Molecular Cloning and Characterization of RGC-32, a Novel Gene Induced by Complement Activation in Oligodendrocytes*

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Sublytic complement activation on oligodendrocytes (OLG) down-regulates expression of myelin genes and induces cell cycle in culture. Differential display (DD) was used to search for new genes whose expression is altered in response to complement and that may be involved in cell cycle activation. DD bands showing either increased or decreased mRNA expression in response to complement were identified and designated Response Genes to Complement (RGC) 1–32. RGC-1 is identical with heat shock protein 105, RGC-2 with poly(ADP-ribose) polymerase, and RGC-10 with IP-10. A new gene, RGC-32, that encodes a protein of 137 amino acids was cloned. RGC-32 has no homology with other known proteins, and contains no motif that would indicate its function. In OLG, the mRNA expression was increased by complement activation and by terminal complement complex assembly. RGC-32 protein was localized in the cytoplasm and co-immunoprecipitated with cdc2 kinase. RGC-32 has no homology with other known proteins, and contains no motif that would indicate its function. In OLG, the mRNA expression was increased by complement activation and by terminal complement complex assembly. RGC-32 protein was localized in the cytoplasm and co-immunoprecipitated with cdc2 kinase. Overexpression of RGC-32 increased DNA synthesis in OLGs C6 glioma cell hybrids. These results suggest that RGC-32 may play a role in cell cycle activation.

Myelin and oligodendrocyte (OLG)† are targets of immune-mediated attack in experimental allergic encephalomyelitis (EAE) and multiple sclerosis (MS). Complement activation plays a critical role in EAE. Activation of complement activation by systemic administration of cobra venom factor or soluble C3b receptor CR1 significantly inhibited inflammatory demyelination (1, 2). Deposition of C9 and C5b-9 in affected brains of EAE and MS (3, 4) as well as decrease in C9 and increase in soluble SC5b-9 in the spinal fluid of MS patients (5, 6) are indicative of terminal complement complex (TCC) assembly within the central nervous system compartment. The complement-activating property of myelin and OLG in the absence of antibody may also play a significant role in myelin damage and functional alteration of OLG in MS (7–9) and EAE induced by adaptive T-cell transfer (1).

Generation of membrane-inserted TCC can stimulate a variety of biological activities in the cell in the absence of lysis. The activities include expression of c-jun and c-fos proto-oncogenes, production of growth factors bFGF (bovine fibroblast growth factor) and PDGF (platelet-derived growth factor), and induction of mitosis (10–13). Increased cytosolic Ca²⁺ and protein kinase C activity are primarily induced by the pore-forming complexes C5b-8 and C5b-9, and generation of sn-1,2-diacylglycerol and ceramide begins at the stage of C5b-7 (14–16). TCC also activate heterotrimeric Gi proteins, and transduce Ras, Raf-1, MEK-1, and ERK1 pathway through the Gβγ subunits (17, 18). This Gβγ-mediated ERK1 signaling pathway is required for TCC to induce cell cycle (18, 19). Assembly of TCC in myelin activates neutral proteinases, and induces hydrolysis of myelin basic protein (MBP) (20), effects that may be responsible for demyelination of central nervous system organ cultures (21). Sublytic complement attack on OLG, on the other hand, induces changes in cellular phenotype, that are potentially beneficial to the cell. These changes include enhancement of mRNA degradation encoding MBP and proteolipid protein, cell cycle induction up to S-phase, and inhibition of OLG apoptosis (10, 22). The molecular mechanisms underlying these effects are poorly understood.

We have used differential display (DD) to screen for genes expressed by OLG in response to complement activation to identify new genes that may be implicated in cell cycle activation and progression. We identified one candidate, RGC-32. In this paper, we report the structure, expression, and the putative biological activity of RGC-32 gene.

MATERIALS AND METHODS

Preparation of Primary Rat OLG by in Vitro Differentiation—O2A (OLG progenitor cell identified by monoclonal antibody A2B5) progenitor cells, isolated by a series of differential shaking of stratified mixed glial culture, were differentiated into OLG for 3 to 4 days in defined medium (DMEM/F12 with 500 ng/ml transferrin (Sigma), 75 ng/ml insulin (Sigma), 75 µg/ml FGF (Collaborative Research Inc., Lexington, MA) and 1 mM sodium pyruvate) (22). At this stage, over 90% of the cells were positive for galactocerebroside (GC) and 85% for MBP.

Activation of Serum Complement and TCC Assembly—Normal human serum (NHS) from several healthy adult donors was pooled and used as a source of complement. The hemolytic activity of complement was inactivated by heating NHS at 56 °C for 45 min (HI-NHS). Cells were exposed to sublytic complement attack by sensitizing OLG with a fixed dose of anti-GC antibody (Ab), then incubating with NHS, HI-NHS (at a final dilution of 1/10) or C7D (120) ≥ C7 (10 µg/ml) for various time periods. The sublytic doses of Ab and NHS were previously determined by measuring the release of cytoplasmic lactate dehydrogenase as an indicator of cell death (10, 15, 22).

Screening by Differential Display—Oligodendrocytes were treated with Ab and NHS, as described above for 1 and 3 h. Total RNA was purified free of contaminating DNA using Message Clean kit (GenHunter Corp., Brookline, MA). The mRNA differential display was

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† The abbreviations used are: OLG, oligodendrocyte(s); Ab, antibody; C7–C9, late-acting complement proteins designated by the number acting sequentially; C7D, sera deficient in the complement component C7; CR1, complement receptor 1; DD, differential display; EAE, experimental allergic encephalomyelitis; EST, expressed sequence tag; GC, galactocerebroside; HI-NHS, heat-inactivated normal human serum; MS, multiple sclerosis; MBP, myelin basic protein; RACE, rapid amplification of cDNA ends; TCC, terminal complement complex representing C5b-7, C5b-8, and C5b-9; RT-PCR, reverse transcriptase polymerase chain reaction; contig, group of overlapping clones; bp, base pair(s); FBS, fetal bovine serum.
performed with RNA image kit 1 (GenHunter Corp.). Primers consist of three 1-base-anchored oligo-dT primers designated as H-T11 (A, -C, -G) to subdivide the mRNA population, and the second primer set represented by arbitrary 13 mer designated as H-AP1 to H-AP7. The OLG mRNA was reverse-transcribed in three separate tubes for each RNA sample; each tube contained one of the three different 1-base-anchored oligo-dT primers and seven H-AP primers, \[ \text{H-AP6} (\text{AAGCTTGCAC-}) \] along with additional seven AAGCT primers.

**TABLE I**

| Organism      | EST no.   | Tissue source          |
|--------------|-----------|------------------------|
| Mouse        | aa028375  | 19.5-day total fetus   |
|              | aa036075  | 4 weeks heart          |
|              | aa433731  | 4 weeks thymus         |
|              | aa125167  | 13.5–14.5-day total fetus |
|              | aa003381  | 4 weeks heart          |
|              | aa49111   | 13.5–14.5-day total fetus |
|              | w83865    | 13.5–14.5-day total fetus |

**Fig. 1. Differential display.** The \[^{32}P\]-labeled PCR products prepared using AAAGC11C in combination with H-AP6 (AAAGCTTGACCAT) were electrophoresed on 6% polyacrylamide gel. Autoradiography of mRNA bands expressed in unstimulated OLG and incubated with Ab and NHS for 1 and 3 h is shown. The mRNA with altered expression was indicated as RGC (Response Gene to Complement). RGC-27 and RGC-30 through -32 are shown. RGC-32 was selected for characterization.

**Fig. 2. Structure of RGC-32 cDNA and predicted amino acid sequence.** A, overall structure of 889-bp RGC-32 nucleotide sequence and positions of selected restriction sites are schematically presented. The hatched box represents the open reading frame. The position of RGC-32 DD clone is also shown. B, the DNA sequence of RGC-32 gene gives rise to a protein of 137 amino acids. The full cDNA sequence can be obtained from GenBank™, accession number AF036548.

**Fig. 3. Northern analysis of RGC-32 mRNA tissue expression.** Expression of RGC-32 mRNA in adult rat tissues (2 mg of mRNA per lane) was examined using multiple choice poly(A)\(^{+}\) RNA Northern blots from OriGene (Rockville, MD). RGC-32 mRNA was detected in kidney, heart, brain, lung, skin, spleen, and thymus but not in testis or liver.

**Cloning of RGC-32—**We have approached the RGC-32 cloning using expressed sequence tag (EST) database homology analysis, RT-PCR, and 5’ rapid amplification of cDNA ends (RACE).

**Bioinformatic**—Relevant ESTs were identified by running the nucleotide sequence of rat RGC-32 DD against EST database and GenBank™ through BLASTN search. The ESTs were retrieved with the ENTREZ service and aligned in a contig.

**PCR and RACE Analysis**—Based on the cDNA sequence of ESTs identified (Table I) and RGC-32 DD mRNA sequence, two PCR primers were designed and used for RT-PCR amplification from mRNA of OLG treated with Ab and NHS for 1 h. The 3’ primer was from RGC-32 3’ DD clone (5’-GCTCTAGAGCTTTAGCTTCGCGG-3’), which was part of the contig. A 572-bp PCR product designated RGC-32A containing the RGC-32 DD sequence was then obtained. To clone the full-length rat RGC-32, we used Marathon RACE amplification kit (CLONTECH). Because rat RGC-32 mRNA was detected during active EAE by Northern blot (data not shown), poly(A) RNA (1 \(\mu\)g) purified from spinal cords of EAE rats on day 19 after immunization was used for cDNA synthesis, and the second strand synthesis was followed by ligation to Marathon cDNA adaptor. The 5’ RACE was performed using the designed gene-specific primer, 5’-GCTCAGATTTAGAAGTGTCCGCGGCGG-3’, together with the AP-1 primer present in the Marathon adaptor. The PCR was performed using XL PCR kit (Perkin-Elmer).

**RGC-32 mRNA Expression**—Total RNA from the spinal cord of EAE rats was examined by Northern analysis for the expression of RGC-32 mRNA. RNA was purified by ultracentrifugation through a 5.7 M CsCl cushion for 18 h at 35,000 rpm using a SW 60 Beckman rotor, fractionated by electrophoresis on 1.2% agarose-formaldehyde gel, and transferred onto Nitrocellulose membrane (Millipore, Bedford, MA) (10, 22). The membrane was then hybridized with \[^{32}P\]-labeled cDNA probe generated from the RGC-32 855-bp cDNA using the oligolabeling kit (Amersham Pharmacia Biotech). Expression of RGC-32 mRNA in primary
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C7D or C7D

1
density was determined by scanning densitometric analysis (Molecular
from three separate experiments.

b
used to quantitate

Blotting, and Indirect Immunoperoxidase—RGC-32
open reading frame
extracted from lysates of
frame with GST gene. Recombinant fusion protein (GST-RGC-32) was
visualized by autoradiography. Time course of
examined by PCR. The amplified cDNA separated on agarose gel was

Two-
shown (Fig. 3). The mRNA band

A

expression was from Stratagene.

polymerase, and 10
Ci of [32P]dCTP. Primers
were then transfected into OLGxC6 glioma cell hybrids, clone ROC-1 (23), by CaCl₂ method (24). [3H]Thymidine incorporation was determined as described (10).

RESULTS AND DISCUSSION

Screening by Differential Display—Using DD-PCR, we have identified 32 bands representing mRNAs with altered expression in response to complement activation. We designated the cDNA bands as RGC-1 to -32 according to the order of identification (Fig. 1). Most of them are mRNAs with increased expression. The nucleotide sequence of seven DD cDNA clones selected for the initial studies was compared with known sequences from GenBank™ Data Bank using BLASTN, TBLASTN, and BLASTX searches. Three clones, RGC-1, -2, and -10, identified as a part of known genes, encode murine heat shock protein 105, poly(ADP-ribose) polymerase, and murine IP-10, respectively. Four clones, RGC-8, -30, -31, and -32, had no matches to any of the GenBank™ Data Bank entries. Initial studies on RGC-32 clone are presented.

Cloning of RGC-32—By running 175-bp rat RGC-32 sequence against EST data base using BLASTN and TBLASTN searches, various ESTs showing significant homology were identified. One group of relevant EST mouse clones formed a contig (Table I), which contains an open reading frame and a putative stop codon. To obtain and confirm the DNA sequence, we amplified the fragment with a set of primers predicted by the consensus sequence derived from the EST contig. The DNA sequence of the 572-bp PCR product was identical to the sequence predicted through bioinformatics and contained the rat RGC-32 DD. To clone the full-length rat RGC-32, Marathon RACE cDNA amplification was carried out. The 5’ of the Marathon RACE reaction yielded a product of 551 bp. This segment together with the previously obtained RGC-32 sequences formed an 889-bp cDNA. To obtain and confirm this DNA sequence, RT-PCR was performed using mRNA derived from OLG stimulated with Ab + NHS for 1 h and two primers, one in the 5’ RACE product (5’TGAACCCACCGCGCGAC-3’), and the other in the RGC-32 DD clone (5’-CAAAAATATTATTATGGGAAAAG-3’). The 855-bp PCR product, which represents the full-length rat RGC-32 (Fig. 2A), contains a start codon in position 101 and encodes a protein of 137 amino acids (Fig. 2B). The rat RGC-32 open reading frame predicted a 14.7-kDa protein. Data bank analysis showed that RGC-32 had no homology with any other proteins known to date, and contained no motif that would indicate its putative biochemical function. Therefore, RGC-32 may represent the prototype of a novel type of genes. RGC-32 does not contain signal sequences. Hydrophobicity analysis indicated no transmembrane domains.

Expression of RGC-32 mRNA in Tissues and Oligodendrocyte—Approximately 1 kb mRNA encoding RGC-32 was expressed by all rat rat kidney, heart, brain, lung, skin, spleen, and thymus, but not in testis or liver, as determined by Northern blot (Fig. 3). By Northern analysis, RGC-32 mRNA expression was observed in rat brains of EAE on day 9 through 23 following immunization (data not shown). In primary rat OLG, RGC-32 mRNA, detected by RT-PCR, showed an increased expression at 3 and 6 h following exposure to Ab + NHS (Fig. 4A), but not with Ab + HI-NHS at 6 h (data not shown).

rat OLG was determined by RT-PCR analysis, using the primers employed for the cloning of RGC-32A. In brief, 2 µg of total RNA was reverse-transcribed using Moloney murine leukemia virus RT in the presence of 2.5 µM oligo(dT) primer and 20 µM dNTP for 1 h at 37° C. PCR amplification was carried out by incubating specific primers with cDNA, 1 unit of Taq polymerase, and 10 µCi of [32P]dCTP. Primers used to quantify β-actin mRNA expression were from Stratagene.

Protein Production, Preparation of Anti-RGC-32 Antibody, Western Blotting, and Indirect Immunoperoxidase—RGC-32 open reading frame, followed by thrombin cleavage. Protein was purified by chromatography using Redipack GST purification mod-}

ure (Amersham Pharmacia Biotech). Antibodies were raised against GST-RGC-32 by immunization of rabbits and IgG fraction of the antisera was screened by Western blot (10). OLG grown on glass coverslips were stained for RGC-32 by indirect immunoperoxidase as described previously (10).

RGC-32 Expression Vector and DNA Synthesis—The RGC-32 cDNA was subcloned into the HindIII and XbaI sites of pcDNA3.1His vector (Invitrogen) according to manufacturer instructions. Expression vectors carrying RGC-32 gene (pcDNA/RGC-32) or empty vectors (pcDNA3.1) were then transfected into OLGxC6 glioma cell hybrids, clone ROC-1 (23), by CaCl₂ method (24). [3H]Thymidine incorporation was determined as described (10).

Expression of RGC-32 in oligodendrocyte determined by RT-PCR. Two-µg aliquot of total RNA was reverse transcribed. Then the expression of RGC-32 (30 cycles) and β-actin (35 cycles) were examined by PCR. The amplified cDNA separated on agarose gel was visualized by autoradiography. Time course of RGC-32 mRNA expression by primary rat OLG following treatment with Ab and NHS is shown (A, upper panel). B, expression of RGC-32 by OLG treated with C7D or C7D + C7 for 3 h is shown (upper panel). The mRNA band density was determined by scanning densitometric analysis (Molecular Dynamics), and the results are presented as RGC-32/β-actin density ratio (A and B, lower panels). Data shown are representative results from three separate experiments.

FIG. 4. Expression of RGC-32 in oligodendrocyte determined by RT-PCR. Two-µg aliquot of total RNA was reverse transcribed. Then the expression of RGC-32 (30 cycles) and β-actin (35 cycles) were examined by PCR. The amplified cDNA separated on agarose gel was visualized by autoradiography. Time course of RGC-32 mRNA expression by primary rat OLG following treatment with Ab and NHS is shown (A, upper panel). B, expression of RGC-32 by OLG treated with C7D or C7D + C7 for 3 h is shown (upper panel). The mRNA band density was determined by scanning densitometric analysis (Molecular Dynamics), and the results are presented as RGC-32/β-actin density ratio (A and B, lower panels). Data shown are representative results from three separate experiments.
18 h, the mRNA level declined below the basal level. C7D + C7 induced an increase from 1.8- to 2-fold in RGC-32 mRNA expression over the C7D level, indicating the requirement of TCC assembly (Fig. 4B). To demonstrate that RGC-32 is indeed expressed in OLG, polyclonal anti-GST-RGC-32 antibody was raised in rabbits. By indirect immunoperoxidase, RGC-32 staining was localized to the cytoplasmic compartment (Fig. 5A). By Western blot, an immunoreactive band of about 15 kDa was detected with recombinant purified RGC-32 (Fig. 5B).

Sublytic complement activation induces OLG to enter cell cycle and an increase in cdc2 kinase activity in G1 (10). Therefore a possible association of RGC-32 and cell cycle kinase was examined. Unstimulated OLG as well as antibody sensitized OLG treated with NHS for 6 and 18 h, or with HI-NHS for 6 h were lysed and immunoprecipitated with anti-cdc2 IgG. By Western blot, an 15-kDa band of immunoreactive anti-RGC-32 was detected in anti-cdc2 immunoprecipitate (Fig. 5B). Intensity of this band was highest at 6 h and declined at 18 h. Similar amounts of RGC-32 were immunoprecipitated from control cells and cells exposed to Ab + HI-NHS (Fig. 5B). The same nitrocellulose membrane, previously examined for the presence of RGC-32 when reacted with anti-cdc2 IgG, revealed a cdc2 34-kDa band, as expected (Fig. 5B). The anti-cdc2 antibody did not react with recombinant purified RGC-32 (Fig. 5B). Antibody-sensitized OLG treated with NHS for 3, 8, and 18 h were also examined for the presence of cdc2 in anti-RGC-32 immunoprecipitate. Cell lysates (200 μg of protein) were immunoprecipitated with anti-RGC-32 IgG in the presence of Protein AG-agarose as described previously (18). The cdc2 protein was present in anti-RGC-32 immunoprecipitate (Fig. 5C), and the intensity of this band was highest at 6 h and declined at 18 h. These results indicated that RGC-32 and cdc2 are co-immunoprecipitated in a specific manner, and their levels are increased by complement activation.

Role of RGC-32 in DNA Synthesis—The increased RGC-32 mRNA expression in response to sublytic C5b-9 assembly in G1 phase and the presence of RGC-32 in anti-cdc2 immunoprecipitate led us to examine its role in cell cycle induction. OLGxG6 glioma cell hybrids were transfected with expression vector carrying full-length RGC-32 cDNA (RGC-32) or empty vector (Control vector). Cells were placed in serum-free medium (SFM) or medium with 10% fetal bovine serum (FBS) and incubated for 18 h in the presence of [3H]thymidine. Overexpression of RGC-32 caused a 2.3-fold increase in DNA synthesis over the level of unstimulated cells and cells transfected with control vector placed in FBS (p < 0.01). Data represent mean ± S.E. from three separate experiments.
glioma cell hybrids were transfected with expression vector carrying full-length RGC-32 cDNA (pcDNARGC32). Cells expressing RGC-32 grown in 10% FBS showed significantly increased DNA synthesis (Fig. 6) when compared with cells transfected with control vector (pcDNA 3.1) and placed in 10% FBS (p < 0.01) or transfected with RGC-32 and placed in serum-free medium (p < 0.01). The results suggest that RGC-32 may play a role in activation and progression of cell cycle in OLG. This is also indicated by the increase in RGC-32 protein level co-immunoprecipitated with cdc2 (Fig. 5, B and C). Therefore, we can speculate that this new protein may play a role in cell cycle by regulating cell cycle-dependent kinases.

We have cloned a novel gene, RGC-32, induced by complement activation in primary rat oligodendrocytes and involved in cell cycle activation. Increased expression in rat brains with active EAE also suggests a role for RGC-32 in inflammatory demyelination. Further exploration is needed to elucidate the biological function of this new gene.

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