RGC-32 Increases p34\(^{CD2}\) Kinase Activity and Entry of Aortic Smooth Muscle Cells into S-phase* 

Proliferation of aortic smooth muscle cells contributes to atherogenesis and neointima formation. Sublytic activation of complement, particularly C5b-9, induces cell cycle progression in aortic smooth muscle cells. RGC-32 is a novel protein that may promote cell cycle progression in response to complement activation. We cloned human RGC-32 cDNA from a human fetal brain cDNA library. The human RGC-32 cDNA encodes a 117-amino acid protein with 92% similarity to the rat and mouse protein. Human RGC-32 maps to chromosome 13 and is expressed in most tissues. Sublytic complement activation enhanced RGC-32 mRNA expression in human aortic smooth muscle cells and induced nuclear translocation of the protein. RGC-32 was physically associated with cyclin-dependent kinase p34\(^{CD2}\) and increased the kinase activity in vitro and in vivo. In addition, RGC-32 was phosphorylated by p34\(^{CD2}\)-cyclin B1 in vitro. Mutation of RGC-32 protein at Thr-91 prevented the p34\(^{CD2}\)-mediated phosphorylation and resulted in loss of p34\(^{CD2}\) kinase enhancing activity. Overexpression of RGC-32 induced quiescent aortic smooth muscle cells to enter S-phase. These data indicate that cell cycle activation by C5b-9 may involve p34\(^{CD2}\) activity through RGC-32. RGC-32 appears to be a cell cycle regulatory factor that mediates cell proliferation, both as an activator and substrate of p34\(^{CD2}\).

C5b-9, the membrane attack complex of complement, causes cell death by forming transmembrane pores (1). When the number of C5b-9 molecules is limited to a sublytic level, nucleated cells are able to escape cell death by eliminating membrane-inserted terminal complement complexes (TCC; C5b-7, C5b-8, and C5b-9) by endocytosis and/or membrane shedding (2–4). Among these complexes, C5b-9 is most potent in activating target cells. C5b-9 causes a Ca\(^{2+}\) influx and generates intracellular second messengers, including phosphatidylinositol triphosphates, diacylglycerol, and ceramide (5–8). Membrane-inserted TCC activates the Gq family of G proteins (9). Activation of Gq/Gi by TCC is responsible for the Gq/Ca\(^{2+}\)-mediated activation of cell cycle through activation of Ras, Raf-1, extracellular signal-regulated kinase-1 (10), and activation of phosphatidylinositol 3-phosphate kinase (11, 12). Cell cycle activation by C5b-9 is associated with an increase in CDK4, CDK2, and p34\(^{CD2}\) activities, and this is followed by an increase in DNA synthesis and cell proliferation (11, 12, 24–26). The C5b-9-induced DNA synthesis is abolished by inhibitors of mitogen-activated protein kinase/extracellular signal-regulated kinase-1 and phosphatidylinositol 3-phosphate kinase (11). Cell cycle activation by C5b-9 in postmitotic cells, such as oligodendrocytes (OLG) and myotubes, is associated with expression of c-JUN and c-FOS protooncogenes and loss of differentiation (12–14).

In an effort to find novel C5b-9-induced genes involved in cell cycle regulation, we cloned the rat Response Gene to Complement (RGC-32) using mRNA differential display PCR in OLG (15, 16). C5b-9 enhanced RGC-32 mRNA expression in primary rat OLG and OLGxC6 glioma cell hybrids (16). This overexpression was associated with an increase in DNA synthesis, suggesting a role of RGC-32 in the cell cycle (16).

In this paper we present the cloning of human RGC-32 and experimental evidence indicating the role of RGC-32 in cell cycle activation through regulation of p34\(^{CD2}\) kinase. In human aortic smooth muscle cells (SMC), complement activation induced nuclear translocation of RGC-32 protein. Overexpression of RGC-32 in aortic SMC increased BrdUrd incorporation and the cell number, and these effects of RGC-32 were further increased when cells were exposed to C5b-9. RGC-32 complexes with p34\(^{CD2}\)-cyclin B1 and increases the kinase activity. This kinase enhancing activity appears to require phosphorylation of RGC-32 at threonine 91 by p34\(^{CD2}\). These findings identify RGC-32 as a substrate and regulator of p34\(^{CD2}\).

MATERIALS AND METHODS

Cloning of Human RGC-32—To clone human RGC-32, we screened the NCBI EST database with the rat rgc-32 sequence. Among several homologous human ESTs, an EcR/BF fragment of the L.M.A.G.E. clone yy46a05 was used to screen 2 \(\times\) 10\(^6\) clones of a Human Fetal Brain 5'-STRETCH PLUS Agt11 cDNA Library (CLONTECH, Palo Alto, CA). In brief, duplicate filters were prepared by Nyttran membranes (Schleicher & Schuell). Membranes were hybridized with rat rgc-32 cDNA and exposed to Kodak Biomax x-ray film. Colonies that were positive on both of the duplicate filters were picked and the colonies were isolated from positive phage clones using Lambda-TRAP PLUS (CLONTECH). The cDNA inserts were amplified by PCR with the Long Distance Insert Screening Amplimer set for Agt11 and KlenTaq polymerase (CLONTECH). The longest PCR product (742 bp) was subcloned into the pBluescript SK(+) vector (Stratagene) and sequenced using the AmpliTaq Sequencing Kit (PerkinElmer).

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into pGEM4 plasmid and amplified in bacteria. The insert was sequenced at the University of Maryland School of Medicine Biopolymer Laboratory.

Cloning of Mouse RGC-32—BLASTN searches of the dbEST database revealed mouse ESTs with a high identity to rat rgc-32. One such EST (ATCC accession number AF036549). The cDNA sequence encodes a 117-amino acid polypeptide, B, alignment of human, mouse, and rat RGC-32 amino acid sequences. Shaded boxes indicate identity. Human RGC-32 lacks 20 amino acids found at the NH₂ terminus of the mouse and rat sequences. Mouse RGC-32, GenBank™ accession number AF276981.

**Cell Cycle Activation by RGC-32**

**A**

| GCAGCC | 6 |
|--------|---|
| GCGGCG | 76 |
| CGGCGC | 146 |
| CACCCG | 216 |
| GACCCG | 24 |
| ATGAAG | 286 |
| CGCCCT | 47 |
| ATGCGG | 356 |
| AGTATG | 426 |
| AGACCA | 496 |
| GTTAGA | 566 |
| CGCACT | 636 |
| GCTATT | 706 |
| ATTCAT | 846 |
| TTTAAT | 895 |

**B**

| hRGC32 | mRGC32 | rRGC32 |
|--------|--------|--------|
| hRGC32 | mRGC32 | rRGC32 |
| hRGC32 | mRGC32 | rRGC32 |
| hRGC32 | mRGC32 | rRGC32 |

![Cell Cycle Activation by RGC-32](https://example.com/cell-cycle-activation.png)

**Fig. 1. Sequence of cDNA encoding human and mouse RGC-32. A, nucleotide and deduced amino acid sequence of human RGC-32 (GenBank™ accession number AF036549). The cDNA sequence encodes a 117-amino acid polypeptide. B, alignment of human, mouse, and rat RGC-32 amino acid sequences. Shaded boxes indicate identity. Human RGC-32 lacks 20 amino acids found at the NH₂ terminus of the mouse and rat sequences. Mouse RGC-32, GenBank™ accession number AF276981.**

**Culture of Primary Aortic Smooth Muscle Cells**—Human aortic SMC from Clonetics (Walkersville, MD) were grown 3–5 passages for 3–4 days in SMC basal medium, containing supplements of 5% fetal bovine serum, 10 ng/ml human epidermal growth factor, 2 ng/ml human fibroblast growth factor, and 5 μg/ml insulin. Prior to the experiments, cells were starved for 24 h in SMC basal medium without serum and growth factor supplements. This method produced over 95% of cells resting in G₀/G₁-phase, as determined by the relative DNA content by propidium iodide staining and fluorescence-activated cell sorter analysis (121). All cells were positive for muscle actin by indirect immunostaining using HHF35 monoclonal IgG (Enzo Diagnostics, Farmingdale, NY).

**Activation of Serum Complement and C5b-9 Assembly**—Pooled normal human serum (NHS) from healthy adult donors was used as a source of serum complement. Aortic SMC were exposed to sublytic complement attack by sensitizing with a fixed dose of anti-HLA class I antibody (Ab) and then incubating with NHS at 1:10 final dilution. The sublytic dose of Ab was predetermined by measuring cell death in the presence of excess NHS (8, 10, 11). To produce a negative control for complement activity of NHS, the hemolytic activity was inactivated by heating NHS at 56°C for 45 min (HI-NHS). C5b-9 was assembled by incubating aortic SMC sequentially with C5b6 complex for 15 min and C7 for 5 min at room temperature, and then with C8 and C9 at 37°C for the indicated period, as previously described (9, 10). Purified C5-C9 proteins were from Quidel (San Diego, CA), and C5b6 was prepared as described (9, 10).

**RNA Isolation and Northern Blot Analysis**—Total RNA was purified from aortic SMC using guanidine isothiocyanate and ultracentrifugation through a 5.7M CsCl₂ cushion for 18 h at 35,000 rpm using an SW60 Beckman rotor (12, 17). RNA was denatured and electrophoresed on a 0.8% agarose-formaldehyde gel and then transferred to nitrocellulose. A multitissue Northern blot (MTN1, Clontech) was separated on 10% SDS-PAGE and then incubated with a fixed dose of anti-HLA class I antibody (Ab) and then incubating with NHS at 1:10 final dilution. The sublytic dose of Ab was predetermined by measuring cell death in the presence of excess NHS (8, 10, 11). To produce a negative control for complement activity of NHS, the hemolytic activity was inactivated by heating NHS at 56°C for 45 min (HI-NHS). C5b-9 was assembled by incubating aortic SMC sequentially with C5b6 complex for 15 min and C7 for 5 min at room temperature, and then with C8 and C9 at 37°C for the indicated period, as previously described (9, 10). Purified C5-C9 proteins were from Quidel (San Diego, CA), and C5b6 was prepared as described (9, 10).

**Production of RGC-32 Protein and Antibody, Western Blotting, and Immunocytochemistry**—The human RGC-32 open reading frame was subcloned into the pGEX-4T-3 vector (Amersham Biosciences) in frame with the GST gene. Recombinant fusion protein (GST-RGC-32) expressed from bacterial lysates was purified by chromatography using Redipack GST purification module (Amersham Biosciences). Antibodies were raised by immunizing rabbits with GST-RGC-32. IgG fractions of antisera were screened by Western blotting (16). To assay tissue expression, protein extracts from human brain, heart, and liver (Protein Medley, CLONTECH) were separated on 10% SDS-PAGE and then examined by Western blotting with rabbit anti-RGC-32 IgG by indirect...
peroxidase method and visualized by ECL (Pierce). For immunostaining, aortic SMC grown on plastic slides in SMC basal medium without serum and growth factor supplements were fixed and stained with anti-RGC-32 IgG by indirect immunoperoxidase method using 3-aminopropylcarbazole. The effect of complement activation was examined by treating cells in plastic chambers with Ab and NHS.

Binding of p34CDC2 to GST-RGC-32—For in vitro binding, recombinant CDC2-cyclin B1 complex (New England Biolabs, Beverly, MA) was mixed with GST-RGC-32 or GST (negative control) at a final concentration of 5 μM. After 30 min at room temperature, samples were mixed with 20 μ1 of 50% slurry of glutathione-Sepharose 4B in phosphate-buffered saline for 30 min, and Sepharose beads were washed three times with phosphate-buffered saline, 0.01% Triton X-100. Protein retained on the beads was eluted with Laemmli buffer and analyzed by Western blot, using anti-p34CDC2 IgG (Santa Cruz Biotechnology, Santa Cruz, CA). For in vivo binding, aortic SMC lysates treated with C5b-9 or C5b6 for 6 and 18 h were immunoprecipitated with anti-p34CDC2 IgG and then examined by Western blotting.

In Vitro p34CDC2 Kinase Assay—Human recombinant p34CDC2-cyclin B1 (New England Biolabs) was used to examine the effect of RGC-32. GST-RGC-32 was incubated with 1 unit of p34CDC2-cyclin B1 for 30 min at 37 °C in the presence of 2 μCi of histone H1 (Roche Molecular Biochemicals) and 1 μCi of [γ-32P]ATP/sample in reaction buffer (25 mM Tris-HCl, pH 7.4, 10 mM MgCl2). Recombinant p27 cell cycle kinase inhibitor (PharMingen, San Diego, CA) (10 μM) was included in some experiments. Phosphorylated histone H1 bands were analyzed by 10% SDS-PAGE and autoradiography (11, 12).

Phosphorylation of RGC-32 by p34CDC2—GST-RGC-32 and the Target Site Analysis by Site-directed Mutagenesis—Purified GST-RGC-32, p34CDC2-cyclin B, and 1 μCi of [γ-32P]ATP/sample were incubated for 30 min at 37 °C, as described above, and proteins were analyzed for phosphorylation on 10% SDS-PAGE and autoradiography. Thr-91 of RGC-32, a possible target for p34CDC2 (19, 20), was mutated using the Transformer Site-directed Mutagenesis kit (Clonetics). The selection primer (GGTTTTCTTAGATATCAGGTGGCAC) converts the AezII site at 1180 in pGEX-4T-3 to EcoRI. A mutagenic primer was CTGCCAC-TGTGCGCTCCACGAGAAAG, which converts Thr-91 to alanine (T91A).

The expression of RGC-32 in the NIH3T3 cell line was confirmed by mRNA and protein analyses. The expression of RGC-32 mRNA was examined by Northern blot using Multiple tissue poly(A)+ RNA Northern blot probed with a full-length human RGC-32 cDNA. β-Actin serves as control. Expression of human RGC-32 protein was examined by Western blot using rabbit anti-human RGC-32 IgG and tissue lysates derived from brain, heart, and liver. As a positive control, GST-cleaved human recombinant RGC-32 (rRGC-32) was used.

RESULTS AND DISCUSSION

Cloning of Human and Mouse RGC-32—In searches of the dbEST and GenBankTM data bases with rat rgc-32 sequence, significant homology was found with two human EST clones (y53e12 and y46a05) derived from a human multiple sclerosis brain library (7). The fact that rat rgc-32 (16) and these human I.M.A.G.E clones shared over 80% DNA sequence identity suggested that the EST sequences may be part of the human RGC-32 gene. We used an EcoRI fragment derived from EST y53e12 to screen a human fetal brain cDNA library. One of the isolated clones had a 722-bp insert containing the 5′-untranslated region and the complete reading frame of rgc-32. Alignment of this clone with the EST sequences produced an 895-bp sequence (Fig. 1A), which approximates the size of the human rgc-32 transcript seen on Northern blots. The nucleotide sequence predicts a 117-amino acid polypeptide with an estimated mass of 12.9 kDa. Human RGC-32 lacks the first 20 amino acids at the NH2 terminus of the rat (16) and mouse protein (Fig. 1B). Excluding this region, human RGC-32 has 92% sequence similarity with the rat protein (16). Mouse RGC-32 (GenBankTM accession number AF276981) is a 965-bp gene with a start codon at position 151 and a poly(A)+ signal at position 902. The gene encodes a 137-amino acid polypeptide with 92% identity to rat RGC-32, including the 20 NH2-terminal amino acids (Fig. 1B). By data base analysis, human and mouse RGC-32 show no homology with any other known proteins and contain no motif that would indicate a putative biochemical function. Human and mouse RGC-32 do not contain signal sequences or transmembrane domains, as determined by hydrophobicity analysis.

Chromosomal Localization and Tissue Distribution of Human RGC-32—Human RGC-32 was assigned to chromosome 13 by screening a monochromosomal somatic cell hybrid panel using PCR, followed by confirmation by Southern blot (Fig. 2). When the human RGC-32 sequence was compared with the marker data base of the sequence-tagged sites using BLASTN, we found an identity with a human sequence-tagged site, SHGC-36498. The radiation hybrid mapping of this sequence-tagged site, available through the Stanford Human Genome project (shgc.stanford.edu), confirmed that human RGC-32 resides on the long arm of chromosome 13 in bin 12 and was annotated in the NCBI data base to locus ID 28894, located in the 13q12–13q14 interval.

The full-length human RGC-32 cDNA was used to probe a human multitissue Northern blot (Fig. 3A). An ~1-kb transcript was abundantly expressed in placenta, liver, skeletal muscle, kidney, and pancreas and was weakly expressed in...
heart and brain, whereas no signal was detected in lung. The mRNA was also detected in human aortic endothelial cells and JY25 B lymphoblastoid line (data not shown). Rabbit anti-human RGC-32 IgG identified a 14-kDa band by Western blot of the GST-cleaved and -purified RGC-32 and also detected RGC-32 protein in brain, heart, and liver (Fig. 3B). Tissue expression of mouse \textit{rgc-32} mRNA was similar to that of rat \textit{rgc-32} mRNA (16) (data not shown).

Expression of RGC-32 mRNA and Nuclear Translocation of the Protein in Aortic SMC in Response to Complement—Although the level of RGC-32 mRNA expressed in aortic SMC was not detectable by Northern blot, it increased to a detectable level by treatment with C5b-9 (Fig. 4A). The mRNA, detected 3 h after exposure to C5b-9, persisted for up to 6 h and was no longer detected at 18 h. C5b6, a negative control for C5b-9, had no effect on RGC-32 expression. Immunostaining, on the other hand, revealed the presence of RGC-32 as a cytoplasmic protein in unstimulated cells in culture (Fig. 4B, upper panel). Upon stimulation with Ab and NHS, nuclear translocation of RGC-32 protein was noted as early as 6 h (middle panel). Nuclear translocation of RGC-32 may play an important role in nuclear regulation of the cell cycle, as nuclear translocation of RGC-32 preceded DNA synthesis and SMC proliferation occurring at 12 and 48 h after complement activation, respectively (11). In view of the protein expression, a Northern blot may not be sensitive enough to detect RGC-32 mRNA in unstimulated SMC. This is in agreement with our previous finding that reverse transcriptase-PCR was required to show an increased \textit{rgc-32} mRNA in rat OLG in response to C5b-9 (16).

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with p34CDC2 in vivo by immunoprecipitation and Western blotting. We found that p34CDC2 was coinmunoprecipitated with RGC-32 both in stimulated and unstimulated aortic SMC (Fig. 5B). By density analysis, the level of RGC-32 immunoprecipitated with anti-p34CDC2 appears slightly higher (1.5-fold) in cells treated with C5b-9 than in the C5b6 control (Fig. 5B). RGC-32 failed to coinmunoprecipitate with CDK2 or with CDK4 (data not shown).

RGC-32 Enhanced p34CDC2 Kinase Activity—Physical association of RGC-32 with p34CDC2 led us to examine the regulation of CDC2 kinase by RGC-32. We mixed GST-RGC-32 with recombinant p34CDC2-cyclin B1 and then assayed kinase activity using histone H1. RGC-32 dramatically increased the p34CDC2 activity in a dose-dependent manner (Fig. 6). Because p34CDC2 activity is inhibited by cell cycle kinase inhibitor p27 (21), we tested the RGC-32 kinase enhancing activity in the presence of p27. RGC-32 failed to coimmunoprecipitate with CDK2 or with CDK4 (data not shown).

Phosphorylation of RGC-32 by p34CDC2 and Identification of the Phosphorylation Site—During the in vitro kinase assay to assess the effect of RGC-32, we noted that RGC-32 was phosphorylated. In the absence of histone H1, p34CDC2-cyclin B1 induced phosphorylation of GST-RGC-32, and the protein complex was detected as a 46-kDa band (Fig. 7, A and B). This p34CDC2-mediated phosphorylation was inhibited by p27, and RGC-32 was not autophosphorylated. Cyclin B1, like RGC-32, was also phosphorylated by p34CDC2 in the absence of histone H1. We searched for a p34CDC2 phosphorylation consensus motif (T/S)P(K/R) in human RGC-32. The p34CDC2 consensus requires a basic residue at the +3 position (19, 20). We identified a putative p34CDC2 consensus sequence, TPKQ, with a threonine at 91, a proline at +1, and a basic residue at the +3 position. A similar sequence was found in the rat and mouse proteins. To test whether Thr-91 is the phosphorylation site, the site was mutated to alanine (T91A), and the corresponding mutated protein was purified and used to examine the role of p34CDC2 phosphorylation of RGC-32. As shown in Fig. 7B, p34CDC2 failed to phosphorylate RGC-32 T91A. These data indicate that Thr-91 is the target for p34CDC2 kinase. These data also indicate that RGC-32 is not required for phosphorylation of cyclin B1 by p34CDC2. Phosphorylation of cyclin B also plays a critical role in nuclear import of the p34CDC2-cyclin B complex (22).

Functional Role of RGC-32 Phosphorylation—Because RGC-32 T91A was not phosphorylated by p34CDC2, we examined whether mutation of Thr-91 affects the ability of RGC-32 to enhance the activity of p34CDC2 (Fig. 8). The T91A mutation markedly reduced the kinase enhancing activity of RGC-32.
an involvement of p34CDC2 in RGC-32 mediated S-phase entry (data not shown). Thus, our findings are consistent with BrdUrd incorporation was reduced to the level of control with 36% S-phase and 29% in G2/M-phase. RGC-32 significantly shifted the cell cycle activation on cells transfected to express S-phase has been reported. p34CDC2-cyclin B is able to activate DNA replication by phosphorylating Thr-376 of Cdk7, a kinase involved in S-phase entry (30, 31). Therefore, p34CDC2 may also affect the S-phase of cell cycle in aortic SMC, in addition to its role in the M-phase (12, 32–34).

In the present paper, we show experimental data to elucidate the role of RGC-32 in the cell cycle. We showed that RGC-32 binds p34CDC2 and increases the activity of the kinase. Both of these activities appear to depend on phosphorylation of RGC-32 by p34CDC2 at a consensus site at Thr-91. Thus, RGC-32 is a new p34CDC2 substrate that can increase the CDC2 kinase activity and enhance S-phase entry (35). Proliferation of aortic SMC plays a critical role in atherogenesis and in restenosis following angioplasty and coronary stenting (36, 37). Proliferation of vascular SMC can be controlled by regulating cell cycle kinetics, including p34CDC2 (38, 39). Therefore, RGC-32 may serve as a target to inhibit the proliferation of vascular SMC, an approach to intervene in the process of atherosclerosis and restenosis.

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