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The transforming activity of activated Gα12

Huiping Jiang, Dianqing Wu, Melvin I. Simon*

Division of Biology, 147-75, California Institute of Technology, Pasadena, CA 91125, USA

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Heterotrimeric GTP-binding proteins transduce receptor-mediated extracellular signals to their effectors. Several activated mutations of Gsα and Giα have been shown to be associated with endocrine tumors. In this report, we have evaluated the transforming activity of an putative activated form of Gα12 subunit. We found that transient expression in NIH3T3 cells of the Gα12 mutant with substitution of glutamine-229 for leucine could lead to focus formation and that stably transfected NIH3T3 cells could form colonies in soft agar and tumors in nude mice

G protein, Transformation; Mutation

1. INTRODUCTION

The constitutive activation of key elements in a variety of signaling systems can lead to oncogenesis. Thus, for example, mutations in the *ras* gene that stabilize it in its GTP-bound form have been associated with a wide variety of tumors, and there is good evidence for a causal role of these mutations in oncogenesis [1]. Other mutations leading to constitutively signaling receptors or to the activation of other components of this pathway such as the Raf serine-threonine kinase have also been shown to be oncogenic [2]. In addition, there are a variety of reports that constitutive signaling through other pathways can lead to oncogenesis, thus, for example, mutation or overproduction of G-Protein Couple receptor proteins has been shown to be oncogenic [3–5] and there are specific examples of activated forms of the heterotrimeric GTP binding protein that act as signal transducers in this pathway such as the Gsα and Giα proteins that appear to be associated with human endocrine tumors [6,7]. There are at least 16 different genes that encode Gα subunits in mammalian systems. They can be arranged into four families on the basis of their amino acid sequence similarities Gs, Gi, Gq, and G12 [8]. It is not clear that activating mutations in each of the different GTP binding Gα subunits can lead to neoplastic transformation. Thus, for example, while a mutant of the α mutant of Gi2 was found to induce transformation in Rat1 cells, it was not very effective in NIH3T3 cells [9–11]. There are also various different reports about the activity of the Cq class of GTP binding alpha subunit proteins. While some groups have found that activated forms of Cαq are associated with transformation [12,13], we have found that activation of Gαq leads primarily to cell death [14]. Surviving cells may be tumorigenic, however, it is not clear whether the Gαq protein acts to select oncogenic cells or if it is involved directly, i.e. as a causal agent in the transformation event. Recently, using an expression cloning technique, it has been found that the overexpression of Gα12 in NIH3T3 cells leads to transformation [15]. In order to further test the oncogenic activity of Gα12, we have prepared an activated mutation in Gα12 cDNA and expressed the cDNA using the CMV promoter. Our results suggest that the activated mutation can efficiently transform NIH3T3 cells and a large fraction of the stable transformed cells can lead to the formation of colonies in soft agar and tumorigenesis in nude mice.

*Corresponding author. Fax: (1) (818) 796 7066.

Abbreviations. G protein; GTP-binding protein, PCR, polymerase chain reaction; FCS, fetal calf serum.

2. MATERIALS AND METHODS

2.1. *Construction of expression plasmids of Gα12 and Gα12QL*

cDNA encoding Gα12 or Gα12QL was carried by a pCMV vector [14], which has a human cytomegaloviral promoter and enhancer. The putative GTPase-deficient mutant Gα12QL with substitution of glutamine-229 for leucine was made by site-directed mutagenesis with PCR [16] and mutations were verified by DNA sequencing.

2.2. *Cell culture and transfection*

NIH3T3 cells (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS). For focus-forming assay, cells were seeded into 10 cm dishes at a density of 5×10^5 the day before transfection Expression plasmid DNAs (20 μg per dish) were introduced into cells with the calcium phosphate precipitation method by using a Stratagene transfection kit. Foci were visualized by staining with Methylene blue 10 days after transfection For stable transfection, cells were transfected in the same way as in the focus-forming assay, except that 2 μg of PGKneo were added to provide antibiotic selection. The stably transfected cell lines were selected with 250 μg/ml of active geneticin (BRL).

2 3. *Growth assay and tumorigenesis in nude mice*

To construct growth curves for stably transfected cell lines, cells were seeded in each well of 24-well plates at a density of 2×10^4. The numbers of cells were counted every other day. For the growth assay in low concentration of FCS, cells were seeded in DMEM containing 10% FCS and then changed into medium containing 0.1% FCS after 5 h. One hundred and fifty cells were seeded for the soft agar assay. The numbers of colonies in soft agar containing more than 20 cells were counted after three weeks.

2.4. *Expression of Gα12 or Gα12QL in the stably transfected cell lines*

Total RNA was isolated from the stably transfected cell lines. After reverse transcription cDNA was amplified by using PCR with a pair of primers corresponding to nucleotides 800–830 of the Gα12 cDNA and to a sequence from the 3′ arm of the pCMV vector. The predicted size of the PCR products should be approximately 470 bp for Gα12 and 750 bp for Gα12QL.

3. RESULTS AND DISCUSSION

To determine whether Gα12 plays a role in regulation of cell growth, we transiently transfected NIH3T3 cells with cDNAs encoding Cα12 and its mutant Gα12QL. We found that Gα12QL could induce focus formation and cells in the foci showed morphology that was characteristic of transformed cells, even though the sizes of

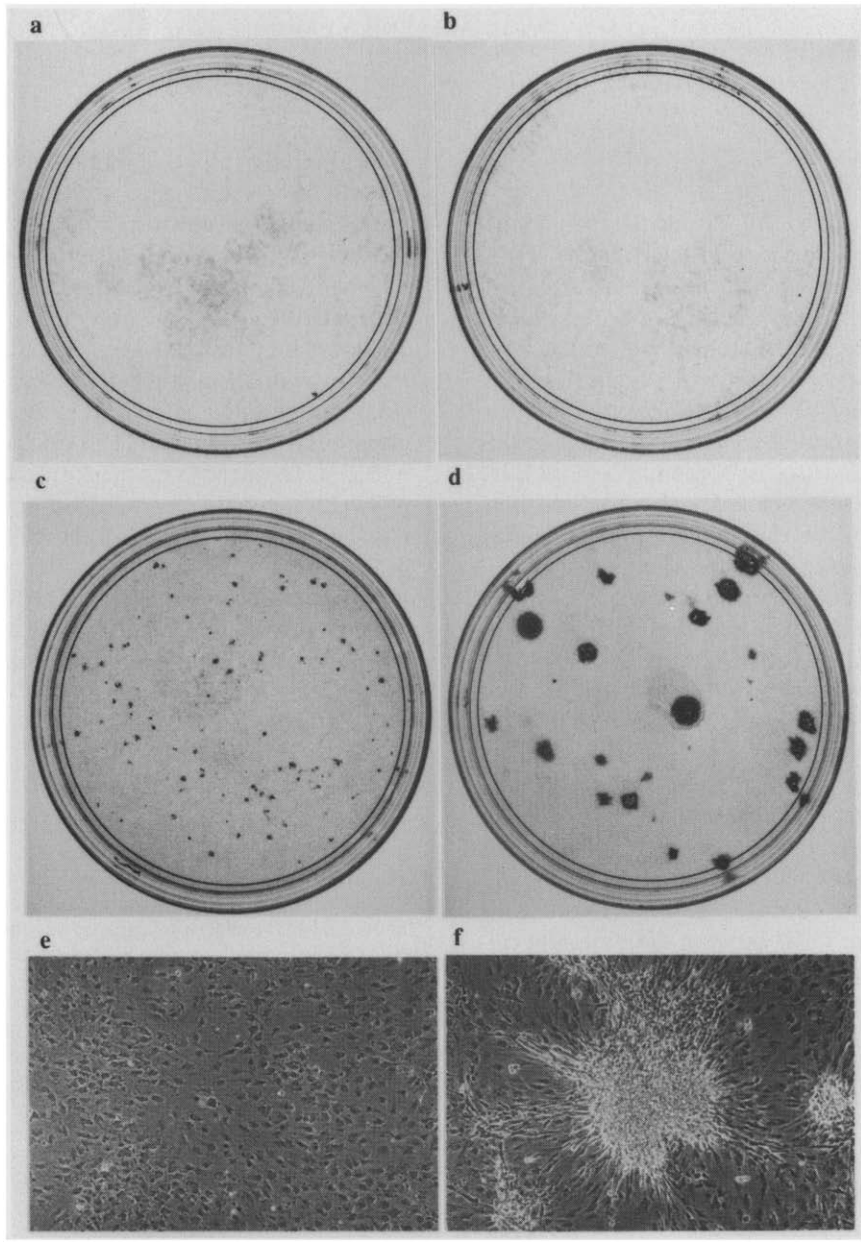

Fig. 1. Gα12QL-induced focus formation in NIH3T3 cells. NIH3T3 cells were transiently transfected with cDNA encoding vector pCMV (a), Gα12 (b), Gα12QL (c) and *ras*Q61L (d). Ten days later foci were stained with Methylene blue. The morphologies of normal NIH3T3 cells (e) and those (f) in a focus from Panel (c) are shown below

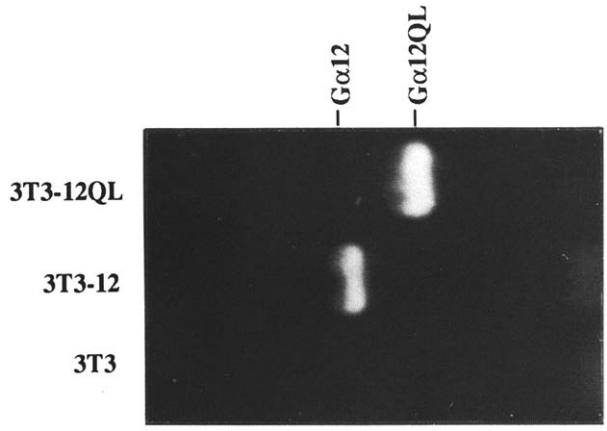

Fig. 2. Expression of Gα12 or Gα12QL in the stably transfected cell lines. Total RNA were isolated form cells transfected with PGKneo (3T3), Gα12 (3T3-12), or Gα12QL (3T3-12QL). After reverse transcription cDNA was amplified by PCR as described in section 2.

foci are generally smaller than those induced by the *ras* mutant with the equivalent substitution mutation (glutamine-61 to leucine) (Fig. 1). Cells transfected with the wild type Gα12 or the pCMV vector did not form foci (Fig. 1). Gα12QL, in which the residue glutamine-229 was replaced by leucine, is presumably activated, since similar mutations in Gαs [17], Gαi2 [18], Gα11, Gαq [14] and *ras* [1] were found to result in constitutively active proteins. The mutation probably leads to the attenuation of GTPase activity, thus fixing the protein in the GTP bound or active form. Therefore, the putative activated form of Gα12 was able to alter growth characteristics of NIH3T3 cells. These results are consistent with the finding that gene expression of the Gα12 was associated with focus formation [15].

To further investigate the potential involvement of Gα12 in transformation and tumorigenesis, we established cell lines stably expressing PGKneo, Gα12 or Gα12QL, designated 3T3-neo, 3T3-12 or 3T3-12QL. The expression of Gα12 or Gα12QL was verified by PCR amplification of the cDNA synthesized from RNA isolated from these stable cell lines. The expected sizes of PCR products were observed for both 3T3-12 and 3T3-12QL (Fig. 2). However, we were not able to clearly detect Gα12 and Gα12QL proteins with a specific antibody in these stable cell lines, though Gα12 and its mutant proteins could be clearly detected with the antibody in COS-7 cells transiently transfected with the same expression vectors. This indicates that the expression levels of Gα12 and Gα12QL driven by the COS-7 promoter in NIH3T3 cells are relatively low.

The stable transfected cell lines were examined by a number of standard tests for transformation. Up to 60% of 3T3-12QL cells formed colonies in soft agar, whereas non-transfected NIH3T3 cells, 3T3-12 and 3T3-neo cells formed few colonies (Table I). In a tumorigenesis test, tumors began to show up in all of the seven mice injected with 3T3-12QL cells after ten days, but did not show up in mice injected with non-transfected cells or cells expressing PGKneo or the wild type of Gα12 even after 40 days (Table I). The doubling time and saturation densities of these stable cell lines are not substantially different (Table I). However, only 3T3-12QL cells were able to survive in medium containing 0.1% FCS (Table I), even though cell number only doubled after six days. The low growth rate may be partially attributed to the poor attachment in the presence of low levels of serum. No live cells of 3T3, 3T3-12 or 3T3-neo were found after six days culture in 0.1% FCS. All of these results indicate that the activated form of Gα12 is capable of inducing transformation and tumorigenesis in NIH3T3 cells.

Aaronson and his co-workers [12] reported that overexpression of the wild type human Gα12 in NIH3T3 cells could induce transformation and tumorigenesis. However, in our hands, expression of Gα12 driven by the CMV promoter was not oncogenic. The inability of Gα12 carried by the pCMV vector to transform NIH3T3 cells may be due to the low expression level of this protein. Gα12 and Gα13 form a class of G proteins that is distinguished by amino acid sequence homology from all of the other G-proteins. The nature of the

Table I

Transformation of NIH3T3 cells by Gα12QL

	Saturation density[a] ($\times 10^5$ cells)	Cell doubling time[a] (h)	Survival in 0.1% of serum[b]	Growth in soft agar (%)[c]	Tumor formation in nude mice[d]
3T3	3	24	No	3	0/4
3T3-neo	6	24	No	3	0/4
3T3-12	6.4	24	No	4	0/5
3T3-12QL	9	20	Yes	60	7/7

[a] 2×10^4 cells were plated in duplicate in each well of 24-well plates. Saturation densities and cell doubling time were determined based on the growth curves.

[b] 2×10^4 cells were seeded in duplicate in each well of 24-well plates. The cells was changed to the medium containing 0.1% FCS 5 h after seeding. The numbers of cells were counted after 6 days.

[c] 150 cells were plated in 0.3% of agar and the colonies containing more than 20 cells were counted after 17 days.

[d] 2×10^5 cells were injected subcutaneously on the back of nude mices. Tumors were observed after 10 days.

effector or of the receptors that interact with these ubiquitously expressed proteins is not known. It is possible that they couple to seven pass membrane receptors that can control growth by modulating the activity of the proteins that control the cell cycle. We have also tested transforming activity of the activated form of Gα13 by using the focus formation assay and we found that activated Gα13 was also capable of inducing focus formation of NIH3T3 cells (data not shown).

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