Thrombin-induced inhibition of myoblast differentiation is mediated by Gβγ

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Abstract Thrombin has been shown to inhibit skeletal muscle differentiation. However, the mechanisms by which thrombin represses myogenesis remain unknown. Since the thrombin receptor couples to G_i, $G_{q/11}$ and G_{12}, we examined which subunits of heterotrimeric guanine nucleotide-binding regulatory proteins ($G\alpha_i$, $G\alpha_{q/11}$, $G\alpha_{12}$ or $G\beta\gamma$) participate in the thrombin-induced inhibition of C2C12 myoblast differentiation. $G\alpha_{i2}$ and $G\alpha_{11}$ had no inhibitory effect on the myogenic differentiation. $G\alpha_{12}$ prevented only myoblast fusion, whereas $G\beta\gamma$ inhibited both the induction of skeletal muscle-specific markers and the myotube formation. In addition, the thrombin-induced reduction of creatine kinase activity was blocked by the C-terminal peptide of β-adrenergic receptor kinase, which is known to sequester free $G\beta\gamma$. These results suggest that the thrombin-induced inhibition of muscle differentiation is mainly mediated by $G\beta\gamma$.

Key words: Thrombin; G protein; Gβγ; Myoblast differentiation; $G\alpha_{12}$; Myotube formation

1. Introduction

Skeletal muscle differentiation is a multistep process that involves the commitment of mesodermal precursor cells to the skeletal muscle lineage, the withdrawal of myoblasts from the cell cycle and the fusion of myoblasts into multinucleated myotubes. These stages are driven by the myogenic basic helix-loop-helix (bHLH) factors that include MyoD, myogenin, Myf5 and MRF4, as well as the myocyte enhancer factor-2. These factors induce the expression of several skeletal muscle-specific genes such as creatine kinase and myosin heavy chain (MHC). Additionally, the irreversible cell cycle arrest is controlled by the induction of cyclin-dependent kinase inhibitor $p21^{CIP1/WAF1}$ [1–3]. The ability of myoblasts to differentiate is negatively regulated by several growth factors including basic fibroblast growth factor (FGF) 2 and transforming growth factor β1 [1–3]. In addition to them, it has been reported that heterotrimeric guanine nucleotide-binding regulatory protein (G protein)-coupled receptor agonists such as thrombin [4–7] and lysophosphatidic acid [8] inhibit skeletal muscle cell differentiation.

Several lines of evidence have suggested that thrombin plays an important role in skeletal muscle development. It has been shown that thrombin is involved in synapse elimination occurring at the neuromuscular junction during muscle development [9,10]. In addition, thrombin causes a delay in skeletal myogenesis [4–7]. The thrombin receptor is expressed in cultured myoblasts and its expression decreases once the myoblasts fuse to form multinucleated myotubes [5]. Thrombin has been reported to act as a mitogen in myoblasts as well as fibroblasts and smooth muscle cells, and induce their proliferation [4,5]. In contrast, it has been reported that thrombin does not induce a mitogenic signal in myoblastic cell line [6], and that it inhibits myoblast apoptosis and functions as a survival factor for myoblasts [7].

Thrombin receptor, which is a member of the G protein-coupled receptor family, is activated by cleavage of its N-terminal extracellular domain with thrombin. The new N-terminus generated by this proteolysis acts as a tethered ligand for the receptor [11,12]. G proteins are composed of α, β and γ subunits, and mammalian G proteins are grouped into four subfamilies: Gs, G_i, G_q and G_{12} [13–15]. Activation of the thrombin receptor leads to inhibition of adenylyl cyclase and activation of phospholipase Cβ through coupling to G_i and $G_{q/11}$, respectively [16]. The receptor also couples to G_{12} in platelets [17] and in Sf9 cells expressing the receptor and $G\alpha_{12}$ [18].

In this study, we investigated which subunits of G proteins are involved in thrombin-induced repression of myogenic differentiation using the transient expression of $G\alpha_{i2}$, $G\alpha_{11}$, $G\alpha_{12}$ and $G\beta\gamma$ in mouse myogenic C2C12 cells. Our results suggest that the thrombin-induced inhibition of myoblast differentiation is mainly mediated by $G\beta\gamma$.

2. Materials and methods

2.1. Materials

Rabbit polyclonal antibody against $G\alpha_{i1/2}$ was generously provided by T. Asano (Institute for Developmental Research, Aichi Human Service Center, Kasugai, Japan). Rabbit polyclonal antibodies against $G\alpha_{11}$ (D-17) and Gβ (T-20) were purchased from Santa Cruz Biotechnology. Rabbit polyclonal antibody against $G\alpha_{12}$ and mouse monoclonal antibody against myogenin (F5D) were obtained from Calbiochem and Pharmingen, respectively. Mouse monoclonal antibody against MHC was obtained from Chemicon International. Mouse monoclonal antibody against c-Myc epitope (9E10) was purchased from BAbCO. Anti-mouse and anti-rabbit Ig antibodies conjugated with horseradish peroxidase were from New England BioLabs. Thrombin and Hoechst 33342 were obtained from Sigma.

2.2. Plasmids

Complementary DNAs encoding rat $G\alpha_{i2}$ [19], mouse $G\alpha_{11}$ [20] and rat $G\alpha_{12}$ [21], and Myc epitope-tagged β-adrenergic receptor kinase C-terminal peptide (βARKct) [22] were prepared as described previously. Constitutively activated mutants of $G\alpha_{i2}$ ($G\alpha_{i2}$Q205L),

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Abbreviations: bHLH, basic helix-loop-helix; MHC, myosin heavy chain; FGF, fibroblast growth factor; G protein, heterotrimeric guanine nucleotide-binding regulatory protein; GM, growth medium; DM, differentiation medium; Csk, C-terminal Src kinase

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$G\alpha_{11}$ ($G\alpha_{11}$Q209L) and $G\alpha_{12}$ ($G\alpha_{12}$Q229L) were made by primer-mediated mutagenesis. Bovine $G\beta_1$ and $G\gamma_2$ cDNAs were generously provided by M.I. Simon (California Institute of Technology, Pasadena, CA, USA) and T. Nukada (Tokyo Institute of Psychiatry, Tokyo, Japan), respectively. RhoA and Rac1 cDNAs were generously provided by K. Kaibuchi (Nara Institute of Science and Technology, Ikoma, Japan). Cdc42Hs cDNA was kindly provided by R.A. Cerione (Cornell University, Ithaca, NY, USA). RhoA(T19N), Rac1(T17N) and Cdc42Hs(T17N) were made by oligonucleotide-directed mutagenesis. C-terminal Src kinase (Csk) and H-Ras(S17N) cDNAs were generous gifts from M. Okada (Institute for Protein Research, Osaka University, Osaka, Japan) and G.M. Cooper (Dana-Farber Cancer Institute, Boston, MA, USA), respectively. All cDNAs were subcloned into mammalian expression vector pCMV5. The isolated cDNAs and the mutations were confirmed by dideoxynucleotide sequencing.

2.3. Cell culture and transfection

Mouse myoblast cell line C2C12 was maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma) containing 15% fetal bovine serum (Life Technologies), 50 U/ml penicillin and 100 μg/ml streptomycin (growth medium, GM). The cells were cultured at 37°C in a humidified 10% CO_2 environment. Differentiation of the cells was induced at 80–90% confluence by changing the culture medium from GM to DMEM containing 2% horse serum (Life Technologies) and antibiotics (differentiation medium, DM). Transfection was performed using LipofectAMINE Plus reagent (Life Technologies) in serum-free medium according to the manufacturer's instructions. At 2 h after transfection, the medium was changed from serum-free medium to GM. After a 24 h incubation in GM, the cells were transferred to DM.

2.4. Creatine kinase assay

The cells were washed twice with phosphate-buffered saline and lysed in lysis buffer (20 mM HEPES–NaOH (pH 7.5), 5 mM $MgCl_2$, 150 mM NaCl, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM EGTA, 1 mM EDTA, 1 mM Na_3VO_4, 10 mM NaF, 25 mM β-glycerophosphate, 10% glycerol and 0.5% Triton X-100) on ice. The cell lysates were centrifuged at 15 000 rpm for 10 min at 4°C and the supernatants were used for creatine kinase assay. Creatine kinase activity was measured with a creatine kinase assay kit (Sigma) and the activity was normalized to protein content, which was determined by the Bradford method with bovine serum albumin as standard protein.

2.5. Evaluation of myoblast fusion

The cells were rinsed twice with phosphate-buffered saline and fixed with 3% paraformaldehyde in phosphate-buffered saline at room temperature. After staining with Hoechst 33342, nuclei were counted and the fusion index (%) was calculated. Fusion index (%) = (number of nuclei in myotubes/total number of nuclei in both myoblasts and myotubes) × 100.

2.6. Immunoblot analysis

Cell lysates were boiled in sodium dodecyl sulfate (SDS) sample buffer. The boiled samples were resolved by SDS–PAGE and transferred to nitrocellulose membranes. The membranes were blocked using 1% bovine serum albumin in phosphate-buffered saline containing 0.1% Tween 20 and incubated with each specific antibody. Immunoreactive bands were visualized by using secondary horseradish peroxidase-conjugated antibodies and chemiluminescence (NEN Life Science Products).

3. Results

3.1. Thrombin has an inhibitory effect on myoblast differentiation

To clarify the involvement of G proteins in skeletal myogenesis, we first examined the effect of thrombin on C2C12 cell differentiation. C2C12 myoblasts were grown to near confluence in GM and then shifted to DM in the absence or the presence of thrombin. In the absence of thrombin, myotube formation typically began 2–3 days following the replacement of medium, whereas the myoblast fusion was markedly inhibited in the thrombin-treated cells (Fig. 1A). We next examined whether thrombin affects the induction of muscle differentiation markers such as creatine kinase and myogenin. The induction of creatine kinase and the expression of myogenin were significantly reduced by thrombin (Fig. 1B,C). These results indicate that thrombin blocks the myoblast differentiation.

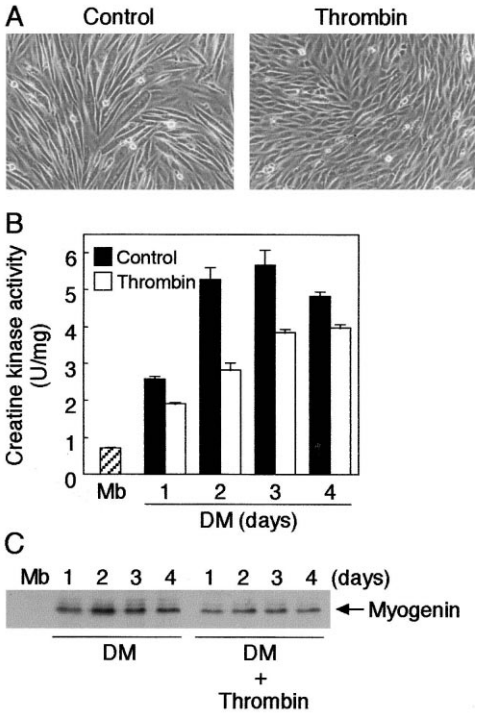

Fig. 1. Effect of thrombin on C2C12 cell differentiation. A: C2C12 myoblasts were grown to 80–90% confluence and then induced to differentiate for 2 days in the absence or the presence of 1 U/ml thrombin. Phase-contrast photomicrographs of representative fields are shown. B: Myoblasts (Mb) were differentiated with DM in the absence or the presence of 1 U/ml thrombin and creatine kinase activity was measured at the times indicated. Values represent the mean ± S.E.M. from at least three independent experiments. C: Western blot analysis of myogenin expression in myoblasts (Mb) or myoblasts cultured in DM for the times indicated in the absence or the presence of 1 U/ml thrombin.

3.2. Inhibitory effect of thrombin on the induction of muscle cell differentiation markers is mediated by Gβγ

Since it has been shown that the thrombin receptor couples to G_i, $G_{q/11}$ and G_{12} [16–18], we investigated which subunits of G proteins ($G\alpha_i$, $G\alpha_{q/11}$, $G\alpha_{12}$ or Gβγ) are implicated in the thrombin-induced inhibition of C2C12 cell differentiation using the transient expression of each subunit. In C2C12 myoblasts, endogenous $G\alpha_{i2}$, $G\alpha_{11}$ and $G\alpha_{12}$ were expressed (Fig. 2A). Although the expression of the thrombin receptor has been shown to decrease during cell differentiation [5], the expression levels of endogenous $G\alpha_{i2}$, $G\alpha_{11}$, $G\alpha_{12}$ and Gβγ did not change up to 7 days after induction of differentiation (data not shown). The cells were transfected with wild-type $G\alpha_{i2}$, $G\alpha_{11}$ and $G\alpha_{12}$, constitutively activated mutants of $G\alpha_{i2}$ ($G\alpha_{i2}$Q205L), $G\alpha_{11}$ ($G\alpha_{11}$Q209L) and $G\alpha_{12}$ ($G\alpha_{12}$Q229L), or Gβγ. Lysates of the transfected cells were resolved by SDS–PAGE and the expression of these proteins was detected by immunoblotting using each specific antibody.

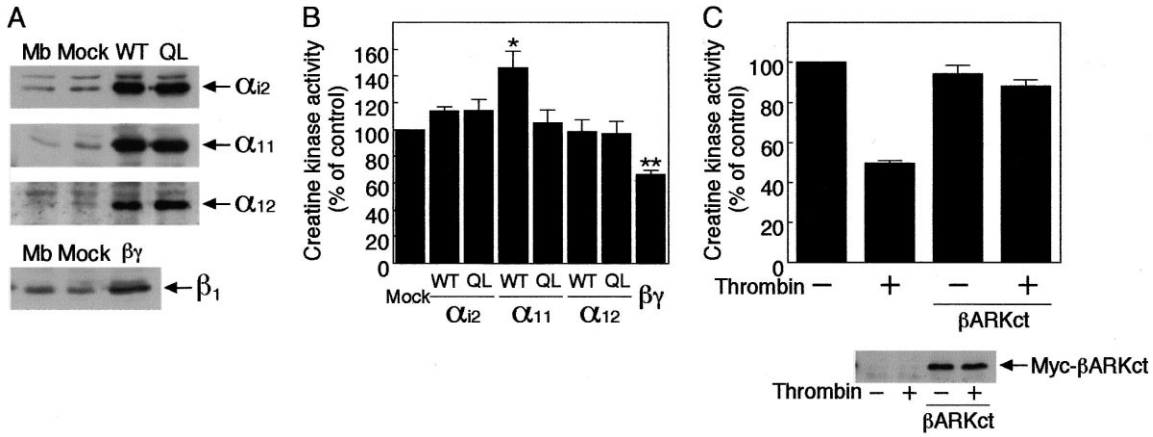

Fig. 2. Effects of $G\alpha_{i2}$, $G\alpha_{11}$, $G\alpha_{12}$ and $G\beta\gamma$ on the induction of creatine kinase activity during C2C12 cell differentiation. A: Cells were transfected with an empty expression vector (Mock), the plasmid of wild-type Gα (WT) or active Gα (QL) (1 μg) as indicated, or the plasmids of $G\beta_1$ (1 μg) and $G\gamma_2$ (1 μg). Equal amounts of cell lysates were subjected to SDS–PAGE and the separated proteins were immunoblotted with each specific antibody. B: Cells were transfected with an empty expression vector (Mock), the plasmid of wild-type Gα (WT) or active Gα (QL) as indicated, or the plasmids of $G\beta_1$ and $G\gamma_2$. Creatine kinase activity was measured at 3 days after initiation of differentiation. The data are expressed as the percent of creatine kinase activity in Mock-transfected cells. C: Cells were transfected with an empty expression vector or the plasmid of Myc-βARKct and cultured in DM in the absence or the presence of 1 U/ml thrombin. Creatine kinase activity was measured at 2 days after initiation of differentiation. The data are expressed as the percent of creatine kinase activity in Mock-transfected cells in the absence of thrombin. The expression level of Myc-βARKct was determined by immunoblotting using anti-Myc antibody. All values represent the mean ± S.E.M. from at least three separate experiments. Mb shows myoblasts. * and ** mean $P < 0.05$ and $P < 0.01$, respectively, as compared to Mock.

Each Gα and Gβ were overexpressed in the transfected cells (Fig. 2A). Wild-type $G\alpha_{i2}$, $G\alpha_{i2}$Q205L, wild-type $G\alpha_{12}$ and $G\alpha_{12}$Q229L did not exhibit any significant effect on the creatine kinase induction as compared to control experiment with Mock-transfected cells (Fig. 2B). Interestingly, wild-type $G\alpha_{11}$, but not $G\alpha_{11}$Q209L, potentiated the induction slightly (Fig. 2B). In contrast, Gβγ suppressed the induction of creatine kinase activity (Fig. 2B). To confirm the involvement of Gβγ in the inhibition of creatine kinase activity by thrombin (Fig. 1B), we further examined the effect of βARKct, which is known to sequester free Gβγ [23], on the thrombin-induced inhibition. Expression of βARKct prevented the thrombin-induced reduction of creatine kinase activity (Fig. 2C). These results suggest that the reduction of creatine kinase activity by thrombin is mediated by Gβγ.

We next investigated the effects of $G\alpha_{i2}$, $G\alpha_{11}$, $G\alpha_{12}$ and Gβγ on the expression of myogenin and MHC. In Mock-transfected cells, the expression of myogenin and MHC was detected at 1 day and 2 days after initiation of differentiation, respectively (Fig. 3). In agreement with the results shown in Fig. 2B, overexpression of wild-type $G\alpha_{i2}$, $G\alpha_{i2}$Q205L, wild-type $G\alpha_{12}$ and $G\alpha_{12}$Q229L had no significant effect on the induction of myogenin and MHC relative to control. In wild-type $G\alpha_{11}$-transfected cells, myogenin has already been expressed under the growth condition (day 0) and MHC was induced 1 day earlier. On the other hand, the expression of myogenin and MHC in Gβγ-transfected cells was reduced and delayed as compared to Mock-transfected cells.

3.3. $G\alpha_{12}$ and Gβγ inhibit myotube formation

We overexpressed $G\alpha_{i2}$, $G\alpha_{11}$, $G\alpha_{12}$ and Gβγ in C2C12 cells and observed the morphology of myoblasts that were induced to differentiate for 4 days (Fig. 4). The ability of wild-type $G\alpha_{i2}$-, $G\alpha_{i2}$Q205L- and $G\alpha_{11}$Q209L-transfected cells to form multinucleated myotubes was comparable to that of Mock-transfected cells (Fig. 4A–C,E). As expected, wild-type $G\alpha_{11}$ accelerated myotube formation, while Gβγ delayed it (Fig. 4D,H). Unexpectedly, wild-type $G\alpha_{12}$ and $G\alpha_{12}$Q229L had an inhibitory effect on myotube fusion (Fig. 4F,G) although they were not able to inhibit the induction of biochemical differentiation markers such as creatine kinase, myogenin and MHC (Figs. 2B and 3). In Mock-transfected cells, approximately 35% of myoblast nuclei were present in myotubes at 5 days in DM. In contrast, the fusion index in wild-type $G\alpha_{12}$- or $G\alpha_{12}$Q229L-transfected cells was reduced to approximately 17 or 7%, respectively. The inhib-

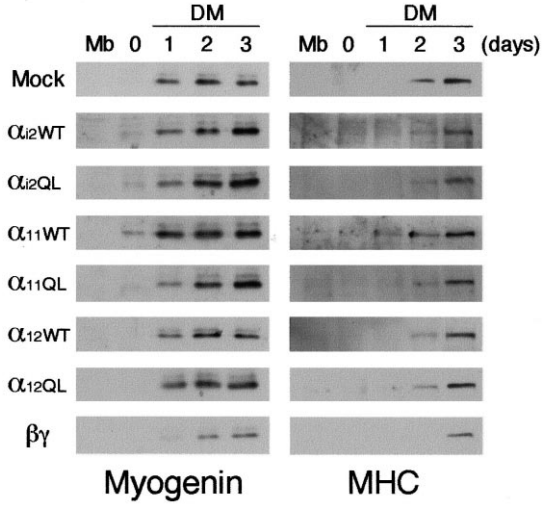

Fig. 3. Effects of $G\alpha_{i2}$, $G\alpha_{11}$, $G\alpha_{12}$ and Gβγ on the induction of myogenin and MHC. Cells were transfected with an empty expression vector (Mock), the plasmid of wild-type Gα (αWT) or active Gα (αQL) (1 μg) as indicated, or the plasmids of $G\beta_1$ (1 μg) and $G\gamma_2$ (1 μg). Cell lysates were resolved by SDS–PAGE, and the expression of myogenin and MHC in myoblasts (Mb) or the transfected cells was detected by immunoblotting using anti-myogenin and anti-MHC antibodies, respectively.

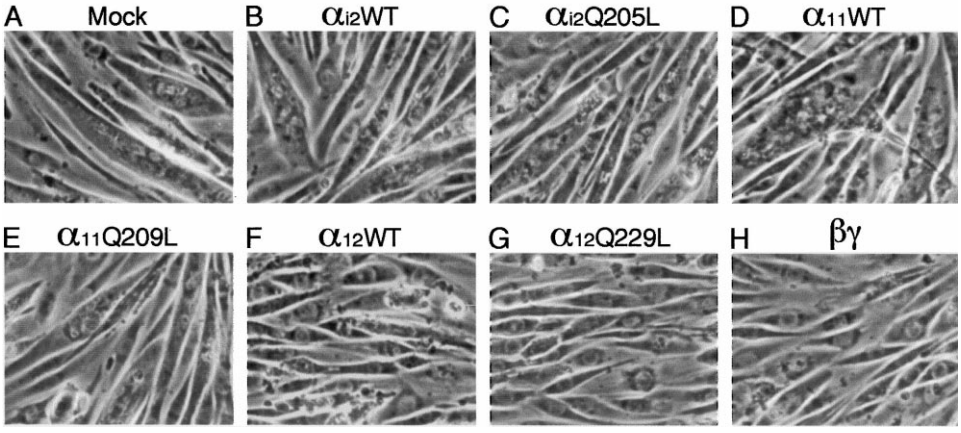

Fig. 4. Effects of $G\alpha_{i2}$, $G\alpha_{11}$, $G\alpha_{12}$ and $G\beta\gamma$ on myotube formation. Cells were transfected with an empty expression vector (Mock) (A), the plasmid of wild-type $G\alpha_{i2}$ (B), $G\alpha_{i2}Q205L$ (C), wild-type $G\alpha_{11}$ (D), $G\alpha_{11}Q209L$ (E), wild-type $G\alpha_{12}$ (F) and $G\alpha_{12}Q229L$ (G), or the plasmids of $G\beta_1$ and $G\gamma_2$ (H), and cultured in DM for 4 days. Phase-contrast photomicrographs of representative fields are shown.

itory effect of $G\alpha_{12}Q229L$ on the myotube formation retained even 7 days in DM (data not shown). We previously reported that Rho and Src family kinase function downstream of $G\alpha_{12}$ in the signaling pathways from $G\alpha_{12}$ to JNK in HEK 293 cells [21]. In addition, several studies indicate that $G\alpha_{12}$ regulates the Rho family GTPases-dependent signaling pathways [24–28]. Therefore, we examined the effects of dominant-negative RhoA(T19N), Rac1(T17N) and Cdc42Hs(T17N), and Csk, a cytoplasmic protein tyrosine kinase which inactivates Src family kinases [29], on the myotube formation of $G\alpha_{12}Q229L$-transfected cells. The expression of RhoA(T19N), Rac1(T17N), Cdc42Hs(T17N) or Csk alone had no significant effect on the myotube formation, and the transfected cells were able to form multinucleated myotubes (data not shown). When the cells were coexpressed with RhoA(T19N), Rac1(T17N), Cdc42Hs(T17N) or Csk together with $G\alpha_{12}Q229L$, they failed to rescue the fusion defect induced by $G\alpha_{12}Q229L$ (data not shown).

4. Discussion

It has been reported that thrombin inhibits skeletal muscle cell differentiation [4–7]. In the present study, we showed that thrombin reduced the induction of muscle differentiation markers such as creatine kinase and myogenin, and delayed myotube formation in C2C12 cells (Fig. 1). However, the mechanisms by which thrombin inhibits muscle differentiation have not been fully elucidated. Since the thrombin receptor has been shown to couple to G_i, $G_{q/11}$ and G_{12} [16–18], we examined which subunits of G proteins ($G\alpha_i$, $G\alpha_{q/11}$, $G\alpha_{12}$ or $G\beta\gamma$) are involved in the thrombin-induced inhibition of C2C12 cell differentiation. $G\alpha_{i2}$ and $G\alpha_{12}$ have no apparent effect on creatine kinase induction (Fig. 2B) and the expression of myogenin and MHC (Fig. 3) as compared to control. In contrast, wild-type $G\alpha_{11}$, but not $G\alpha_{11}Q209L$, enhanced the creatine kinase induction (Fig. 2B) and induced the earlier expression of myogenin and MHC (Fig. 3). Although the regulatory mechanisms by which wild-type $G\alpha_{11}$ promotes the induction remain to be clarified at present, some of $G_{q/11}$-coupled receptors other than the thrombin receptor may participate in the induction of muscle cell differentiation. Since $G\alpha_{11}Q209L$ failed to promote the creatine kinase induction (Fig. 2B) and the induction of myogenin and MHC (Fig. 3), the transient activation and/or deactivation of $G\alpha_{q/11}$ at some stage during myogenesis might be required for the muscle differentiation.

$G\beta\gamma$ repressed the creatine kinase induction (Fig. 2B) and the expression of myogenin and MHC (Fig. 3). In addition, βARKct, which sequesters free $G\beta\gamma$, blocked the thrombin-induced attenuation of creatine kinase activity (Fig. 2C). Moreover, the expression of $G\beta\gamma$, as well as thrombin, delayed myotube formation (Figs. 1A and 4H). These data suggest that the inhibitory effect of thrombin on myogenesis is mediated by $G\beta\gamma$. Consistent with our observation, it has been reported that the βγ subunits of pertussis toxin-sensitive G proteins are involved in FGF-mediated repression of myogenesis in mouse skeletal muscle satellite cell line MM14 [30]. However, the molecular mechanisms leading to activation of pertussis toxin-sensitive G proteins by the stimulation of the FGF receptor which is a receptor tyrosine kinase but not G protein-coupled receptor remain unknown. The $G\beta\gamma$-mediated signaling pathways involved in repression of myogenic differentiation are currently under investigation.

Wild-type $G\alpha_{12}$ and $G\alpha_{12}Q229L$ prevented myotube formation (Fig. 4F,G) despite no inhibitory effect on the induction of creatine kinase, myogenin and MHC (Figs. 2B and 3). Some reports have shown that myoblast fusion is independent of the expression of biochemical differentiation markers [31–34]. Rho, Rac and Cdc42 have been shown to be required for myogenic differentiation [35,36]. In contrast, it has recently been reported that the transient expression of constitutively activated Rac and Cdc42 in primary myoblasts causes the inhibition of muscle cell differentiation [37]. In *Drosophila*, the expression of activated Rac in muscle precursors also disrupts myoblast fusion [38]. Furthermore, it has been shown that Src family tyrosine kinase inhibits skeletal myogenesis [39]. However, Rho family GTPases and Src family kinase are unlikely to participate in the $G\alpha_{12}$-mediated inhibition of myoblast fusion since dominant-negative mutants of Rho, Rac and Cdc42, and Csk failed to restore the $G\alpha_{12}Q229L$-induced fusion defect (data not shown). It has been reported that adhesion molecules including N- and M-cadherins, N- and V-CAMs, and integrins are involved in myoblast fusion [40]. $G\alpha_{12}$ in myoblasts may regulate these adhesion molecules through Rho family GTPases- and Src family kinase-independent mechanisms. Further studies are necessary to eluci-

date the molecular mechanisms by which $G\alpha_{12}$ inhibits myoblast fusion.

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References

[1] Lassar, A.B., Skapek, S.X. and Novitch, B. (1994) Curr. Opin. Cell Biol. 6, 788–794.
[2] Ludolph, D.C. and Konieczny, S.F. (1995) FASEB J. 9, 1595–1604.
[3] Molkentin, J.D. and Olson, E.N. (1996) Curr. Opin. Genet. Dev. 6, 445–453.
[4] Kelvin, D.J., Simard, G., Sue-A-Quan, A. and Connolly, J.A. (1989) J. Cell Biol. 108, 169–176.
[5] Suidan, H.S., Niclou, S.P., Dreessen, J., Beltraminelli, N. and Monard, D. (1996) J. Biol. Chem. 271, 29162–29169.
[6] Guttridge, D.C., Lau, A., Tran, L. and Cunningham, D.D. (1997) J. Biol. Chem. 272, 24117–24120.
[7] Chinni, C., de Niese, M.R., Tew, D.J., Jenkins, A.L., Bottomley, S.P. and Mackie, E.J. (1999) J. Biol. Chem. 274, 9169–9174.
[8] Yoshida, S., Fujisawa-Sehara, A., Taki, T., Arai, K. and Nabeshima, Y. (1996) J. Cell Biol. 132, 181–193.
[9] Liu, Y., Fields, R.D., Festoff, B.W. and Nelson, P.G. (1994) Proc. Natl. Acad. Sci. USA 91, 10300–10304.
[10] Zoubine, M.N., Ma, J.Y., Smirnova, I.V., Citron, B.A. and Festoff, B.W. (1996) Dev. Biol. 179, 447–457.
[11] Vu, T.-K.H., Hung, D.T., Wheaton, V.I. and Coughlin, S.R. (1991) Cell 64, 1057–1068.
[12] Vu, T.-K.H., Wheaton, V.I., Hung, D.T., Charo, I. and Coughlin, S.R. (1991) Nature 353, 674–677.
[13] Gilman, A.G. (1987) Annu. Rev. Biochem. 56, 615–649.
[14] Kaziro, Y., Itoh, H., Kozasa, T., Nakafuku, M. and Satoh, T. (1991) Annu. Rev. Biochem. 60, 349–400.
[15] Simon, M.I., Strathmann, M.P. and Gautam, N. (1991) Science 252, 802–808.
[16] Hung, D.T., Wong, Y.H., Vu, T.-K.H. and Coughlin, S.R. (1992) J. Biol. Chem. 267, 20831–20834.
[17] Offermanns, S., Laugwitz, K.-L., Spicher, K. and Schultz, G. (1994) Proc. Natl. Acad. Sci. USA 91, 504–508.
[18] Barr, A.J., Brass, L.F. and Manning, D.R. (1997) J. Biol. Chem. 272, 2223–2229.
[19] Itoh, H., Kozasa, T., Nagata, S., Nakamura, S., Katada, T., Ui, M., Iwai, S., Ohtsuka, E., Kawasaki, H., Suzuki, K. and Kaziro, Y. (1986) Proc. Natl. Acad. Sci. USA 83, 3776–3780.
[20] Umemori, H., Inoue, T., Kume, S., Sekiyama, N., Nagao, M., Itoh, H., Nakanishi, S., Mikoshiba, K. and Yamamoto, T. (1997) Science 276, 1878–1881.
[21] Nagao, M., Kaziro, Y. and Itoh, H. (1999) Oncogene 18, 4425–4434.
[22] Sun, Y., Yamauchi, J., Kaziro, Y. and Itoh, H. (1999) J. Biochem. 125, 515–521.
[23] Koch, W.J., Hawes, B.E., Inglese, J., Luttrell, L.M. and Lefkowitz, R.J. (1994) J. Biol. Chem. 269, 6193–6197.
[24] Buhl, A.M., Johnson, N.L., Dhanasekaran, N. and Johnson, G.L. (1995) J. Biol. Chem. 270, 24631–24634.
[25] Collins, L.R., Minden, A., Karin, M. and Brown, J.H. (1996) J. Biol. Chem. 271, 17349–17353.
[26] Voyno-Yasenetskaya, T.A., Faure, M.P., Ahn, N.G. and Bourne, H.R. (1996) J. Biol. Chem. 271, 21081–21087.
[27] Fromm, C., Coso, O.A., Montaner, S., Xu, N. and Gutkind, J.S. (1997) Proc. Natl. Acad. Sci. USA 94, 10098–10103.
[28] Katoh, H., Aoki, J., Yamaguchi, Y., Kitano, Y., Ichikawa, A. and Negishi, M. (1998) J. Biol. Chem. 273, 28700–28707.
[29] Nada, S., Okada, M., MacAuley, A., Cooper, J.A. and Nakagawa, H. (1991) Nature 351, 69–72.
[30] Fedorov, Y.V., Jones, N.C. and Olwin, B.B. (1998) Mol. Cell. Biol. 18, 5780–5787.
[31] Hu, J.-S. and Olson, E.N. (1990) J. Biol. Chem. 265, 7914–7919.
[32] Crescenzi, M., Crouch, D.H. and Tatò, F. (1994) J. Cell Biol. 125, 1137–1145.
[33] Bennett, A.M. and Tonks, N.K. (1997) Science 278, 1288–1291.
[34] Russo, S., Tomatis, D., Collo, G., Tarone, G. and Tatò, F. (1998) J. Cell Sci. 111, 691–700.
[35] Takano, H., Komuro, I., Oka, T., Shiojima, I., Hiroi, Y., Mizuno, T. and Yazaki, Y. (1998) Mol. Cell. Biol. 18, 1580–1589.
[36] Wei, L., Zhou, W., Croissant, J.D., Johansen, F.-E., Prywes, R., Balasubramanyam, A. and Schwartz, R.J. (1998) J. Biol. Chem. 273, 30287–30294.
[37] Gallo, R., Serafini, M., Castellani, L., Falcone, G. and Alemà, S. (1999) Mol. Biol. Cell 10, 3137–3150.
[38] Luo, L., Liao, Y.J., Jan, L.Y. and Jan, Y.N. (1994) Genes Dev. 8, 1787–1802.
[39] Alemà, S. and Tatò, F. (1994) Semin. Cancer Biol. 5, 147–156.
[40] McDonald, K.A., Horwitz, A.F. and Knudsen, K.A. (1995) Semin. Dev. Biol. 6, 105–116.