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RGS2: Regulation of Expression and Nuclear Localization

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RGS2, a Regulators of G-protein Signaling family member, regulates signaling activities of G-proteins, and RGS2 itself is controlled in part by regulation of its expression. This investigation extended previous studies of the regulation of RGS2 expression by examining the effects of stress, differentiation, and signaling activities on RGS2 mRNA level in human neuroblastoma SH-SY5Y cells. Cell stress induced by heat shock rapidly and transiently increased RGS2 mRNA levels, whereas differentiation to a neuronal phenotype reduced basal RGS2 mRNA levels by 50%. RGS2 mRNA levels were increased in differentiated cells by heat shock, carbachol, and activation of protein kinase C. After transient transfection of GFP-tagged RGS2, a predominant nuclear localization was observed by confocal microscopy. Thus, RGS2 expression is regulated by stress and differentiation, as well as by second messenger signaling, and transfected GFP-RGS2 is predominantly nuclear. © 2001 Academic Press

Key Words: **RGS2; heat shock; G-protein; muscarinic receptors.**

RGS2 is a member of a family of Regulators of G-protein Signaling (RGS) proteins that regulate signaling cascades initiated by activation of G-protein-coupled receptors (1–3). RGS proteins facilitate the intrinsic inactivating GTPase reaction of G-protein α-subunits, thereby limiting the actions of activated α-subunits. Regulated expression appears to be an important mechanism for controlling the actions of RGS2. The expression of RGS2 is regulated by neuronal activity (4), by certain psychotropic agents (4–6), and by receptor-coupled second messenger systems (7–10). Stimulation of muscarinic receptors coupled to the phosphoinositide signal transduction system rapidly and transiently induced robust increases in RGS2 mRNA levels in human neuroblastoma SH-SY5Y cells (9). Activation of protein kinase C accounted for a significant portion of the muscarinic receptor-stimulated RGS2 expression (9). However, cell type-selectivity in the mechanisms regulating RGS2 expression are evident, since phorbol ester-induced activation of protein kinase C did not increase RGS2 mRNA levels in blood mononuclear cells (11). RGS2 mRNA levels also have been reported to be increased by concanavalin A in blood mononuclear cells (11, 12) and by elevation of cyclic AMP in PC12 cells, human embryonic kidney cells, and T cells (7, 8, 13), but not in human neuroblastoma SH-SY5Y cells (9). The rapid and robust changes induced by a variety of agents associated with several intracellular signaling cascades suggests that modulation of RGS2 expression may be an important mechanism by which cells regulate RGS2 function.

The present study extends previous investigations of the regulation of RGS2 expression by signaling systems. These experiments used human neuroblastoma SH-SY5Y cells, a widely used neuronal model system, to examine the effects of cell stress (induced by heat shock) and of differentiation of the levels of RGS2 mRNA, to examine the regulation by several stimuli of RGS2 mRNA in differentiated cells, and to identify the intracellular distribution of green fluorescent protein (GFP)-tagged RGS2 after transient transfection. The results show that RGS2 mRNA levels are increased by heat shock, decreased by differentiation, and increased in differentiated cells treated with carbachol to activate endogenously expressed M3 muscarinic receptors, by heat shock, and by activation of protein kinase C. Furthermore, a predominant nuclear localization of GFP-tagged RGS2 was observed by confocal microscopy.

MATERIALS AND METHODS

Cell culture. Human neuroblastoma SH-SY5Y cells were grown in RPMI medium (Cellgro, Herndon, VA) supplemented with 10% horse serum (Life Technologies, Gaithersburg, MD), 5% fetal clone II

Abbreviations used: GFP, green fluorescent protein; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; RGS, regulators of G-protein signaling; TBS, Tris-buffered saline.

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(Hyclone, Logan, UT), 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. To induce differentiation, SH-SY5Y cells were grown in Neurobasal medium supplemented with B-27 (Life Technologies), as described previously (14). Cells were maintained in humidified, 37°C chambers with 5% CO_2. Experimental agents used include carbachol, atropine, and phorbol 12-myristate 13-acetate (PMA) from Sigma (St. Louis, MO). For heat shock treatments, dishes of cells were incubated at 45°C for 30 min and then returned to a 37°C chamber for the indicated periods of time. Cyclic AMP levels were measured using a kit according to the supplier's instructions (Amersham, Arlington Heights, IL).

Northern blots. RGS2 cDNA was generously provided by Dr. D. R. Forsdyke (Queen's University, Kingston, Ontario, Canada). Total RNA was extracted using Trizol reagent (Life Technologies) according to the manufacturer's instructions. RNA (10 μg) was separated by electrophoresis in 1.2% agarose gels containing formaldehyde and transferred to nitrocellulose membranes. cDNA was random prime-labeled with [^{32}P]dCTP (Amersham Pharmacia Biotech). Blots were hybridized with labeled probes at 42°C for 18 h and then washed in two changes of 2× saline-sodium citrate and 0.1% SDS at 20°C for 20 min and once in 1× saline-sodium citrate and 0.1% SDS at 55°C for 10 min. Results were obtained using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA), and bands were quantitated using ImageQuant.

Imaging of GFP-RGS2. Full-length RGS2 cDNA was amplified by PCR from pBR323/RGS2 DNA (primers: 5′-AGAGTCGACATGCAAAGTGCTATGTTCTT-3′ and 5′-GGTGGATCCCTCGAGATGTAGCATGAGG-3′), digested with *Sal*I and *Sma*I, and inserted into the *Sal*I/*Sma*I sites of the pEGFP vector (Clontech, Palo Alto, CA) to make pEGFP/RGS2. All plasmids were verified by DNA sequencing. Cells were grown on poly-D-lysine/laminin-coated coverslips and transiently transfected with pEGFP-RGS2 or pEGFP using Fugene 6 (Roche) according to the manufacturer's protocol. After 24 h, cells were washed with PBS, and fixed with 2% paraformaldehyde, 0.2% glutaraldehyde in PBS for 30 min at room temperature. To identify nuclei, cells were incubated with 1 μg/ml DAPI for 10 min at room temperature. Cells were examined with a Nikon Diaphot 200 Epifluorescence microscope and an Olympus confocal scanning microscope.

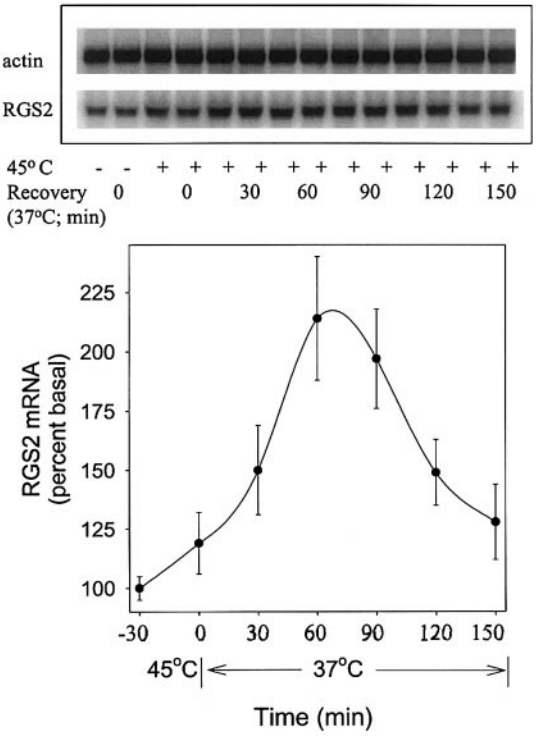

FIG. 1. Heat shock treatment increases RGS2 mRNA levels in SH-SY5Y cells. SH-SY5Y cells were incubated at 45°C for 30 min, followed by a recovery incubation at 37°C for a further 30 to 150 min. RGS2 and actin mRNA levels were measured in cells that were harvested at 30 min intervals. The top panel shows Northern blots with two independent samples for each treatment, and the lower panel shows quantitative values obtained in four independent experiments expressed as the percent of basal RGS2 mRNA levels in untreated cells. Means ± SEM.

RESULTS

RGS2 mRNA levels were measured in human neuroblastoma SH-SY5Y cells subjected to heat shock at 45°C for 30 min, followed by a recovery incubation at 37°C for 30 to 150 min (Fig. 1). RGS2 mRNA levels were increased by this treatment protocol, with a small increase in occurring during the 30 min incubation at 45°C, and a large and transient rise in RGS2 mRNA levels evident during the subsequent recovery incubation at 37°C. Thus, cell stress induced by heat shock increases RGS2 mRNA levels.

To examine RGS2 in differentiated cells, SH-SY5Y cells were grown in Neurobasal media which rapidly terminates cell division and induces a neuronal phenotype within a day of treatment, and causes progressive increases in neurite outgrowth during successive days (14). Induction of differentiation left the basal RGS2 mRNA levels unchanged initially, but this was followed by successive decreases during the subsequent days of differentiation (Fig. 2), leading to a level of RGS2 mRNA after 4 and 5 days of differentiation that was approximately 50% of the level in proliferating SH-SY5Y cells. Thus, the expression RGS2 is subject to regulation by neuronal differentiation.

To assess the regulation of RGS2 mRNA levels in differentiated cells, SH-SY5Y cells that had been differentiated for 4 days were treated with several test agents. The results of these experiments (Fig. 2) revealed that carbachol, heat shock, and PMA caused substantial increases in RGS2 mRNA levels, while forskolin was without effect (although forskolin treatment raised cyclic AMP levels from a basal level of 47 ± 3 to 355 ± 61 nmol/μg protein; $n = 4$). These effects of each agent on RGS2 mRNA levels were qualitatively the same in differentiated SH-SY5Y cells as reported in proliferating SH-SY5Y cells previously (9); however, the magnitudes of the increases in RGS2 mRNA levels caused by effective stimuli were substantially greater in differentiated than undifferentiated SH-SY5Y cells. Thus, increases caused by carbachol, heat shock plus recovery, and PMA in differentiated SH-SY5Y cells (approximately 19-, 6-, and 8-fold, respectively) were greater than in undifferentiated SH-SY5Y cells (approximately 4-, 2-, and 4-fold, respectively) (9).

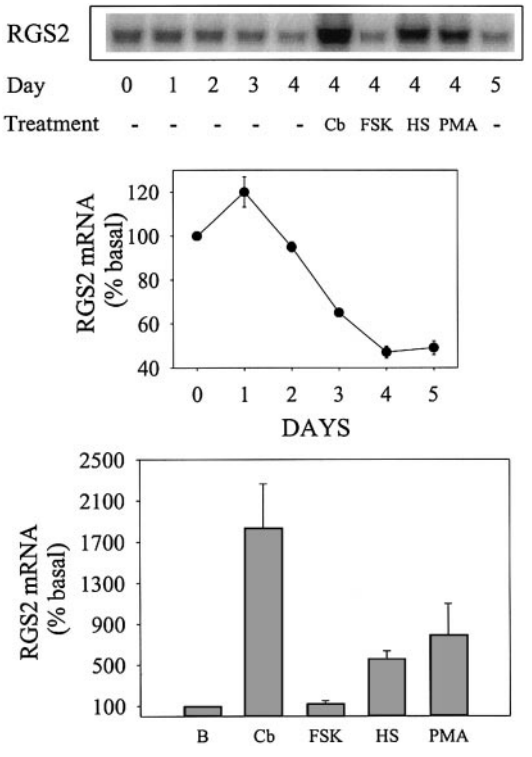

FIG. 2. Basal and stimulated RGS2 mRNA levels in differentiated SH-SY5Y cells. SH-SY5Y cells were grown in differentiating Neurobasal media for 1 to 5 days and RGS2 mRNA levels were measured in cells harvested each day (means ± SEM; $n = 3$). Additionally, cells grown in Neurobasal media for 4 days were treated with carbachol (Cb; 1 mM; 60 min), forskolin (FSK; 10 μM; 60 min), heat shock (HS; 30 min at 45°C followed by 90 min at 37°C), or PMA (0.2 μM; 90 min), and RGS2 mRNA levels were measured. Means ± SEM ($n = 3$).

Due to the lack of specific antibodies that recognize endogenous RGS2 in western blots, we used transiently transfected GFP-tagged RGS2 to assess the intracellular localization in SH-SY5Y cells. Figure 3 shows epifluorescent images of GFP-RGS2 which uniformly demonstrated nuclear accumulation of GFP-RGS2, whereas relatively less appeared to be free in the cytosol. Identical results were obtained with both N-terminal and C-terminal GFP-tagged RGS2 (data not shown), whereas expressed GFP alone was distributed throughout the cells. Confocal microscopy confirmed that GFP-RGS2 was located within nuclei in SH-SY5Y cells.

DISCUSSION

RGS2 is a member of the Regulators of G-protein Signaling family of proteins which act with some selectivity on certain subtypes of G-protein α-subunits to attenuate their activities (1–3). Considering the abundance and importance of G-protein coupled receptors in mammalian cells, regulatory influences on the expression of RGS proteins are likely to have a significant impact on cell function. This investigation found that RGS2 mRNA levels were increased by cell stress induced by heat shock, and that differentiation of neuroblastoma cells led to a 50% reduction in RGS2 mRNA levels. Furthermore, stimulation of differentiated SH-SY5Y cells with carbachol, heat shock, and PMA increased RGS2 mRNA levels. The exquisite control of the expression of RGS2, the rapidity and magnitude of the changes, and the multiple stimuli that are modulatory suggest that the action of RGS2, and thus its control by regulated expression, serves a vital role in cell function. This may derive from the ubiquitous use by cells of G-protein-coupled receptors to mediate responses to extracellular stimuli and the necessity to control these responses through the actions of RGS proteins, as well as other functions of RGS proteins yet to be discovered. That other functions of RGS2 await discovery is suggested by the finding that transfected GFP-RGS2 was found to be predominantly localized in the nucleus.

This investigation revealed for the first time that expression of an RGS protein is subject to modulation by cellular stress. Specifically, treatment of cells with heat shock increased RGS2 mRNA levels. Cellular stress is well known to increase the expression of classical immediate early genes such as *c-fos* (15), thus the finding that these stressors also cause a rapid induction of RGS2 expression adds further to the previously noted similarities between RGS2 and immediate early genes. Siderovski *et al.* (16) first noted similarities in the regulated expression of RGS2 and immediate early genes such as *c-fos*. Ingi *et al.* (4) further substantiated the similarity by their finding that in brain, stimulation such as with electroshock caused rapid and robust increases in RGS2 mRNA levels, a response well known to occur with classical immediate early genes. Many immediate early genes were first classified as such when it was found that stimulators of protein kinase C caused rapid increases in expression (15). We have noted that RGS2 also is controlled by this classical mechanism, as activation of protein kinase C directly with PMA, or indirectly through muscarinic receptors, caused a rapid increase in RGS2 mRNA levels (9). Finally, Fos and Jun as well as many other immediate early gene protein products exert their functional effects in the nucleus. As discussed further below, GFP-RGS2 also was predominantly in the nucleus. Overall, these findings demonstrate a growing number of similarities between RGS2 and classical immediate early genes.

There have been a limited number of previous reports addressing the intracellular localization of members of the RGS family of proteins. Most of these have used subcellular fractionation methods and have found RGS proteins associated with the membrane fraction or distributed between the membrane and cytosolic

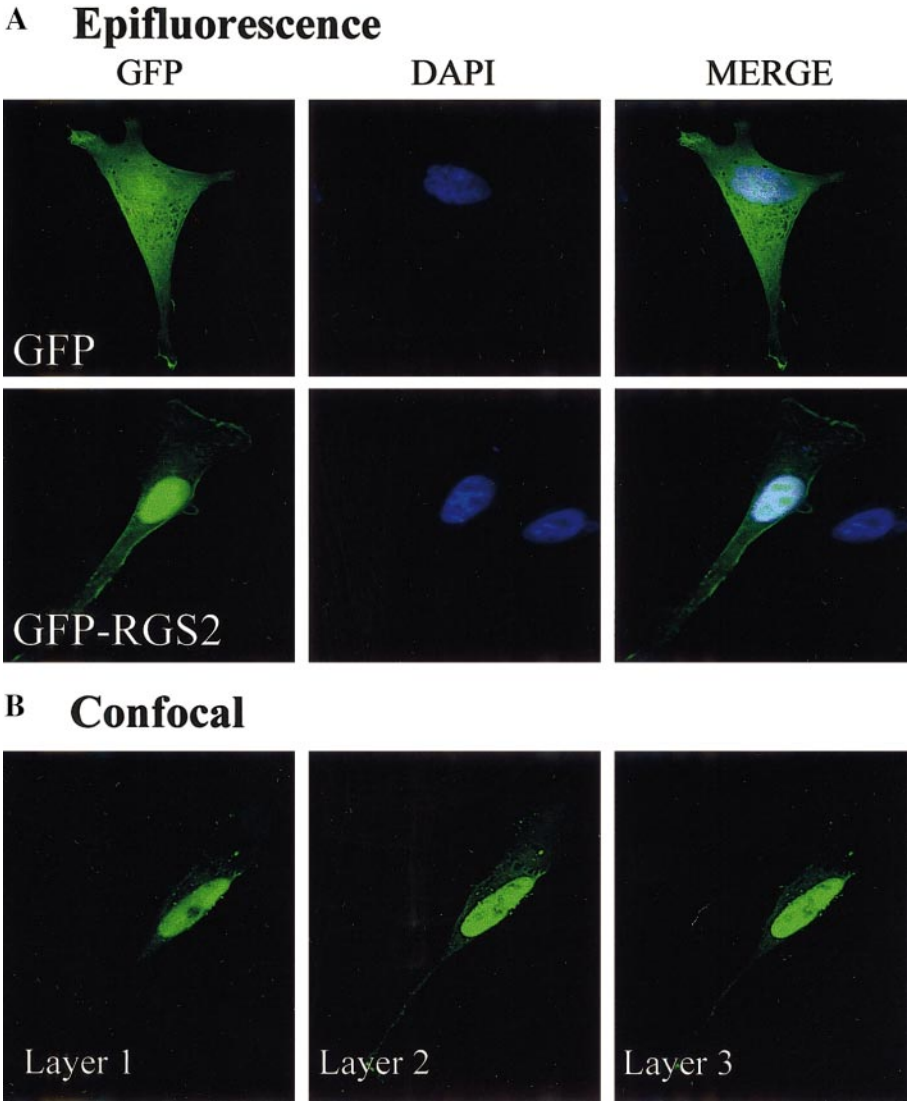

FIG. 3. Nuclear localization of GFP-RGS2. SH-SY5Y cells were transiently transfected 24 h prior to imaging. (A) Epifluorescent images of cells expressing GFP or GFP-RGS2 are shown. The figures on the left show green fluorescence images, the figures in the middle show blue DAPI-stained nuclei, and the figures on the right show merged images. (B) Confocal images after transient transfection with pEGFP/RGS2. The images show layers at 0.8-μm intervals. Epifluorescence and confocal images show the predominant nuclear localization of GFP-RGS2.

fractions (reviewed in 3). Because of the low basal level of expression of RGS2, little is known about the localization of endogenous RGS2. However, many previous investigations have utilized GFP-tagged proteins to assess intracellular distribution, and using this strategy Bowman *et al.* (17) reported that the majority of GFP-RGS2 expressed in lymphoid cells was in the nucleus. This surprising finding was extended in recent reports. Transfection of CHO cells with a myc-tagged truncated variant of RGS3, RGS3T, revealed its predominant nuclear localization (18). GFP-tagged RGS2 and RGS10, but not RGS4 and RGS16, were predominantly located in the nucleus in COS-7 cells (19). Certain splice variants of RGS12 also showed predominantly nuclear localization (20). The present report further supports the conclusion that RGS2 is predominantly, although not solely, a nuclear protein. A consistent predominance of RGS2 in the nucleus was observed following expression of GFP-RGS2 in SH-SY5Y cells and examination with confocal microscopy confirmed that the GFP-RGS2 was within the nucleus. As noted previously (18–20), the nuclear localization of RGS proteins raises the possibility that they contribute more to cellular functions than solely as regulators of receptor-coupled heterotrimeric G-protein GTPase activities.

In summary, RGS2 mRNA levels were found to be subject to rapid regulation by stress induced by heat shock, by cellular differentiation, and by treatment of differentiated cells with carbachol, PMA, and heat

shock. The functional impacts of these effects remain to be established, but in addition to regulating the activities of G-proteins there may be other, as yet undiscovered, actions of RGS2 which are associated with its nuclear localization. Taken in conjunction with the unique mechanisms regulating RGS2 (e.g., 4), it appears possible that RGS2 has specialized functions underlying its sensitivity to controlled expression by a wide variety of stimuli, and its localization in the nucleus.

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