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Cellular stress increases RGS2 mRNA and decreases RGS4 mRNA levels in SH-SY5Y cells

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Abstract

Modulation of the expression of regulator of G-protein signaling (RGS) proteins is a major mechanism used to modulate their actions. Besides control by second messengers, the expression of RGS proteins, particularly RGS2, can be regulated by cell stress. Because RGS2 and RGS4 expression can be regulated by the cell cycle, we examined if cell cycle signals are involved in their regulation following stress. Treatment of SH-SY5Y cells with camptothecin increased RGS2 mRNA and decreased RGS4 mRNA levels. This effect on RGS2 mRNA was blocked by the cyclin-dependent kinase-2 (cdk2) inhibitors roscovitine and purvalanol. Cell cycle arrest was further implicated in regulating RGS mRNA levels because geldanamycin, which causes cell cycle arrest by inhibiting the actions of heat shock protein 90, caused changes in the mRNA levels of RGS2 and RGS4 similar to, and additive with, the effects of camptothecin. Overall, these results indicate that cell cycle arrest regulates the expression of RGS2 and RGS4, and that the expression of these two RGS family members is oppositely regulated by stress that causes cell cycle arrest.

Keywords

RGS2; RGS4; Camptothecin; Roscovitine; cdk2; Geldanamycin; Cell cycle arrest

Regulator of G-protein signaling (RGS) proteins attenuate the signaling activities of many G-protein-coupled receptors through their action as GTPase activating enzymes to deactivate G-proteins [10]. RGS proteins themselves are regulated in a number of ways, one of the best characterized being by control of their transcription. Thus, many RGS proteins, such as RGS2 and RGS4, are expressed at a low level but this can be increased by activation of G-protein-coupled receptors, presumably to provide a feedback mechanism to attenuate receptor-mediated signaling [5,14].

In addition to their classical association with plasma membrane receptor-coupled signal transduction systems, emerging evidence suggests that RGS proteins have additional actions and are regulated by additional cellular stimuli. In this respect, RGS2 is especially interesting because it has often been found to be located in the nucleus rather than at the plasma membrane associated with G-protein-coupled receptors [3]. Additionally, the expression of RGS2 has been shown to be regulated by several cell stressors [15,21,22], by the cell cycle [22], and by the differentiation state of cells [15]. Since some cell stressors can cause cell cycle arrest, and RGS2 expression changes during the cell cycle in unstressed cells [22], these characteristics raised the possibility that cyclin-dependent kinases associated with the cell cycle may have a regulatory influence on the expression of RGS2. We examined this by using the topoisomerase 1 inhibitor camptothecin to stress cells, which activates the tumor suppressor p53 and increases

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RGS2 mRNA levels in human neuroblastoma SH-SY5Y cells [22], and roscovitine, an inhibitor of cyclin-dependent kinases (cdk). Roscovitine is a purine analog which competitively binds at the ATP binding site [11,16] and at concentrations up to approximately 10 μ M roscovitine is a specific inhibitor of cdk2, while at higher concentrations it can inhibit other kinases [1]. Furthermore, we compared changes in RGS2 mRNA to those of RGS4 mRNA because we recently reported that the expression level of RGS4 is regulated by cell stress in an opposite direction from that of RGS2 [22].

Human neuroblastoma SH-SY5Y cells were grown in RPMI medium (Cellgro, Herndon, VA) supplemented with 10% horse serum (Invitrogen, Carlsbad, CA), 5% fetal clone II (Hyclone, Logan, UT), 2 mM L-glutamine, 100 units/ml penicillin and 100 μ g/ml streptomycin, and were maintained in humidified, 37 °C chambers with 5% CO₂ prior to incubation in serumfree media overnight before treatments. Experimental agents used included camptothecin, roscovitine, purvalanol, LiCl, kenpaullone (Sigma, St. Louis, MO), and indirubin-3'-monoxime (Alexis Biochemicals, San Diego, CA).

RGS2 cDNA was generously provided by Dr. D.R. Forsdyke (Queen's University, Kingston, Ont., Canada) and RGS4 cDNA was obtained from the Guthrie cDNA Resource Center (Guthrie, Sayre, PA). The methods for measuring mRNA levels using Northern blots were as described previously [14]. Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA was separated by electrophoresis in 1.2% agarose gels containing formaldehyde and transferred to nitrocellulose membranes. cDNA was random prime-labeled with [³²P]dCTP (Amersham Pharmacia Biotech). Blots were hybridized with labeled probes at 42 °C for 18 h and then washed in two changes of 2 $\times$ 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.

DNA damage was induced by treatment with the topoisomerase 1 inhibitor camptothecin, which we previously reported causes a concentration-dependent increase in the levels of p53 and p21, indicative of DNA damage, with a maximal effect produced by 1 μ M camptothecin in SH-SY5Y cells [17]. Treatment with camptothecin causes a large, rapid increase in RGS2 mRNA levels and reduction in RGS4 mRNA levels [22]. Since regulation of cdk2 is a key component of the DNA damage response [2], we tested if the cdk2 inhibitor roscovitine affected these responses to DNA damage. Fig. 1A shows the concentration-dependent inhibitory effect of roscovitine in SH-SY5Y cells treated with camptothecin to induce the expression of RGS2. As previously reported [22], treatment with camptothecin (1 μ M; 2 h) caused a large (3-fold) increase in the level of RGS2 mRNA. Pretreatment with roscovitine (1 h) caused a concentration-dependent inhibition of camptothecin-induced increases in RGS2 mRNA, with an IC₅₀ near 1 μ M. This potency indicates that the effect of roscovitine is highly likely due to inhibition of cdk2 [1]. A high concentration (10 μ M) of roscovitine completely eliminated RGS2 mRNA expression, but this strong effect may be due to the inhibition of additional proteins besides cdk2. Furthermore, the inhibitory effect of 1 μ M roscovitine on camptothecin-induced RGS2 mRNA levels was relatively fast and long-lasting, as it was evident at the shortest time tested after camptothecin treatment (1 h) and throughout a 4 h time course of camptothecin treatment (Fig. 1B).

We noted previously that the expression of RGS4 is regulated oppositely to that of RGS2 following cell stress [22]. In agreement with this observation, camptothecin treatment decreased RGS4 mRNA levels, as opposed to the increase in RGS2 mRNA levels (Fig. 1A and B). However, treatment with roscovitine initially had little effect on the camptothecin-induced reduction in RGS4 mRNA levels (Fig. 1A), although reductions were observed with longer treatment times of 3 and 4 h (Fig. 1B). Neither camptothecin nor roscovitine altered

actin mRNA levels, indicating that the inhibitory influence of roscovitine on RGS2 mRNA is a selective effect.

To test if the inhibitory effect of roscovitine on stimulated RGS2 expression could be due to a toxic effect of the drug, we examined its effects on carbachol-induced increases in RGS2 mRNA. As reported previously [14], by activating endogenous muscarinic receptors in SH-SY5Y cells carbachol stimulated a large increase in RGS2 mRNA levels (Fig. 1C). In contrast to its effect with camptothecin, roscovitine pretreatment did not inhibit the carbachol-stimulated increase in RGS2 mRNA levels except at the highest concentration tested (10 μ M). This indicates that roscovitine inhibited RGS2 expression in a context-specific manner, being effective following cell stress but not following second messenger-induced expression of RGS2. Neither carbachol nor roscovitine (except at the highest concentration tested) altered RGS4 mRNA levels (Fig. 1C).

To confirm that the inhibition by roscovitine of camptothecin-induced increases in RGS2 mRNA levels was due to inhibition of cdk2, experiments were carried out with purvalanol, another selective inhibitor of cdk2 [8]. Pretreatment with purvalanol also concentration-dependently inhibited the camptothecin-induced increase in the mRNA level of RGS2 (Fig. 2A). The higher concentrations of purvalanol also decreased the expression of RGS4. The inhibitory effect of purvalanol on camptothecin-induced increases in RGS2 mRNA levels also was rapid and long-lasting (Fig. 2B). We further confirmed the dependence of RGS2 mRNA increases on cdk2 by using other less specific inhibitors, including 10 μ M indirubin-3'-monoxime and 10 μ M kenpaullone, both of which inhibit cdk enzymes but also inhibit glycogen synthase kinase-3 [6]. Both kenpaullone and indirubin-3'-monoxime blocked camptothecin-induced increases in RGS2 expression, whereas the glycogen synthase kinase-3 inhibitor lithium did not, supporting the conclusion that this effect was due to cdk inhibition, not inhibition of glycogen synthase kinase-3. Taken together, it is clear that the camptothecin-induced increase in the expression of RGS2 is blocked by inhibition of cdk2.

Heat shock protein-90 (Hsp90) has been reported to modulate the actions of many proteins, including p53 which is activated following camptothecin treatment in SH-SY5Y cells and most other types of cells [12,17] to induce arrest of the cell cycle to allow repair of DNA damage, and/or to activate cell death by apoptosis [9]. Geldanamycin is a selective inhibitor of Hsp90, a chaperone for many cellular proteins, including p53 [13,18] and induces cell cycle arrest [4]. Therefore, we tested if geldanamycin mimicked the effects of camptothecin on the mRNA levels of RGS2 or RGS4. With both RGS2 and RGS4 expression, geldanamycin increased the camptothecin-induced changes although the direction of the changes caused by camptothecin were opposite for the two RGS proteins (Fig. 3A). Thus, there was a greater increase in RGS2 mRNA with both 1 μ M camptothecin and 10 μ M geldanamycin together than with either agent alone, and there was a greater decrease in RGS4 mRNA with both camptothecin and geldanamycin together than with either drug alone. With both RGS2 and RGS4 the effects of camptothecin and geldanamycin appeared to be approximately additive, indicative that geldanamycin alone had a regulatory influence. To examine this, cells were incubated with 10 μ M geldanamycin for 0.5-3.5 h. Treatment with 10 μ M geldanamycin caused time-dependent increases in RGS2 mRNA levels and decreases in RGS4 mRNA levels (Fig. 3B and C). Thus, following treatment with camptothecin or geldanamycin, or with both agents together, the RGS2 and RGS4 mRNA levels were oppositely modulated.

RGS proteins are most renowned for their regulation of G-protein-coupled receptor signaling at the plasma membrane. Not only do RGS proteins regulate signaling activities of these many receptors, but also second messengers emanating from these activated receptors in turn regulate RGS proteins, especially by regulating their expression [5,10]. However, mysteries remain about the actions of RGS proteins and regulatory mechanisms influencing RGS proteins. For

example, many RGS proteins are located in the nucleus where their actions remain to be more fully clarified [3]. Additionally, we have been particularly interested in the robust regulatory influences of cell stress on RGS2 expression, which is increased by heat shock [15,21], oxidative stress [21], microtubule-disrupting agents [22], and DNA damage [22]. As well as stress, differentiation and the cell cycle regulate RGS2 mRNA levels [15,22]. Therefore, we sought to identify mechanisms that could contribute to this regulation. In the present study we present evidence that stress-induced cell cycle arrest, and the regulatory kinase cdk2, are important regulators of RGS2 and RGS4 expression, and furthermore, we extended previous indications that the mRNA levels of RGS2 and RGS4 are oppositely regulated in a variety of conditions.

Treatment of cells with camptothecin, a topoisomerase 1 inhibitor, causes DNA damage, accumulation of p53, and cell cycle arrest which can lead to apoptosis [17]. Following exposure of SH-SY5Y cells to camptothecin, there were also large, rapid changes in the mRNA levels of RGS2 and RGS4. Notably, however, these were regulated in opposite directions as the mRNA level of RGS2 increased while that of RGS4 decreased. The same opposite effects on RGS2 and RGS4 mRNA levels were observed following treatment with geldanamycin which caused increased RGS2 and decreased RGS4 mRNA levels. We [22] and others [7] have noted previously that the expression of RGS4 can be regulated in an opposite direction to that of RGS2. A functional reason for these opposite regulations of the mRNA levels of RGS2 and RGS4 awaits further understanding of their actions, especially concerning the cell cycle. At present, although the expression of RGS2 especially has been shown to be regulated by many conditions that affect the cell cycle, as well as the cell cycle itself in unstressed cells, the functional outcome of this link remains unknown. Previously [22], we have speculated that the link may be via G-proteins that are nuclear and may contribute to the cell cycle [19], but this remains to be explored further.

In addition to finding that camptothecin treatment oppositely regulated RGS2 and RGS4 mRNA levels, we also discovered that cdk2 inhibition abrogated the effect of camptothecin on RGS2, but not RGS4, mRNA levels. This is the first kinase shown to modulate the stress-induced increase in RGS2 mRNA. It is perhaps notable that recently DNA damage was reported to increase cdk2 in SH-SY5Y cells [20]. Furthermore, since cdk2 inhibition only affected RGS2, not RGS4, expression, this finding indicates that different stress-associated mechanisms are responsible for the opposite effects of stress on these two RGS proteins.

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 significant impacts on cellular signaling and functions. Our research has shown that not only do cell stressors increase the expression of RGS2, but they also have the opposite effect to decrease RGS4 expression. At least part of this regulation of RGS expression by cell stress is associated with changes in the cell cycle. These associations between the cell cycle and control of the expression of RGS2 and RGS4 suggest that these proteins have as yet undetermined cellular actions, and further, that RGS2 and RGS4 may serve distinct functions as opposed to their very similar actions in regulating plasma membrane receptor-coupled G-protein signaling systems.

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Fig. 1.

Inhibition of camptothecin-stimulated RGS2 expression by roscovitine. (A) SH-SY5Y cells were pretreated for 1 h with 0-10 μM roscovitine, followed by treatment with 1 μM camptothecin (Campto) for 2 h and measurements of RGS2 (ROS), RGS4, and actin mRNA levels by Northern blots. Quantitative values are presented as the percent of mRNA levels in cells not treated with camptothecin or roscovitine. (B) Cells were pretreated with 1 μM roscovitine for 1 h followed by treatment with 1 μM camptothecin for 1-4 h and measurements of RGS2, RGS4, and actin mRNA levels by Northern blots. (C) Cells were pretreated for 1 h with 0-10 μM roscovitine followed by treatment with 1 mM carbachol (Carb) for 1 h and measurements of RGS2 and RGS4 mRNA levels by Northern blots.

Fig. 2.

Inhibition of camptothecin-stimulated RGS2 expression by purvalanol. (A) SH-SY5Y cells were pretreated for 1 h with 0-10 μ M purvalanol (Purv), followed by treatment with 1 μ M camptothecin for 2 h and measurements of RGS2 and RGS4 mRNA levels by Northern blots. (B) Cells were pretreated with 1 μ M purvalanol for 1 h followed by treatment with 1 μ M camptothecin for 1-4 h and measurements of RGS2 and actin mRNA levels by Northern blots. (C) Cells were pretreated for 1 h with 20 mM LiCl, 10 μ M indirubin-3-monoxime (ind), or 10 μ M kenpaullone (kpl), followed by treatment with 1 μ M camptothecin for 2 h and measurements of RGS2 and RGS4 mRNA levels by Northern blots.

Fig. 3. Regulation of RGS2 and RGS4 mRNA levels by geldanamycin. (A) SH-SY5Y cells were pretreated with 10 μ M geldanamycin (Geld) for 1 h followed by treatment with 1 μ M camptothecin for 2 h and measurements of RGS2, RGS4, and actin mRNA levels by Northern blots. (B) Cells were incubated with 10 μ M geldanamycin for 0.5-3.5 h followed by measurements of RGS2, RGS4, and actin mRNA levels by Northern blots. (C) Quantitation of results shown in (B).