Coordinate expression of the α and β subunits of heterotrimeric G proteins involves regulation of protein degradation in CHO cells

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Abstract Individual cell types express a characteristic balance between heterotrimeric G protein α and βγ subunits, but little is known about the regulatory mechanism. We systemically examined the regulatory mechanism in CHO cells. We found that expression of Gαs, Gαi2, and Gαq proteins increased in direct proportion to the increase of Gβ1γ2 overexpressed transiently. Expression of Gβ protein also increased following overexpression of Gαs, Gαi2, and Gαq. The Gβγ overexpression stimulated degradation of Gβ in contrast to reduction of Gαs degradation. We conclude that coordinate expression of the G protein subunits involves regulation of protein degradation via proteasome in CHO cells.

Key words: Heterotrimeric GTP-binding protein; G protein α subunit; G protein βγ subunit; Coordinate expression; Protein degradation; Proteasome

1. Introduction

Heterotrimeric GTP-binding regulatory proteins (G proteins) are composed of α, β, and γ subunits, and transduce extracellular signals into intracellular signals by coupling receptors and effectors [1]. When a signaling molecule such as a hormone binds to a G protein-coupled receptor, the receptor undergoes a conformational change. Such a conformational change leads to the activation of the G protein by facilitating the replacement of GDP with GTP on the α subunit of the protein (Gα), which then induces the dissociation of Gα-GTP from its βγ dimer (Gβγ) [2,3]. Both the dissociated Gα-GTP and Gβγ subunits are able to elicit cellular responses by interacting with numerous effectors, including adenylate cyclases, phospholipases, phosphodiesterases, and ion channels [4,5]. G protein signaling is terminated by the hydrolysis of GTP on the Gα subunit into GDP by intrinsic GTPase, which is followed by the formation of the inactive heterotrimeric structure, composed of GDP-Gα and Gβγ.

Individual cell types are known to express a characteristic balance of Gα and βγ subunits [6], which may reflect a continuously repeating association and dissociation of Gα and Gβγ due to the GTPase cycle. The presence of Gα in excess of Gβγ or that of Gβγ in excess of Gα would result in the continuous activation of effectors even in the absence of the stimulating signals [7]. Therefore, a cell needs to maintain a steady Gβγ/Gα ratio, which requires a regulatory mechanism to coordinate the expressions of Gα and Gβγ. However, little is known about the molecular mechanisms involved in the regulation of the coordinate expressions of Gα and Gβγ. Only a few studies have reported changes in the expression of Gβ following changes in Gα expression [7,8], but no report has been issued on the effect of Gβγ on the expression of endogenous Gα in mammalian cells. Therefore, we examined systemically whether the expressions of Gα and Gβ are coordinately regulated, and probed what regulatory mechanism is involved in this expression. We found that the expressions of Gα and Gβ are coordinately regulated in Chinese hamster ovary (CHO) cells by a mechanism involving the differential regulation of the protein degradation rate.

2. Materials and methods

2.1. Expression plasmids

The cDNAs encoding the hemagglutinin (HA)-tagged wild type and mutant Gαs were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Constitutively active mutants, Gαs R201E (Gαs RE) and Gαs Q227L (Gαs QL), were constructed by replacing arginine at 201 with glutamic acid [9] and glutamine at 227 with leucine [10], respectively. Dominant negative mutants, Gαs G226A (Gαs GA) and Gαs A366S/G226A/E268A (Gαs TM), were formed by replacing glycine at 226 with alanine [11] and by combining the three mutations, respectively [12]. The plasmids containing the cDNAs of Gβ1 and Gγ2 were kindly provided by Dr. William F. Simonds (NIDDK, NIH, Bethesda, MA, USA). All the constructs were subcloned into pCDNA3 vector (InVitrogen Life Technologies).

2.2. Cell cultures

CHO cells stably expressing the ecdysone receptor were purchased from InVitrogen Life Technologies, and grown in F-12 Ham medium containing 10% fetal bovine serum in a CO_2 incubator at 37°C. The cells were treated with 100 μM cycloheximide (Sigma Chemicals) to inhibit protein synthesis or 20 μM MG-132 (Biomol Research Laboratories) to inhibit proteasome.

2.3. Establishment of stable cell lines expressing various types of Gαs

The CHO cells were transfected with plasmids containing wild type or mutant type Gαs or pcDNA3 vector alone by using FuGENE6 transfection reagent (Roche Molecular Biochemicals). Transfected

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Abbreviations: CHO, Chinese hamster ovary; G protein, heterotrimeric GTP-binding regulatory protein; Gα, α subunit of G protein; Gαs, α subunit of stimulatory G protein; Gβ, β subunit of G protein; Gβγ, βγ dimer of G protein; HA, hemagglutinin

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cells were grown in a medium containing 500 μg/ml G418. Clones expressing the transfected Gαs were selected by examining their mRNA expression by reverse transcription-polymerase chain reaction (RT-PCR), using primers specific for the HA tag region of the transfected Gαs. The expression of the mutant protein was confirmed by Western blot, using anti-HA antibody to detect overexpressed Gαs and RM antibody to detect both endogenous and overexpressed Gαs protein [13].

2.4. Transient expression of Gαs and Gβγ

To induce the transient expression of Gαs and Gβγ, the CHO cells were passaged in 10 cm dishes 1 day prior to transfection, and the subconfluent (70–80%) cells were transfected with 10 μg of each plasmid containing Gαs, Gαi2, Gαq, Gβ1, or Gγ2 using Lipofectamine (InVitrogen Life Technologies). The overexpression of Gβγ was induced by cotransfecting the plasmid containing Gβ1 (10 μg) and that containing Gγ2 (10 μg) with a molar ratio of 1.0. Control cells were transfected with reagents containing no DNA or pcDNA3 vector DNA. The transfected cells were replated 24 h after transfection, and harvested 48 h later.

2.5. Immunoblot analysis

Immunoblot analysis was performed as described previously using antibodies specific to Gαs (RM) [14], Gαi (AS7), Gαq (QL), Gβ (SW) [15,16], HA (Roche Applied Science), or actin (Santa Cruz). Band densities were quantified with an image analyzer (Model GS-700, Bio-Rad), and expressed as a ratio to the corresponding band density in non-transfected CHO cells or vector-transfected cells.

2.6. Data analysis

At least three independent experiments were conducted. The non-parametric Mann–Whitney *U*-test was used to analyze average values, and a P value of <0.05 was considered statistically significant. Pearson analysis was used to analyze the correlation between the expressions of Gα and Gβ proteins.

3. Results and discussion

3.1. The expression of Gβ protein increased in CHO cells stably overexpressing various types of Gαs

CHO cell clones stably expressing wild type or various mutant types of Gαs were established, and the amounts of overexpressed wild type Gαs, Gαs QL or Gαs RE were between 1.5- and 2.1-fold that of vector-transfected cells. Cells expressing Gαs GA produced the largest amount of Gαs protein, which was more than 3.3-fold that of the vector-transfected cells (Fig. 1).

The expression of Gβ protein was increased in CHO cells stably overexpressing wild type Gαs, the constitutively active forms of Gαs (Gαs RE and Gαs QL), and the dominant negative forms of Gαs (Gαs GA and Gαs TM) (Fig. 1). In addition, all the cell lines expressing wild type or mutant types of Gαs displayed comparable Gβ/Gαs density ratios in the range 0.51–0.78. The strength of this correlation between the expression of Gαs and Gβ was confirmed by Pearson's correlation analysis (correlation coefficient 0.918, $P<0.01$).

3.2. The expression of Gβ protein increased in proportion to the amount of Gα proteins transiently overexpressed in CHO cells

The expression of Gβ protein was found to increase in CHO cells transiently transfected with wild type Gαs, constitutively active Gαs RE and Gαs QL, or dominant negative Gαs GA and Gαs TM (Fig. 2A). The expression levels of transiently transfected Gαs protein varied less than those of the stable cell lines, and their Gβ/Gαs density ratios ranged from 0.79 to 1.09.

To test whether the expression level of Gβ is dependent on the expression level of Gαs proteins, Gβ protein levels were assessed in CHO cells transiently transfected with increasing amount of Gαs expression plasmids. The expression of Gβ protein increased in direct proportion to the amount of the wild type Gαs ($r=0.938$, $P<0.01$, data not shown), constitutively active Gαs QL ($r=0.744$, $P<0.01$, data not shown), or dominant negative Gαs GA ($r=0.932$, $P<0.01$) (Fig. 2B).

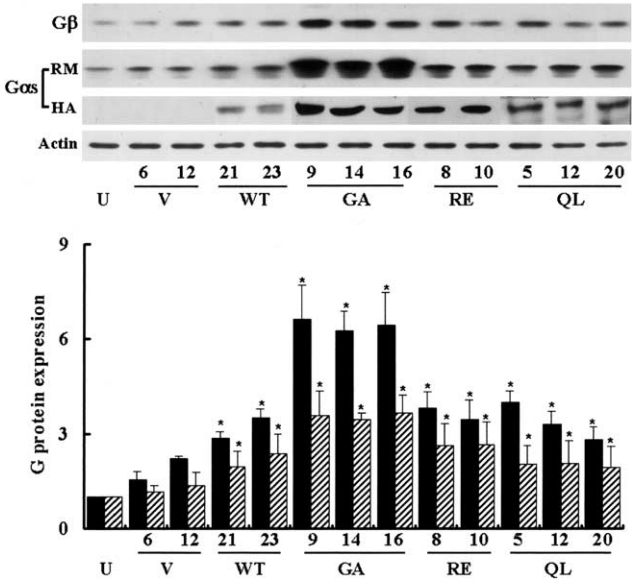

Fig. 1. Increased expression of Gβ proteins in CHO cells stably overexpressing Gαs. Representative immunoblots and densitometric results for Gαs and Gβ proteins are presented. The lysates of CHO cells stably overexpressing wild type (WT), a dominant negative (GA), or constitutively active (QL and RE) Gαs were analyzed by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot (10 μg for Gβ and actin, 20 μg for Gαs detected with RM, 50 μg for Gαs detected with HA). Gαs protein was detected with RM or HA antibody, Gβ with SW antibody, and actin with an anti-actin antibody, and visualized by incubating the blot with an enhanced chemiluminescence substrate mixture followed by exposure to an X-ray film. The amount of protein is expressed as a multiple of the band density in untransfected CHO cells, and the histograms and error bars represent the average and standard deviations of at least three independent experiments. The filled bar represents Gαs protein detected by RM antibody, and the hatched bar Gβ protein. The number denotes the respective clone number, and U represents untransfected cells. The asterisk indicates a statistically significant difference from the vector-transfected control (V) ($P<0.05$, Mann–Whitney *U*-test).

In order to determine whether other Gα subunits can induce Gβ expression, as did Gαs, the effect of the transient overexpression of the α subunit of an inhibitory G protein isoform (Gαi2) and that of Gq protein (Gαq) on the expression of Gβ protein was determined in CHO cells. The increased expression of Gαi2 or Gαq also resulted in increased Gβ expression (Fig. 3), suggesting that all Gα proteins share the Gβ-inducing effect.

3.3. The expression of Gα proteins increased in proportion to the amount of Gβ protein transiently overexpressed in CHO cells

We examined whether overexpression of Gβγ can induce the expression of Gαs in CHO cells. Gβ1γ2 overexpression resulted in the increased expression of Gαs, Gαi2, and Gαq proteins in CHO cells (Fig. 4A), which suggested that the

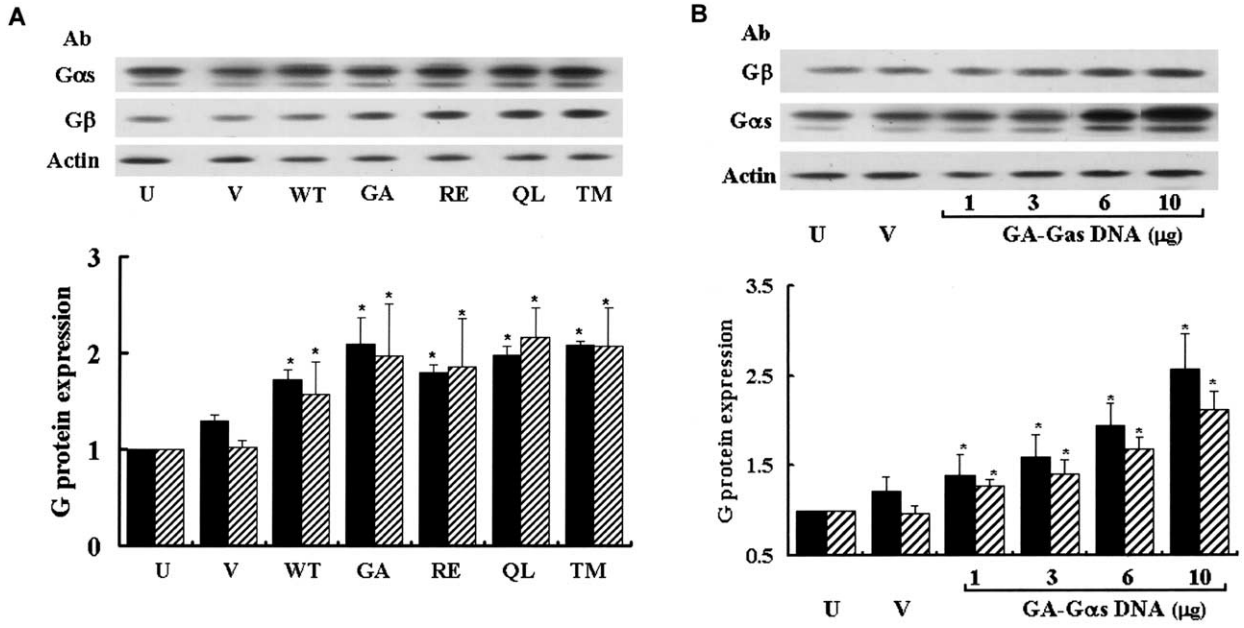

Fig. 2. The expression of Gβ protein increased in proportion to the amount of Gα proteins transiently overexpressed in CHO cells. A: Increased expression of Gβ proteins in CHO cells transiently overexpressing Gαs. B: Proportional increase in Gβ protein expression following the transient expression of Gαs GA. Subconfluent CHO cells in 10 cm dishes were transfected with 10 μg of plasmid containing wild type (WT), constitutively active (QL and RE), or dominant negative (GA and TM) Gαs using Lipofectamine. The control cells were untransfected (U), or transfected with the pcDNA3 vector but without the insert (V). CHO cells were also transfected with various amount of the expression plasmid Gαs GA (B). The transfected cells were harvested after 72 h, and analyzed by SDS–PAGE and Western blotting. See the legend of Fig. 1.

overexpression of Gβγ might induce all the α subunits in CHO cells. The expression of Gαs was found to increase in proportion to the amount of Gβγ protein expression achieved by co-transfecting increasing amounts of Gβ1 and Gγ2 plasmid DNA (Fig. 4B), and the Gβ/Gαs density ratios ranged from 1.00 to 1.07.

In this experiment, transient overexpression of Gβγ was found to induce the expression of Gα proteins, including Gαs, Gαi2, and Gαq in CHO cells. This is the first report, to our knowledge, showing that Gβγ can induce the expression of endogenous Gα subunits in mammalian cells, though loss of cpgb-1 Gβ protein was reported to lead to lower levels of the Gα proteins in the fungus *Cryphonectria parasitica* [17]. In the same way, stable or transient overexpression of Gαs proteins increased Gβ protein expression in CHO cells, and transient overexpression of Gαi2 or Gαq resulted in increased Gβ expression. These results show clearly that the expressions of Gα and Gβγ are coordinately regulated in CHO cells, and that, therefore, any stable or transient change in the expression of either Gα or Gβγ protein might result in parallel changes in the corresponding heterotrimer-forming endogenous subunits. This conclusion agrees well with a previous report showing that stable increases in expression of Gαi1, Gαi2, Gαi3 or Gαq are always accompanied by an increase in Gβ protein levels in NIH3T3 fibroblasts [18]. It is also compatible with reports that the abrogation of Gαi2 expression, by knocking out the gene, decreased Gβ expression in embryonic murine fibroblasts [8], and that the deletion of Gαo decreased Gβ expression in the mouse brain [7].

The GDP-bound α subunit of G proteins has a high affinity for Gβγ, and it forms a stable heterotrimer. Then, the Gβγ subunit turns off activated Gα by inducing conformational changes [19]. The formation of an inactive heterotrimer helps

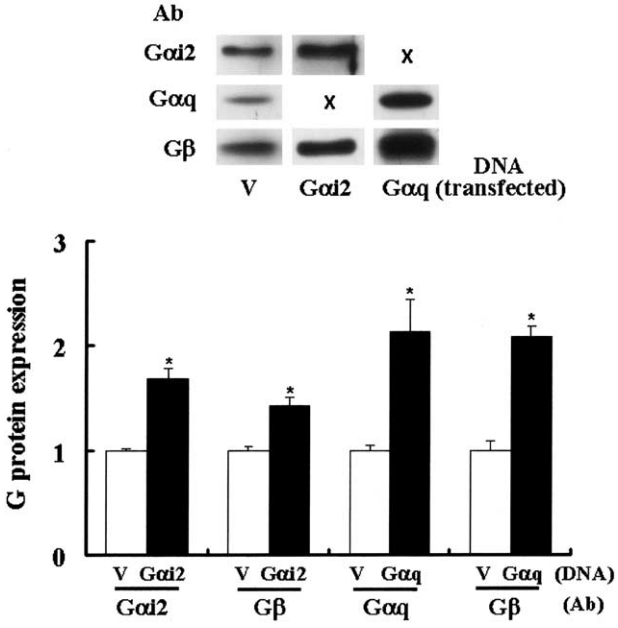

Fig. 3. Increased expression of Gβ proteins in CHO cells following the transient expression of Gαi2 or Gαq. Subconfluent CHO cells were transfected with an expression plasmid containing Gαi2 or Gαq. Transfected cells were harvested 72 h after transfection, and analyzed by SDS–PAGE and Western blotting. The filled bar represents G proteins expressed in the Gα-transfected cells, and the empty bar G proteins expressed in the vector-transfected cells. See the legend of Fig. 1.

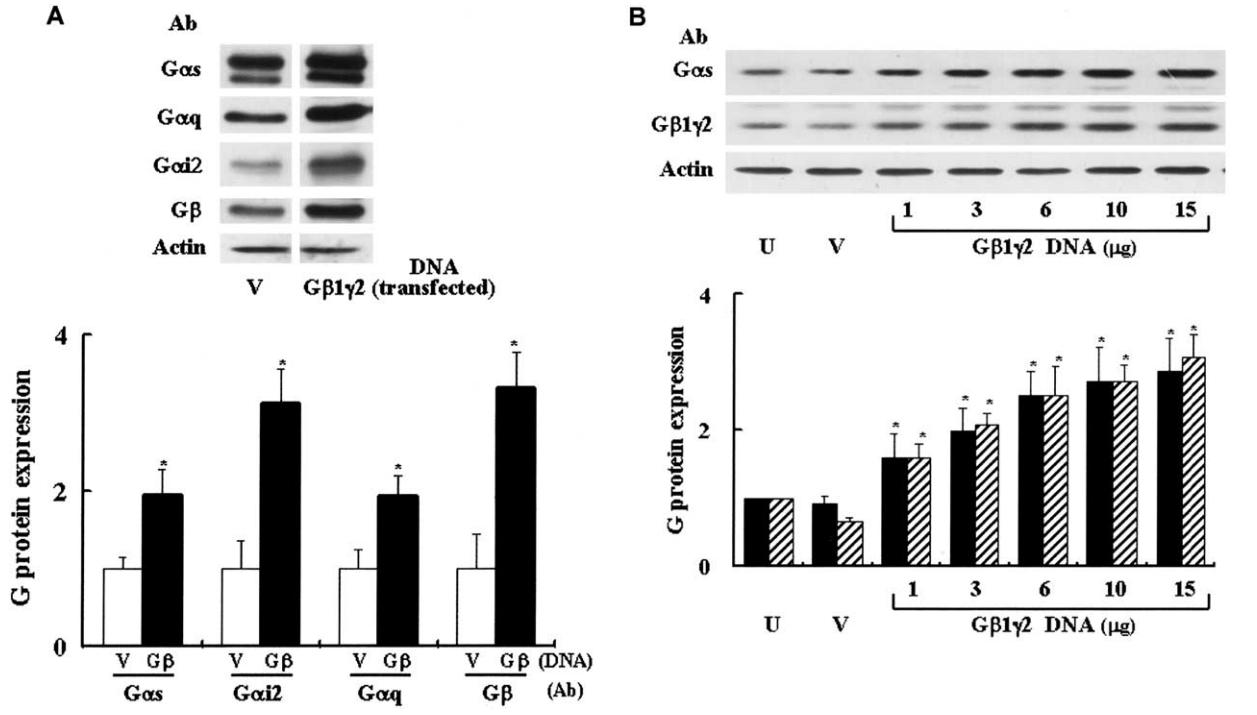

Fig. 4. The expression of Gα proteins increased in proportion to the amount of Gβ protein transiently overexpressed in CHO cells. A: Increased expression of Gα proteins in CHO cells following the transient overexpression of Gβ1γ2. B: Proportional increase in Gαs protein expression following transient overexpression of Gβ1γ2. Subconfluent CHO cells were co-transfected with fixed or increasing amounts of expression plasmid containing Gβ1 and that containing Gγ2. The transfected cells were harvested after 72 h, and analyzed by SDS–PAGE and Western blotting. A, the filled bar represents G proteins expressed in the Gβ1γ2-transfected cells, and the empty bar G proteins expressed in the vector-transfected cells. B, the filled bar represents Gαs protein and the hatched bar Gβ protein. See the legend of Fig. 1.

to maintain a low noise level and enhances the interaction of Gα with its receptors [5]. Thus, the presence of Gα in excess of Gβγ or that of Gβγ in excess of Gα would result in the continuous activation of effectors even in the absence of the stimulating signals [7]. Therefore, cells need to have mechanisms for strict coordinate regulation of Gα and Gβγ protein expression to maintain cellular homeostasis, and this study provides evidence of such mechanisms.

The expression levels of Gα proteins were found to increase in proportion to the Gβ protein levels in CHO cells overexpressing Gβγ, and those of Gβ protein were also found to increase in proportion to Gαs protein levels in CHO cells overexpressing Gαs. In other words, the more Gβγ expressed, the more Gα induced, and vice versa. Consequently, the expression ratio Gβ/Gαs was comparable among CHO cells stably overexpressing various mutant forms of Gαs, among CHO cells transiently transfected with various amounts of Gαs, and among CHO cells transiently transfected with various amounts of Gβγ plasmids. A change in the Gβ/Gα ratio is suggested to play an important role in altering cellular functions, because an altered Gβ/Gα ratio has been associated with differentiation [20] and development [21], for example. These findings en masse support the notion that the protein expression ratio Gβ/Gα could be an important parameter in the coordination of Gα and Gβγ expression. However, the difference in the Gβ/Gα ratio between the cells stably and transiently overexpressing Gαs cannot be explained clearly, but the difference can be speculated to result from the adaptation of stably expressing cells to a lower Gβ/Gα ratio during the long selection period. However, overexpression of Gαi2 was reported not to induce Gβ in cardiac myocytes [22], suggesting a cell type-specific regulatory mechanism for maintaining the Gβ/Gα ratio.

All the types of Gαs examined, i.e. wild type, constitutively active or dominant negative mutant types, were found to have similar capacities to induce the expression of Gβ, suggesting that the difference in Gαs functions might not play a significant role in the coordinate regulation of Gαs and Gβ in CHO cells. However, the different ability of mutant Gα to induce Gβ expression has been reported. The overexpression of a dominant negative mutant of Gαi2 increased the expression of Gβ proteins, but a constitutively active mutant failed to induce Gβ proteins in NIH3T3 cells [18]. It is not known why Gβ expression is differently regulated following the overexpressing of the constitutively active forms of Gαs and Gαi2. Nevertheless, we speculate that a difference of cell lines could probably be the cause, because different cell lines may have different compositions of G proteins and different regulatory mechanisms for coordinating the expression of Gα and Gβγ proteins [7].

3.4. The degradation rate of overexpressed Gαs or Gβ proteins increased, but that of the heterotrimer-forming endogenous Gαs or Gβ proteins decreased in CHO cells

To probe the regulatory mechanism for the coordinate expression of Gα and Gβγ, we examined the degradation rate of G proteins following the overexpression of Gα or Gβγ proteins. The degradation rate was assessed by measuring the remaining G proteins at various time points after blocking protein biosynthesis with cycloheximide. In CHO cells transiently overexpressing Gα, the endogenous Gβγ proteins degraded more slowly than in the mock-transfected control,

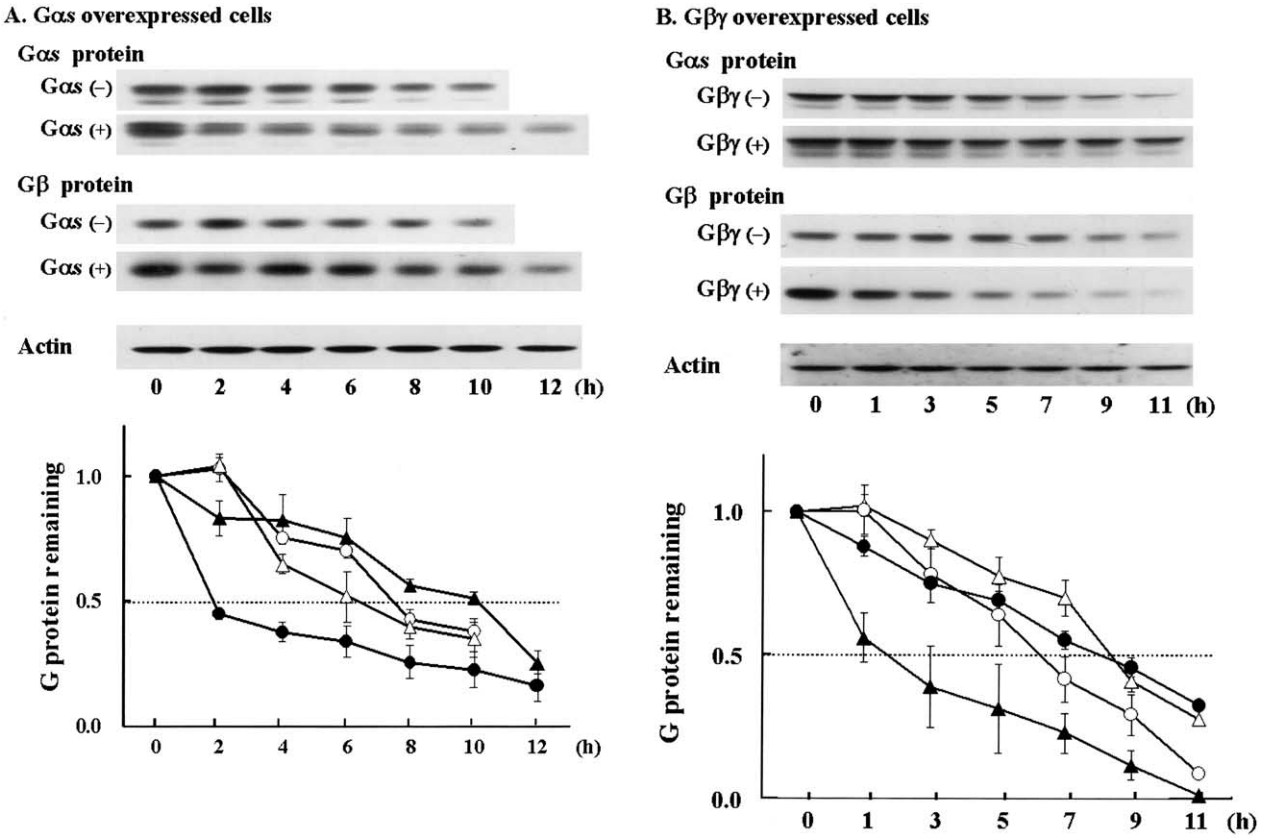

Fig. 5. Differential regulation of the degradation rate of G protein subunits following Gαs and Gβγ overexpression. Degradation of Gα and Gβ protein in CHO cells transiently overexpressing wild type Gαs (A) or Gβγ (B). ○, Gαs in the absence of overexpression; ●, Gαs in the presence of Gαs or Gβγ overexpression; △, Gβ in the absence of overexpression; ▲, Gβ in the presence of overexpression of Gαs or Gβγ. Seventy-two hours after transfecting CHO cells with Gαs or Gβγ, the cells were treated with 100 μM cycloheximide (CHX). Then, the remaining G protein subunit was monitored at the indicated times by SDS–PAGE and Western blot. See the legend of Fig. 1.

and the half-life of Gβ increased to 9.7 ± 0.58 h as compared with 6.7 ± 0.58 h of the control. On the other hand, the Gαs protein degraded more rapidly with a decreased half-life of 1.9 ± 1.8 h compared to 7.3 ± 0.25 h of the control (Fig. 5A). In contrast, in CHO cells transiently overexpressing Gβγ, the Gβ proteins degraded more rapidly with a half-life of 2.7 ± 1.3 h compared to 7.3 ± 0.58 h of the control, but the endogenous Gαs protein degraded more slowly with a half-life of 9.0 ± 0.30 h compared to 6.8 ± 0.61 h of the control (Fig. 5B). Transfection of vector only did not induce significant changes in the degradation rate of Gαs and Gβ (data not shown), and the degradation rate of actin did not change significantly with transfection of either Gαs or Gβγ.

This result indicates that the degradation rates of the overexpressed Gα or Gβγ increase in contrast with the decreased degradation rates of the heterotrimer-forming endogenous Gα or Gβγ. Such differential regulation of degradation rates could contribute to minimizing the number of subunit proteins that fail to form stable heterotrimers and to the maintenance of an optimal Gβγ/Gα ratio in the cell, though constitutively active Gαs QL bound to a smaller amount of Gβ than wild type Western blot analysis of Gβ bound to the transfected Gαs, which was immunoprecipitated by anti-HA antibody, showed Gβ/HA density ratio of 1.0, 1.4, and 0.2 for wild type, GA, and QL Gαs, respectively, in CHO cells. Therefore, the differential regulation of degradation rates of Gα and Gβ might be an underlying mechanism for the coordinate expression of Gα and Gβ. Such a post-transcriptional mechanism has been previously suggested for the regulation of Gα and Gβ expression. The overexpression of Gαi2 QL resulted in the increased expression of Gβ mRNA, but this failed to increase Gβ protein expression, suggesting that post-transcriptional regulation increased Gβ protein degradation in the cell [18]. Similarly, the expression of Gβ mRNA remained unchanged, but its protein concentration decreased to about one-third in Gαo−/− mouse brain. This discrepancy between the expressions of mRNA and protein was also attributed to rapid protein deg-

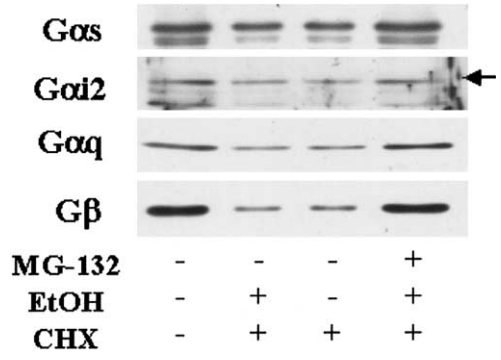

Fig. 6. Inhibition of G protein degradation by a proteasomal inhibitor, MG-132. The untransfected CHO cells were treated with 100 μM cycloheximide (CHX) and 20 μM MG-132 in ethanol (EtOH) for 12 h, and then the remaining G protein subunits in the treated cells were monitored by SDS–PAGE and Western blot. See the legend of Fig. 1.

radation, though the increase in the degradation rate of Gβγ was not analyzed [7].

To test whether Gαs and Gβ are degraded by the proteasomal system, we examined the effect of MG-132, a proteasomal inhibitor, on the degradation of G protein subunits. It was found that treatment with MG-132 prevented the degradation of both Gαs, Gαi, Gαq and Gβ in CHO cells (Fig. 6), which indicates that Gα and Gβ subunits are also degraded via proteasome pathways. Though several studies have reported changes in the degradation rate of G proteins, the mechanism of degradation is not clearly understood [23]. However, the Gαo subunit was found to be degraded via the proteasome pathway recently [24]. Thus, we speculate that degradation via the proteasome pathway might be a common mechanism for degradation of G protein subunits, and that regulation of proteasomal degradation of G protein subunits might play an important role in the coordination of Gα and Gβ subunits.

The question addressed in the present study was whether the expressions of Gα and Gβ proteins are coordinately regulated, and what regulatory mechanism is involved in this coordinate expression. From this study, we conclude that the expressions of Gα and Gβγ are coordinately regulated in CHO cells, that is, an increase in Gβ proteins results in an increase in Gα proteins, and vice versa. It is also concluded that the differential regulation of the degradation rates of G protein subunits is involved in the coordinate expression of Gα and Gβγ in CHO cells.

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