Purification, cDNA cloning, and secretory properties of FLRG protein from PC12 cells and the distribution of FLRG mRNA and protein in rat tissues*

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Summary. A 35 kD protein was isolated and purified from conditioned media of Bcl-2 cDNA-transfected PC12 cells and its cDNA cloned. A database analysis showed that the 35 kD protein is a rat homologue of the human FLRG protein. The biochemical as well as morphological properties of the rat FLRG protein in PC12 cells were examined and its distribution in rat tissues determined. The levels of FLRG mRNA expressed were low during the fetal period, compared with those of follistatin mRNA. The distribution of FLRG and follistatin mRNAs differed from each other after birth; the expression levels of FLRG mRNA were abundant in the adrenal gland and testis, whereas those of follistatin mRNA and activin A were markedly high in the ovary. The presence of FLRG mRNA and/or protein was confirmed in spermatocytes at various differentiating stages and in endocrine cells of both the adrenal cortex and medulla. When overexpressed in PC12 cells, the FLRG protein was found to be stored in secretory granules of the cells and largely secreted by a regulated pathway, while activin A enhanced the constitutive secretion of the FLRG protein from wild-type PC12 cells, indicating that the FLRG protein possesses dual properties in secretory pathways. The different distribution between

FLRG and follistatin mRNA suggests that, like follistatin in the ovary, the FLRG protein may be involved in the maintenance of spermatogenesis in the testis and the growth and function of adrenal tissue cells, probably by regulating the functions of its binding partners such as the TGF- β superfamily members.

Introduction

FLRG (follistatin related gene) has been identified in B cell chronic lymphocytic leukemia as a t(11; 19) (q13; p13) translocation, and its corresponding cDNA encodes a secretory glycoprotein which is highly homologous to follistatin due to two follistatin modules at the C-terminal region of the protein (Hayette *et al.*, 1998). Follistatin, which has been identified as an activin-binding protein (Ying *et al.*, 1987; Nakamura *et al.* 1990), contains three cysteine-rich (CR) domains at the C-terminal region, known as follistatin modules (FM) (Patthy and Nikolics, 1993), through which it binds to activin and is involved in the regulation of various biological events by acting as an activin antagonist (Corrigan *et al.*, 1991; Kogawa *et al.*, 1991; Hashimoto *et al.*, 1992). One group of proteins has been shown to contain conserved FM/CR domains in their structures and are referred to as the follistatin-module-protein family (Mason *et al.*, 1986; Campanelli *et al.*, 1992; Tsim *et al.*, 1992; Shibamura *et al.*, 1993; Sage and Vernon, 1994; Lane and Sage, 1994; Mendis and Brown, 1994; Mendis *et al.*, 1996; Sumitomo, 2000). FLRG also belongs to this family of proteins (Hayette *et al.*, 1998), and similar to follistatin, FLRG is capable of binding to biologically active proteins such as activin A, and bone morphogenetic protein (BMP)-2, 6 and 7 through FM/CR domains (Tsuchida *et al.*, 2000; Tortorello *et al.*, 2001).

Received October 18, 2003

*This study was supported by grants from the Japan Society for the Promotion of Science and the Japanese Ministry of Education, Science, Sport, and Culture.

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PC12 cells, a rat pheochromocytoma cell line, are known to proliferate when cultured in the presence of serum, while they differentiate into neuron-like cells in the absence of serum but in the presence of nerve growth factor (NGF), and undergo apoptosis following trophic factor deprivation (Batistatou *et al.*, 1992; Batistatou and Greene, 1993). We have previously shown that PC12 cells transfected with Bcl-2 cDNA (*bcl-2*) survive in serum-deprived cultures and, interestingly, differentiate into neuron-like cells in the absence of NGF (Sato *et al.*, 1994). In the course of biochemical as well as morphological studies of *bcl-2*-transfected PC12 (*bcl-2*/PC12) cells, we noted that the cells secrete a FLRG protein, when cultured in the absence of serum. To date, no study has reported on the secretory properties of the FLRG protein from cells and its tissue distribution during the pre- and postnatal life of rats.

In the present study, therefore, we first purified the protein from the culture medium of *bcl-2*/PC12 cells and cloned its cDNA, while the intracellular distribution and secretory properties of FLRG protein were examined in wild-type (wt) PC12 cells and cells that overexpress the protein. Moreover, tissue distribution of FLRG mRNA was examined in rats during embryonic and postnatal stages, using quantitative polymerase chain reaction (QPCR), *in situ* hybridization, and immunohistochemistry.

Materials and Methods

Animals

The present study used Wistar rat embryos obtained at embryonic days (E) 9, 10, 11, 12, 13, 14, 15, 16 and 18, and Wistar rats obtained at birth, and 1, 4, 6, and 8 weeks of age. All animal experiments were done under the Guideline of Osaka University Graduate School of Medicine for the Care and Use of Laboratory Animals.

Cell culture and transfection

PC12 cells were cultured in Dulbecco's modified Eagle medium containing 4,500 mg/l glucose (HDMEM, Sigma, St Louis, MO), supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 mg/ml). For the preparation of transfected cells, cDNAs of human *bcl-2* and rat FLRG tagged with or without enhanced green fluorescent protein (EGFP) cDNA at the 3'-terminus of the FLRG coding region were inserted into pcDNA3 plasmid (Invitrogen, Madison, WI). These constructs were transfected into PC12 cells using the calcium phosphate precipitation method (Chen and Okayama, 1987); transfected cells were selected

by G418 (Geneticin, Gibco BRL, NY) and were confirmed to stably express mRNAs and the proteins of the constructs. Highly expressive clones were selected and used in the experiments described here.

Purification and cloning of rat FLRG

To purify substances responsible for cell survival, one liter of conditioned media was collected from the serum-free culturing of *bcl-2*/PC12 cells. After centrifugation of the conditioned medium at 3,000 g to remove debris, the supernatant was concentrated 100 fold by cutting off molecular sizes in excess of 50 kD and below 10 kD using gas-pressured membrane filters (Stirred ultrafiltration cell Mdoe 8010, Amicon Inc., MA). The concentrated medium was applied to a reversed-phase HPLC using a Phenyl 5PW-RP (ϕ 5.0 mm $\times$ 5.0 cm, Tosoh, Tokyo) column equilibrated with a buffer A containing 0.05% trifluoroacetic acid (TFA) and eluted with a gradient of 0–60% acetonitrile in the buffer A. The resulting elutes were separated into 30 fractions which were further lyophilized and applied to cell death assays and SDS-PAGE. A common band found in the fractions which showed a high survival activity had a molecular weight of 35 kD. The concentrated conditioned medium was also fractionated by gel filtration using a Shodex KW-803 column (ϕ 8.0 mm $\times$ 300.0 mm, Hitachi, Tokyo) in a 0.1 M phosphate buffer, pH 7.4, containing 0.1 M Na₂SO₄, and the separated fractions were applied to cell death assays to estimate the fraction showing peak cell surviving activity.

The fraction with the highest survival activity prepared by HPLC was then applied to 7.5% native PAGE and stained with CBB (Nakarai, Kyoto). The stained bands were cut out and homogenized and extracted with a 20 mM Tris-HCl buffer, pH 7.5, for 12 h at 4°C. After centrifugation, the supernatants were dialyzed with HDMEM for 12 h at 4°C and applied to SDS-PAGE. After SDS-PAGE, candidate proteins were transferred to an Immobilon PSQ membrane (Millipore, Bedford, MA, USA) and their amino terminal sequences determined using a protein sequencer HPG100A (Hewlett Packard, Palo Alto, CA, USA). A protein having a molecular mass of 35 kD was further analyzed to obtain the internal amino acid sequences by cleavage with cyanogen bromide. The resulting amino terminal and one internal sequence analyzed were VMGSGDSVPG GVVWLQQGKEAT and YRGR, respectively.

For the cDNA cloning of the 35 kD protein, partial cDNA fragments were amplified using degenerate primers corresponding to the amino acid sequences; the forward and reverse primers used were 5'-ca (ag) ca (ag) gg (acgt) aa (ag) ga (ag) gc (acgt) ac-3' and 5'-c (gt) (acgt) cc (acgt) c (gt) (ag) ta-3', respectively and the cDNA fragment was generated

from *bcl-2*/PC12 cells mRNA by RT-PCR using an LA RNA PCR kit (Takara, Tokyo). The coding sequence obtained possessed 366-bps; to identify the presumed full-length rat cDNA, a rapid amplification of the cDNA ends (RACE) with adapter ligated *bcl-2*/PC12 cDNAs was performed using a Marathon Race Kit (Clontech, USA). The forward (5'-tcagaattcgtgctgaagacacaggt cagcc-3') and reverse (5'-gactctagagctcgaggacgcgcaggtctgggtgtcc-3') primers were used for the 3'- and 5'-RACE, respectively. To obtain the complete full-length cDNA of the 35 kD protein, a λ gt11 cDNA library of *bcl-2*/PC12 cells was constructed using a SuperScript Choice System for cDNA synthesis (Gibco BRL, NY, USA). The library (5×10^5 recombinants/screening) was screened with a 32P labeled fragment using a Random Primer DNA Labeling Kit (Takara), and positive clones were analyzed by constructing deletion mutants using a Kilo sequence deletion kit (Takara). DNA sequencing was analyzed using a DNA sequencer SQ-5500 (Hitachi, Tokyo).

Antibodies and immunoblotting

Monoclonal antibodies against synaptophysin and GFP were obtained from commercial sources (CHEMICON, CA, USA). Rabbit polyclonal antibodies against two rat synthetic peptides corresponding to VMGSGDSVPGGV (24–35) and RTARCRGHPDLRVMY (147–161) were raised and purified by affinity chromatography. Antibody specificity was evaluated by Western blot analysis using the conditioned medium of the *bcl-2*/PC12 cells. The conditioned medium of the cells was electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride membranes (Millipore, Tokyo). The membranes were blocked with 10% bovine serum albumin (BSA) and probed with the primary antibodies at various concentrations for 1 h at room temperature. After washing with PBS containing 0.05% Tween 20 (TPBS), the membranes were further incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase for 1 h at room temperature. Subsequent visualization was performed using an ECL reagent (Amersham Pharmacia, Piscataway, NJ, USA).

Determination of secretory pathways of FLRG in PC12 cells

Wild-type (wt) PC12 cells and those overexpressing the FLRG or FLRG-EGFP proteins were passaged into 6-well-plates at densities of 5×10^5 cells per well, respectively. After 24 h, the culture medium was changed to HDMEM containing 1.0% FBS, and the cells were cultured for an additional 4 h. The cells were then incubated in the fresh

medium twice for 30 min each. To stimulate the secretory pathways, cultures were further incubated in a fresh medium containing 55 mM KCl for 30 min. Proteins in the culture media obtained at each step from each experimental group were precipitated by the addition of 10% trichloroacetic acid (TCA, Sigma, St. Louis, MO, USA) at 4°C for 10 min. After centrifugation at 15,000 g for 10 min, the resulting pellets were dissolved in 100 μ l of an SDS sample buffer and boiled. Cells in each experimental group after stimulation with KCl were lysed with a lysis buffer consisting of 0.05 M Tris-HCl, pH 7.5, 0.15 M NaCl and 1% Triton X-100 and prepared for SDS-PAGE. A 20 μ l aliquot of each sample was subjected to immunoblotting as described above. The density of each protein band in the blotting was measured using NIH image software and the amount of secreted and cellular FLRG protein was calculated as a percentage of the values obtained in the case of cellular FLRG protein without EGFP. The amount was the mean $\pm$ standard errors of the mean (SEM) of three independent experiments.

Regulation of secretion of FLRG protein from PC12 cells

For Western blot analyses, wt PC12 cells were passaged into 6-well-plates at densities of 5×10^5 cells per well, respectively. After 24 h, the culture medium was changed to HDMEM containing 1.0% FBS, and the cells were then cultured for 0, 1, 3, 6, 12, 24, 36, 48 and 72 h in the absence or presence of 50 ng/ml activin A (Promega, Madison, WI, USA). Proteins in the culture media obtained at each time point from each experimental group were precipitated by treatment with 10% TCA (Sigma) at 4°C for 10 min. After centrifugation at 15,000 g for 10 min, the pellets were dissolved in 100 μ l of an SDS sample buffer and boiled. A 20 μ l aliquot of each sample was subjected to immunoblotting as described above. The density of each protein band in the blotting was measured using NIH image software, and the amount of secreted FLRG protein was calculated as the percentage of the values that were obtained in the case of 48 h-cultured media with 50 ng/ml activin A. The amount was the mean $\pm$ SEM of three independent experiments.

Immunohistochemistry and in situ hybridization

To define the localization of the FLRG protein in wild-type and FLRG-EGFP cDNA-transfected PC12 cells, they were cultured for 7 days in the absence of serum but in the presence of 100 ng/ml NGF and fixed with 4% paraformaldehyde containing 4% sucrose buffered with a 0.1 M phosphate buffer, pH 7.2, for 2 h. The cells were thoroughly

washed with PBS and treated with 0.3% H₂O₂ in methanol for 30 min. For double immunostaining, the cells were incubated with an anti-rat FLRG protein (40 mg/ml) and monoclonal anti-GFP or anti-synaptophysin for 24 h at 4°C and further incubated with Texas red-conjugated anti-rabbit IgG and FITC-conjugated anti-mouse IgG for 1 h at room temperature (Sigama, St. Louis, MO). The stained cells were viewed with a confocal laser scanning microscope (LSM510, Carl Zeiss, Germany).

To examine the tissue distribution of FLRG mRNA and protein, 10 adult Wistar rats (8 weeks of age) were deeply anesthetized with pentobarbital (25 mg/kg i.p.) and fixed by cardiac perfusion with 4% paraformaldehyde buffered with a 0.1 M phosphate buffer, pH 7.2, containing 4% sucrose, for light microscopic immunohistochemistry. Adrenal and testicular tissues were quickly excised from the animals and further immersed in the same fixative for 2 h. The samples were processed for paraffin embedding, with 5 µm sections cut with a microtome and placed on silane-coated glass slides, while some samples were frozen in liquid nitrogen after cryoprotection with sucrose solutions, and 10 µm sections were cut with a cryostat and placed on the glass slides.

Deparaffinized and frozen sections from each sample were immunostained, as described by Nitatori *et al.* (Nitatori *et al.*, 1995) or Koike *et al.* (2002). Briefly, they were treated with 0.3% H₂O₂ in methanol for 30 min and incubated with 2% normal goat serum for 20 min at room temperature. They were then incubated at 4°C with an anti-FLRG protein (40 µg/ml) for one to three days. Further incubations were performed with biotinylated goat anti-rabbit IgG for 1 h and peroxidase-conjugated streptavidin (VECTASTAIN ABC KIT, Vector Laboratories, Burlingame, USA) for 1 h at room temperature. After each step, the sections were rinsed thoroughly in 0.1 M phosphate-buffered 0.5 M saline (pH 7.2) (PBS), containing 0.1% Tween 20 (TPBS) (Sigma, St. Louis, MO, USA). Staining for peroxidase was performed using 0.0125% 3, 3'-diaminobenzidine tetrahydrochloride and 0.002% H₂O₂ in a 0.05 M Tris-HCl buffer (pH 7.6) for 10 min.

In-situ hybridization was conducted using digoxigenin-labelled riboprobes to localize mRNA encoding for FLRG in the testicular samples. Riboprobes were synthesized from the same plasmid preparations used for cDNA cloning, using a DIG RNA labeling mix (Roche) with SP6/T7/T3 polymerase, and were thereafter purified by centrifugation through Chromaspin columns (Clontech, Palo Alto, CA, USA).

Deparaffinized sections were digested with 12 µg/ml of proteinase K for 30 min at 37°C. Antisense and sense DIG-labelled probes (1 ng/µl) were applied to hybridization buffer containing 10% dextran sulphate, 200 mg/ml tRNA

(Roche, Tokyo), 0.25% SDS (Sigma), 1 × Denhardt's solution, 600 mM NaCl, and 1 mM EDTA (pH 8.0), and incubated for 17 h at 50°C. For post-hybridization, tissue sections were washed 2 × SSC at 50°C, and non-specific binding was abolished by treatment with 20 mg/ml RNase A for 30 min at 37°C. The specifically bound probe was subsequently visualized using an anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche) diluted in 1.5% blocking reagent (Roche) in 100 mM Tris-HCl (pH 7.5), 150 mM NaCl, with 5-bromo-4-chloro-3-indoxyl phosphate/nitro blue tetrazolium chloride (NBT/BCIP) (Roche) as a chromogen, to produce a deep bluish purple stain. No counterstain was used, and sections were mounted with Glycerol Gelatin (Sigma). Sense probes were applied at matching concentrations to serial tissue sections and treated identically, thus serving as negative controls.

RT-PCR analysis

Total RNAs were extracted from various tissues of adult rats including the brain, lung, liver, intestine, heart, kidney, ovary and testis, from whole embryos obtained at embryonic days 9 to 12 (E9 to E12), and from wild-type PC12 cells, using a Polytron homogenizer for 1 min in Isogen. To avoid contamination with genomic DNA, 40–50 µg of the total RNAs were treated with 0.2 U/ml DNase I (Nippon Gene) for 20 min at 37°C. Then 2.0 µg/ml of the total RNAs were used for cDNA synthesis using AMV reverse transcriptase (Takara) and amplification of the FLRG and GAPDH cDNAs were performed by PCR using the same primers as mentioned above.

Quantitative RT-PCR (QPCR)

The total RNAs extracted from various rat tissues including the brain, lung, kidney, adrenal gland, testis and ovary at E18, at birth (P0), and at 1, 4, and 6 weeks after birth, and from whole embryos from E9 to E16 in TRIzol (Invitrogen, Carlsbad, CA) were further used for the determination of mRNA concentrations. The cDNAs of FLRG, follistatin, and activin A were synthesized by using 5.0 µg of total RNA in 20 µl reverse transcript solutions (1 × reverse transcription buffer, 500 ng oligo-dT primer, 40 U RNaseOUT recombinant RNase Inhibitor, 500 µM dNTP mix, 10 mM dithiothreitol, 5 mM MgCl₂ and 50 U SuperScript II) using a SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). The reaction was performed at 42°C for 50 min. The reverse transcript reactions were then stopped by heating to 70°C for 15 min, and RNA templates were digested with RNase H at 37°C for 20 min. cDNA reactions were used for real-time quantitative PCR (QPCR) using an ABI PRISM 7700 Sequence Analyzer (Applied

Biosystems, Tokyo). The gene-specific forward/reversed primers and fluorescein labeled probes are given below. PCR was carried out with a Platinum Quantitative PCR SuperMix-UDG (Invitrogen, Carlsbad, CA, USA) using 1.25 μ l of cDNA, 200 nM Fam (6-carboxy-fluorescein) labeled gene-specific TaqMan probes, and 200 nM gene-specific forward and reverse primers. The PCR amplification was performed using a 96-well optical tray and caps with a 25 μ l final reaction mixture at 50°C for 2 min and at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and at 60°C for 1 min.

The primer pairs and probes were as follows: activin A (inhibin β -A) (GenBank accession number NM_017128) forward: 5'-CAAGGATATTGGCTGGAATGACT-3', reverse: 5'-GGCACTCACCTCACAATAGTTG-3', TaqMan probe: 5'-TCATTGCTCCCTCTGGCTATCATGCC-3'; follistatin (GenBank accession number NM_012561) forward: 5'-CCGGAACCCTCATCTTCAGA-3', reverse: 5'-CCCTCATAGGCTAATCCAATGG-3', TaqMan probe: 5'-CCTGAGAAAGGCCACCTGGCTTGCTG-3', FLRG (GenBank accession number AB021295) forward: 5'-CCAACCCTGGCCAAGAACT-3', reverse: 5'-AATGGAA CGGCCAGAAAG-3', Taqman probe: 5'-CTCGTCGTGT CACTTGCGCCAGG-3', TGF- β 1 (GenBank accession number NM_012578) forward: 5'-GAATACAGGGCTTTC GCTTCA-3', reverse: 5'-TCGGTTCATGTCATGATGGG T-3', TaqMan probe: 5'-TCAGTCCCAAACGTCGAGGTG ACCTG-3'.

To determine the absolute concentration of the target transcripts, conventional PCR for activin A, follistatin, FLRG, and TGF- β 1 was performed by EX Taq polymerase (Takara) using a rat embryo cDNA and each gene-specific forward and reverse primers—which were the same primers as those used for QPCR. The purified PCR products were diluted at 0.1, 10, 100 fg and 10 pg, and used for QPCR as templates to generate standard curves. The concentrations of mRNAs in each sample were calculated with the Sequence Detection System software (Applied Biosystems, Tokyo). The crossing line intercept of an unknown sample was subsequently compared with the standard curve to generate a quantitative amount of starting materials. The mRNAs of each specific gene were calculated and indicated in fmol units which were contained in 1 μ g of total RNA. The final amount was presented as the mean $\pm$ SEM of three independent experiments.

Statistics

Statistics were performed according to analysis of variance (ANOVA)

Results

CDNA of FLRG and its deduced amino acid sequences

As evidenced by native-PAGE analysis, the RP fraction with the highest survival activity contained several protein bands. One of these bands, with a molecular weight of 35 kD, was further analyzed. After an amino acid sequence determination at the aminoterminal region, the protein was digested by cyanogen bromide, and a cDNA from the *bcl*-2/PC12 cell cDNA library was cloned and confirmed to encode the amino acid sequence of the protein (Figure 1A). The cDNA encoded a 265 amino acid sequence which contained a 23 amino acid signal sequence and two potential N-linked glycosylation sites (Fig. 1A, C). A data bank analysis indicated that the 35 kD protein was a rat homologue (rFLRG) of human FLRG (hFLRG) protein (Hayette *et al.*, 1998). These FLRG proteins contained two cysteine rich domains corresponding to the follistatin module (Fig. 1C). The rFLRG protein showed a diffuse band when examined by SDS-PAGE, and its positive band in Western blotting shifted to a lower band (approximately 28 kD) after digestion with glycopeptidase (data not shown), suggesting that the rFLRG protein is glycosylated. The predicted amino acid sequences of rat, mouse, and human FLRG proteins reported (Hayette *et al.*, 1998; Tsuchida *et al.*, 2000) revealed high degrees of conservation; in a comparison of the amino acid sequences of FLRG proteins among the three species, the rFLRG protein shared 93.6% and 72.7% homologies with mouse (m) FLRG and hFLRG proteins, respectively (Fig. 1B).

Using purified FLRG protein, the survival activity was examined, but no clear-cut survival activity was detected when wt PC12 cells in serum-deprived cultures were exposed to the protein at various concentrations (data not shown).

Distribution of rFLRG in embryos and various tissues during postnatal life

We examined the expression of FLRG mRNA in embryos at various stages and various tissues during postnatal life by RT-PCR and QPCR analyses. By RT-PCR, the gene expression was ubiquitously detected in adult tissues, and a relatively abundant expression was observed in the lung, testis, ovary, and adrenal gland (Fig. 2A). In central nervous system tissues, mRNA expression was found to be low in the cerebrum and cerebellum but high in the spinal cord. Moreover, in whole embryos, the gene expression was detected as early as E9 and became high at E10 (Fig. 2A).

Since the rFLRG protein possesses two follistatin

A

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ATGCGTCCTGGGGCACTGTGGCCGCTGCTTTGGGGAGCCCTGGTCTGGGCGGTGGGATCC
M R P G A L W P L L W G A L V W A V G S
GTGGGCGCCGTGATGGGCTCGGGGATTCTGTGCCGGTGGTGTGTGCTGGCTCCAGCAG
V G A V M G S G D S V P G G V C W L Q Q
GGCAAGGAGGCCACCTGCAGTCTGGTGCTGAAGACACAGGTACGCCGGGAGGAGTGCTGC
G K E A T C S L V L K T Q V S R E E C C
GCTTCCGGCAACATCAACACAGCCTGGTCCAATTACCCACCCGGGCAATAAAATCAGC
A S G N I N T A W S N F T H P G N K I S
CTGCTAGGATTCCTGGGCCTTGTCCACTGCCTACCATGCAAAGATTCTTGCACGGAGTG
L L G F L G L V H C L P C K D S C D G V
GAGTGCGCCCCCGGCAAGGCGTGCCGATGTTGGGGGCGTCCACTGCGAGTGCCTG
E C G P G K A C R M L G G R P H C E C V
TCCAACCTGCGAGGGGTTCCCGCGGGCTTCCAGGTCTGCGGCTCTGATGGCGCCACCTAC
S N C E G V P A G F Q V C G S D G A T Y
CGGGACGAATGCGAACTGCGCACCGCGCTGTGCGGACACCCAGACCTGCGCGTCATG
R D E C E L R T A R C R G H P D L R V M
TACCGCGCGCTGCCAAAAGTCTGCGCTCAGGTAGTGTGCCCGCGCCCGCAGTCGTC
Y R G R C Q K S C A Q V V C P R P Q S C
CTGTGGATCAGACTGGCAGCGCACACTGCGTGGTGTGTCGCGCTGCACCTGCCAGTA
L V D Q T G S A H C V V C R A A P C P V
CCTCCCAACCTGGCCAAGAACTCTGCGGCAACAACAGTTACCTACATCTCGTCGTGT
P P N P G Q E L C G N N N V T Y I S S C
CACTTGCAGCCAGCCACTTGCTTTCTGGGCGTTCATTGGAGTTCGGCACCCGGGCATC
H L R Q A T C F L G R S I G V R H P G I
TGACAGGTGGCCCCAAAGTACCAGCAGAGGAGGAAGAGAAGTCTCGTGTGA
C T G G P K V P A E E E E N F V *

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B

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rFLRG  MRPGA---LWPLLW GALVWAVGSV GAVMGS GDSVPGGV CW
mFLRG  **S*---*****T*****E*****
hFLRG  *****PGP*****P*****A*****F*-SS*****NPA*****

rFLRG  LQQGKEATCSLV LKTQVSRECCASGNINTAWSNFTHPGN
mFLRG  ***R*****R*****
hFLRG  ****Q*****Q*D*T*A*****D*****L*****

rFLRG  KISLLGFLGLVHCLPCKDSCDGV ECGPGKACRMLGGRPHC
mFLRG  *****
hFLRG  **N*****

rFLRG  ECVSNCEGVPA GFQVCGSDGATYRDECELRTARCRGHPDL
mFLRG  *****L*****
hFLRG  **A*D*S*L**RL*****A*****

rFLRG  RVMYRGRCQKSCAQVVCPRPQSC LVDQTGSAHCVV CRAAP
mFLRG  *****
hFLRG  S*****R***EH*****V*****

rFLRG  CPVFPNPQQLCGNNNTYIISCHLRQATCF LGRSIGVRH
mFLRG  ****S*****
hFLRG  ****SS*****M*****

rFLRG  PGICTGGPKVP----AEEEEENFV 256
mFLRG  *****-----***** 256
hFLRG  A*S*A*T*EE*PGGES***** 263

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C

Fig. 1. Structure of rat FLRG. **A:** Nucleotide sequence of an FLRG cDNA open reading frame and the deduced amino acid sequence. A lined portion shows the nucleotide and amino acid sequences corresponding to a signal sequence. **B:** Comparison of amino acid sequences of rat (r), mouse (m) and human (h) FLRG. In the consensus alignment, identical amino acids of mFLRG and hFLRG to rFLRG are shown by asterisks. Total numbers of amino acids are noted at the ends. **C:** Schematic demonstration of the FLRG protein. SS shows the signal peptide (1–23), and FM I and FM II show the follistatin module (cysteine-rich domain) I (97–165) and II (169–241). Possible glycosylation sites (flags) are noted at positions 71 and 213.

modules (cysteine rich domains) and is considered to be a member of the follistatin family of proteins, tissue mRNA expression was compared between FLRG and follistatin, and activin A as their binding partner, using the QPCR method. In whole embryos from E9 to E15, the expression levels of follistatin mRNA were consistently high at any stages, compared with those of FLRG mRNA, although the levels of FLRG mRNA were relatively high at E10 (Fig. 2B).

As we have previously demonstrated expression levels of FLRG and follistatin mRNAs at 8 weeks of age, the levels of FLRG mRNA were high in the testis and adrenal gland, while those of follistatin mRNA were conspicuous in the ovary (Zhang *et al.*, 2003). In brain (cerebral) tissue, the concentrations of FLRG and follistatin mRNAs were low at all stages examined, compared with those in other peripheral tissues (Fig. 3A). Concentrations of FLRG mRNA in the lung increased substantially from one to four weeks after birth and then decreased (Fig. 3B), while they reached high levels in the adrenal gland after 4 weeks of age (Fig. 3D). In the kidney, relatively high expression levels were detected for FLRG mRNA at one week of age and in follistatin mRNA after four weeks of age (Fig. 3C). The concentrations of FLRG and follistatin mRNAs showed opposite results between the testis and ovary; the expression level of FLRG mRNA predominated in the testis, and that of follistatin mRNA in the ovary after birth (Fig. 3E, F). The expression levels of activin A mRNA were low in all tissues except for the ovary, where they reached high levels after 4 weeks of age (Fig. 3A – F). Thus, the expression levels of FLRG mRNA differed depending on developmental stages as well as tissues, and from those of follistatin mRNA in peripheral tissues from E16 to 6 weeks of age.

To confirm the high level of expression of FLRG mRNA in the testis and adrenal gland, in situ hybridization and/or immunostaining were performed in these tissues. In the testis of adult rats, positive signals for FLRG mRNA were localized intensely in spermatocytes located in the inner part but not in the large parts of spermatocytes, while they were found in spermatogonia and primary spermatocytes (Fig. 4A, B). Positive immunostaining for the FLRG protein was also localized in these cells in seminiferous tubules, and cap-like structures in more differentiated spermatocytes were intensely positive for the protein (Fig. 4C). In adult rat adrenal tissue, positive staining for FLRG protein was distinct in both cortical and medullary cells (Fig. 4D). In particular, the immunoreactivity for the protein was localized in some medullary endocrine cells (Fig. 4E), while it was intensely detected in cortical cells of the zona glomerulosa (Fig. 4F).

Fig. 2. Expression of FLRG and follistatin mRNAs. **A:** A RT-PCR study. The expression of FLRG mRNA is ubiquitous and relatively abundant in the lung, adrenal gland, testis, and ovary from adult rats, and in a whole embryo at E10 (upper panel). Lower panel shows GAPDH mRNA, used as internal controls. **B:** A quantitative PCR study. Expression levels of FLRG mRNA are detectable in whole embryos at E9 and transiently increase in embryos at E10, whereas concentrations of follistatin mRNA are significantly higher in embryos from E9 to E15 than those of FLRG mRNA. The mRNAs of FLRG and follistatin were indicated in fmol numbers which were contained in 1 μ g of the total RNA. The final amount is presented as the mean $\pm$ SEM of three independent experiments.

Fig. 3. Changes in the expression levels of FLRG, follistatin, and activin A mRNAs in various rat tissues during pre- and postnatal lives. Total RNAs were obtained from the brain (A), lung (B), kidney (C), adrenal gland (D), testis (E), and ovary (F) of rat embryos at E18 and rats at birth (P0), and at 1 week (1W), 4 weeks (4W), and 6 weeks (6W) after birth. The mRNAs of FLRG, follistatin, and activin A were indicated in fmol numbers which were contained in 1 μ g of total RNA. The final amount is presented as the mean $\pm$ SEM of three independent experiments.

Fig. 4. Localization of FLRG mRNA (A, B) and protein (C–F) in the testis (A–C) and the adrenal gland (D–F) of adult rats. A–C: Positive signals for FLRG mRNA are intense in spermatocytes located in the inner part but not in large parts of the spermatocytes, while they are also seen in spermatogonia and primary spermatocytes (A, B). Immunoreactivity for the FLRG protein is also seen intensely in spermatogonia and some of the spermatocytes, but weakly in primary spermatocytes (C). D–F: Positive staining of FLRG protein is detected in cells located in both the cortex and medulla (D). The immunoreactivity was intense in some medullary endocrine cells (E), while it was strongly positive in endocrine cells through the cortex, especially in those of the zona glomerulosa (F). Bars: 20 μ m (A–C), 100 μ m (D), 10 μ m (E, F)

Secretory properties of FLRG protein in PC12 cells

Since the FLRG protein possesses a signal sequence of 23 amino acids and has been found in the medium of *bcl-2*/PC12 cells, it is clearly a secretory protein. To investigate the secretory pathway for the FLRG protein, wt PC12 cells and cells overexpressing FLRG or FLRG-EGFP protein were prepared. The cells were cultured in HDMEM containing 1.0% FBS for 4 h. They were then incubated in a fresh medium twice for 30 min each to examine secretion of the protein in the absence of stimulation, while cultures were further incubated in a fresh medium containing 55 mM KCl for 30 min to stimulate the secretory pathway. As shown in Figure 5A, no FLRG protein was detected in the media of wt PC12 cells after 4-h or each of the 30-min incubations or even after the stimulation with 55 mM KCl, although the protein was present in low levels in the cells after KCl

stimulation. When PC12 cells overexpressing the FLRG or FLRG-EGFP protein were incubated for a longer time span, 4 h, clear-cut protein bands for FLRG/FLRG-EGFP proteins were detected in the media (Fig. 5A, B). Over a short time span, however, no protein accumulated in the media after each of the 30-min incubations, but dense bands corresponding to FLRG/FLRG-EGFP proteins were detected in the media and cells after KCl stimulation (Fig. 5A, B). These results indicate that the FLRG protein is synthesized in PC12 cells while it is secreted from the cells by both regulated and constitutive pathways.

To confirm the presence of the FLRG protein in PC12 cells, immunohistochemistry was applied to wt PC12 cells and those overexpressing the FLRG-EGFP protein. In wt PC12 cells, no positive staining for FLRG protein was found (data not shown), but dual staining with anti-FLRG and anti-GFP showed that granular staining for the FLRG protein

Fig. 5. Secretion of the FLRG protein from PC12 cells is mediated by both the regulated and constitutive pathways. **A:** Western blots of FLRG/FLRG-EGFP protein in media of wild-type PC12 cells (wild), and those overexpressing the FLRG protein (FLRG) and FLRG-EGFP protein (FLRG/GFP), respectively. Wild-type PC12 cells and those overexpressing FLRG or FLRG-EGFP proteins were first cultured at a density of 5×10^3 per well in HDMEM containing 1.0% FBS for 4 h, and then incubated in a fresh medium two times for 30 min each to examine the secretion of the protein without stimulation, while cultures were further incubated in fresh medium containing 55 mM KCl for 30 min to detect the proteins in the media (KCl med) and in cells (KCl cell) after secretagogue. **B:** Quantification of protein densities in A. Columns indicate amounts of the FLRG protein in cultured media of wild-type PC12 cells (white), and those overexpressing the FLRG protein (grey) and FLRG-EGFP (black). Vertical bars indicate $\pm$ standard deviation.

completely coincided with that for GFP in the transfected cells cultured in the presence of serum (Fig. 6A-C), indicating that antibodies raised against synthetic fragments of rat FLRG protein can be utilized for immunostaining. Since the FLRG protein is secreted from cells by the regulated pathway, the protein is assumed to be stored in secretory granules which accumulate in the cells. To confirm this, transfected cells cultured in the absence of serum but in the presence of NGF for 7 days were used for the staining of synaptophysin, a marker of secretory granules, and of FLRG-EGFP. Positive signals for synaptophysin were localized in the perinuclear Golgi area and the terminal region of the neuritis (Fig. 6D, F), whereas immunoreactivity for the FLRG-EGFP protein was detected largely in the perinuclear Golgi region of the cell bodies and also in the terminal region of the processes (Fig. 6E, F). A high power view of the terminal swelling demonstrated that the synaptophysin-immunoreactivity was largely co-localized in granular structures with GFP immunoreactivity (Fig. 6G). Collectively, these lines of evidence suggest that the FLRG protein is not only secreted from PC12 cells by the constitutive pathway but also sorted into secretory granules from the trans-Golgi network, which are mostly secreted by the regulated pathway.

Regulation of FLRG protein secretion from wild-type PC12 cells by activin A

PC12 cells overexpressing FLRG or FLRG-EGFP protein were shown to secrete the protein without stimulation when observed over a long time span, 4 h, but wild-type cells were not. The expression of FLRG mRNA has been shown to be up-regulated in hematopoietic cells and astrocytes by TGF- β 1 (25-27), and in HepG2 cells by activin A (28). Since PC12 cells do not express TGF- β 1 receptors, the effect of activin A on the expression of the FLRG protein in wt PC12 cells was examined by detecting the FLRG protein secreted from the cells. For this, wt PC12 cells were passaged into 6-well-plates at a density of 5×10^5 cells per well, respectively. After 24 hr, the culture medium was changed to HDMEM containing 1.0% FBS, and the cells were then cultured for 0, 1, 3, 6, 12, 24, 36, 48 and 72 h in the absence or presence of 50 ng/ml activin A. As shown in Figure 7, even in cultures that were not stimulated by activin A, a weak protein band corresponding to the FLRG protein appeared 12 h after the beginning of the cultures, and this band became distinct at 24 h and thereafter. In stimulated culturing with activin A, a weak protein band corresponding to the protein was distinct at 12 h, while the protein band became much more intense at 24 h and thereafter than that without stimulation (Fig. 7A, B). Thus, wt PC12 cells secrete the FLRG protein without stimulation and its expression in the cells is enhanced by activin A.

Fig. 6. Presence of the FLRG protein in secretory granules of PC12 cells. **A – C:** Dual staining of FLRG-EGFP protein in PC12 cells. Granular immunostaining of FLRG-EGFP which was stained with an anti-GFP (**A**) and anti-FLRG protein (**B**), respectively, perfectly coincides with each other (**C**). **D – F:** Colocalization of FLRG-EGFP and synaptophysin in secretory granules. PC12 cells overexpressing FLRG-EGFP cultured in the absence of serum but in the presence of NGF for 7 days were immunostained for synaptophysin (**E**), a marker for secretory granules, and of FLRG-EGFP with anti-GFP (**D**). Positive signals for synaptophysin are localized in the perinuclear Golgi area and the terminal region of the neurites (**E, F**), whereas immunoreactivity for the FLRG-EGFP protein is detected largely in the perinuclear Golgi region of the cell bodies and also in the terminal region of processes (**D, F**). **G:** A higher magnification of the terminal swelling in **F**. Positive signals of synaptophysin are colocalized well in secretory granules with those of FLRG-GFP. Bars: 10 μ m (**A – F**), 5 μ m (**G**)

Discussion

We have previously shown that *Bcl-2* cDNA-transfected PC12 cells survive following serum deprivation and differentiate into neuron-like cells (Sato *et al.*, 1994). In a search for trophic factors of PC12 cell survival/differentiation, we purified a 35 kD protein from conditioned media of the cells and cloned its cDNA. A database analysis showed that the 35 kD protein is a rat homologue of the hFLRG protein. Like the hFLRG protein, the rFLRG protein was a glycoprotein, while the rFLRG protein consisted of 256 amino acids with 23 amino acids of a signal sequence and shared a sequence similarity of 93.6% and 72.7% with mFLRG and

hFLRG proteins, respectively (Hayette *et al.*, 1998; Tsuchida *et al.*, 2000).

The present study examined the distribution of FLRG mRNA and protein in rat tissues during embryonic and/or postnatal stages and the secretory properties of the protein in PC12 cells. Our results demonstrate that: 1) the expression of FLRG mRNA is relatively low during embryonic life, compared with that of follistatin mRNA, while its postnatal expression is age-dependent, being abundant in the lung from 1 to 4 weeks of age, in the adrenal gland after 4 weeks of age, and in the testis after birth; 2) the FLRG protein is secreted from PC12 cells by both regulated and constitutive pathways; and 3) the secretory activity of FLRG using the constitutive pathway is up-regulated by activin A.

Fig. 7. Up-regulation of FLRG expression in wild-type PC12 cells by activin A. **A:** Western blots of FLRG protein in the media of PC12 cells cultured in the presence (+) and absence (-) of 50 ng/ml activin A for the indicated time periods (Time). **B:** Quantification of protein densities in **A**. Block and white columns indicate amounts of the FLRG protein with and without activin A, respectively. Vertical bars indicate $\pm$ standard deviation. *shows $P < 0.05$.

Tissue distribution of FLRG

We previously demonstrated that the distribution of FLRG mRNA in adult rat tissues is similar to that in human and mouse tissues but largely different from the distribution of follistatin mRNA (Zhang *et al.*, 2003). Although FLRG mRNA was detected from E9 and increased at E10, the expression levels of follistatin mRNA were higher than those of FLRG mRNA during embryonic development. In fact, a loss of function study on follistatin has shown that it is essential for the development of the musculoskeletal system and the epidermis (Matzuk *et al.*, 1995). Our previous data showed that the expression level of FLRG mRNA is low in the adult and embryonic brains, but is conspicuously high in primary embryonic astroglial cells (Zhang *et al.*, 2003). This indicates that the identification of topographical sites and cells expressing FLRG is important to further understand the role of FLRG during embryonic development.

In contrast to the embryonic stages, it is interesting to note that expression sites of FLRG and follistatin mRNAs began to differ from each other during postnatal life, and the expression levels of follistatin and activin A mRNAs are conspicuous in the ovary. In cultured human granulosa-luteal cells, the expression of follistatin mRNA is regulated by gonadotrophins through both protein kinase A and C dependent pathways, and exogenous follistatin inhibits the effect of activin A (Cataldo *et al.*, 1994; Tuuri *et al.*, 1996), whereas that of FLRG mRNA is regulated by protein kinase C and the prostaglandin E2 signaling pathways but not by gonadotrophins (Liu *et al.*, 2002). Moreover, the expression level of FLRG mRNA varies diurnally in the rat ovary, with the highest level during day time, but its change does not depend on the estrus cycle, as evidenced by an RNase protection assay (Arai *et al.*, 2003). When overexpressed in one line of transgenic mice, the FLRG protein has been suggested to interrupt follicular development and fer-

tility in females (Schneyer *et al.*, 2001). Collectively, these lines of evidence suggest that the effect of activin A on steroidogenesis in ovarian follicles is mainly modulated by follistatin through the estrus cycle and, to some extent, by the FLRG protein through gonadotrophin-independent pathways.

Our present study using QPCR, however, demonstrated that the expression level of FLRG mRNA in the ovary is low, nearly one fourth that in the testis, where follistatin and activin A mRNAs are expressed at very low levels. The presence of the FLRG protein and mRNA has been immunohistochemically confirmed in spermatogonia and spermatocytes at various stages in seminiferous tubules, as well as by *in situ* hybridization. This suggests the importance of the FLRG protein in spermatogenesis. In particular, the FLRG protein can bind to several members of the TGF- β superfamily including activin A, and BMP-2, -6 and -7 (Tsuchida *et al.*, 2000; Tortoriello *et al.*, 2001). In these TGF- β superfamily members, BMP-7 has been shown to play a role in both the maintenance of spermatogenesis and epididymal function (Zhao *et al.*, 2001), suggesting that the FLRG protein may also be involved in spermatogenesis by regulating the functional role of BMP-7 as a possible binding partner. In addition to testicular tissue, FLRG mRNA was highly expressed in the lung during early postnatal development and in the adrenal gland during late postnatal development. In these tissues, follistatin and activin A mRNAs were also expressed at low levels. It is therefore reasonable to assume that certain binding partners of the FLRG protein are present in the adrenal gland and lung to maintain the growth and functions of the tissues.

Intracellular transport of FLRG protein and its secretion from PC12 cells

The present immunohistochemical results showed that the FLRG protein is localized in adrenal cortical and medullary cells. Since adrenal endocrine cells store secretory granules for peptide hormones and amines in the medulla and do not possess such secretory granules in the cortex, our data indicate that the FLRG protein has dual properties in the secretory pathways. Indeed, we have previously shown that the FLRG protein is secreted from primary embryonic astroglial cells and C6 cells, a rat astrogloma cell line, by the constitutive pathway (Zhang *et al.*, 2003). Until recently, however, no study has reported on the precise localization and secretory properties of the FLRG protein in peptide hormone-producing endocrine cells.

It is well known that regulated secretory proteins are stored in secretory granules, while constitutive secretory proteins are transported from the trans-Golgi network to cell membranes and secreted without storage in secretory

granules. The sorting signals and the mechanisms of secretory proteins from the trans-Golgi network to secretory granules have been studied (Gumbiner and Kwilly, 1982; Gorra *et al.*, 2001); several candidates responsible for selective sorting have been demonstrated, including the granin family of proteins (Tooze *et al.*, 1998, 2001; Hosaka *et al.*, 2002) and the membrane-associated form of carboxypeptidase E (Cool *et al.*, 1997), although a comprehensive mechanism for this selective sorting remains largely unsettled (Goor *et al.*, 1999; Cowley *et al.*, 2000). As stated above, the FLRG protein was found in adrenal medullary endocrine cells and conditioned media of wt PC12 cells, a rat adrenal pheochromocytoma, although its localization was not detected in wt PC12 cells. We therefore prepared PC12 cells overexpressing FLRG or FLRG-EGFP protein to examine its secretory properties and found that the FLRG protein is vigorously secreted from the PC12 cells by the regulated pathway and is sorted into secretory granules, which are largely accumulated in the terminal swelling of the neuron-like processes of the cells. At present, the sorting mechanism responsible for the FLRG protein remains unknown in endocrine cells. Moreover, when the secretory activity from wt and transfected PC12 cells was observed for longer time spans, these cells were found to secrete the protein by the constitutive pathway. The current results suggest that the FLRG protein possesses dual properties in secretory pathways, both constitutive and regulated.

Regulation of FLRG protein secretion from wild-type PC12 cells by activin A

As stated above, the FLRG protein was secreted from PC12 cells via the regulated pathway when stimulated by a secretagogue, KCl, while it was also secreted from the cells through the constitutive pathway to some extent. The secretion of proteins by the constitutive pathway is regulated by the synthesis of proteins in the cells which is largely dependent on their mRNA expression. In human HepG2 cells, the expression of FLRG mRNA is rapidly induced by TGF- β and activin A through Smad proteins (Bartholin *et al.*, 2001, 2002). We have previously shown that TGF- β 1 enhances both the expression levels of FLRG mRNA in and the secretion of its protein from primary astroglial cells (Zhang *et al.*, 2003). BMP-2 and activin A induce the neuronal differentiation of PC12 cells without activating MAP kinases and MEK (Iwasaki *et al.*, 1996), whereas TGF- β 1 shows no effect on the differentiation of these cells since detectable amounts of TGF- β 1 receptors are not expressed (Cosgaya *et al.*, 1997). We therefore examined whether activin A facilitates the secretion of the FLRG protein from PC12 cells through the constitutive pathway. Although we failed to confirm that activin A, a member of the TGF- β super-

family, induces the neuronal differentiation of PC12 cells or enhances the survival of the cells (data not shown), it distinctly facilitated the secretion of the FLRG protein from wt PC12 cells. At present, the question of why activin A cannot induce the neuronal differentiation of PC12 cells remains unanswered. However, these lines of evidence suggest that the expression of FLRG is regulated in PC12 cells by activin A, probably through the activation of Smad proteins.

Collectively, our current data—showing that rFLRG cloned from a PC12 cell cDNA library is highly expressed in several rat tissues such as the testis and adrenal gland where follistatin and activin A mRNAs are expressed at very low levels—argue for the presence of its binding partner which may belong to the TGF- β superfamily of proteins in these cells. Moreover, our previous and current data on the secretion of the FLRG protein indicate that this protein is secreted by either constitutive or regulated pathway, and that this differs depending on the cell types expressed. However, further studies will be required to define the physiological role of the FLRG protein in tissue cells.

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