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Regulator of G protein signaling 9-2 (RGS9-2) mRNA is up regulated during neuronal differentiation of mouse embryonic stem cells

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Abstract

In this study we demonstrate up-regulation of mRNA for Regulator of G protein Signaling (RGS) 6, 7, 9 and 11, R7 family RGS binding protein (R7BP) and RGS9 anchor protein (R9AP) during neuronal differentiation of mouse embryonic stem cells (mESCs). This expression pattern was most robust for RGS9 whose transcript level was low in undifferentiated mESCs but increased over 125 fold when differentiating mESCs began to exhibit a neuronal precursor cell (NPC) phenotype. In addition, we demonstrate that RGS9 mRNA is expressed in neuronal stem cells isolated from embryonic mouse cortex. The expression of RGS9 in two distinct populations of NPCs suggests that RGS9 and its accessory proteins may play an important role in neuron development.

Introduction

G protein coupled receptors (GPCR) transduce extra cellular signals to intracellular responses through trimeric G-protein complexes by catalyzing the exchange of GDP for GTP at the $G\alpha$ subunit. GPCR signal termination occurs at a rate critically determined by the GTPase activity of the $G\alpha$ subunit which can be accelerated by RGS proteins [1]. In this way, RGS proteins have been shown to modulate the shape and duration of GPCR responses and alter coupling properties to second messengers including cAMP and mitogen activated protein kinase pathways [2].

The R7 family of RGS proteins, including RGS-6, 7, 9 and 11, contain multiple modular domains: the $G\gamma$ -like domain (GGL), Disheveled/Engrailed/Plextrin homology (DEP) domain and GTPase accelerating protein domain (GAP). R7 RGS proteins interact with the accessory protein $G\beta 5$ via the GGL domain, which enhances their GAP activity and is important for stability [3]. The DEP domain is required for interactions with other proteins including specific GPCRs [4-6].

Interestingly, Liu et al., (2002) demonstrated that RGS6 enhanced Neuronal Growth Factor (NGF) dependent neuronal differentiation of PC12 cells [6]. This enhancement was not dependent on the GAP activity of RGS6 but required an interaction with SCG10 mediated

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by the DEP domain of RGS6. SCG10 is a member of the stathmin protein family that regulate cytoskeletal dynamics by modulating intermediate filament polymerization [6, 7]. Whether this novel interaction between RGS6 and SCG10 extends to other R7 RGS proteins and other stathmin protein family members has not yet been determined. However, these findings suggest that the modular domains of the R7 family RGS proteins may mediate additional signaling processes distinct from their role in GPCR signal termination; signaling that may influence neurogenesis.

To investigate the potential role of R7 family RGS proteins in neuron development we used all-trans retinoic acid (RA)-induced neuronal differentiation of mouse embryonic stem cells (mESCs) as a model system. Here, we compared the relative mRNA expression levels of R7 RGS proteins and related proteins throughout differentiation.

Materials and Methods

Chemicals

Ascorbic acid was obtained from Fisher Scientific (Waltham, MA). leukemia inhibitory factor (LIF), and B27 were obtained from Invitrogen (Carlsbad, CA); all other chemicals were from Sigma Chemical (St Louis, MO).

mESC culture

Undifferentiated mESCs were obtained from ATCC and expanded as previously described [8]. 5×10^5 mESCs were plated in 15ml of ES media (knockout DMEM, 15% fetal bovine serum, supplemented with L-glutamine, penicillin/streptomycin, LIF, non-essential amino acids and beta-mercapto-ethanol) in 10cm bacterial petri dishes. Every 2 days, non adherent mESCs colonies were collected by centrifugation, dissociated with trypsin-EDTA, and re-plated as above or used to initiate neuronal differentiation as described below.

Neuronal Differentiation of mESCs

10 cm petri dishes were seeded with 2×10^6 dissociated mESCs in ES media (10ml) without LIF and reduced serum (10% FBS) for four days. Resulting embryoid bodies (EB) were collected by centrifugation and plated in ES media with reduced serum (10%), without LIF and supplemented with 400nM all trans retinoic acid (RA). Resulting EBs were collected by centrifugation and RA containing media was replaced midway through the four day RA treatment period. Following four days of RA treatment, EBs were dissociated with trypsin and plated on poly-D-lysine coated vessels at a concentration 5×10^5 cells/ml in Neural Basal A media (Invitrogen) supplemented with B27, L-glutamine, and penicillin/streptomycin.

Neuronal Stem cell culture

Neuronal stem cells derived from embryonic day 14 mouse cortex were obtained from Stem Cell Technologies (Vancouver, Canada) and expanded in culture according to manufacturer's instructions including manufacturer's recommended media formulations and procedures for maintaining undifferentiated NSCs.

Relative gene expression reverse transcriptase PCR

Cell samples were collected by centrifugation and washed with phosphate buffered saline (PBS). Total RNA was isolated using Trizol from Invitrogen according to manufacturer's instructions. Mouse tissue was homogenized and RNA also extracted using Trizol. RNA samples were treated with DNASE 1 (Roche, Indianapolis, USA) at 37 degrees for 30 minutes followed by phenol chloroform extraction. 2 μ g of total cellular RNA was reverse transcribed at 37°C for 50 minutes in a 20 μ l reaction volume primed with random hexamer

oligonucleotides (Sigma, Saint Louis, USA). “- RT” samples were treated identically except no RT enzyme was added. All RT-PCR products shown in figure 2A resulted from 25 cycles of amplification using 0.5ul of cDNA synthesized from 2ug of total RNA in a 25ul reaction with the following exceptions: cDNA was diluted 1:10 prior to 25 cycles of amplification using the OCT-4 and Nestin-1 primers. Standard PCR reactions were carried out using TAQ (Promega, Madison, WI) and annealing temperatures between 55 and 60° C.

Quantitative reverse transcriptase PCR (qPCR)

A real time RT-PCR protocol was applied using a Light Cycler 480 (Roche) instrument and SYBR Green Fluorescent dye (Roche Diagnostics, Mannheim, Germany). The reaction mixture was prepared according to manufacturer's instructions and using 1 µl cDNA template prepared from 2ug total RNA (diluted 5 fold). Cycling conditions were as follows: 95°C for 5min, and 40 cycles at 95°C for 10sec, 55°C for 30 sec 72°C for 30sec. Relative copy number was estimated as the efficiency (E) raised to the difference in cycle number required to reach threshold as previously described [9]. Efficiency was calculated by performing the RT-PCR reactions with increasing amounts of RGS9 or GAPDH cDNA. By plotting the cycle number required to reach threshold versus the log of the dilution of cDNA the slope was calculated and the reaction efficiency was estimated as: $E=10^{-1/\text{slope}}$ [9]. Product specificity was confirmed from product melting curves and threshold values were determined using EXOR4 software package (Roche Diagnostics, Mannheim, Germany).

Immunocytochemistry

Following the differentiation protocol described above and four days following plating on poly D coated cover slips, resulting neurons we prepared for fluorescence microscopy as previously described [10], using the following reagents: rabbit anti-MAP2 (Sigma Aldrich, 1:300), Alexafluor 488 conjugated anti-rabbit antibody (Invitrogen, 1:500), and DAPI (0.2ug/ml). Fluorescence was imaged using a Nikon microscope equipped with Nikon objectives, epi-fluorescence filters and NIS-elements imaging software (Nikon USA, Melville, NY).

Results

Neuronal differentiation of mESCs with RA resulted in cultures in which greater than 90% of the cells stained positively for the neuronal marker, MAP2 (fig. 1B). Additional characterization of the resulting neurons has previously been described [8]. Representative images of live mESCs at different stages of differentiation are shown in figure 1A.

To examine relative gene expression, we isolated total RNA from differentiating mESCs at six time points (1-6) that spanned the differentiation spectrum from undifferentiated mESCs to primitive neurons (fig. 1). Total RNA was isolated, reverse transcribed and resulting cDNA was used to amplify and compare levels of target genes at each time point using the primers shown in table 1. Reverse transcriptase PCR (RT-PCR) products were resolved by agarose gel electrophoresis, visualized with UV/ethidium bromide and reflect relative levels of gene transcripts for the targets indicated during neuronal differentiation of mESCs (fig. 2A).

Interestingly, mRNA for members of the R7 family of RGS proteins and their anchor proteins demonstrated distinct patterns of expression generally characterized by low expression in undifferentiated cells with marked increases upon withdrawal of the mESCs from LIF. In contrast, the stem cell marker OCT-4 expression steadily decreased and nestin-1 increased. mRNA levels for the house keeping gene, GAPDH, was similar throughout. Furthermore, the non R7 family RGS protein, RGS2, was expressed similarly

throughout differentiation with a slight increase upon LIF withdrawal. None of the primers amplified products from corresponding samples prepared without reverse transcriptase and the sizes of all resulting PCR products were consistent with the predicted amplification target size listed in table 1. Specificity of RT-PCR products was further confirmed using restriction enzyme fragment analysis (data not shown).

Of the RGS family members and related genes detected, RGS9 mRNA expression demonstrated the most robust change in expression during neuronal differentiation. To confirm and quantify this change in RGS9 mRNA, we used quantitative real time RT-PCR (qPCR). The relative change in copy number of RGS9 mRNA was calculated as described in the *materials and methods* and expressed as a fold increase compared to undifferentiated mESCs in figure 2B. RGS9 mRNA copy number increased ~125 fold in cells withdrawn from LIF for 4 days compared to undifferentiated cells. Relative transcript levels for GAPDH were not significantly different in the same samples. Average GAPDH mRNA expression levels were 95.1 +/- 36.7% and 85.0 +/- 46.7% (% +/- stdev) in two and four days with out LIF, respectively, compared to undifferentiated cells.

To determine the isoform of RGS9 expressed during differentiation of mECs, we utilized primers specific for either the retinal or striatal splice variants of RGS9, RGS9-1 and RGS9-2, respectively. RGS9-1 specific primers amplified the predicted size fragment from cDNA synthesized from mouse eye RNA extracts. Under the same conditions we were unable to detect RGS9-1 from cDNA synthesized from total RNA isolated from mESCs during stage 3 of neuronal differentiation (withdrawn from LIF for four days). In contrast, RGS9-2 specific primers amplified products of appropriate size from cDNA synthesized from RNA extracts of mouse brain, eye, and differentiating ESCs harvested near the peak of RGS9 expression (fig. 2C).

The temporal expression pattern of RGS9 suggested that RGS9 may be associated with neuronal progenitor cells (NPC) previously described during neuronal differentiation of mESCs [11, 12]. Neuronal differentiation of mESCs however results in heterogeneous populations of NPCs and other cell types. Therefore, coincident detection of different mRNAs within these populations of cells does not necessarily assign their origin to the same cell type [12]. Thus we sought to determine whether RGS9 was also expressed in a more homogenous population of related NPCs. To this end, neuronal stem cells (NSCs) were cultured from the cortex of embryonic day 14 mice. Here RGS9 mRNA was readily detected along with R7BP, nestin-1, and PAX-6 but not OCT-4, RGS2, RGS6, RGS7, RGS11, or R9AP (fig.3). RT-PCR products shown in figure 3, resulted from 25 cycles of amplification except PAX-6 which was from 30 cycles of amplification. Further increasing cycle number to 40 did not lead to detectable products for the other gene targets (data not shown). Identical results were obtained from two separate NSC cultures.

Discussion

In this study we demonstrate distinct mRNA expression patterns for R7 RGS proteins during neuronal differentiation of mESCs. For example RGS9 mRNA exhibited robust changes in expression for which qPCR revealed greater than a 125 fold increase in RGS9 mRNA copy number once mESC began to exhibit characteristics of NPCs. RT-PCR results using primers specific for the two RGS9 variants implicated RGS9-2 as the major isoform expressed during neuronal differentiation of mESCs. Furthermore, RGS9 and R7BP mRNA were also detected in NSCs derived from embryonic mouse cortex.

Although it is difficult to specifically define *in vivo* cell counterparts to the cell types derived during neuronal differentiation of mESCs, they differentiate in a manner that shares

characteristics with neurogenesis in the cerebral-cortex [13]. In addition, the transcription factor PAX-6 is a marker for radial glial cells and has been used to distinguish NPCs *in vivo* which give rise to specific neuron subtypes in the cortex. RA induced mESCs have previously been shown to differentiate into neurons through a PAX-6 expressing NPC [14-16]. We also observed PAX-6 expression during neuronal differentiation of mESCs and detected PAX-6 mRNA in cortical NSCs. Thus two distinct populations of NPCs with related properties express RGS9 mRNA and the R7 anchor protein, R7BP.

However, there were clear distinctions in mRNA expression during neuronal differentiation of mESCs and cortical NSCs. For example, we did not detect expression of multiple RGS proteins in cortical NSCs despite overlapping expression patterns with RGS9 in differentiating mESCs. Although mESC cultures in this study gave rise to homogenous neuron cultures defined by near uniform MAP-2 expression, the cultures at previous time points are likely heterogeneous. For example, the multi-potent stem cell marker OCT-4 steadily decreased, but was detected throughout most of the differentiation time points suggesting the continued presence of primordial cell types even towards the end of the differentiation protocol. These cell types however, do not persist under serum free conditions [12]. In addition, others have demonstrated that neuronal differentiation of mESCs recapitulates the temporal pattern of cortical development where differentiation of NPCs and neurons that have characteristics of deep cortical layers develop earlier than higher layers [17, 18]. Thus the expression of specific RGS proteins may be associated with distinct subtypes of NPCs or other more primordial cell types. RGS7 and RGS11 mRNA expression for example, demonstrated significant up regulation during neuronal differentiation of mESCs but their expression peaked earlier than RGS9 and was not detected in cortical NSCs.

In addition to RGS9 mRNA up regulation during neuronal differentiation, the mRNA for the anchor proteins R7BP and R9AP were similarly regulated (fig. 2). R7BP and R9AP regulate R7 family RGS protein stability, cellular localization, and activity [19, 20]. The coincident up regulation of R7 RGS proteins and their anchor proteins underscores a potential role for R7 RGS proteins in NPCs.

In general, previous studies of RGS9, RGS11, R7BP and R9AP protein have demonstrated expression patterns that increase during postnatal development of the CNS [19, 21-24]. For RGS9, RGS11 and R9AP, postnatal expression becomes increasingly specific to discrete CNS regions [19, 21, 22, 24, 25]. For example, RGS9 protein has previously been detected in embryonic mouse brain, but with more significant postnatal protein levels detected for RGS9-1, and RGS9-2 predominately in the retina and striatum, respectively [21].

Although *in vivo* studies of RGS9, RGS11, and R9AP, have not described expression that would be consistent with expression in NPCs, both models used in this study produced an enrichment of NPCs in culture. In contrast, related cell types *in vivo* represent only a small fraction of CNS tissue and in adult animals are largely restricted to discrete regions [16, 26]. For example, primary culture of NSCs is dependent on expansion of EGF and/or FGF responsive cells from brain and Louis et al, 2008 estimated less than 0.2% of cells harvested from embryonic mouse brain generate self renewing neurosphere's in culture [27]. Thus previous studies may not have recognized R7 RGS gene expression in NPCs due to limitations in detection.

Importantly, progenitor cells largely determine the regenerative capacity of specific tissues. An increase in proliferation and mobilization of neuronal stems cells for example, is a common response to a variety of brain insults [28, 29]. Tissue specific stems cells hold promise for treating degenerative diseases but a key to their study and use are additional

markers that clearly define specific progenitor cell types. With the largely restricted expression pattern of RGS9 in the adult brain, our data suggests that RGS9-2 may be a useful marker to aid in identifying distinct NPCs.

In addition, RGS6 has previously been shown to modulate cytoskeletal reorganization important in neuronal differentiation by interacting with SCG10 [7]. Although we did not investigate the expression of stathmin genes, our data suggests a mechanism by which SCG10 or other stathmin family members' activity could be altered during neuron development by the changes in expression of R7 RGS proteins like RGS9. Additional study of the function of R7 RGS proteins in NPCs may provide important new insights into understanding NPC function, neurogenesis and the expanding role of RGS proteins in regulating cell signaling processes. In addition R7 RGS proteins and their accessory proteins may provide novel markers for distinguishing between NPC subtypes.

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Abbreviations used

GAP	GTPase accelerating protein
GGL	G γ -like
GPCR	G protein coupled receptor
PBS	phosphate-buffered saline
RGS	regulator of G protein signaling
mESC	mouse embryonic stem cell
NPC	neuronal precursor cells
NSC	neuronal stem cell
FGF	fibroblast growth factor
EGF	epidermal growth factor
LIF	Leukemia inhibitory factor
RT-PCR	reverse transcriptase polymerase chain reaction

Research Highlights

- The mRNA for the R7 Regulator of G protein family members demonstrate distinct expression patterns during neuronal differentiation of mouse embryonic stem cells
- RGS9 mRNA is up regulated 125 fold in cells with characteristics of neuronal precursor cell types derived from mouse embryonic stem cells
- RGS9 and R7BP mRNA are also expressed in neural stem cells isolated from embryonic mouse cortex

Fig. 1. Neuronal differentiation of mESCs

A. Representative images of live mESCs at various stages of differentiation, including undifferentiated and acutely dissociated mESCs (**a**), mESCs grown without LIF for 3 days (**b**), mESCs grown without LIF for 4 days, and treated with RA for 3 days (**c**), and mES cell derived neurons plated in NBA media for 3 days on poly-D lysine coated vessels (**d**). **B.** Immunocytochemistry for the neuronal marker, MAP-2 in mESC-derived neurons. Four days following plating on poly-D lysine coated cover slips mESC-derived neurons were fixed and permeabilized. Rabbit anti-MAP2 staining was visualized using an Alexa-488 labeled secondary antibody and nuclei were stained with, DAPI. Fluorescence was imaged using a Nikon microscope equipped with epi-fluorescence filters (Nikon USA, Melville, NY) and captured with either a 20 $\times$ objective (**a,b, & c**) or 100 $\times$ objective (**d,e,&f**). Scale bars in **A** and **B** represent 100 μ m and 20 μ m, respectively.

Fig. 2. Gene expression during neuronal differentiated of mESCs

A. Representative images of ethidium bromide stained RT-PCR products resolved by agarose gel electrophoresis. Total RNA was isolated and reverse transcribed from mESCs harvested at 6 time points during neuronal differentiation: **1**, undifferentiated; **2**, two days without LIF; **3**, four days without LIF; **4**, two days with RA; **5**, four days with RA; and **6**, two days following plating on poly-D coated vessels. Target genes were amplified using the gene specific primers indicated and are listed in table 1. Images represent results obtained from at least three independent cultures. **B.** qPCR measurements of the relative RGS9 mRNA copy number from total RNA isolated from undifferentiated mESCs and mESCs in which neuronal differentiation was initiated by LIF withdrawal for 2 and 4 days. Bars

represent average fold increase in copy number compared to undifferentiated cells performed in duplicate from 4 independent populations of mESCs. * indicates $p < 0.05$ compared to cells withdrawn from LIF for two days, error bars represent standard deviation. **Inset**, representative melt curve for the RGS9 RT PCR product. **C.** As in **A** except, total RNA was isolated and reverse transcribed from mouse eye, brain and stage 3 of neuronal differentiation of mESCs (4 days – LIF). Target genes were amplified using the gene specific primers indicated.

Fig. 3. RGS9 mRNA is expressed in mouse cortical NSCs

Representative images of ethidium bromide stained RT-PCR products resolved by agarose gel electrophoresis and visualized using UV/ethidium bromide. Total RNA was isolated from cortical NSCs, reverse transcribed and the target genes indicated were amplified using standard procedures with Taq DNA polymerase (+RT). No PCR products were detected using identical template samples prepared without reverse transcriptase (-RT). Identical results were obtained from two separate NSC cultures.

Table 1
Gene specific primers

5' and 3' primers used to amplify target genes indicated are listed along with the predicted product size.

Gene target	5' primer	3' primer	product length (bp)
RGS2	AAGGATTGGAAGACCCGTTT	GAGGACAGTTTTTGGGTGA	275
RGS6	TCTCTCCAAAATCCCCAGTG	TTGCTTTGTTCTGCATCGTC	278
RGS7	GTGTTCTCCGGTCAGACAT	CTAGCCTTGCCTTGTTTTGC	265
RGS9	TTTCAACTTCAGCGAACTGATTCG	TGATGTCCACGGTGTTGGCTGCTTC	503
RGS9*	GAGGATGGCATTCTCTCCAAA	TGGGGTCTTGCAAAGGATAG	233
RGS9-1	CAACATAGACGGCAAAACCA	GCAGCTCCTTTTGAGTTGG	356
RGS9-2	CAACATAGACGGCAAAACCA	AGCGGCAGGATGGTGAGAAG	407
RGS11	CGGCTACATCTACCCACTGC	CTCGAGCCTGCATCAATACC	251
R7BP	GACTGCAAGATGCTTGTTCCA	ACTGAAGAGACCCCAGCAGA	298
R9AP	AACAAGACCACAGCGTGCTA	CGCAGCTACAGCAGAGGAG	339
MAP2	CAGGTCACAGGGCACCTATT	TGTGGCTGTTTGTTCTGAGG	262
Nestin-1	AGTCAGAGCAAGTGAATGG	AGAAACAAGATCTCAGCAGG	600
PAX-6	TCTAATCGAAGGGCCAAATG	GGTTGCATAGGCAGGTTGTT	260
OCT-4	CACGAGTGGAAGCAACTCA	AGATGGTGGTCTGGCTGAAC	246
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	452

* Indicates RGS9 primer set used in qPCR.