Maintenance of cellular levels of G-proteins: different efficiencies of α_s and α_o synthesis in GH₃ cells

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G-proteins couple membrane-bound receptors to intracellular effectors. Each cell has a characteristic complement of G-protein α , β and γ subunits that partly determines the cell's response to external signals. Very little is known about the mechanisms that set and maintain cellular levels of G-proteins or about potential points of regulation. We have assayed the steady-state levels of mRNA and protein for two types of G-protein subunits, α_s and α_o , in rat brain, heart and GH₃ cells, and found that in all these cases, it takes 9- to 20-fold more mRNA to produce a given amount of α_s protein than to produce the same amount of α_o protein. Such a situation could arise from a relatively rapid rate of α_s protein degradation, requiring rapid protein synthesis to compensate, or from relatively inefficient translation of α_s mRNA compared with α_o mRNA. The latter appears to be the case in

GH₃ cells. These cells contain 94 times more mRNA for α_s than for α_o , yet the rate of α_s protein synthesis is only 9 times greater than α_o protein synthesis. The degradation rates of the two proteins are similar (13 h for α_s and 18 h for α_o). To begin to define the mechanism that accounts for the fact that it takes more mRNA to synthesize a given amount of α_s than α_o , we asked whether there is a pool of α_s mRNA that does not participate in protein synthesis. We found that virtually all α_s and α_o mRNA is associated with ribosomes. Therefore, all the mRNA is likely to be capable of directing protein synthesis. Since the rate-limiting step in protein synthesis is usually binding of the ribosome to mRNA at initiation, our results suggest that the relatively slow rate of α_s protein synthesis is regulated by a mechanism that acts beyond initiation at peptide elongation and/or termination.

INTRODUCTION

Cell surface receptors are coupled to intracellular signalling pathways by a family of heterotrimeric G-proteins composed of α , β and γ subunits. Many subtypes of G-proteins are present in individual cells and transmit signals from receptors to ion channels and intracellular enzymes. The G_s proteins activate adenylate cyclase and regulate some Ca²⁺ channels. Subunits of the G α_1 family (α_{11} , α_{12} , α_{13} , α_o and α_z) are responsible for inhibition of adenylate cyclases, activation of phospholipases and regulation of ion channels [1,2]. Multiple species of mRNA for α_s and α_o are generated by alternative splicing and have been identified in various cells [3–7]. In GH₃ cells, two alternatively spliced forms of α_o were shown to couple to different receptors [8]. It is not known whether the variants of α_s also have different functions.

Both the amount and the type of G-proteins expressed in an individual cell affect the cell's ability to respond to hormones and other extracellular signals. For example, in the genetic disorder pseudohypoparathyroidism, the affected patients have half the normal amount of α_s and are resistant to the action of parathyroid and other hormones [9–11]. In cardiac development, changes in G-protein levels correlate with changes in hormone responsiveness [12–14]. Changing the levels of G-protein subunits, genetically or by antisense methods, changes cellular responses to external stimuli [8]. Despite the biological importance of regulating the amount of G-protein subunits in cells, very little is known about how the cell brings this about.

The starting point for the study reported here was our observation that there seemed to be more mRNA relative to protein for α_s than for α_o . In this study, we have documented this

discrepancy and have begun to define the processes that determine the cellular levels of α_s and α_o . Our studies show that α_s and α_o are synthesized with different efficiencies in GH₃ cells, and suggest that the mechanisms that regulate the rate of α_s protein synthesis may be at the peptide elongation or termination steps.

MATERIALS AND METHODS

Cell culture, RNA isolation and RNase protection

The GH₃ cells were maintained in Dulbecco's modified Eagle's medium with 9% (v/v) fetal bovine serum (FBS) at 37 °C in 5% CO₂/95% air. RNA and protein were extracted from 28-day old Sprague–Dawley rats. Total cellular RNA was isolated from rat heart, brain or cultured GH₃ cells by the method of Chomczynski and Sacchi [15]. Total RNA (12–25 μ g) was used in the RNase protection assay. Fragments of cDNA clones of various G-protein subunits were subcloned into pBluescript vectors and used as templates for *in vitro* transcription of antisense RNA probes (see Table 1). Protection experiments were performed essentially according to the method of Ausubel et al. [16]. RNA and antisense probes were hybridized at 42 °C, then digested with 10 μ g/ml RNase A (United States Biochemical Corporation) and 200 units/ml RNase T1 (United States Biochemical Corporation). The riboprobes are uniformly labelled with [³²P]CTP; all probes to be used in an assay were synthesized at the same time so that their specific activity is proportional to the cytidine content (given in Table 1). The amount of mRNA was compared by densitometry of the autoradiograms, or by cutting out the radioactive bands, counting the radioactivity and correcting for differences in specific activity of the probe due to

Abbreviations used: FBS, fetal bovine serum; TCA, trichloroacetic acid.

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Table 1 Probes for RNase protection assay to quantify mRNA

RNase protection experiments were performed essentially according to the method described by Ausubel et al [16]. The 251 bp band (*) was the major protected band in RNA from brain and GH₃ cells. The other spliced variants of α_s were barely detected, although the probe covered the alternatively spliced junction in α_s mRNA. However, in total RNA from rat heart, we detected at least two forms of the spliced variants.

	Subunit	Probe length (bp)	Protected length (bp)	Number of cytidines	Probe location
	α_o	389	331	101	Amino acid sequence 12–123 (common to α_{oa} and α_{ob})
	α_{i2}	348	290	105	First 92 amino acids and 14 bp 5' non-coding sequence
	α_s	309	251*	69	Amino acid sequence 62–146
	β_1	347	275	74	80 C-terminal amino acids and 176 bp 3' non-coding sequence

Table 2 Comparison of mRNA and protein levels for G-protein subunits

Column A: the protein levels were measured by quantitative Western blots as described in the Materials and methods section. Column B: the average value for each protein (column A) was used to calculate the ratio. Column C: the relative mRNA levels were determined by the RNase protection assay as described in the Materials and methods section. Ratios were calculated from assays done together and analysed on the same gel. The radioactive bands were cut out and counted. The numbers shown are means $\pm$ S.E.M. for the number of determinations shown in parentheses.

Tissue or cell	A Amount of subunit (μ g/mg)		B Relative protein (α_s/α_o)	C Relative mRNA (α_s/α_o)	Ratio mRNA/protein (C/B)
	α_s	α_o			
Brain	0.6 $\pm$ 0.1 (6)	1.8 $\pm$ 0.3 (4)	0.3	6 $\pm$ 2 (5)	20
GH ₃	0.3 $\pm$ 0.09 (7)	0.03 $\pm$ 0.008 (8)	10	94 $\pm$ 25 (3)	9.4
Heart	0.2 $\pm$ 0.06 (3)	0.06 $\pm$ 0.01 (4)	3	46 $\pm$ 3 (3)	15

different cytidine content. The ratios were calculated from the relative radioactivity of the bands for each of the subunits analysed simultaneously in the same sample of mRNA. When the amount of labelled probe is in excess (as in our studies), the signal of the protected band is proportional to the amount of the specific mRNA. The 140 bp U3 probe was made from an *EcoRI*–*PvuII* fragment of rat U3 cDNA [17,18].

Assay of steady-state protein level

Tissue was homogenized in TMSDE buffer [50 mM Tris/HCl, pH 7.6/6 mM MgCl₂/75 mM sucrose/1 mM dithiothreitol/1 mM EDTA] with 3 mM benzamidin and 1 μ g/ml each of leupeptin, soybean and lima bean trypsin inhibitors. Cells were harvested into TMSDE buffer, and passed 2–3 times through a 23-gauge needle. The protein concentration of the homogenate was determined by a modified Lowry method [19]. The amount of α_s and α_o was determined by Western blots using purified α_s and α_o standards. The α_s and α_o subunits were purified from bovine brain as described previously [20,21]. The concentration of α_o was determined by amino acid analysis performed in duplicate. Since the α_s sample contained some $\beta\gamma$ and a small amount of α_1 , we could not determine the concentration by amino acid analysis. Therefore, we analysed known amounts of α_o and three dilutions of α_s on the same 11% SDS/PAGE, stained with Coomassie Blue, and measured the bands by laser densitometry. The amount of 52 kDa α_s in the sample was calculated by comparing the area of the α_s bands with the area

of the bands containing known amounts of α_o . The amino acid composition of the two proteins is similar, so it is reasonable to consider that they bind the Coomassie Blue dye equally. To measure α_s and α_o in cell and tissue homogenates, at least three dilutions of each sample and standard were loaded. In different experiments, the concentrations ranged from 1 to 5 μ g for brain, and from 5 to 19 μ g for heart and GH₃ cell lysates. In a few experiments, we analysed tissue and cell homogenate on a single 11% gel, loading an equal amount of protein for each (15 μ g) and three different dilutions of standards. The results were similar; the pooled results are shown in Table 2. The standards, as well as the tissue and cell samples, were simultaneously transferred onto nitrocellulose membranes [22] and probed separately with specific antibodies; antibody binding was detected using a chemiluminescence kit (NEN/Du Pont) according to the manufacturer's recommendations. We used short exposure times to stay within the linear range. The polyclonal antibody to α_s was raised against a peptide corresponding to amino acids 384–394 of α_s (Gramsch, Inc.) and was used at a 30000-fold dilution. The antibody to α_o was a polyclonal antibody raised by this laboratory against the purified α_o protein, as described previously [23], and was used at a 3000-fold dilution.

Assays of the rate of protein synthesis and degradation

The GH₃ cells were plated at a density of 0.5×10^6 /well in 6-well plates. After 48 h, the cells were washed with medium free of methionine and cysteine and incubated in this medium with 5% (v/v) dialysed FBS for 30 min. [³⁵S]Methionine and [³⁵S]cysteine (Translabel, ICN) were added to a final concentration of 100 μ Ci/ml. The degradation rate was measured by a modification of our previously described method [23]. The cells were quickly washed and harvested at various time intervals as noted. For measuring the degradation rate, the cells were starved of methionine and cysteine as described above, then incubated in medium with [³⁵S]methionine and [³⁵S]cysteine (Translabel, Amersham) and 5% (v/v) FBS for 3.5 h to label the proteins. The cells were washed three times with Dulbecco's modified Eagle's medium, and the radioactive cysteine and methionine chased for the indicated times with complete medium containing cysteine and methionine. Washed cells (0.8 ml) were harvested in RIPA buffer [0.1% SDS/1% Triton X-100/0.5% (v/v) deoxycholate/150 mM NaCl/4 mM EDTA/25 mM Tris/HCl, pH 7.6, with 1 mM PMSF added just before use]. The cell lysates were homogenized by passing through a 23-gauge needle and centrifuged at 16000 g for 10 min in a microcentrifuge. The supernatant was used for immunoprecipitation. A 0.5–0.8 ml volume of the cell lysate was precleared by incubation with

Protein A-Sepharose beads at 4 °C for 30 min, and the supernatant was recovered. The antibodies to α_s (10 μ l at 1:30 dilution) and α_o (10 μ l of serum without dilution) were added together to the supernatant, and the mixture was incubated at 4 °C for 60 min. Protein A-Sepharose [300 μ l; 10% (v/v)] was added and shaken at 4 °C for 60 min. The sample was spun and washed 4–5 times with RIPA buffer and then the antigen and antibody were eluted with SDS/PAGE sample buffer and analysed by 11% SDS/PAGE followed by autoradiography [24]. We titrated the antibodies and the Protein A-Sepharose concentration to ensure that the quantity of antibody and Protein A-Sepharose used was sufficient to precipitate all the α_s and α_o in the sample. For protein synthesis, the label was added and cells were harvested at 0, 10, 20, 30 and 60 min. The 35 S-labelled total cellular proteins were measured by incorporation of 35 S-amino acids into the trichloroacetic acid (TCA)-insoluble fraction. Lysate (15 μ l) was mixed with 1 ml of 5% (v/v) TCA and the insoluble fraction was collected on a glass-fibre filter (Whatman). The filter was washed twice with 2.5 ml of 5% TCA and 5 ml of ethanol and then counted in a scintillation counter. Because there is much more α_s than α_o , a smaller amount of lysate was used for α_s immunoprecipitation. In order to keep the signals of equal intensity, we used 15–25 times as much lysate for α_o immunoprecipitation as for α_s . The volume was equalized with RIPA buffer. The areas of each band were quantified by densitometry. The areas were corrected for the different volumes of lysate used, as well as for the difference in cysteine and methionine composition of α_s (7 Met, 8 Cys) and α_o (7 Met, 10 Cys). About 18% of the 35 S-amino acids in Translabel is cysteine and 82% is methionine. Since the ratio of methionine to cysteine is similar in α_s and α_o , we corrected for the total difference in methionine plus cysteine.

Fractionation of polyribosomal and non-polyribosomal RNA

Cells were extracted at 4 °C in 20 mM Tris/HCl, pH 7.6/0.25 M sucrose/5 mM MgCl_2 /25 mM KCl/1 mM dithiothreitol/0.5% Triton X-100/0.5% deoxycholate/500 μ g/ml heparin. The lysate was centrifuged at 12000 g for 10 min. The supernatant was loaded onto a 12 ml 15–40% sucrose gradient with a 2 ml 60% sucrose cushion and centrifuged for 6.5 h at 25000 rev./min ($r_{av.} = 122$ mm) in a Beckman SW27 rotor at 4 °C [25]. The UV absorption of the fractions (0.4 ml each) was measured at 260 and 280 nm. Fractions were pooled into three or four larger fractions according to their A_{260} . RNA was isolated from each pool by phenol/chloroform extraction and was used for the RNase protection analysis, as described above.

RESULTS AND DISCUSSION

Steady-state level of α_s and α_o mRNA and protein

In order to compare the steady-state mRNA levels of different G-protein subunits, we made a group of antisense RNA probes and used an RNase protection assay as described in Table 1. To compare protein levels, we used Western blots (Table 2). In all cases, we used unfractionated homogenates to determine absolute protein levels to avoid any losses on fractionation. The protein concentration of the homogenates was determined by the Lowry assay [19], and the amount of both α_s and α_o was measured in the same homogenate. Representative RNase protection and Western blot results are shown in Figures 1 and 2 respectively. The α_s mRNA is more abundant than α_o mRNA in all the cells and tissues studied. For comparison, we also measured the mRNA levels for β_1 and α_{i2} . The level of β_1 mRNA is approximately equal to that of α_o mRNA in brain and GH₃ cells. The amount of α_{i2} mRNA is less than that of α_o mRNA in brain but more

Figure 1 Comparison of mRNA levels of α_o , α_s , α_{i2} and β_1 in brain and GH₃ cells

The probes used for RNase protection assays are described in Table 1. Total RNA extracted from rat brain or cultured GH₃ cells was used for analysis. The undigested probes (not shown) are at least 50 bp longer than the protected portion of the probes (see Table 1). The upper band in the doublet seen when the α_o and β_1 probes were mixed was also seen in the tRNA lane and is probably due to a secondary structure in the probe. The smear beneath the major protected α_s bands in brain and GH₃ cells is due to overexposure of the film. Since α_s mRNA in GH₃ cells is approx. 100 times more abundant than α_o , the exposure needed to see α_o resulted in overexposure of the bands. None of the minor bands were included in calculating the relative amount of mRNA.

abundant than α_o mRNA in GH₃ cells. In rat brain and GH₃ cells, the major species of α_s mRNA detected by RNase protection corresponds to the mRNA encoding the long form (52 kDa) of α_s protein. This is also the major form of the protein seen on Western blots (Figure 2). In rat heart, at least two forms of alternatively spliced mRNA were detected (results not shown), but the major form of α_s protein was 45 kDa, in agreement with previously reported results [26,27].

The content of α_s and α_o proteins in brain, heart and GH₃ cells is given in Table 2. The amount of α_s protein varies much less in different tissues or cells than that of α_o , consistent with the previous finding that α_s functions universally in tissues or cells while α_o is expressed in a tissue-specific fashion. In all these systems, there is more α_s mRNA for a given amount of α_s protein than α_o mRNA for a given amount of α_o protein. In rat brain, the amount of α_s protein is 3-fold less than that of α_o protein while the level of α_s mRNA is 6-fold higher than that of α_o mRNA (Table 2). The absolute amount of α_o protein we measured in rat brain is in good agreement with values reported by Asano et al. [28] using a different method. In the heart, the α_s : α_o mRNA ratio is 46, while the α_s : α_o protein ratio is only 3. In GH₃ cells, the level of α_s mRNA is 94-fold higher than that of α_o mRNA, while the level of α_s protein is only 10-fold higher than that of α_o protein. Thus, in brain, heart and GH₃ cells, it takes between 9- and 20-fold more RNA to make a given amount of α_s protein than α_o protein.

The discrepancy is not due to an artifact of an RNase protection assay that uses a probe corresponding to only a part of the full-length mRNA. The α_s pre-mRNA is known to be alternately spliced between exons 2 and 3 and exons 2 and 4 to generate the long and short forms of the protein [29]. In addition, two novel forms of α_s mRNA have been identified, both of which

Figure 2 Comparison of protein levels of α_s and α_o in rat brain, heart and GH₃ cells

Top panel: Western blot of α_s or α_o proteins from GH₃ cells. A 15 μ g portion of the tissue extract or GH₃ cell lysate was loaded in each lane for SDS/PAGE. After separation, the proteins were transferred onto nitrocellulose and probed with specific antibodies as described in the Materials and methods section. Middle and bottom panels: Western blots of α_s or α_o proteins from GH₃ cells. The indicated amounts of cell lysate and standard protein were fractionated by SDS/PAGE, transferred to nitrocellulose and probed with specific antibodies as described in the Materials and methods section.

presumably encode truncated forms of α_s proteins [30,31]. Since the probes used for RNA protection correspond to only a portion of the mRNA, it is possible that some α_s mRNA that we detect does not encode the full-length protein. The 'excess' α_s mRNA would then be due to mRNA that cannot direct synthesis of active protein. To test this hypothesis, we made a series of probes that covered the entire coding region. The sizes of the protected bands corresponded well with the sizes of α_s antisense-RNA portion in the probes, and the amount of mRNA determined by each of these probes was also similar. Thus, the results of RNase protection assays with our probes accurately reflect the level of the predominant form of α_s mRNA in these cells (Figure 3).

Degradation rates of α_s and α_o proteins in GH₃ cells are similar, while the rates of synthesis differ

A discrepancy between the amount of mRNA and protein for different G-proteins in the same cell type (GH₃ cells) could arise

Figure 3 RNase protection analysis of α_s mRNA in GH₃ cells

Top panel: Schematic representation of α_s mRNA and the probes used for RNase protection analysis. Probe G is the α_s probe listed in Table 1. Middle and bottom panels: RNase protection analysis of α_s mRNA with various probes that cover the entire coding region of α_s mRNA. The RNase protection assay was performed as described in the Materials and methods section. The calibration is shown on the right in the middle panel and on the left in the bottom panel.

from a relatively rapid rate of α_s degradation, with a compensatory requirement for rapid protein synthesis to maintain steady-state levels, or from relatively inefficient translation of α_s mRNA compared with α_o mRNA. To distinguish between these possibilities, we measured the relative rate of α_s and α_o degradation and synthesis using GH₃ cells as a model. Rates of synthesis and degradation must necessarily be measured over different periods of time and under somewhat different conditions. However, in all experiments we compared the degradation rate or the synthesis rate of α_s and α_o in the same cells at the same time. As shown in Figure 4, the $t_{1/2}$ values for α_s and α_o

Figure 4 Degradation of α_s and α_o proteins in GH3 cells

Top: autoradiography of immunoprecipitated α_s and α_o from ^{35}S -labelled GH3 cells. Cells were labelled with $[\text{35S}]\text{methionine}$ and $[\text{35S}]\text{cysteine}$ (Translabel, Amersham) for 3.5 h and the label was chased with complete medium containing cysteine and methionine before harvesting at the times indicated. The labelled α_s and α_o were simultaneously immunoprecipitated by specific antibody against α_s and α_o , which were added to the same lysate. The difference in α_s and α_o signals reflects the different amounts of each protein in these cells. Bottom: degradation curves for α_s and α_o proteins. The signal of the band at each time point is expressed as a percentage of the signal at time zero. Data from four experiments were averaged and the average decrease in α_s and α_o (mean $\pm$ S.E.M.) was plotted. The linear correlation coefficients of α_s and α_o degradation rates were -0.98 and -0.94 respectively. The $t_{1/2}$ values for α_s and α_o are 13 h and 18 h respectively. The arrows indicate 50% degradation.

proteins in GH3 cells are 13 h and 18 h respectively. We had previously found the $t_{1/2}$ for α_o degradation to be 28 h in a closely related cell line, GH4. The difference in the $t_{1/2}$ of α_o is probably due to the differences between the cell lines [23].

In some cases, the levels of α subunits are partially regulated by changes in the degradation rate. As neuroblastoma cells differentiate in culture, the degradation rate of α_o decreases from 28 h to 58 h [32]. The degradation rate of α_o in primary cerebellar granule cells is lower still, 154 h [32]. The level of α_o protein increases during rat brain development. We have determined protein and mRNA levels for α_o in the developing rat brain and found that the protein increased 8- to 10-fold, while the mRNA increased less than 2-fold, in agreement with previously reported results [33,34]. Perhaps movement of α_o during development to sites such as neuronal growth cones [35] may protect it from degradation. Control of the degradation rate of α_s is also important, although on a different time scale. Levis and Bourne [36] found that activation of the epitope-tagged α_s decreased its $t_{1/2}$ from 22 h to 6.2 h in S49 cells. In our study, we measured the degradation rate of α_s without hormonal stimulation, and we detected no rapidly degraded pool of α_s .

At steady-state, the rate of protein synthesis is equal to the rate of degradation. Because the steady-state level of α_s protein is 10-fold higher than that of α_o protein, and the rates of degradation for α_s and α_o are 13 h and 18 h respectively, the rate of protein synthesis for α_s would have to be higher than for α_o to maintain the difference that we measured in steady-state protein levels. To determine whether this is in fact the case, we measured the rates of protein synthesis of α_s and α_o . We compared the synthesis of the two proteins in the same cells at the same time so that the rate

Figure 5 Synthesis of α_s and α_o proteins in GH3 cells

Top: autoradiograph of immunoprecipitated α_s and α_o from ^{35}S -labelled GH3 cells. Because there is much more α_s than α_o in cells, a smaller amount of lysate was used for α_s immunoprecipitation to keep the signals of equal intensity. We used 15–25 times as much lysate for α_o as for α_s . The volume was equalized with RIPA buffer. The immunoprecipitated ^{35}S -labelled α_s and α_o were separated by SDS/PAGE. The areas of each band of α_s and α_o on the same gel were quantified by densitometry. The autoradiogram shown was produced by using 25-fold more lysate for α_o than for α_s . Middle: the time course of synthesis of α_s and α_o proteins. Data shown is from one experiment out of a total of six. The line was fitted to the points using a least-squares fit. The correlation coefficients for α_s and α_o are 0.89 ± 0.06 and 0.94 ± 0.01 (means $\pm$ S.E.M.; $n = 6$) respectively. Bottom: the time course of Translabel incorporation into TCA-insoluble fraction of GH3 cells. Incorporation into total protein was measured as described in the Materials and methods section. The amount ^{35}S -labelled protein at 60 min was taken as 100%. Data from three experiments was averaged (mean $\pm$ S.E.M.) and plotted.

of uptake of labelled amino acids and the rate of general protein degradation should affect both proteins similarly. We measured the relative rates of α_s and α_o synthesis by immunoprecipitation of labelled α_s and α_o proteins (Figure 5, top and middle panels). The incorporation was corrected for the number of cysteines and methionines in α_s and α_o . We were particularly careful to measure incorporation of label into α_s and α_o from gels and films exposed simultaneously and containing similar amounts of radioactivity to minimize any effects of non-linear response in the X-ray film. The rate of total protein synthesis was measured by counting the incorporation of ^{35}S -amino acids into TCA precipitable protein (Figure 5, bottom). We found that the rate of α_s synthesis is 8.9-fold (8.9 ± 1.4 ; $n = 6$) greater than the rate of α_o synthesis (Table 3). From this value and the degradation rates, we calculated that the steady-state level of α_s should be 6.5-fold higher than that of α_o , which is similar to our measured value (see Tables 2 and 3).

Table 3 Summary of rates of synthesis and degradation of α_s and α_o in GH₃ cells

Subunit	A* Relative protein	B* Relative mRNA	C† Relative rate of synthesis	D Relative rate of synthesis/mRNA (C/B)	E‡ $t_{1/2}$ of proteins (h)
α_o	1.0	1.0	1.0	1.0	18
α_s	10	94	8.9 ± 1.4 ($n = 6$)	0.1	13

* Values are from Table 2.

† The relative rates of synthesis were calculated from the slopes of linear regression of the data as shown in Figure 5. The relative rate of α_s and α_o synthesis was always calculated from experiments in which the rates of synthesis were determined simultaneously. The mean ratio $\pm$ S.E.M. ($n = 6$) is given.

‡ Half-lives determined in Figure 4.

The similarity of the calculated and measured relative steady-state levels of α_s and α_o confirms the validity of our determination of the relative rates of their synthesis and degradation.

Analysis of the distribution of α_s and α_o mRNA in non-polyribosomal and polyribosomal fractions

Since the mRNA level of α_s is 94-fold higher than that of α_o , but the rate of α_s protein synthesis is only 9-fold higher than α_o , either α_s mRNA is translated 10-fold less efficiently than α_o mRNA or no more than 10% of α_s mRNA is used for α_s protein synthesis. In oocytes, the majority of the maternal mRNA that will be utilized during early development is sequestered during oogenesis into storage ribonucleoprotein particles. Assembly of these storage ribonucleoprotein particles is believed to prevent the recruitment of mRNA to ribosomes for translation until the correct developmental time [37,38]. It is possible that a similar mechanism could mask α_s mRNA and regulate its translation. To test this hypothesis, we prepared polyribosomes under conditions that extract all ribosomes, whether free or bound to membrane or cytoskeleton [39]. We fractionated the polyribosomal and non-polyribosomal mRNA from GH₃ cells by sucrose-density-gradient centrifugation and performed RNase protection analysis of mRNA in pooled fractions. For comparison, we also measured the α_{12} mRNA. Most of the α_s , α_o and α_{12} mRNA can be detected in the ribosomal and polyribosomal fraction, and only small amounts can be detected in non-ribosomal fractions (Figure 6). As a control, the U3 RNA, a non-translatable RNA component for pre-ribosomal RNA processing, was found primarily at the top of the gradient, showing that not all RNA was in large structures. We did not attempt to measure polysome size, because in these studies we only wanted to determine how much of the α_s and α_o mRNA was free. In the presence of 10 mM EDTA, which dissociates polyribosomes [40], all of the RNA was found at the top of the gradient (Figure 6). The amount of mRNA in the gradient without EDTA appears to be less than that in the gradient with EDTA, probably because it is spread over several fractions. The fact that the U3 RNA is equal under both conditions shows that there are no major losses. These results suggest that most of the α_s , α_o and α_{12} mRNA is able to bind ribosomes and is capable of engaging protein translation.

Protein synthesis is initiated by binding mRNA to ribosomes. Because we find that most of the α_s and α_o mRNA is bound to ribosomes and polyribosomes, our results suggest that the translation of α_s mRNA may be controlled in the elongation and

Figure 6 RNase protection analysis of RNA from sucrose density gradient with or without EDTA

Top: A_{260} profile of the sucrose density gradient. 12 ml 15–40% sucrose gradients with a 2 ml 60% sucrose cushion were used to separate polysomal mRNA and non-polysomal mRNA. Fractions were pooled into four fractions according to A_{260} . The 80 S ribosome sediment in the middle of the gradient and the polyribosome at the bottom of the gradient [25]. Bottom: RNase protection analysis of RNA from the four pools. Because of the dramatic difference in mRNA abundance between α_s or U3 and α_o or α_{12} , the protected α_o and α_{12} bands were exposed for 8 days at -70°C and the protected α_s and U3 bands were exposed for 2 h at -70°C .

termination steps rather than at initiation (the most common site of translational control). The synthesis of heat-shock protein 70 in chicken reticulocytes appears to be regulated at the elongation step [41]. A similar phenomenon has been seen with thymidine kinase, which is expressed in a replication-dependent manner. Proliferating cells have more thymidine kinase than their growth-arrested quiescent cells or terminally differentiated counterparts. The synthesis of thymidine kinase increases when cells enter the cell cycle, without any change in thymidine kinase mRNA levels nor in the thymidine kinase degradation rate. Regardless of the rate of thymidine kinase synthesis, all the thymidine kinase mRNA is associated with polyribosomes both in proliferating and quiescent cells [42,43].

Possible mechanism for the regulation of intracellular G-protein synthesis

Individual cells contain many subtypes of G-proteins. Both the amount and subtypes of G-proteins in individual cells affect the

cell's response to extracellular signals. Conversely, the intracellular levels of G-proteins may also depend on the type, strength and frequency of external signals. An α_s subunit containing a mutation that keeps it permanently activated is degraded faster than wild-type α_s [36]. If activated α_s (GTP- α_s) is degraded faster than inactive α_s (GDP- α_s), more α_s protein would have to be synthesized to maintain a constant intracellular level of α_s after cells are stimulated with a ligand-receptor complex. Therefore, the regulation of the translation rate of α_s mRNA might be a critical step to maintain the intracellular α_s level. In transgenic animals, in which the α_s gene was controlled by a cardiomyocyte-specific promoter, the level of α_s mRNA was 38-fold higher than that of wild-type animals, while the level of α_s protein was only 2.8-fold higher than that of wild-type animals [44]. Similarly, there is a 79-fold increase in the α_s mRNA level in cardiomyocytes infected with adenovirus carrying α_s cDNA, but only 5-fold increase in the α_s protein level [45]. These results are consistent with our suggestion that translation of α_s mRNA may be a step at which the intracellular level of α_s protein is regulated. Our results, and the results cited above, re-emphasize that for α_s it may be misleading to assume that changes in mRNA levels accurately reflect changes in protein levels.

Several mechanisms might account for the difference in the efficiency of α_s and α_o synthesis. Our results suggest that differences in elongation rate may be one of the factors. The elongation rate is partially dependent on specific tRNA abundance and therefore is sensitive to codon usage. However, we could find no major difference in codon usage between α_s and α_o mRNA. The difference in the efficiency of mRNA translation might be the consequence of sequence difference in the 5' or 3' non-coding region. Finally, α_s and α_o might be synthesized in different compartments. The α_o protein is myristoylated and palmitoylated, while the α_s protein is only palmitoylated. Myristoylation occurs during the early stage of translation and is not reversible. Possibly, myristoylation of α_o allows an α_o mRNA-polyribosome complex to anchor to a particular compartment, such as endoplasmic reticulum. The mRNAs for α_s and α_o might be differentially distributed in the cells, like the mRNA for some other proteins that are bound to the cytoskeleton or to membranes [39]. Taken together, our study has demonstrated that the α_s and α_o mRNAs are translated at different efficiencies in GH₃ cells, and that the different rates of α_s and α_o synthesis play an important role in setting the relative levels of different subunits of G-proteins. These mechanisms may be important for controlling the response of cells to external signals.

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