma). Cell monolayers were washed with ice-cold PBS twice and lysed with ice-cold Nonidet P-40-containing lysis buffer (10 mm Tris–HCl, pH 7.5, 137 mm NaCl, 2 mm EDTA, 10% glycerol, 1% Nonidet P-40, 0.25% sodium deoxycholate, 60 mm β-octylglucoside) supplemented with cocktail of protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany) and a mixture of phosphatase inhibitors (1 mm sodium orthovanadate, 20 mm sodium fluoride). For immunoprecipitation, cell lysates were incubated with corresponding antibodies plus 20 μL of Protein A/G–Sepharose bead slurry (Amersham Pharmacia) overnight at 4°C. The beads were washed five times with lysis buffer and resuspended in SDS/β-mercaptoethanol sample buffer. Whole-cell lysates and immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. The rinsed and blocked membranes were separately incubated with various antibodies followed by appropriate secondary antibodies conjugated with horseradish peroxidase (HRP) (Jackson Immuno-Research Laboratories, West Grove, PA, USA). The blots were again washed and developed with the SuperSignal WestPico® chemiluminescent substrate (Pierce, Rockford, IL, USA). To determine protein phosphorylation the blots were stripped and reprobed with various anti-phospho- IgG and developed. Optical densities of phosphorylated proteins were quantified using SCION IMAGE (version B4.02, Scion Corporation, Frederick, MD, USA).

Statistical analysis

Results were expressed as mean ± SEM. Statistical analysis used commercially available statistical software (PRISM v4.0, GraphPad Software Inc.). The data were submitted to either one-way or two-way ANOVA followed by the Student–Newman-Keul’s test. In all cases, significance was set up at p < 0.05.

Results

Identification and cloning of full-length cDNA encoding GRAL

In an effort to search for novel members of the GFRα family, we used the sequences of known GFRα proteins as queries to blast against the GenBank mouse genome draft sequence database. The similarity search yielded a mouse bacterial artificial chromosome (BAC) clone (GenBank Accession no. AC079244) containing a segment of sequence showing similarity with the conserved cysteine-rich domain of known GFRα proteins. Subsequent sequence analysis of the BAC clone using GENSCAN software predicted a novel gene spanning over several exons and containing the GFRα-like segment corresponding to the third exon predicted. Based on this sequence information, primers were designed to specifically amplify the full-length cDNA of the novel gene using 3’- and 5’-RACE approaches. Sequence analysis of the resulting RACE fragments derived from an adult mouse brain cDNA library revealed the existence of cDNAs of several splice variants. Comparison of these sequences with the mouse genomic sequence showed that most of these transcripts were variants that retained unspliced introns and had the GFRα-like open reading frame (ORF) interrupted. However, two of these cDNAs contained a full and uninterrupted ORF and thus could represent functional transcripts. We designated the novel gene as GRAL (GDNF receptor alpha-like) and its two splice variants, GRAL-A (2080 bp) and GRAL-B (1833 bp), respectively (GenBank Accession nos AY457637 and AY457638).

The coding regions of GRAL-A and GRAL-B encode proteins of 393 and 238 amino acid residues with calculated molecular mass of 43.9 and 26.6 kDa, respectively. Two possible sites for N-linked glycosylation are predicted in both variants (i.e. NKTD at position 101–104 and NLTT at position 115–118 of the amino acid sequence of GRAL) (Fig. 1). Both variants have a N-terminal putative signal peptide of 20 amino acid residues (MLVFIFLA VTLSSENESSSP) (Fig. 2a). GRAL-A contains a further putative transmembrane region (at position 350–370 amino acids) near the C-terminus (Figs 1 and 2a), indicating that these proteins may have similar three-dimensional structures. Sequence comparison and secondary structure analysis enabled us to propose a model for the domain structure of GRAL proteins (Fig. 2). GRAL-A is comprised of three cysteine-rich domains (C1, C2 and C3). GRAL-B lacks the third cysteine-rich domain C3 compared with GRAL-A. Interestingly, GFRα proteins have also been proposed to contain three cysteine-rich domains (Airaksinen et al. 1999; Masure et al. 2000). Importantly, the alignment of mouse GRAL and known GFRα proteins demonstrated...
that most of the homologous residues are located in the C2 domain (Figs 1 and 2a). In C1 and C3 domains, although the distribution pattern of cysteine residues in terms of number and positions is very similar between GRAL and GFRα proteins, the overall similarity in amino acid sequence in these domains is low.

GFRα-1–4 are characterized by a C-terminal sequence typical of a GPTdIns-anchored membrane protein, consisting of a hydrophobic region of 17–31 amino acids preceded by a hydrophilic sequence containing a stretch of three small amino acids (Gerber et al. 1992). However, we predict that no GPTdIns cleavage site exists in the C-terminal sequence of both GRAL variants. Moreover, several short peptide motifs, such as SPYE, ERRR, TPN, and SGN, which are highly conserved within known GFRα proteins, are absent in GRAL. Instead DKWN and THY motifs appear in corresponding positions in GRAL. Several N-glycosylation sites are conserved between different GFRα proteins. For example, GFRα-1 and GFRα-2 share glycosylation sites at positions 365 and 427 (Jing et al. 1997). Similarly, two potential N-glycosylation sites were predicted in GRAL-A and GRAL-B (see above and Fig. 1).

Taken together, these results suggest that, although sharing a significant number of features with members of GFRα family, the GRAL ORF has also several characteristics that distinguish it from known GFRα proteins, thus rendering it a distant homolog of GFRα family. This hypothesis is supported by phylogenetic comparison (Fig. 2b).

**Computational analysis of the genomic structure of GRAL**

Bioinformatic analysis was used to analyze the genomic features of GRAL. The exon/intron structure of the mouse GRAL gene was characterized by alignment of the two splice sites.
variants and the genomic sequence. The gene contains nine exons and eight introns, with all of the introns conforming to the GU–AG rule. The sixth exon can be spliced out alternatively, resulting in the two splice variants A and B (Fig. 3a). This alternative splicing event results in a different C-terminus of the translated protein.

BLAST search using the full-length mouse GRAL cDNA against GenBank human genome draft sequence database revealed a genomic sequence entry (GenBank Accession no. AC026838) containing the human homolog of mouse GRAL. Taking advantage of GENSCAN prediction and pairwise comparison, both mouse cDNA and human genomic sequence were analyzed in parallel. All exons encoding the hypothetical human GRAL were identified. The putative human GRAL gene spans over 60-kb in the human genome, comprising nine exons and eight introns. Moreover, the numbers and lengths of exons appear to be highly conserved between mouse and human GRAL genes (Fig. 3a). The putative human GRAL shares relatively high similarity with its murine homolog GRAL (81% similarity and 70% identity, respectively, data not shown).

The transcription factor binding sites were explored by means of comparative genomics analysis. The CONSITE program was used to compare and analyze 1000 bp genomic region of both mouse and human GRAL gene. The bottom line shows a scale in bp depicting the distance from the site where the transcription starts (0 bp). Transcription factors are described by different symbols and the arrows indicate their binding sites. The regulatory modules (a subregion with a high density of cis-elements) are highlighted. S, Sox-5; B, broad-complex 1; H, hepatocyte forkhead homolog 1 (HFH-1); A, acute myeloid leukaemia 1 (AML-1) protein; C, CCAAT-box/Enhancer binding protein; T, TATA-box binding protein.

**Fig. 2** Structural and phylogenetic comparison of mouse GRAL and GFRα family members. (a) Proposed domain structures of mouse GFRα proteins and GRAL proteins. Black boxes represent domains composed of ~100 amino acid residues. SP, signal peptide; GPtIns, glycosylphosphatidylinositol anchor region; TM, transmembrane region; GFRα-1, GFRα-2 and GFRα-3 (GFRα 1–3) have three cysteine-rich domains (C1, C2 and C3), whereas GFRα-4 lacks the first cysteine-rich domain. GRAL-A also has three cysteine-rich domains, whereas GRAL-B contains only domains C1 and C2. The conserved motifs that were used to mark the boundaries of adjacent domains are shown. Note that GFRα family members and GRAL proteins use different boundary markers. (b) Phylogenetic comparison of mouse GFRα proteins with GRAL. Comparisons of the mature protein sequences of mouse GFRα and GRAL were made using CLUSTAL W multiple alignment program, version 1.8.

**Fig. 3** Genomic analysis of mouse GRAL and putative human GRAL gene. (a) Structural diagrams of the genomic and mRNA structures of mouse GRAL. The genomic structure of putative human GRAL gene is also shown. Exons are represented by numbered boxes, and intron sequences are indicated as lines. The sizes (in bp) of the respective exons are indicated above. The positions of the putative translation start and stop codons are indicated by arrowheads. The organization of exons is highly conserved between mouse and human. (b) Comparative analysis of transcription factor binding sites on putative mouse and human promoters. The panel represents upstream 1000 bp of the 5′-genomic region of both mouse and human GRAL gene. The bottom line shows a scale in bp depicting the distance from the site where the transcription starts (0 bp). Transcription factors are described by different symbols and the arrows indicate their binding sites. The regulatory modules (a subregion with a high density of cis-elements) are highlighted. S, Sox-5; B, broad-complex 1; H, hepatocyte forkhead homolog 1 (HFH-1); A, acute myeloid leukaemia 1 (AML-1) protein; C, CCAAT-box/Enhancer binding protein; T, TATA-box binding protein.
regions immediately before the putative transcription start sites of mouse and human GRAL (Fig. 3b). In both cases, a highly conserved TATA-box motif was found at about -30 bp. This motif, together with the upstream CCAAT-box motif, could form the basal promoter region (the RNA polymerase II binding site). Importantly, several transcription factor binding sites were indicated with high probability further upstream. Five of these putative cis-elements, i.e. binding sequences for sox-5, broad-complex 1, hepatocyte forkhead homolog 1 (HFH-1), acute myeloid leukaemia 1 protein and CCAAT-box/enhancer binding protein, were located within a 200 bp subregion (Fig. 3b). The high density of transcription factor binding sites in this subregion is typical of regulatory modules, which are relatively independent cis-regulatory units known to mediate complex developmental patterns of expression (Yuh et al. 1998). The identification of at least one regulatory module in both mouse and human GRAL promoter suggests that transcription of GRAL during development may be regulated by complex, module-based cis-regulatory systems.

To identify the human chromosomal location of GRAL gene, we performed computational localization analysis based on human genome draft sequence database. The analysis of markers present on the GRAL-containing BAC clone AC026838 placed the GRAL gene on human chromosome 6p11–12, flanked by markers D6S294-GRAL-D6S428 (telomeric to centromeric).

Temporal and spatial characteristics of GRAL mRNA expression

Expression of GRAL mRNA was analyzed in various embryonic and adult mouse tissues. In Northern blot analysis performed on whole brain from mouse embryos (embryonic days 12 and 17), no marked GRAL gene transcript was detectable. Also, there was no apparent hybridization signal in adult mouse tissue mRNA blots (mouse MTN blot, Clontech, data not shown). However, GRAL gene transcript was detected in neonatal brain. As shown in Fig. 4(a), GRAL mRNA was expressed in the hippocampus and cerebrum, whereas in the cerebellum and striatum no hybridization signal could be detected by Northern blotting. The size of the bands was close to 18 S RNA (1.9 kb). This was in agreement with the predicted size of the mouse GRAL mRNA (2080 bp for GRAL-A, 1833 bp for GRAL-B). Because the sizes of the two splice variants are very close, they cannot be visually distinguished from each other in the Northern blot. However, as GRAL-A mRNA is far more abundant than GRAL-B (see below), we suggested that the signals in the Northern blot largely represent mRNA expression level of GRAL-A, the full-length form of GRAL.

The tissue distribution patterns of GRAL mRNA in the CNS and various peripheral tissues of adult mouse were revealed by RT-PCR analysis. The expression of GRAL in adult mouse brain could be detected by RT-PCR analysis using primers spanning the full-length of its mRNA (Fig. 4b). However, as the product is relatively long (~2000 bp), another pair of primers was re-designed to amplify a shorter fragment in order to determine the tissue distribution pattern of GRAL mRNA. The primers were designed to amplify the region between the first and seventh exons, spanning over 50 kb in the mouse genome, thus eliminating the possibility that the PCR products were generated from immature unspliced transcripts or contaminating genomic DNA. Through this approach, both isoforms of GRAL mRNA were detected in the CNS of adult mouse (Fig. 4c). In contrast, there was no detectable GRAL mRNA in peripheral organs examined, such as the heart, liver, spleen, lung, kidney, placenta, skeletal muscle and small intestine. Among the various regions in the CNS, GRAL mRNAs were relatively more abundant in the substantia nigra, the hippocampus and the spinal cord (Fig. 4d). The distribution patterns of GRAL-A and -B mRNA were almost identical, with the expression level of GRAL-A mRNA being consistently higher than that of GRAL-B in all brain regions expressing GRAL mRNA (Fig. 4c,d). Expression of GRAL mRNAs was also examined in vitro in cultured hippocampal cells. RT-PCR analysis demonstrated that GRAL mRNA was expressed in cultured neurons but not in cultured astrocytes (data not shown), suggesting that GRAL expression observed in the CNS is primarily contributed by neuronal cells.

GRAL mRNA expression was further analyzed in the developing brain. The level of GRAL mRNA fluctuated during brain development. Both isoforms appeared as two faint bands in the cerebrocortex of embryonic day 17. Remarkably, their expression level was increased over fivefold at postnatal day 0 and then markedly declined in adult. GRAL-B mRNA became almost undetectable in adult. Notably also, the ratio of GRAL-A to GRAL-B increased to 3 : 1 at postnatal day 0 from ~1 : 1 at embryonic day 17 (Fig. 4g). In the hippocampus, the mRNA levels of both GRAL isoforms fluctuated in a similar manner as in the cerebrocortex. GRAL-A mRNA level at postnatal day 0 was approximately fourfold higher that the level at embryonic day 17, whereas the ratio of GRAL-A to GRAL-B mRNA changed from ~3 : 1 at embryonic day 17 to 5 : 1 at postnatal day 0 (Fig. 4h). This pattern of expression suggests that GRAL may be developmentally regulated in mouse brain, with both GRAL-A and GRAL-B potentially playing important roles during development.

Subcellular localization of GRAL protein

PC12 cells were transfected with pFLAG–GRAL-A and immunofluorescent analysis was performed, either with or without permeabilization using 0.5% Triton X-100. In non-permeabilized cells, the FLAG-tagged GRAL protein, readily detected by anti-FLAG IgG, appeared on the cell surface (Fig. 5a), suggesting that at least a portion of GRAL is localized in the plasma membrane with an extracellular
N-terminus. However, after permeabilization, the immuno-reactivity was present in the whole cell body but not the nucleus (Fig. 5a). These results indicate that GRAL may be localized both in plasma membrane and cytoplasm. The integrity of the FLAG-tagged GRAL protein was confirmed by western blot analysis with an anti-FLAG IgG which detected a 44 kDa protein (Fig. 5b). To further verify the subcellular localization of GRAL protein, PC12 cells were
transfected with a pGRAL-A–EGFP encoding C-terminal EGFP-tagged GRAL. Green fluorescence was observed predominantly in the cell membrane (colocalizing with the membrane tracer DiI) and partly in the cytosol (Fig. 5c). The GRAL-A-EGFP fusion protein was detected by western blotting using an anti-GFP antibody as a 71 kDa protein (Fig. 5d), consistent with the sum of the two fused proteins (44 and 27 kDa, respectively). The GRAL-A–EGFP fusion protein was not detectable in lysates of cells transfected with EGFP vector only. Taken together, these results suggest that GRAL is most likely a membrane protein with a possible, conditional cytoplasmic localization.

**Overexpression of GRAL protected PC12 cells from serum deprivation-induced apoptosis**

In order to provide insights into the biological function of GRAL, the effect of GRAL overexpression on cell survival was investigated in vitro in PC12 cells. PC12 cells express low levels of endogenous cRET tyrosine kinase and neither GRFα-1 nor GRFα-2 is detectable in these cells (Treanor et al. 1996; Wang et al. 1998). RT-PCR analysis revealed a very low level of GRAL mRNA in both parental PC12 cells and empty vector-transfected PC12 clones (Fig. 6a). Three PC12 cell line clones that stably and constitutively overexpressed FLAG tagged GRAL-A, both at mRNA and protein level, were established (clones 10, 11 and 18, Fig. 6a,b). Remarkably, all three GRAL-A-overexpressing clones exhibited several prominent phenotypic changes compared with their parental or empty vector-transfected PC12 cell lines, with the most striking being the enhanced cell survival following trophic support (serum) deprivation.

It is well established that serum withdrawal in cultures of PC12 cells induces cell apoptosis (Rukenstein et al. 1991) and this in vitro model is widely used to study the molecular mechanisms of apoptosis (Hetman and Xia 2000). Consistently with this, serum withdrawal resulted in noticeable apoptosis of cultured PC12 cells, as evidenced by the detection of marked DNA fragmentation in empty vector-transfected PC12 cells (Fig. 6c). In contrast, PC12 GRAL-A-overexpressing cells had significant reduction of DNA fragmentation (Fig. 6c), suggesting a protective effect of GRAL expression on PC12 cells against apoptosis. To quantify this effect, a series of cell survival assays were

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**Fig. 4** Temporal and spatial expression pattern of GRAL mRNA in mouse. (a) Northern blotting analysis of GRAL mRNA in the hippocampus, cerebellum, cerebrum and the striatum from newborn mice. GRAL mRNA is expressed at a relatively high level in the hippocampus, whereas its level in the cerebral cortex is lower. No GRAL transcript could be detected in the striatum and cerebellum. Re-probing of GAPDH indicates approximately equal loading of mRNA. (b) RT-PCR analysis using primers spanning from the first to ninth exons revealed the expression of full-length GRAL-A and GRAL-B mRNA in the adult mouse brain. M, DNA marker. (c) RT-PCR analysis showed selective expression of GRAL mRNA in adult mouse brain, but not in peripheral tissues. Isoforms GRAL-A (1347 bp) and -B (1097 bp) are indicated by arrows. The 550-bp β-actin fragment indicates equal loading of cDNA samples. GRAL-A mRNA is more abundant than GRAL-B in adult mouse brain. (d) Distribution pattern of mouse GRAL transcripts in various brain regions in the adult mouse. GRAL transcripts were relatively abundant in the substantia nigra, the hippocampus and the spinal cord. (e–h) Developmental regulation of GRAL mRNA expression level in the mouse cerebrocortex (e, g) and hippocampus (f, h). (e, f) Semi-quantitative RT-PCR amplification of GRAL and β-actin. (g, h) Quantitative data of (e) and (f), respectively. The results were presented as ratios of optical densities of amplified band versus β-actin (mean ± SEM, n = 3). *p < 0.05, compared with E17 and adult, using one-way ANOVA followed by the Student-Newmann-Keuls test. E17, embryonic day 17; P0, postnatal day 0. The data in (b) to (h) represent at least three independent experiments.
performed. Using fluorescein diacetate staining, it was estimated that ~80% of empty vector-transfected (control) PC12 cells underwent apoptosis two days following serum withdrawal (Fig. 6e). The number of surviving cells in control cultures was further decreased with time. Only 10.3% of cells remained viable at day 4 and almost none survived at day 6 (Fig. 6f). Conversely, overexpression of GRAL in PC12 cells markedly promoted the survival of these cells. Similar to the finding in DNA ladder analysis, using FACS analysis, it was found that, at 24 h post serum withdrawal, the percentage of apoptotic cells (sub-G₁ cells) was markedly reduced in GRAL-overexpressing PC12 cells compared with the control cells transfected with the empty vector (26% apoptotic cells in pFLAG–GRAL-A-transfected cell population vs. 44% apoptotic cells in empty vector-transfected cell population) (Fig. 6d). Moreover, an increase of up to threefold (59.3%) in the number of surviving cells (indicated by fluorescein diacetate staining) was detected in GRAL-overexpressing PC12 cell cultures (clone 18) at day 2, compared with the empty vector-transfected cells. Significant
percentages of surviving cells were still observed by day 4 and 6 (41.1 and 27.1%, respectively) (Fig. 6f). Similar results were obtained in cell-survival assays of PC12 GRAL-A-expressing cell clones 10 and 11.

To exclude the possibility that the phenotypic change observed was due to unexpected cell transformation during clone selection with G418 and/or clonal diversity, non-clonal pools of stable transfectants were also generated. Following transfection with either empty vector (pcDNA3.0) or pFLAG–GRAL-A construct, PC12 cells were maintained under the pressure of G418 selection for two more weeks without further cloning. The cultures were then subjected to serum starvation and the numbers of live/dead cells were counted. The number of surviving cells in GRAL-A-transfected PC12 cell cultures was ~2.4-fold higher than in the control (46.4% in GRAL-A-transfected cells vs. 18.9% in control cells) (Fig. 6g). Thus the ability of GRAL to increase survival of PC12 cells in conditions of trophic support deprivation was confirmed by two independent experimental approaches.

cRet tyrosine kinase is activated efficiently by GDNF family ligands when the GFRα-receptors are coexpressed with Ret in the same cells (Jing et al. 1996, 1997), indicating a functional ‘in cis’ interaction. It has been shown that cRet is present in PC12 cells whilst coreceptor GFRα1 is absent in these cells (Treanor et al. 1996). Moreover, PC12 cells can become responsive to GDNF treatment when exogenous GFRα1 cDNA is introduced into the cells. These genetically modified cells possess improved ability to tolerate trophic factor deprivation (Chen et al. 2001). In the light of these considerations, we addressed the question whether the increased survival of GRAL-overexpressing PC12 cells can be further elevated by the addition of exogenous GDNF family ligands. PC12 GRAL-expressing cell clones 10, 11 and 18 were treated separately with GDNF, neurturin, persephin, artemin (50–200 ng/mL) or a combination of these factors for up to 7 days. None of the treatments resulted in any marked improvement in the cell survival compared with untreated cells following serum withdrawal (Fig. 7). We also determined whether GRAL-A can bind cRet in PC12 cells using co-immunoprecipitation. No detectable interaction was observed between the two proteins was observed (data not shown).

As the protective effect of GRAL overexpression was rather modest, we were interested in whether the survival-promoting effect is dependent on the dosage of transfected plasmids. PC12 cells were transfected with different amounts of EGFP-tagged GRAL-A (0, 1.6, 3.2 μg/mL) followed by serum-withdrawal for 24 h. The percentage of apoptotic cells among the fluorescence-positive cells was determined by staining with Hoechst 33342 dye. The apoptotic cells showed crimped cell bodies and condensed/fragmented nuclei (Fig. 8a). Whereas percentage of apoptotic cells in the pEGFP-transfected cell cultures (served as negative control) was up to 60%, transfection with lower amount of GFP–GRAL-A (1.6 μg/mL) reduced the percentage to <30%. The percentage was further reduced to ~15% in PC12 cells transfected with higher level (3.2 μg/mL) of GFP–GRAL-A (Fig. 8b). Western blot analysis confirmed increased expression levels of GRAL-A (Fig. 8c). This suggested that the survival promoting effect induced by GRAL overexpression is dose dependent.

Pro-survival effect of GRAL on hippocampal neurons in primary cultures

We found that GRAL is expressed in the hippocampus of mice (Fig. 4d,f) and rats (data not shown). Next, we determined the anti-apoptotic activity of GRAL in primary cultures of hippocampal neurons. Rat hippocampal neuronal cultures were separately cotransfected with pFLAG–GRAL-A and pEGFP—N1 or pEGFP—N1 and pcDNA3.0. EGFP expression in the transfected cells was used to visualize whole-cell morphology, including the presence of neurites of hippocampal neurons in culture. Immunofluorescent analysis using anti-FLAG IgG allowed visualization of transfected GRAL-expressing hippocampal neurons (Fig. 9a).

In the presence of serum in culture medium, both control and pEGFP—N1/pFLAG–GRAL-A-transfected hippocampal neurons presented normal neuronal morphology with branching neurites. Forty-eight hours after serum starvation, up to 79% of transfected neurons in control cultures showed typical characteristics of apoptotic neurons, such as fragmented nuclei, retraction of neurites and shrinkage of cell bodies (Fig. 9). However, apoptosis was distinctly reduced in neurons overexpressing GRAL-A, with only 56% of these
neurons presenting neurites retraction (Fig. 9a,b). Furthermore, whereas 73% of the control neurons showed condensed and/or fragmented nuclei (as demonstrated by Hoechst 33342 staining), only 53% of GRAL-A-overexpressing neurons had such apoptotic profile (Fig. 9a,c). Thus, GRAL-A appears to be able to promote survival of hippocampal neurons by inhibiting apoptosis.

Overexpression of GRAL-A reduced serum deprivation-induced apoptosis by inhibiting JNK activation in PC12 cells

To obtain further insights into the mechanisms underlying the anti-apoptotic effect of GRAL, several potential signaling pathways were investigated. As mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K) pathways were reported to be responsible for the viability of PC12 cells (Hetman and Xia 2000; Liu et al. 2003), we first examined the involvement of these two signaling pathways in GRAL-mediated anti-apoptosis mechanisms. No marked differences on the phosphorylation levels of either extracellular-signal-regulated kinase (ERK) 1/2 or AKT were detected between control and GRAL-A-overexpressing cells at 6, 12 or 24 h following serum deprivation (Fig. 10a), suggesting that MAPK and PI3K pathways are not associated with GRAL-A-mediated anti-apoptotic effect. By contrast, Jun–N-terminal kinase (JNK) pathway is known to be potently and preferentially activated during the programmed cell death of PC12 cells by a variety of stimuli (a,b).

Overexpression of GRAL-A protected cultured hippocampal neurons from serum deprivation-induced apoptosis. The postnatal rat hippocampal neurons were plated at a density of $3 \times 10^5$ cells/cm$^2$ followed by cotransfection with pEGFP–N1/pFLAG–GRAL-A. Co-transfection with empty vectors pEGFP–N1/pcDNA3.0 was used as control. Cell apoptosis was induced by a 48-h serum starvation. (a) Photomicrographs showing hippocampal neurons cotransfected with pEGFP–N1/pFLAG–GRAL-A (i, ii) or empty vectors pEGFP–N1/pcDNA3.0 (iii, iv) 48 h after serum starvation. (i, iii) Immunostaining with anti-FLAG IgG; (ii, iv) EGFP fluorescence; Insets in (ii) and (iv) show corresponding Hoechst 33342 staining. Scale bar, 10 µm. (b) Quantitative data from (a) Data are from three independent experiments. *$p < 0.05$, Student’s t-test, compared with controls (pEGFP–N1/pcDNA3.0-transfected). All data represent three independent repeated experiments. **$p < 0.05$, Student’s t-test, compared with PC12 cells transfected with 1.6 µg/mL pGRAL-A–EGFP. (c) Western blotting analysis of PC12 cells transfected with different amount of pGRAL-A–EGFP. 1.6 µg/mL pEGFP was transfected into negative control cells. Tubulin western blot shows approximately equal loading of samples.
environmental stresses, such as serum starvation and neurotoxins (Yang et al. 2004). Thus, we examined whether JNK pathway is involved in the GRAL's anti-apoptotic activity. As shown in Fig. 10(b) the phosphorylation level of JNK was dramatically increased in empty vector-transfected PC12 cells 6 h following serum starvation. The level was further increased at 12 h and sustained for at least 24 h after the treatment. In contrast, up-regulation of the JNK1/2 phosphorylation level induced by serum deprivation in PC12 GRAL-overexpressing cells (clone 18) was markedly reduced at 6, 12 and 24 h after treatment (Fig. 10c). At 12 h following serum withdrawal, the expression level of the 54 kDa isoform of JNK was reduced by 82% in pFLAG–GRAL-A-transfected cells (0.14 ± 0.025 arbitrary units) compared with empty vector-transfected cells (0.78 ± 0.043 arbitrary units) (Fig. 10c). Furthermore, treatment of PC12 cells with SP600125 (C14H8N2O, A.G. Scientific Inc., San Diego, CA, USA), a selective JNK inhibitor, could partially responding control cells (empty vector).

**Fig. 10** GRAL prevented serum deprivation-induced apoptosis by inhibiting activation of JNK signaling pathway. (a) Western blot detection of phosphorylated form of ERK1/2 and AKT in empty vector-transfected or pFLAG–GRAL-A-transfected (clone 18) PC12 cells at various time points following serum deprivation. Whole (non-phosphorylated and phosphorylated) ERK and AKT levels served as loading controls. (b) Western blot detection of phosphorylated JNK1/2. Whole (non-phosphorylated and phosphorylated) JNK1 served as loading control. Vector, empty vector-transfected PC12 cells; GRAL, clone 18. (c) Quantitative data of phosphorylation of JNK1/2 presented in (b). The optical densities of the bands were determined and the results were expressed as ratios of optical densities of p-JNK1/2 versus JNK1 (mean ± SEM, n = 3). *p < 0.05, compared with corresponding control cells (empty vector). (d) Photomicrographs of empty vector-transfected and pFLAG–GRAL-A-transfected PC12 cells (clone 18) with or without treatment of selective JNK inhibitor SP600125. The PC12 cells were serum-starved for 24 h with (+) or without (–) addition of 50 µM SP600125 followed by staining with Hoechst 33342. Arrows indicate apoptotic cells with condensed/fragmented nuclei. Scale bar, 10 µm. (e) The PC12 cells were serum-starved for 24 h in the presence of various concentrations of SP600125 (0, 5, 10 and 50 µM) and the percentages of apoptotic cells in the cultures were determined. *p < 0.05, compared with untreated cultures (n = 3). All data of (a) to (e) represent three independent repeated experiments.
mimic the anti-apoptotic effect of GRAL-A in a dose-dependent manner (Fig. 10d,e). Thus, in the presence of 10 μM of SP600125, the percentage of apoptotic cells (revealed by staining with Hoechst 33342) in empty vector-transfected PC12 cell population was reduced by 32.3% (34.1 ± 3.9% apoptotic cells in JNK-inhibitor-treated cells vs. 50.4 ± 3.6% apoptotic cells in untreated cell population). In contrast, the percentage of apoptotic cells in GRAL-transfected PC12 cells (clone 18) remained approximately the same level. These experiments were also performed on other GRAL-transfected clones (10 and 11) and parental PC12 cells, and similar results were obtained (data not shown). Hence the anti-apoptotic function of GRAL appeared associated with decreased JNK activation.

Discussion

In this study, we report the molecular cloning and characterization of a novel gene, named GDNF receptor-alpha-like (GRAL). Sequence analysis and functional characterization suggest that this gene is a distant homolog of members of GFRα family. GRAL expression is highly selective to brain tissues and its overexpression promotes neuronal cell survival.

Sequence analysis indicates that GRAL shares important structural features with GFRα proteins. They are all composed of cysteine-rich domains and 24 cysteine and several other amino acid residues are well conserved between GRAL and GFRα proteins. In particular, the second cysteine-rich domain (C2) is highly conserved. Even in the more divergent C1 and C3 domains, the number and spacing of cysteine residues are highly conserved. Like GFRα, GRAL presents a highly hydrophobic N-terminal signal peptide. GFRα proteins are primarily membrane-anchored proteins, but alternative splicing of GFRα1–4 can produce soluble forms (Lindahl et al. 2001). Similarly, GRAL also has two splice variants, one is membrane-anchored (GRAL-A) and the other is putatively secreted (i.e. GRAL-B).

However, GRAL differs significantly from a typical GFRα. First, all GFRα proteins have a stretch of 17 hydrophobic amino acid residues at the C-terminus preceded by a group of three small amino acids (Gly, Ser, Asn), which is a typical cleavage/binding site for a GPTdIns linkage. In GRAL, the single transmembrane region replaces the GPTdIns anchor. Furthermore, several short peptide motifs, such as SPYE, ERRR, TPN, and SGN, are highly conserved between known GFRα proteins. These motifs, however, are absent from GRAL. Instead, DKWN and THY motifs are present. Second, GFRα proteins are present in a variety of tissues. For example, GFRα-1 mRNA is widely distributed in neuronal and non-neuronal tissues and is expressed throughout embryonic development to adulthood, implying a broad spectrum of biological functions (Treanor et al. 1996). GRAL, by contrast, is primarily expressed in the CNS.

Third, GFRα proteins function in GDNF family ligand-activated signal transductions involving direct binding of a ligand to GFRα. In contrast, the anti-apoptotic function of GRAL characterized in this study is independent of GDNF family ligands. Taken together, these findings render GRAL a remote relative of GFRα proteins.

In this study, based on the high conservation in the numbers and sequences of GRAL exons and exon/intron junctions between mouse and human homologs, we identified a putative human homolog of GRAL (sharing 81% similarity and 70% identity with the murine sequence) in silico. Interestingly, we found that the human ortholog of GRAL (IVF19356) has been identified by Clark et al. (2003) in an extensive characterization of novel human secreted and transmembrane proteins. The current human genome assembly locates the human GRAL ortholog exactly at 6:55.3M (6p12.1). Our predicted human GRAL gene shows 98% identity with the previously identified human ortholog of GRAL (GenBank Accession no. AY3581989). The high degree of identity between the two sequences supplies a credible experimental confirmation of the human GRAL sequence proposed herein.

Comparative genomic analysis revealed that several putative cis-elements, highly conserved between mouse and human, may exist in the genomic regions containing the GRAL promoter. The cis-regulatory system of GRAL is likely to be modular in organization, because at least one typical regulatory module containing multiple target sites for DNA binding factors was identified in the putative GRAL promoter (Yuh et al. 1998). In addition to transcriptional regulation, GRAL expression may be modulated by complex post-transcriptional mechanisms, because a variety of GRAL pre-mRNA isoforms were identified in the mouse brain during the process of GRAL gene cloning. The occurrence of multiple (at least six) unspliced mRNA species required RT-PCR analysis to determine the temporal and spatial expression pattern of functional GRAL transcripts. Analysis of this pattern suggested that most of these sequence variants represent immature unspliced mRNA of GRAL. Similar developmental regulation was reported for human β-like globin genes (Kollia et al. 1996). Our findings regarding the highly selective expression of mouse GRAL in the CNS and its developmental regulation support the hypothesis of a tight regulation of GRAL expression, both at the level of transcription initiation and in the processing of multiple mRNA species.

This study revealed that the expression of GRAL in both the cerebrocortex and the hippocampus are developmentally regulated. GRAL expression reached its peak at postnatal age and declined thereafter, suggesting its involvement in brain development. Cell survival analysis demonstrated that GRAL can indeed partially rescue hippocampal neurons deprived of trophic support in culture. Given the selective tissue- and developmental expression pattern of GRAL, we speculated that GRAL may play a role in supporting the survival of central neurons during the processes of establishing and
refining the neuronal circuits, which are primarily completed during the postnatal development of the CNS.

How might GRAL function in promoting neuronal survival against apoptosis? The fact that overexpression of GRAL in PC12 cells and hippocampal neurons promotes their survival in the absence of known GDNF family members indicates that GRAL utilizes a signaling cascade which is distinct from those activated by GDNF-family ligands and receptors. Using western blot analysis in combination with pharmacological approaches, neither MAPK nor PtdIns-3K signaling pathways – two signaling pathways known to play important roles in rescuing neuronal cells from apoptosis (Chen et al. 2001) – were found to be activated by overexpression of GRAL in PC12 cells following serum withdrawal. However, overexpression of GRAL in PC12 cells reduced the level of JNK activation, a signaling pathway that has been shown to take part in serum starvation-induced apoptosis in PC12 cells (Yang et al. 2004). Moreover, administration of the selective JNK inhibitor SP600125 could partially prevent empty vector-transfected and parental PC12 cells from serum-deprivation-induced apoptosis. Thus it is rational to attribute at least part of GRAL’s anti-apoptosis effect to down-regulated activation of JNK. However, the fact that the number of apoptotic cells in SP600125-treated cultures was higher than that in pFLAG–GRAL-A-transfected cultures suggests that there might be other signaling pathways involved in the GRAL-mediated anti-apoptotic effect.

In summary, we have cloned and characterized GRAL, a novel brain-enriched, developmentally regulated gene. We found that the full-length form of GRAL (GRAL-A) can promote the survival of PC12 cells and of primary cultured hippocampal neurons in vitro. Further studies are required to characterize the signaling mechanisms by which GRAL regulates the survival of central neurons and the role it plays during the development of the CNS.

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References


