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Dual role of Response gene to complement-32 in multiple sclerosis

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ABSTRACT

Response gene to complement (RGC)-32 is a novel molecule that plays an important role in cell proliferation. We investigated the expression of RGC-32 in multiple sclerosis (MS) brain and in peripheral blood mononuclear cells (PBMCs) obtained from patients with relapsing–remitting multiple sclerosis. We found that $CD3^+$, $CD68^+$, and glial fibrillar acidic protein $(GFAP)^+$ cells in MS plaques co-localized with RGC-32. Our results show a statistically significant decrease in RGC-32 mRNA expression in PBMCs during relapses when compared to the levels in stable MS patients. This decrease might be useful in predicting disease activity in patients with relapsing–remitting MS. RGC-32 expression was also correlated with that of FasL mRNA during relapses. FasL mRNA expression was significantly reduced after RGC-32 silencing, indicating a role for RGC-32 in the regulation of FasL expression. In addition, the expression of Akt1, cyclin D1, and IL-21 mRNA was significantly increased during MS relapses when compared to levels in healthy controls. Furthermore, we investigated the role of RGC-32 in TGF-β-induced extracellular matrix expression in astrocytes. Blockage of RGC-32 using small interfering RNA significantly inhibits TGF-β induction of procollagen I, fibronectin and of the reactive astrocyte marker α-smooth muscle actin (α-SMA). Our data suggest that RGC-32 plays a dual role in MS, both as a regulator of T-cells mediated apoptosis and as a promoter of TGF-β-mediated profibrotic effects in astrocytes.

Introduction

We have recently identified a novel gene product, Response Gene to Complement (RGC)-32, induced by complement activation, and have demonstrated its activity, primarily as a cell cycle regulator (Badea et al., 1998, 2002; Fosbrink et al., 2009). We have shown that overexpression of RGC-32 leads to an increase in DNA synthesis and causes cell cycle progression from the G1/G0 to G2/M phase (Badea et al., 2002). Both of these responses can be abolished by transfecting the cells with RGC-32-specific siRNA (Fosbrink et al., 2009). We have also found that the RGC-32 protein forms complexes with CDC2/cyclinB1 and enhances CDC2 kinase activity (Badea et al., 2002). In addition, RGC-32 binds and modulates the activity of Akt (Fosbrink et al., 2009). Thus, RGC-32 appears to be a previously unrecognized regulator of Akt and CDC2, critical kinases involved in cell cycle regulation. In some tumor cells, RGC-32 acts as a tumor suppressor and has been found to inhibit cell cycle activation (Saigusa et al., 2006; Vlaicu et al., 2010). Since complement activation induces RGC-32 expression (Badea et al., 2002) and is also involved in the pathogenesis of multiple sclerosis (MS) lesions (Rus et al., 2006), we asked how RGC-32 might play a role in MS.

It is well documented that autoreactive memory T cells play an important role in MS pathogenesis. Adoptive transfer of encephalitogenic $CD4^+$ T cells in naïve animals induces experimental autoimmune encephalomyelitis (EAE), an animal model of MS. In particular, $CD4^+$ myelin basic protein (MBP)-reactive T cells induce extensive central nervous system (CNS) inflammation and mild demyelination in EAE (Sospedra and Martin, 2005; Steinman, 2001). In addition $CD8^+$ cytotoxic T cells recognizing MBP-derived peptides directly contribute to severe CNS demyelination in EAE, presumably by inducing injury of oligodendrocytes (Sospedra and Martin, 2005; Steinman, 2001). The number of infiltrating T cells increases dramatically during the acute phase of EAE but then declines significantly in remission, as a result of apoptotic cell death (Suvannavejh et al., 2000). Several studies have demonstrated impaired apoptosis of T cells in MS patients (Aktas et al., 2006; Sharief, 2000; Zipp et al., 1998). Furthermore, relapses may be

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associated with the persistent presence of myelin-activated T-cells resulting from impaired T-cell apoptosis (Aktas et al., 2006; Sharief, 2000; Zipp et al., 1998).

T-cell apoptosis in both EAE and MS is in part regulated by the Fas-FasL system (Aktas et al., 2006), and ex vivo studies have demonstrated an increased resistance by T cells to Fas-mediated apoptosis during MS relapses (Okuda et al., 2006). In addition, levels of FasL expression have been found to be low during relapses, consistent with the increased resistance of the T cells to apoptosis (Lopatinskaya et al., 2003). FasL expression on T cells is regulated by multiple factors, including the CDC2/cyclin B1 complex (Torgler et al., 2004). This effect of the CDC2/cyclin B1 complex has been found to be dependent on cyclin B1 because overexpression of cyclin B1 enhances FasL promoter activity, whereas a dominant-negative version of CDC2 blocks the induction of the Fas ligand promoter (Torgler et al., 2004). Since RGC-32 binds to and up-regulates CDC2/cyclin B1 kinase activity (Badea et al., 2002; Fosbrink et al., 2009), it is possible that RGC-32 regulates T-cell survival by modulating the expression of FasL.

In this study, we examined the expression of RGC-32 in MS brain and found that $CD3^+$, $CD68^+$, and $GFAP^+$ cells in MS plaques co-localized with RGC-32. To gain more insight into the role of RGC-32 in relapsing–remitting (RR) MS, we investigated the levels of RGC-32 mRNA expression in PBMCs of MS patients in conjunction with FasL and CDC2 mRNA expression, in order to determine whether an association exists between these genes and MS clinical activity. Our data indicate that RGC-32 and FasL mRNA expression are up-regulated in PBMCs of stable patients with RR MS. In contrast, during periods of clinical exacerbation, RGC-32 and FasL mRNA expression was significantly decreased in these patients when compared to that in stable patients. FasL expression was significantly reduced after RGC-32 silencing, indicating a role for RGC-32 in FasL expression. Moreover, RGC-32 is induced by TGF- β in astrocytes, and plays an important role in TGF-β-mediated extracellular matrix production in vitro. These data indicate a significant role for RGC-32 in regulating T-cell mediated apoptosis in MS and extracellular matrix production by astrocytes.

Materials and methods

Brain tissue

Frozen brain tissue specimens acquired at autopsy from eight patients with a definite diagnosis of MS were obtained from the Human Brain and Spinal Fluid Resource Center, Veterans Affairs West Los Angeles Health Care Center. Active lesions contained abundant infiltrates consisting of T cells and macrophages, with detectable myelin degradation products. Inflammation was restricted to the lesion margins in chronic active lesions. Regions of normal-appearing white matter (NAWM) and normal-appearing gray matter lesions (NAGM) that lacked macroscopic or histological evidence of demyelination were also used. The samples were derived from patients between the ages of 38 and 51, with a mean age of 47. Four healthy control brain samples between the ages of 36 and 72, with a mean age of 49, were obtained from the Cooperative Human Tissue Network, Charlottesville, VA.

Immunohistochemical staining for RGC-32, CD3, and CD68

Immunohistochemical staining of brains from MS patients was performed as previously described (Cudrici et al., 2007). The air-dried cryostat brain sections (4–6 μm) were fixed for 10 min in acetone containing 0.3% H_2O_2 to remove endogenous peroxidase. $CD4^+$ T-cells were deposited on glass slides in a cytospin centrifuge then fixed in acetone:methanol (2:1) in the presence of 0.3% H_2O_2. Tissue sections and cytospins were blocked for 10 min with 2.5% horse serum, then incubated overnight at 4 °C with rabbit anti-RGC-32 as previously described (Fosbrink et al., 2005). The slides were washed three times for 3 min with PBS, pH 7.4, and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (Jackson Immunoresearch Labs, West Grove, PA), diluted 1/250. Specific reactions were developed using NovaRED (Vector Labs, Burlingame, CA) as a substrate; slides were then counterstained with Harris's hematoxylin (Sigma, St. Louis, MO) and mounted with permanent mount. For CD3 and CD68 detection, brain cryosections were processed as described above and then incubated with mouse monoclonal anti-CD3 antibody (NovoCastra, Newcastle upon Tyne, U.K.), diluted 1/50, for 2 h at room temperature (RT) or with mouse monoclonal CD68 (Dako, Carpenteria, CA), diluted 1/200, for 1 h at RT. The sections were washed with PBS, pH 7.4, and then incubated for 1 h at RT with HRP-conjugated goat anti-mouse IgG (Jackson Immunoresearch). Specific reactions were again developed using NovaRED. MBP immunostaining (rabbit IgG anti-MBP from Genescript, Piscataway, NJ) and Luxol Fast Blue stain were performed as previously described (Fosbrink et al., 2005).

Double-staining immunohistochemistry

Frozen sections of brains from adult patients with MS were double-stained for RGC-32 and CD3, CD68, or GFAP as previously described (Rus et al., 2005). Cryosections were initially processed for RGC-32 immunostaining as described above, and the reaction developed with NovaRed. The sections were treated with 0.3% H_2O_2 to remove excess peroxidase and then incubated for 2 h at RT with mouse monoclonal anti-CD3 (NovaCastra), diluted 1/50. The slides were washed several times in PBS and reacted with goat anti-mouse (Santa Cruz Biotech) for 1 h at RT. The slides were then exposed to diaminobenzidine (Pierce, Rockford, IL) and mounted with permanent mount. For double-staining for RGC-32 and or CD68, GFAP, mouse monoclonal anti-GFAP (eBiosciences, San Diego, CA) or monoclonal antibody anti-CD68 (Dako), diluted 1/200 were followed by alkaline phosphatase-conjugated goat anti-mouse (Sigma), diluted 1/400. The reaction was developed using a Vector alkaline phosphatase substrate kit III (Vector Labs). Control sections were prepared by immunostaining without the primary antibody or by using control isotype IgG instead of the primary antibody. The immunostained slides were independently evaluated by two investigators.

Patients and controls

We enrolled a total of 34 patients with RR MS (20 patients with stable disease and 14 with relapses). The criteria for inclusion of MS patients in the study were: (i) age 18 to 65 years; (ii) fulfillment of McDonald criteria for definite MS (McDonald et al., 2001; Polman et al., 2005); (iii) relapsing–remitting course; (iv) having newly diagnosed MS, or MS not treated with currently used immunomodulatory drugs (interferon-β or glatiramer acetate) for 3 months prior to study entry; (v) no exacerbations in the 4 weeks before the study; (vi) no iv or po steroids for 4 weeks prior to study enrollment; (vii) no treatment with Tysabri, Gilenya, mitoxantrone, cyclophosphamide, or investigational drugs during the past year; and (viii) a disability score of 0–5.5, as defined by the expanded disability status scale (EDSS) (Kurtzke, 1983). Exclusion criteria for MS patients were: (i) a history of autoimmune disorders, vascular disease, or active acute or chronic infections; (ii) use of antibiotics in the last 30 days; (iii) a history of intracranial or intraspinal tumor or metabolic myelopathy; or (iv) a history of alcohol or drug abuse.

Twenty-eight healthy, age-, gender-, and race-matched healthy controls were also enrolled in the study. Exclusion criteria for controls were: the presence of (i) overt acute or chronic disease(s) or (ii) other autoimmune disease(s).

Collection of PBMCs, isolation of $CD4^+$ cells, total RNA purification, and cDNA synthesis

PBMCs were collected using BD Vacutainer CPT tubes (Becton Dickinson, Franklin Lakes, NJ). The mononuclear cells were isolated

from fresh blood according to the manufacturer's protocol. PB $CD4^+$ cells were isolated by negative selection using magnetic microbeads (Miltenyi Biotec, Auburn, CA). The purity of human T-cell subsets obtained using this technique has, in our experience, been consistently > 95%, as assessed by FACS analysis (Rus et al., 2005). RNA isolation and cell lysate preparation for protein analysis were performed the same day (Niculescu et al., 1997). Total RNA was purified using the RNeasy Mini Kit (Qiagen, Santa Clarita, CA) according to the manufacturer's instructions. RNA (0.5 μg per sample) was mixed with RT buffer, dNTP, and oligo-dT primer (Invitrogen). RNA was denatured by incubation at 65 °C for 5 min. The reverse transcriptase (Promega) and RNase inhibitor (Invitrogen) were then added, and the reaction mixture was incubated at 37 °C for 1 h. The reaction was terminated by incubating the mixture at 95 °C for 5 min.

Real-time PCR

Real-time PCR was performed using a StepOne real-time PCR system (Applied Biosystems, Foster City, CA). The primers for the genes investigated were designed and synthesized by TIB Mol Biol LLC (Adelphia, NJ) or IDT (Coralville, IA) (Table 1) and used in conjunction with LightCycler FastStart DNA Master SYBR Green I (Roche) according to the manufacturer's protocol. As a negative control for each real-time PCR assay, the same reaction was performed in the absence of cDNA or reverse transcriptase. For each gene, the cycle threshold (C_T) values were determined in the exponential phase of the amplification plot and normalized by subtraction of the C_T value for 18S (generating a ΔC_T value). The results were normalized to L13 ribosomal protein. A standard curve was generated using serial dilutions of qPCR Reference Total cDNA (Clontech, Mountain View, CA), and the normalized mRNA value (NRV) was calculated according to the following formula for relative expression of target mRNA: NRV (TarS/L13), where TarS represents the level of mRNA expression of the target gene, and L13 corresponds to that of the amplified L13 mRNA.

In some cases, the -fold change in target gene samples, after normalization with the housekeeping gene (18S), was calculated using the $2^{-\Delta\Delta CT}$ value, where $\Delta\Delta C_T = \Delta C_T$ (sample) $- \Delta C_T$ (control) and ΔCT is the C_T value of target gene normalized to the C_T value of the housekeeping gene (Cudrici et al., 2008).

Table 1
Primers used for real-time PCR.

Gene symbol	Primers sequence	Product (bp)
RGC-32	For: 5′-AGGAACAGCTTCAGCTTCAG-3′ Rev: 5′-GCTAAAGTTTTGTCAAGATCAGCA-3′	152
Akt1	For: 5′-ACGCCAAGGAGATCATGC-3′ Rev: 5′-CTCCATGCTGTCATCTTGGTC-3′	185
FasL	For: 5′-GCCCATTTAACAGGCAAGTC-3′ Rev: 5′-ATCACAAGGCCACCCTTCTT-3′	110
CDC2	For: 5′-TTTTCAGAGCTTTGGGCACT-3′ Rev: 5′-AGGCTTCCTGGTTTCCATTT-3′	100
CCND1	For: 5′-CCCAACAACTTCCTGTCCTACT-3′ Rev: 5′-GTCGGTGGGTGTGCAAG-3′	210
CDKN1A	For: 5′-GAAGACCATGTGGACCTGTCAC-3′ Rev: 5′-AGGGCTTCCTCTTGGAGAAGAT-3′	172
COL1A1	For: 5′-CTCAAGATGTGCCACTCTGA-3′ Rev: 5′-CGCTTCCATACTCGAACTG-3′	238
FN1	For: 5′-GCAAGCCTGAACCTGAAGAG-3′ Rev: 5′-GCAGGTACAGTCCCAGATCA-3′	110
ACTA2	For: 5′-GAAGCCCAGCCAGTCGCCATC-3′ Rev: 5′-TTGCTCTGCGCTTCGTCCCC-3′	235
L13	For: 5′-CGTGCGTCTGAAGCCTACA-3′ Rev: 5′-GGAGTCCGTGGGTCTTGAG-3′	227
18S	For: 5′-GTAACCCGTTGAACCCCATT-3′ Rev: 5′-CCATCCAATCGGTAGTAGCG-3′	151

For, forward primer; Rev, reverse primer; Bp, base pairs; RGC-32, response gene to complement 32; FasL, fas ligand; CCND1, cyclin D1; CDKN1A, cyclin-dependent kinase inhibitor 1A; COL1A1, collagen, type I, alpha 1; FN1, fibronectin 1; L13, ribosomal protein L13; 18S, 18S ribosomal RNA; IL21 (interleukin 21) expression was assessed using SABiosciences RT qPCR Primers, reference positions 384–404, catalog number PPH01684A.

Astrocyte isolation

Neonatal astrocytes were purified from the brains of 1-day-old Sprague–Dawley rats as previously described (Rus et al., 1992). After removal of the meninges, the brain was minced and sequentially passed through nylon meshes. The dissociated cell suspension was plated onto 75-cm^2 plates in DMEM/Ham's F-12 medium containing 10% FBS. Oligodendrocyte precursor cells (OPC) and glial cells were separated from the astrocyte monolayer by shaking overnight at 200 rpm on a rotary shaker. The OPC and glial cell suspension was discarded, and adherent astrocytes were kept in DMEM/Ham's F-12 medium containing 10% FBS. More than 97% of the cells isolated expressed the astrocyte marker GFAP.

RGC-32 silencing and transfection of cells

Transfection of PBMCs

PBMCs were plated in RPMI-10% FBS in 12-well plates 24 h prior to viral infection. After 24 h, the medium from the plate wells was removed and replaced with 1 ml of Polybrene/RPMI-10% FBS medium mixture per well. Cells were then infected by adding the RGC-32 shRNA (Santa Cruz Biotech) or control lentiviral particles (Santa Cruz Biotech) to the culture medium overnight, according to the manufacturer's instructions. The next day, the culture medium was removed and replace with 1 ml of RPMI-FCS 10%, and after 24 h, the transfected cells were analyzed for the expression of RGC-32, FasL and L13 mRNA by real-time PCR as described above. FasL expression by CD4 cells was determined using FACS analysis as previously described (Cudrici et al., 2006).

Transfection of astrocytes

Astrocytes were plated at 3×10^5 cells/well in 6-well plates and incubated at 37 °C in a CO_2 incubator until they reached 80% confluence. Cells were then transiently transfected with RGC-32 siRNA (Santa Cruz Biotech) or control siRNA in triplicate by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. Two hundred pmol siRNA and 5 μl of Lipofectamine 2000 were diluted separately in Opti-MEM I medium (Invitrogen), incubated for 5 min, then combined and incubated for 30 min at RT. At 24 h after transfection, astrocytes were changed into DMEM/10% FBS for another 24 h, then starved in serum-free DMEM for 18 h and treated with 10 ng/ml TGF-β or vehicle for the indicated periods of time.

Western blotting

Western blotting was performed as previously described (Rus et al., 1996). Astrocytes were washed with PBS, then lysed in a buffer consisting of 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 1% Triton X-100, 0.1% SDS, 100 mM NaCl, 10% glycerol, 0.5% sodium deoxycholate, 1 mM Na_3VO_4, and complete mini protease inhibitor mixture (Roche Applied Science, Indianapolis, IN), which was added just prior to use. Protein concentrations were determined using a BCA protein assay kit (Pierce, Rockford, IL). Samples were fractionated on an SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Millipore, Bedford, MA). The membrane was blocked with 0.1%Tween-TBS containing 1% bovine serum albumin (BSA) for 1 h and incubated with rabbit anti-CTGF IgG (Santa Cruz Biotech) or rabbit anti-RGC-32 IgG overnight at 4 °C. Goat anti-rabbit IgG-HRP conjugated antibodies (Santa Cruz Biotech) were applied for 1 h at RT. After washing, the immune complexes were detected using enhanced chemiluminescence (Pierce).

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed as previously described (Singh et al., 2010). In brief, astrocytes were treated with 1% formaldehyde in PBS for 10 min to crosslink protein to protein and protein to DNA. Cells were then pelleted by centrifugation and lysed in 1 ml ice-cold lysis buffer with 5 μl proteinase inhibitor cocktail (Roche) and 5 μl phenylmethylsulfonyl fluoride (Sigma). The mixture was homogenized to release the nuclei from the cells. The nuclei were sonicated in 1 ml of shearing buffer at a 2.5 power level for 20 min with 20-s sonications and 30-s intervals using a Fisher Sonic Dismembrator Model 60. The resulting DNA fragments had an average length of ~100–500 bp. A portion (10%) of the volume was frozen and kept aside as input before the addition of the antibodies. Chromatin was immunoprecipitated overnight at 4 °C with anti-RUNX1 antibody (5 μg/reaction; Calbiochem, La Jolla, CA), anti-p65 NF-kB (Santa Cruz Biotech), or normal rabbit IgG (5 μg/reaction; Santa Cruz Biotech). Immune complexes were precipitated by incubation of the samples with protein G-Sepharose equilibrated with salmon sperm DNA at 4 °C for 2 h. The protein G-Sepharose beads were washed with cold ChIP sonication buffer containing no protease inhibitors, then washed sequentially with low-salt wash buffer, high-salt wash buffer, and LiCl wash buffer (0.25 M LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA and 10 mM Tris, pH 8.1). The immune complexes were eluted with 1% SDS and 0.1 M $NaHCO_3$. The cross-linking was reversed by incubation at 65 °C for 4 h, followed by ethanol precipitation of the proteins and DNA. Proteinase K digestion was performed at 55 °C for 1 h. The immunoprecipitated chromatin and 1:10 dilution of input chromatin were analyzed by real-time PCR using primers designed to amplify fragments of the RGC-32 promoter (For: 5′-CCCCTAGATCGTCTGGGACT -3′, Rev: 5′-GCTTCATGGAGAGGTTGCTC -3′).

Statistical analysis

Comparisons between multiple groups were performed using two-way ANOVA. P values <0.05 were considered significant. Pearson correlation analysis was conducted to examine the association between variables. Statistical analysis was performed using SAS software version 9.2. All values are shown as mean ± SEM and are representative of three experiments unless otherwise noted.

Results

Localization of RGC-32 in MS brain

Since effector T cells migrate into the brain at the time of an MS relapse, we investigated the expression of RGC-32 in the MS brain in relation to that of T cells and macrophages. We first examined the localization of RGC-32 in 20 areas from 8 patients with MS (Table 2). MS brain samples from active lesions contained abundant inflammatory cell infiltrates, consisting of $CD4^+$ and $CD8^+$ T cells as well macrophages. Acute active lesions contained inflammatory cells throughout the entire lesion, whereas the inflammation was restricted to the lesion margins in chronic active lesions (Cudrici et al., 2007). Using indirect immunoperoxidase, we were able to show that RGC-32 was expressed in both acute and chronic active lesions (Figs. 1–3, Table 2). RGC-32 was also found to be present on cells in both perivascular (Figs. 1A and 2A) and parenchymal areas (Figs. 1D, 2D, 3A, B). The RGC-32 deposition was confined not only to MS plaques but was also present in areas of NAWM and NAGM (Table 2). In general, the expression of RGC-32 was higher in the MS plaques than in NAWM and NAGM. Controls for the immunoperoxidase reaction using control isotype IgG instead of the primary antibody were all negative (Figs. 1C, 2C, 3D). Both MBP staining (Fig. 1E) and Luxol fast blue (Fig. 2E) clearly indicated the presence of demyelination in the MS plaques examined.

Table 2
RGC-32 expression in MS brain.

Case (age, sex)	Lesion (no)	Lesion type	Perivascular	Parenchymal
1 (47, F)	Frontal plaque (3)	Chr. active	+++	+
		NAWM	++	++
		NAGM	+	++
2 (50, M)	Frontal plaque (1)	NAGM	+++	−
3 (50, M)	Temporal plaque (3)	Acute	++	+++
		NAWM	++	++
		NAGM	+	+
4 (50, M)	Occipital plaque (1)	NAWM	+/++	+++
5 (51, F)	Frontal plaque (3)	Acute	++	++
		NAWM	++	+++
		NAGM	++	++
6 (51, F)	Occipital plaque (3)	Chr. active	+++	+++
		NAWM	+++	+++
		NAGM	++	+
7 (38, F)	Parietal plaque (3)	Chr. active	++	++
		NAMW	+	++
		NAGW	+	+
8 (38, F)	Occipital plaque (3)	Chr. active	+++	+++
		NAWM	+++	+++
		NAGM	++	++
9–12 (mean age 49, M, F)	Normal brain (8)	GM	−	−/+ rare positive neurons
		WM	−	−

F, female; M male; NAWM, normal appearing white matter; NAGM, normal appearing grey matter; GM, grey matter; WM, white matter; Chr., chronic; −, negative; +, slightly positive; ++, positive; +++, highly positive.

Colocalization of RGC-32 with CD3-, CD68-, and GFAP-positive cells

On serial sections, the pattern of RGC-32 (Figs. 1A, 2A) expression was found to be compatible with that of CD3 (Fig. 1B) and CD68 (Fig. 2B). To further establish that CD3- and CD68-positive cells also express RGC-32, we carried out double-labeling experiments using specific antibodies. We were able to show that some of the RGC-32 deposits co-localized with $CD3^+$ cells (Fig. 1D). We also found that some of the $CD68^+$ cells in MS plaques co-localized with RGC-32 (Fig. 2D). In addition, RGC-32 was found to be present on $CD68^+$ cells that had morphology suggestive of glial cells (data not shown). In addition to CD3- and CD68-positive cells, GFAP-positive cells also co-localized with RGC-32 (Fig. 3C), indicating that astrocytes in MS brain also express RGC-32.

Expression of RGC-32, FasL, and CDC2 in PBMCs of RR MS patients and controls

We next examined the expression of RGC-32 in unstimulated PBMCs in relation to disease activity. We also investigated the expression of FasL and CDC2 mRNA, since is known that RGC-32 binds to and regulates CDC2, which in turn has a regulatory effect on FasL expression (Badea et al., 2002; Fosbrink et al., 2009; Torgler et al., 2004). Expression of RGC-32, FasL, CDC2, and L13 (a housekeeping gene) mRNA was measured by real-time PCR. MS samples were divided into those from stable periods in which no clinical activity was present and those from periods of relapse in which clinical activity was present and had been detected by a neurologist. Stable RR MS patients had statistically significant higher levels of RGC-32 mRNA expression than did controls ($p<0.0001$) (Fig. 4A). Patients with stable MS had also significant higher levels of FasL than did control subjects ($p=0.0093$) (Fig. 4B). On the other hand, CDC2 mRNA levels were not significantly different between stable MS patients and controls (Fig. 4C). Next we analyzed whether the mRNA expression of the studied genes was affected by the occurrence of relapse. Statistically

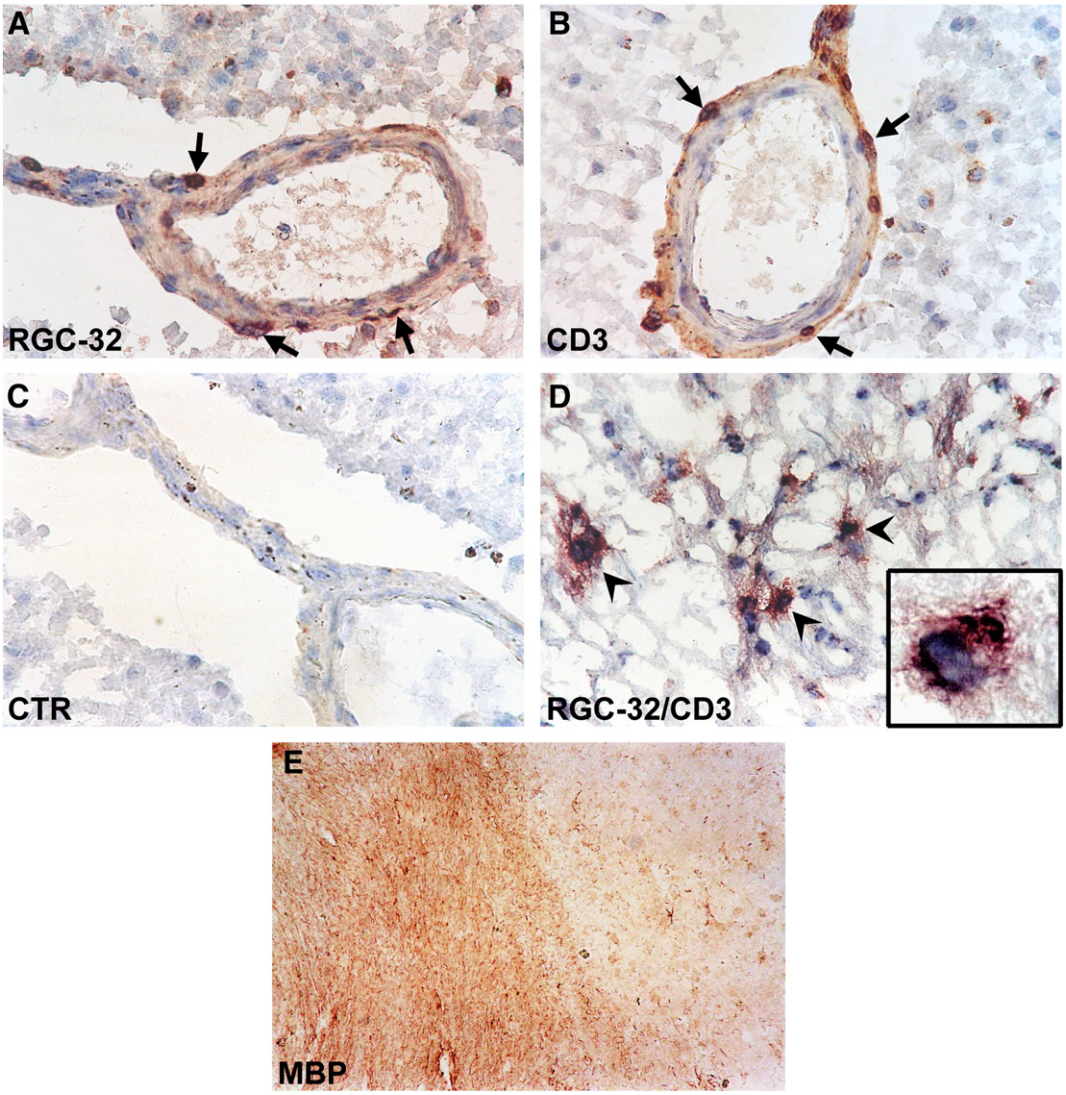

Fig. 1. Expression of RGC-32 and CD3 in MS brain. Cryostat sections were immunostained for RGC-32 (A) and CD3 (B) using the indirect immunoperoxidase method. A. Cells that stained positively for RGC-32 (arrows) were seen in perivascular areas; B. On serial sections, the pattern of RGC-32 expression was found to be compatible with that of $CD3^+$ cells C. Control of the immunoperoxidase reaction. By double-staining (D), RGC-32 (red deposits) is seen to be co-localized (arrowheads) with CD3 (dark brown deposits) in an MS plaque. E. The same area as in A–D, stained for myelin basic protein, shows significant absence of MBP and demyelination in an MS plaque. Original magnification: A–D (×400), D: insert ×1000, E: ×100.

significantly lower levels of RGC-32 mRNA were found in MS patients with relapses than in those who were clinically stable ($p<0.0001$) (Fig. 4A). Patients with relapses also had significantly lower FasL levels than did stable MS patients ($p=0.0206$) (Fig. 4B). CDC2 mRNA levels in MS patients with relapses were similar to those in stable patients and controls (Fig. 4C). As shown in Fig. 4D, RGC-32 mRNA expression was correlated with that of FasL mRNA during relapses ($r=0.89141$, $p=0.0002$). Interestingly, FasL expression was also positively correlated with that of CDC2 mRNA expression during relapses ($r=0.67963$, $p=0.015$).

We next investigated by immunostaining RGC-32 expression in $CD4^+$ T-cells isolated from PB. We found that RGC-32 was present in $CD4^+$ cells mostly in the cytoplasm and sometimes in the nucleus (Fig. 5A). Controls of immunoperoxidase reaction were negative (Fig. 5B). Significantly higher number of $CD4^+$ cells expressing RGC-32 was found in stable MS patients when compared to controls ($p<0.001$) and to patients with relapses ($p<0.003$) (Fig. 5C). The expression pattern of RGC-32 protein in $CD4^+$ is similar to that of mRNA in PBMCs with low levels of expression during relapses when compared to stable MS patients.

Expression of cyclin D1, Akt1, and p21 mRNA in PBMCs in RR MS patients and controls

Since RGC-32 plays an important role in cell cycle regulation (Badea et al., 2002; Fosbrink et al., 2009), we investigated the mRNA expression of cyclin D1, Akt1, and p21 (an Akt substrate and cell cycle inhibitor), which are known to be involved in cell cycle activation (Liang and Slingerland, 2003; Stacey, 2003). When compared to those of healthy controls, the levels of cyclin D1 were significantly higher in both stable ($p=0.0342$) and relapsing MS patients ($p=0.0129$) (Fig. 6A). No significant changes in cyclin D1 levels were found when we compared stable MS patients to those with active disease (Fig. 6A). Similar results were obtained for Akt1 mRNA expression. Patients with relapses had significantly higher levels of Akt1 than did controls ($p=0.0106$) (Fig. 6B). Statistically significantly higher levels of Akt1 were also seen stable MS patients than in healthy controls ($p=0.0326$) (Fig. 6B). Cell cycle inhibitor p21 mRNA levels were not significantly changed in MS patients when compared with controls (Fig. 6C). Interestingly, the expression of IL-21, a cytokine expressed by activated $CD4^+$ T-cells (Parrish-Novak et al., 2000; Tzartos et al., 2011), was higher in

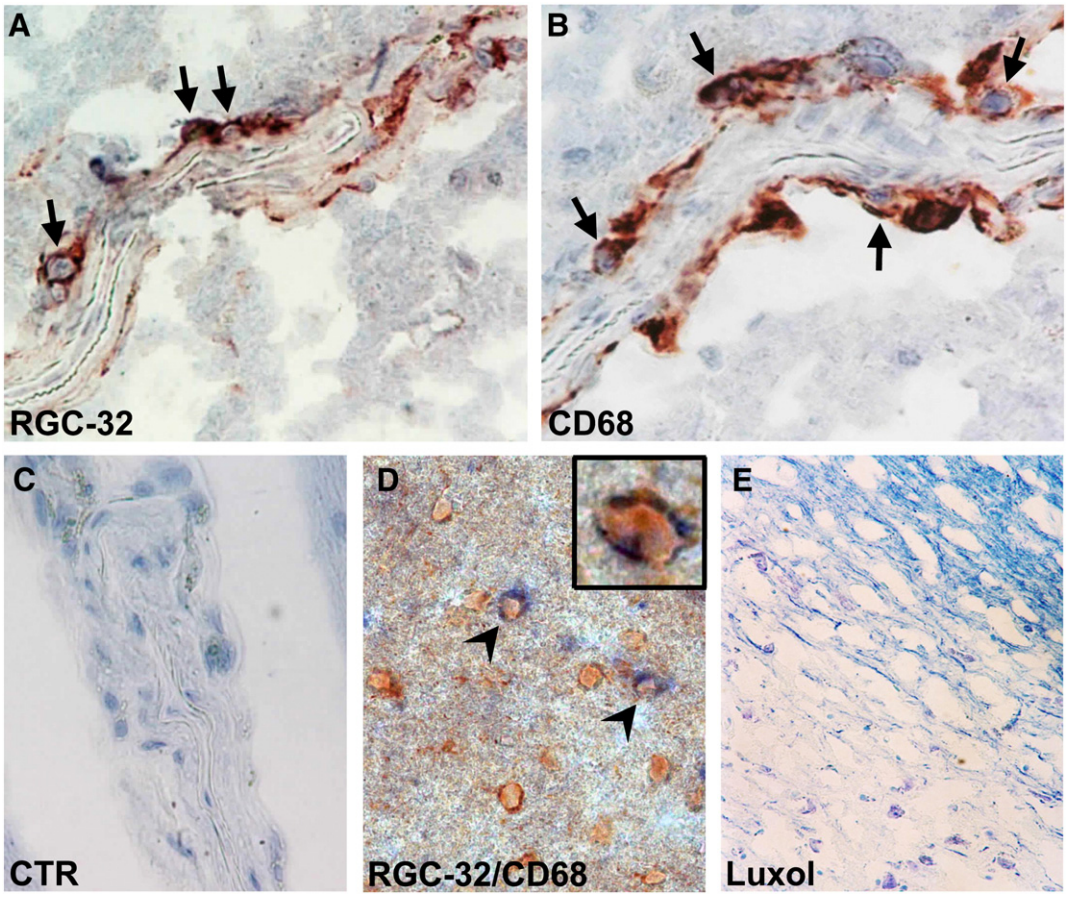

Fig. 2. Expression of RGC-32 and CD68 in MS brain. Cryostat sections were immunostained for RGC-32 (A) and CD68 (B) using the indirect immunoperoxidase method. A. RGC-32-expressing cells (arrows) are seen in perivascular areas; B. On serial sections, the pattern of RGC-32 expression is similar to that of $CD68^+$ cells. C. Control of the immunoperoxidase reaction. D. By double-staining, RGC-32 (red deposits) is seen to be co-localized with $CD68^+$ cells (blue deposits) in the MS plaque (arrowheads). E. The same area as in A–D, stained with Luxol fast blue, shows significant demyelination. Original magnification: A–D (×400), D: insert: ×1000, E: ×100.

MS-relapse patients than in either stable patients ($p = 0.0208$) or healthy controls ($p = 0.0369$) (Fig. 6D).

Effect of RGC-32 silencing on FasL expression

To investigate the requirement for RGC-32 for FasL mRNA expression, we used shRNAs targeting RGC-32 to silence its expression. PBMCs were transfected with RGC-32 shRNA lentivirus and then tested for their effectiveness in blocking the endogenous expression of RGC-32 mRNA using real time-PCR. RGC-32 shRNA effectively decreased mRNA RGC-32 expression by 72% when compared to cells transfected with control shRNA (shCTR) (Fig. 7A). We then examined the ability of RGC-32 shRNA to block FasL mRNA expression. As shown in Fig. 7B, FasL expression was reduced by 47% after RGC-32 silencing, indicating an important role for RGC-32 in FasL mRNA expression. In addition FasL expression on the surface of $CD4^+$ cells was significantly reduced (by 48%) after RGC-32 silencing (Fig. 7C).

TGF-β induces RGC-32 expression in astrocytes

We have previously shown that RGC-32 is expressed by astrocytes in MS lesions. In view of the important role of TGF-β in promoting gliosis and of the fact that RGC-32 is a downstream target of TGF-β (Huang et al., 2009), we were interested in determining whether or not RGC-32 plays a role in TGF-β-induced production of extracellular matrix (ECM) in astrocytes. We selected rat neonatal astrocytes for our in vitro experiments, since we were able to obtain enough primary cells for the required experiments. We first investigated whether RGC-32 is expressed in rat astrocytes in culture and whether RGC-32 expression is regulated by TGF-β. As shown in Fig. 8A, RGC-32 is normally expressed at a low level in astrocytes and is induced by TGF-β. After 3 h of TGF-β stimulation, mRNA expression was significantly increased, and both RGC-32 mRNA and protein expression levels were robustly increased after 18 h of treatment when compared to controls ($p < 0.02$) (Figs. 8A, B). These data indicate that TGF-β is a strong inducer of RGC-32 expression in astrocytes.

Effect of TGF-β on RUNX1 recruitment at the RGC-32 promoter

Previous studies have shown that the transcription factor RUNX1 is required for RGC-32 expression in the ovaries (Park et al., 2008) and that TGF-β induces RUNX1 activation (Wildey and Howe, 2009). An analysis of the RGC-32 promoter has revealed the presence of several putative RUNX1 binding sites, with three consensus binding sites (Park et al., 2008). These findings suggest that RUNX1 might transcriptionally regulate RGC-32 gene expression in astrocytes. To determine whether RUNX1 specifically binds to these candidate sites in the RGC-32 promoter in vivo, ChIP assays were performed using astrocytes treated with vehicle (CTR) or stimulated with TGF-β for 3 or 5 h, the times reported for maximal RUNX1 protein expression (Ito and Miyazono, 2003) and high RGC-32 gene expression, respectively (Fig. 8A). Immunoprecipitations were performed with anti-RUNX1 antibody or with normal rabbit IgG as a negative control. DNAs were analyzed by real-time PCR. DNA fragments containing RUNX1 transcription factor binding sites were found

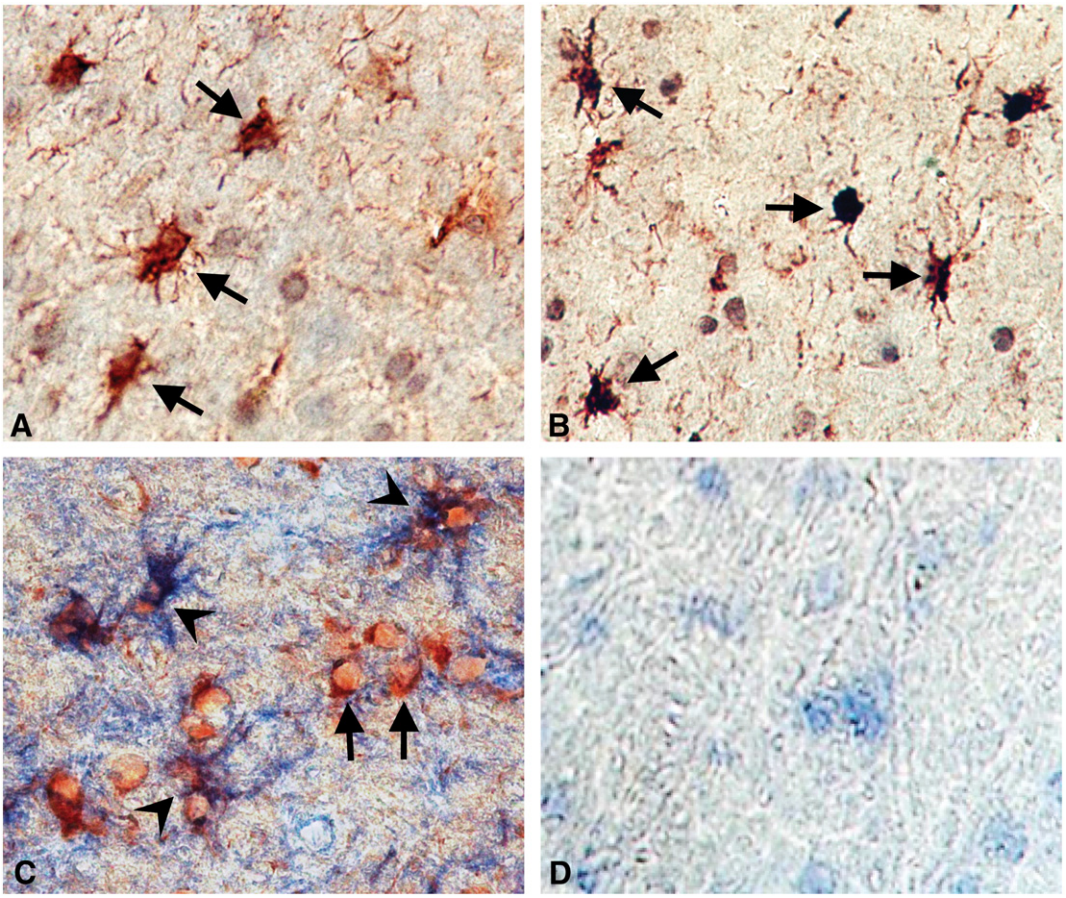

Fig. 3. Co-localization of RGC-32 and GFAP in MS brain. Cryostat sections were stained for RGC-32 using the indirect immunoperoxidase method. A, B. RGC-32-expressing cells (arrows) are seen in parenchymal areas and have morphology suggestive of astrocytes. C. By double-staining, some of the RGC-32 (red deposits) are seen to be co-localized with GFAP (blue deposits) in an MS plaque (arrowheads). Not all the RGC-32-positive cells in parenchymal areas express GFAP (arrows). D. Control of the immunoperoxidase reaction. Original magnification: A–D (×400).

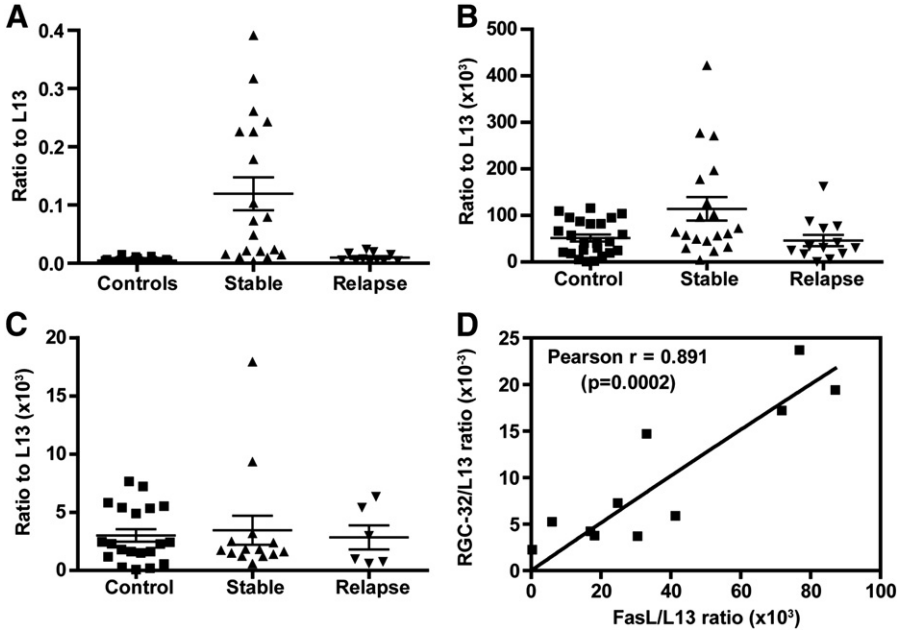

Fig. 4. Expression of RGC-32, FasL, and CDC2 mRNAs in MS patients and controls. The expression of mRNAs was measured by real-time PCR and expressed as a ratio to L13. A. Stable MS patients had significantly higher levels of RGC-32 mRNA expression than did controls ($p<0.0001$). Significantly lower levels of RGC-32 mRNA were found in MS patients with relapses than in those who were clinically stable ($p<0.0001$). B. Patients with stable MS also had significantly higher levels of FasL than did control subjects ($p=0.0093$). A significant decrease in FasL ($p<0.0206$) was seen in patients with relapses when compared to stable MS patients. C. CDC2 mRNA changes were not statistically significant. D. RGC-32 mRNA expression levels were correlated with those of FasL mRNA during relapses ($r=0.89141$, $p=0.0002$).

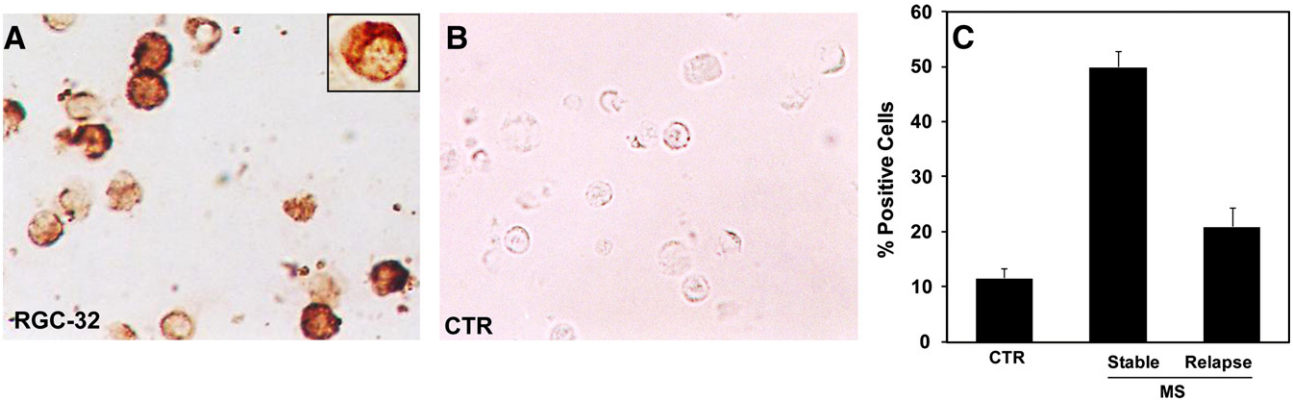

Fig. 5. Expression of RGC-32 in $CD4^+$ T-cells form MS patients and controls. $CD4^+$ T-cells were isolated by negative selection and deposited on glass slides in a cytospin centrifuge then fixed in acetone: methanol (2:1). RGC-32 expression was determined by immunocytochemistry as described in Materials and Methods. A. RGC-32 was present in $CD4^+$ cells mostly in the cytoplasm and less in the nucleus. B. Control of the immunoperoxidase reaction using isotype control instead of the primary antibody was negative. Original magnification: A,B (×400), A: insert (×1000). C. Significantly higher number of $CD4^+$ cells expressing RGC-32 was found in stable MS patients when compared to controls ($p<0.01$) and to MS patients with relapses ($p<0.003$).

to be enriched in the chromatin samples immunoprecipitated with RUNX1 antibody ($p<0.01$) (Fig. 8C), indicating that TGF-β induced RUNX1 recruitment to the RGC-32 promoter in vivo.

We also investigated if NF-kB specifically binds to the NF-kB candidate sites in the RGC-32 promoter (Park et al., 2008). Similar TGF-β- induced recruitment to the RGC-32 promoter was found for p65 NF-kB ($p<0.01$) (Fig. 8D). On the other hand we did not find p53 binding to the RGC-32 promoter using ChIP assay (data not shown). Our findings suggest a complex interplay between RUNX1 and other transcription factors such as NF-kB that may participate in the TGF-β- induced transcriptional activation of RGC-32 in rat astrocytes.

Effect of RGC-32 on TGF- β-induced procollagen I, fibronectin, α-SMA and CTGF

To determine whether RGC-32 is involved in TGF-β-induced ECM production, we investigated whether RGC-32 silencing had an effect on TGF-β-induced expression of procollagen I, fibronectin, and CTGF. We choose to test these three proteins because their expression has been found to be increased in MS lesions (Holley et al., 2003; Mohan et al., 2010). First, we investigated the effect of TGF-β on ECM expression and found that procollagen I ($p<0.003$), fibronectin ($p<0.02$), and CTGF ($p<0.02$) were significantly induced at 18 h of stimulation (Figs. 9A, C). The effect of TGF- β on α-smooth muscle actin (α-SMA) expression was also investigated since α-SMA was found to be re-expressed by reactive astrocytes (Moreels et al., 2008). We found that TGF-β induced α-SMA was significantly increased at 18 h of treatment ($p<0.05$) (Fig. 9B). Next, we silenced RGC-32 expression by transfecting rat astrocytes with siRGC-32 and compared the effect of this treatment to that of control siRNA. Our results clearly showed that a significant knockdown of RGC-32 expression was obtained after exposure to RGC-32 siRNA ($p<0.004$). The RGC-32 siRNA effectively decreased the mRNA RGC-32 expression by 92% and that the protein expression by 87% when compared to cells transfected with control siRNA (siCTR) (Figs. 9D,E). Post-transfection astrocytes were treated with TGF-β for 18 h. This

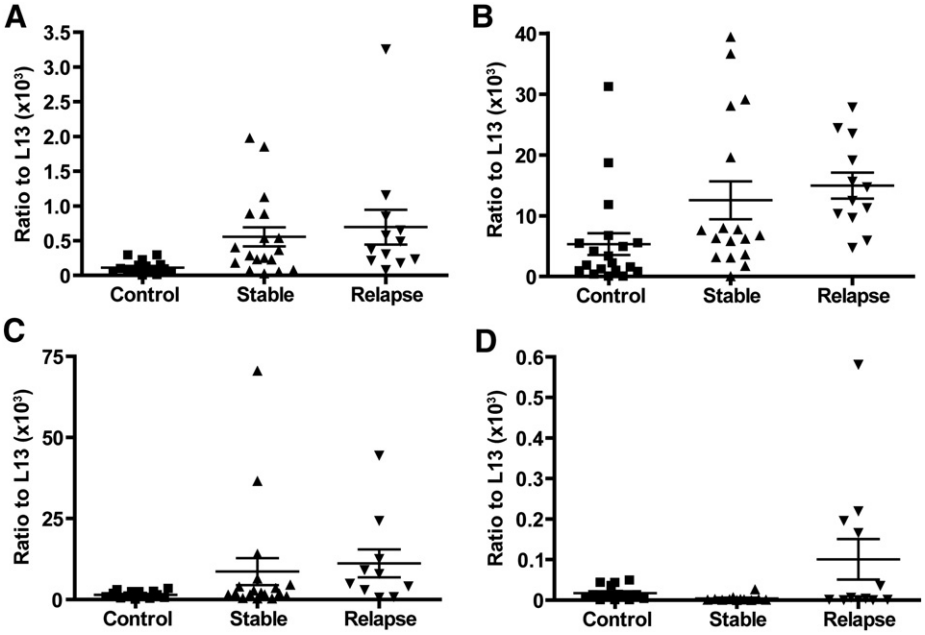

Fig. 6. Expression of cyclin D1, Akt1, and p21 mRNAs in MS patients and controls. The expression of mRNAs was measured by real-time PCR and expressed as a ratio to L13. A. When compared to those in healthy controls, the levels of cyclin D1 were significantly increased in both stable ($p=0.0342$) and relapsing MS patients ($p=0.0129$). B. Stable MS patients ($p=0.0326$) and those with relapses ($p=0.0106$) had significantly higher levels of Akt1 than did controls. C. Cell cycle inhibitor p21 mRNA levels were not significantly changed in MS patients when compared to controls. D. IL-21 mRNA expression was higher in MS patients with active disease than in stable patients ($p=0.0208$) or healthy controls ($p=0.0369$).

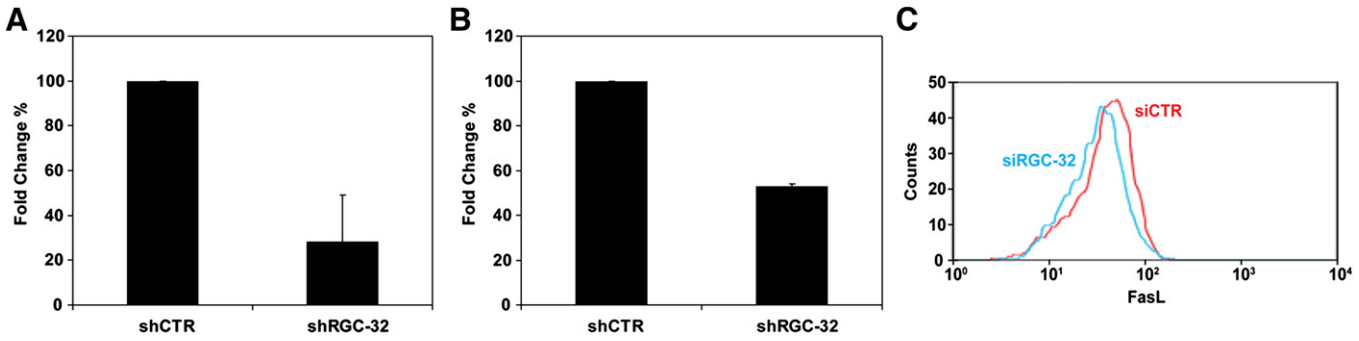

Fig. 7. FasL expression is mediated through RGC-32. PBMCs from patients with stable MS were transfected with lentivirus RGC-32 shRNA or shCTR for 48 h. The expression of RGC-32 and FasL mRNA was then determined using real-time PCR. A. RGC-32 shRNA treatment effectively decreased mRNA RGC-32 expression by 72% when compared to shRNA control (shCTR)-transfected cells. B. FasL mRNA expression was reduced by 47% ($p = 0.01$), indicating an important role for RGC-32 in mediating FasL mRNA expression. C. FasL expression on the surface of $CD4^+$ cells was analyzed by FACS analysis and was significantly reduced (by 48%) after RGC-32 silencing.

time point was selected because we had found significantly higher levels for procollagen I, fibronectin, α-SMA and CTGF at 18 h after TGF-β stimulation (Figs. 9A–C). Our data indicated that RGC-32 knockdown also resulted in a significant reduction in TGF-β induced procollagen I ($p<0.01$) and fibronectin ($p<0.05$) expression when compared with siCTR TGF-β stimulated cells (Fig. 9F). α-SMA was also significantly reduced ($p<0.05$) after RGC-32 silencing (Fig. 9G).On the other hand, RGC-32 silencing had no effect on CTGF expression (Fig. 9H). These data suggest that RGC-32 plays an important role in the TGF-β-mediated induction of ECM expression in astrocytes.

Discussion

The aim of our study was to evaluate the role played by RGC-32 in MS. Using immunohistochemistry, we were able to show that inflammatory cells (T-cells and macrophages) and astrocytes express RGC-32 in the MS brain. The expression of RGC-32 was not confined to the MS plaques but was also present in NAWM and NAGM areas, indicating a widespread distribution of cells expressing RGC-32. To gain more insight into the role played by RGC-32 in MS, we then examined the expression of RGC-32 mRNA in relation to disease activity

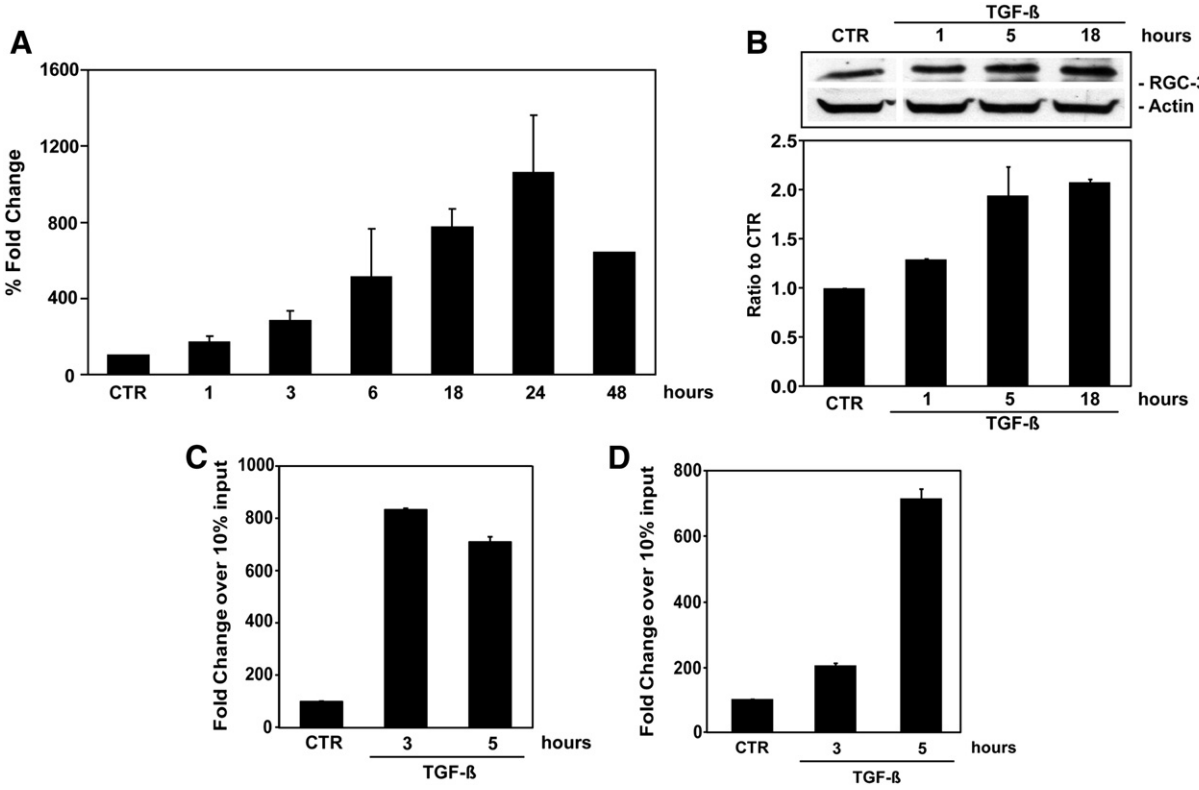

Fig. 8. TGF-β induces the expression of RGC-32 in astrocytes. Astrocytes were treated with 10 ng/ml TGF-ß for the times indicated and examined for the expression of mRNA and protein. A. RGC-32 mRNA expression was determined by real-time PCR. The expression of the mRNA at the beginning of the experiment (CTR) was considered to be 100%. Results are expressed as means ± SEM, relative to the CTR. B. RGC-32 protein expression was quantified by Western blotting and normalized to β-actin (right panel), and the results are presented as -fold increase over control (CTR). C, D. Effect of TGF-β on RUNX1 and NF-kB recruitment at the RGC-32 promoter. ChIP assays were used to detect the binding of transcription factors to the RGC-32 promoter region in astrocytes. ChIP assays were performed using DNA extracted from astrocytes after TGF-β treatment. Immunoprecipitations were performed with anti-RUNX1 antibody (C) and anti-p65 NF-kB antibody (D), or with normal rabbit IgG as a negative control. DNA fragments containing RUNX1 and p65 NF-kB transcription factor binding sites were found to be enriched in the chromatin samples immunoprecipitated with specific antibodies.

in MS patients. We performed this analysis in unstimulated PBMCs in order to mimic the in vivo situation as closely as possible; alterations in mRNA expression in unstimulated cells are more likely to have a predictive value for clinical exacerbations (Lopatinskaya et al., 2003). We have now demonstrated that the levels of RGC-32 significantly increase in the PBMCs and $CD4^+$ T-cells of clinically stable MS patients as compared to healthy controls and significantly decline during clinical relapse. The changes in RGC-32 mRNA expression levels that occurred in the PBMCs from MS patients during relapses were positively correlated with those of FasL expression, which has previously been shown to decrease significantly during relapses (Lopatinskaya et al., 2003). To further investigate this relationship, we silenced the expression of RGC-32 using RGC-32 shRNA and found that the expression of FasL mRNA and protein was significantly reduced, indicating that FasL expression is RGC-32-dependent, at least in part.

The synchronized changes in RGC-32 and FasL expression in PBMCs that we observed may be explained by the known role of CDC2 in the regulation of FasL transcription (Torgler et al., 2004). Since RGC-32 binds to and up-regulates CDC2/cyclin B1 kinase activity (Badea et al., 2002; Fosbrink et al., 2009), and since RGC-32 silencing significantly decreased FasL mRNA expression, it is possible that RGC-32 regulates FasL expression by modulating the activation of the CDC2/cyclin B1 complex. These data also suggest that RGC-32 is involved in regulating T-cell mediated apoptosis by modulating the expression of FasL. Our results suggest that a decrease in the levels of RGC-32 mRNA signals the onset of a relapse, and thus may serve as a marker that could be used to predict disease activity.

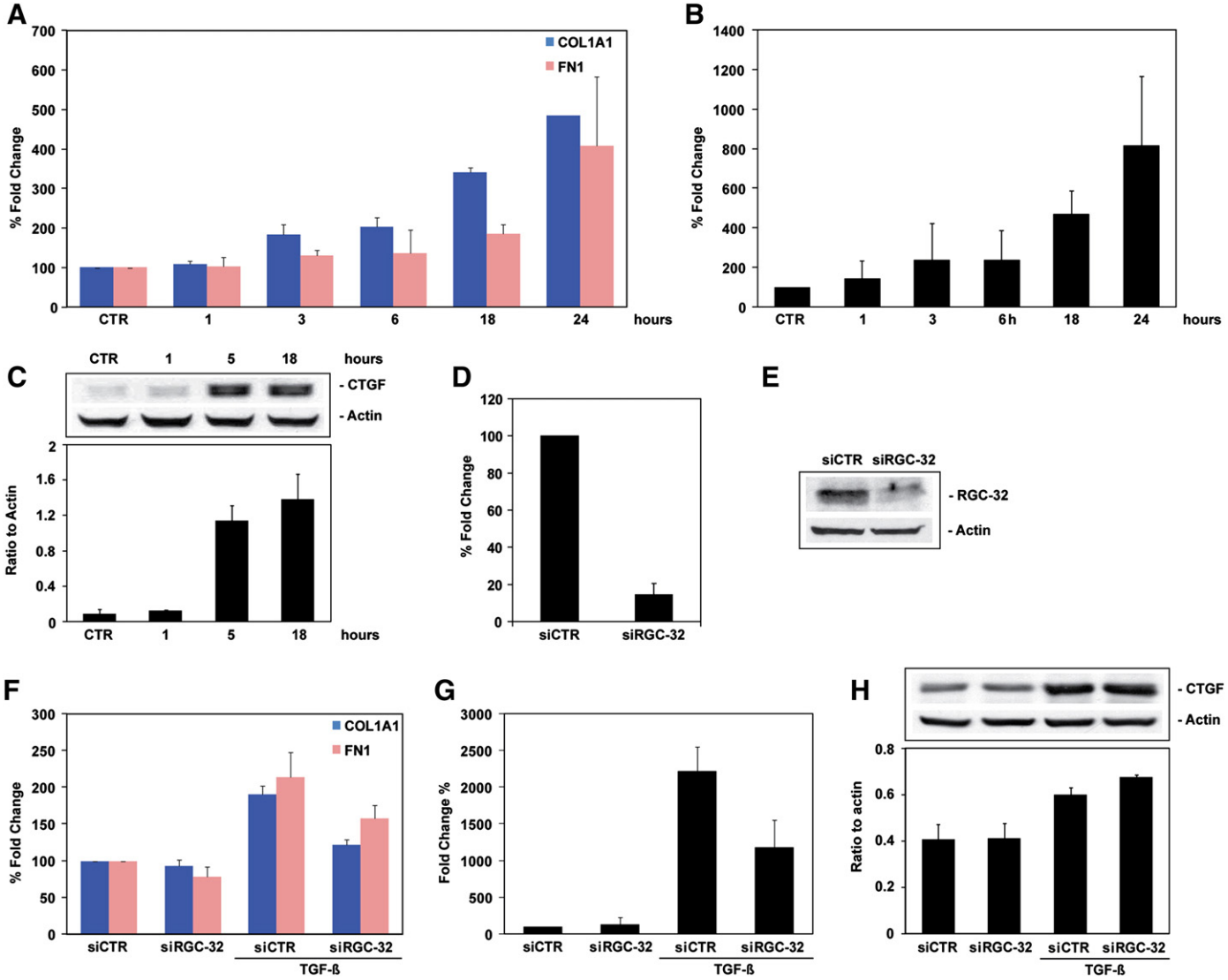

Fig. 9. Effect of RGC-32 silencing on procollagen I, fibronectin, α-SMA and CTGF expression. A,B,C. TGF-β-induced expression of procollagen I, fibronectin, α-SMA and CTGF. Astrocytes were exposed to TGF-β (10 ng/ml) for the indicated periods of time. Expression of procollagen type I (A), fibronectin (A), α-SMA (B) was determined by real-time PCR and expression of CTGF by Western blotting (C). The expression of the mRNA at the beginning of the experiment (CTR) was considered to be 100%. Results are expressed as percent ± SEM, relative to the CTR. CTGF was expressed as a ratio to β-actin expression. D,E. RGC-32 silencing using siRNA. Astrocytes were transfected with siRGC-32 or siCTR using Lipofectamine 2000. After 48 h, total RNA was extracted and analyzed for RGC-32 and 18S mRNA expression by real time-PCR. SiRGC-32 reduced the RGC-32 mRNA expression by 90% when compared with siCTR (D). A similar reduction in RGC-32 at the protein level (by 87%) was found at 72 h by Western blotting (E). F, G, H. Effect of RGC-32 silencing on procollagen I, fibronectin, α-SMA and CTGF expression. Astrocytes were transfected with siRGC-32 or siCTR using Lipofectamine 2000. After 48 h, cells were stimulated with TGF-ß (10 ng/ml) and total mRNA was purified and analyzed for the expression of procollagen I, fibronectin and α-SMA mRNA. After 72 h posttransfection, cells were stimulated with TGF-ß (10 ng/ml) and total protein was extracted and analyzed for CTGF expression by Western blotting. RGC-32 siRNA significantly reduced the procollagen I (F), fibronectin (F) and α-SMA (G) mRNA expression induced by TGF-β, but had no effect on CTGF expression (H).

The increase in Akt1 and cyclin D1 mRNA expression seen in the PBMCs of MS patients suggests that these cells are actively cycling; this result is also in agreement with the reduction in FasL expression. In addition, the high levels of IL-21 seen during relapses may be related to the role of this protein in promoting the proliferation of human $CD4^+$ and $CD8^+$ T cells (Jones et al., 2009).

Our data also show for the first time that RGC-32 is expressed by astrocytes in the MS brain and that its transcription is regulated by RUNX1 in conjunction with NF-kB. In addition, our in vitro experimental evidence also points to a major role for RGC-32 in TGF-β-mediated ECM production, as shown by the reduction in procollagen I, and fibronectin expression that we observed after RGC-32 silencing. This effect of RGC-32 on α-SMA is significant since α-SMA is re-expressed by reactive astrocytes (Moreels et al., 2008) which are the major cellular component of the glial scar. The effect on α-SMA suggests that RGC-32 is also required for the transition of astrocytes to a reactive state. On the other hand, RGC-32 silencing had no effect on CTGF expression, indicating that these two mediators of TGF-β effects work independently. These in vitro experiments point to a possible role for RGC-32 in the gliosis seen in MS and EAE. Further in vivo experiments are clearly needed to clarify the role of RGC-32 in gliosis.

RGC-32 seems to play a role in regulating both T-cell survival through the regulation of FasL expression and in regulating TGF-β-mediated profibrotic effects in astrocytes, indicating a possible role in gliosis. Hence, this molecule may play a dual role in MS, both as a regulator of $CD4^+$ T-cells mediated apoptosis and as a mediator of TGF-β-mediated profibrotic effects. RGC-32 may therefore represent a useful new target for therapeutic intervention in MS.

Abbreviations

ChIP	Chromatin immunoprecipitation assays
CTGF	connective tissue growth factor
EAE	experimental autoimmune encephalomyelitis
ECM	extracellular matrix
EDSS	expanded disability status scale
GFAP	glial fibrillar acidic protein
HRP	horseradish peroxidase
MBP	myelin basic protein
MS	multiple sclerosis
NAGM	normal-appearing gray matter
NAWM	normal-appearing white matter
OPC	oligodendrocyte precursor cell
RGC-32	Response gene to complement 32
RR	relapsing–remitting
RT	room temperature
siRNA	short interfering RNA
shRNA	short hairpin RNA
TGF-β	transforming growth factor β

Conflict of Interest statement

The authors declare that there are no conflicts of interest.

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