Korean J Biol Sci 1: 93-98, 1997

Colocalization of α-Subunit of Gq Protein with Actin Filaments in L8E63 Cells

Sungsuk Chae and Dongeun Park*

Department of Life Science, Kwang-Ju Institute of Science and Technology, Kwang-Ju 506-303, Korea

Key Words:
Gαq
F-actin
Colocalization
L8E63
Differentiation
Immunofluorescence

The present study investigated the cellular localization of α-subunit of Gq (Gαq) protein in developing L8E63, rat skeletal muscle cell line. The colocalization of Gαq with actin cytoskeleton was demonstrated by double-labeling experiments. In mononucleated myoblasts, the immunofluorescence staining pattern of Gαq was almost identical with that of F-actin visualized with rhodamine-conjugated phalloidin. However, this colocalization of Gαq with cytoskeleton was not maintained in multinucleated myotubes. The staining pattern of Gαq in myotubes did not match with any specific subcellular structure, but appeared as a uniformly distributed diffuse staining throughout the whole cell surface. Interestingly, change in the expression level of Gαq was not detected during myoblast differentiation, suggesting that actin-associated Gαq protein might dissociate from the cytoskeleton as cells differentiate. Immunocytochemical experiments using specific antibodies directed against several G proteins indicated that the subcellular localizations of Gαi1, Gαi2, Gαi3, and Gαo were different from those obtained with Gαq.

Guanine nucleotide-binding proteins (G protein) are involved in many signal transduction processes in a variety of cells. Many different kinds of transmembrane receptors, such as hormone, neurotransmitter, and sensory receptors, are coupled via G proteins to a variety of effectors, including adenylyl cyclase, phospholipases, phosphodiesterases, and potassium and calcium channels (Birnbaumer, 1992; Neer, 1995). Heterotrimeric G protein, which is composed of α, β, and γ subunits, constitutes a large family of proteins that relay signals from cell surface receptor to the intracellular effector. Gq class of G proteins include four different α subunit members, α_q, α_{11}, α_{14}, and α_{16} (Simon et al., 1991). All the α subunits of the Gq class of G proteins are known to activate phospholipase C-β isozymes (Blank et al., 1991, Jhon et al., 1993) which generate two important second messengers, diacylglycerol (DAG) and inositol 1, 4, 5-triphosphate (IP_3) by hydrolyzing phosphatidyl inositol 4, 5-bisphosphate (PIP_2). Gαq is widely distributed in tissues and cell lines. Gαq is known to mediate responses to many stimuli such as vasopressin, bombesin, angiotensin II, m_1, m_3 muscarinic agonists and, α_2 adrenergic agonists which activate phospholipase C via a pertussis toxin insensitive manner (Lee and Rhee, 1995). Although the mechanisms of receptor-mediated Gαq activation and coupling to Phospholipase C-β isozymes have been extensively studied, little information is available on the celluar localization of Gαq protein. Recently, Ibarrondo et al. (1995) reported close association of Gαq protein with actin filaments in WRK1 cell, a rat mammar tumor cell line. However, Vaziri and Downes (1992) reported that Gαq was readily detectable in plasma membranes and in ghosts from turkey erythrocytes but was absent from cytoskeleton preparations. Similiarly, Wilson et al. (1994) reported that in semithin sections of rat pituitary, anti-Gαq antibodies predominantly labeled the plasma membrane of most cells and only weakly stained the Golgi region. Therefore, the localization of Gαq protein appears to be different depending on cell types. G proteins have been reported to interact with cytoskeletal proteins. Drugs that disrupt cytoskeletal structures, such as cytochalasin or colchicine, were shown to alter the generation of second messengers in a variety of cell types like neutrophils, platelets, lymphocytes, myometrial cells, and frog adrenocortical cells (Painter et al., 1987; Nakano et al., 1989; Leiber et al., 1993). There is evidence for a compartmentation of the α subunit of the G protein involved in the adenylyl cyclase inhibition ($G\alpha_i$) in the coupling of muscarinic, opiate, and α_2-adrenergic receptors to their effectors in NG108-15 cells, probably mediated by the cytoskeleton (Graeser and Neubig, 1993). Lateral cosegregation of receptors and Gαi during capping in lymphocytes has been shown to be dependent on cytoskeletal reorganization (Bourguignon et al., 1990). Gαi in human neutrophils and β subunits of G proteins in S49 lymphoma cells have been found to be associated with cytoskeletal structures (Calson et al., 1986; Sarndahal et al., 1993).

* To whom correspondence should be addressed.
Tel: 82-62-970-2486, Fax: 82-62-970-2484

The fusion of mononucleated myoblasts to form multinucleated myotubes is a striking morphologic event that is unique to the differentiation of skeletal muscle. Because the differentiation of many cloned myogenic cell lines grown *in vitro* rather faithfully mimics *in vivo* development, such cells have provided a convenient model system in which to examine developmental regulation, gene expression, and biochemical differentiation. As cells differentiate, their signaling characteristics change, cytoskeletons are reorganized, and new pathways are expressed and integrated into the function of the cell. The aim of the present study was to investigate cellular localization of Gαq in L8E63 rat myogenic cells which progressively differentiate from mononucleated myoblasts to multinucleated myotubes in culture, and the possible changes in cellular distribution of Gαq during myogenesis.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), trypsin, antibiotics (penicillin-streptomycin solution and fungizone) were obtained from Gibco BRL. Horse serum was purchased from Biofluids. Rhodamine-conjugated phalloidin was from Molecular probes. Cytochalasin D, and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antiserum were from Sigma. Alkaline phosphatase-conjugated IgGs were from Jackson Immunology. Nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were from Promega.

Antibodies

The carboxyl-terminal dodecapeptide of Gαq, ILQLNLKEYNLV, was synthesized, cross-linked to Keyhole lympet hemocyanin (KLH) using glutaraldehyde, and used to generate antibodies in New Zealand White rabbit. Other G protein antibodies, RM1 for $G\alpha_s$ and $G\alpha_{olf}$, AS/7 for $G\alpha_{i1}$ and $G\alpha_{i2}$, and EC2 for $G\alpha_{i3}$ and $G\alpha_o$ were obtained from Du Pont.

Cells and culture conditions

E63 cells, a myogenic clone isolated from L8 rat skeletal myoblasts, were grown at 37℃ in a humidified atmosphere containing 10% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B. For maintaining cells in proliferating myoblast stage, cells were subcultured every three days. Cells cultured for 6-8 days without changing the medium started the differentiation process and formed multinucleated myotubes.

Immunofluorescence staining

Cells were grown on glass coverslips coated with 0.1% gelatin. Cells on coverslips were rinsed in Dulbecco's phosphate-buffered saline, pH 7.5 (DPBS), fixed in 3.7% paraformaldehyde in PBS for 10 min at room temperature, rinsed three-times in PBS. The cells were permeabilized with 0.1% Triton X-100 in PBS for 2 min at room temperature. The cells were rinsed three-times in PBS and incubated for 1h at 37℃ with the primary antisera in 3% BSA. After incubation with the primary antisera, the cells were rinsed with PBS and then incubated with FITC-conjugated goat anti-rabbit immunoglobulin for 40 min at room tempature. The cells were washed again with several changes of PBS, mounted in mounting solution (Sigma), and observed with a fluorescence microscope (Leica).

Preparation of cell extracts

Cells were cultured in 100 mm culture dishes for the indicated times and washed twice with cold PBS. The cells were scraped into microcentrifuge tube and lysed by sonication. The cell lysates were quantified using the BCA protein assay kit (Pierce. Co.). Same amounts of proteins were boiled in SDS sample buffer for 5 min and electrophoresed in 10% SDS-polyacrylamide gel. The separated proteins were transferred to nitrocellulose paper for immunoblotting.

Immunoblotting

The nitrocellulose membrane was washed three times with Tris-buffered saline, pH 7.4, containing 0.05% Tween 20 (TBST) and incubated with primary antibody for 3 h followed by alkaline phosphatase conjugated goat anti-rabbit immunoglobulin for 1 h at room temperature. After three washes with TBST, immunoreactive proteins were visualized with NBT and BCIP in 150 mM NaCl, 5 mM EDTA, and 100 mM Tris HCl, pH 9.5.

Results

Indirect immunofluorescence staining combined with confocal microscopy was used to determine the cellular distribution of Gαq in L8E63 myogenic cells in culture. Cells cultured for 3 days were actively dividing mononucleated myoblasts. Images of horizontal sections of these cells obtained by using confocal microscopy revealed that Gαq is associated with the plasma membrane and intracellular structures resembling actin filaments (Fig. 1). To examine whether this staining pattern is maintained during myogenesis, the cellular distributions of Gαq in mononucleated myoblasts (Fig. 2A) and in multinucleated myotubes (Fig. 2B) were compared. Interestingly, the actin filament-like pattern of Gαq-immu-

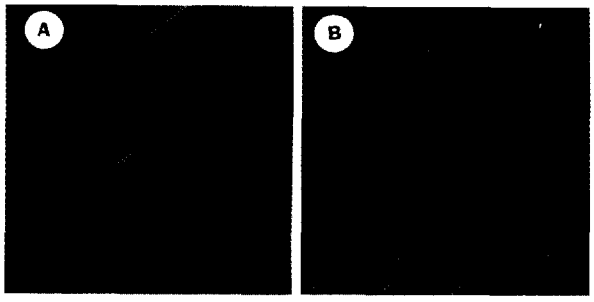

Fig. 1. Localization of Gαq in L8E63 cells. L8E63 cells cultured for 3 days were fixed, permeabilized and immunostained with anti-Gαq antiserum. The cells were visualized by FITC-conjugated anti-rabbit IgG. Images of serial horizontal sections of cells in 1 μm-interval were obtained by using confocal microscopy (B). Images from top to bottom of cells are arranged from upper-left to lower-right of the panel. The enlarged image of a horizontal section obtained from bottom of cells is shown in (A).

noreactive structures disappeared in multinucleated myotubes (Fig. 2B). However, the loss of staining pattern seemed not due to the loss of immunoreactivity. The Gαq-immunoreactivity appeared uniformly distributed over the whole cell surface in multinucleated myotubes. This observation complied with the results obtained in immunoblots where anti-Gαq antibody did not detect noticeable changes in the expression level of Gαq protein during myogenesis from myoblasts to myotubes (Fig. 3). Judging from the expression level of myosin heavy chain (MHC)

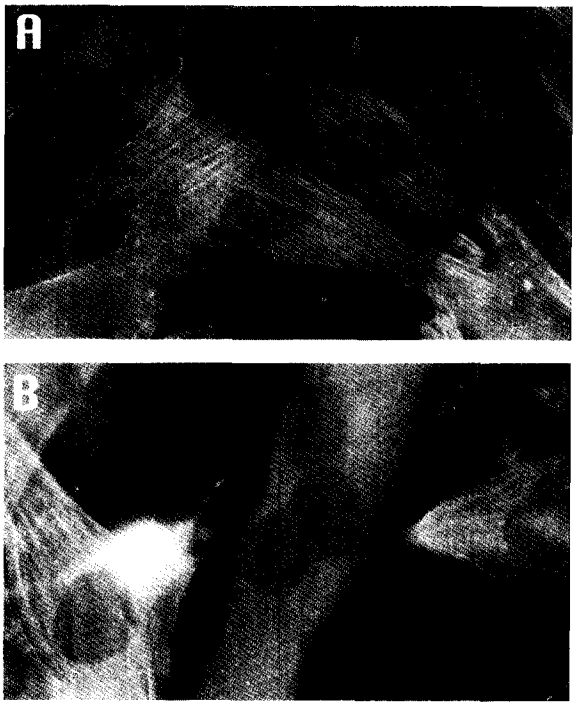

Fig. 2. Changes in the distribution of Gαq in L8E63 cells during myogenesis. L8E63 cells cultured for 3 days (A), or 9 days (B) were immunostained with anti-Gαq antiserum, and visualized by FITC-conjugated anti-rabbit IgG.

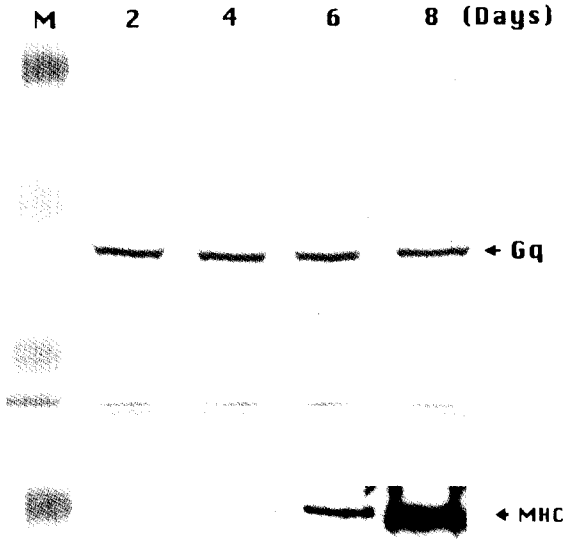

Fig. 3. Immunoblotting of Gαq in L8E63 cells during myogenesis. Lysates were prepared from L8E63 cells cultured for indicated times. The samples (60 μg each) were separated by SDS-PAGE and transfered to nitrocellulose membrane. The membrane was incubated with antibodies against Gαq or myosin heavy chain (MHC). MHC was used as a marker molecule to indicate the degree of myoblast differentiation.

and morphological changes, L8E63 cells started differentiation process after 6 days in culture and formed fully differentiated myotubes after 8 days in culture. The disappearance of the actin filament-like staining pattern of Gαq was not due to the decreased expression of Gαq protein in myotubes but due to the change of cellular localization of Gαq protein during myogenesis. To confirm whether Gαq and actin filaments are colocalized in the myoblast, double-labeling experiments were performed using anti-Gαq antibody and rhodamine-conjugated phalloidin which specifically binds to F-actin. The double-labeling experiments by anti-Gαq antibody and phalloidin revealed almost identical distribution patterns of Gαq and F-actin in L8E63 myoblasts (Fig. 4A and B). In a situation where F-actin filaments were disorganized by treatment with 1.5 μM cytochalasin D, the distribution of Gαq and F-actin filaments was markedly altered, but their colocalization was preserved (Fig. 4C and D). To examine whether the colocalization of Gαq protein with actin filaments is a general feature for heterotrimeric G proteins, the cellular localizations of other heterotrimeric G proteins were analyzed by immunofluorescence staining. The staining pattern with antibody RM1, which is directed against $G\alpha_s$ and $G\alpha_{olf}$, displayed a similiar pattern with the Gαq staining patterns in myoblasts but less apparent (Fig. 5A). Again, the actin filaments-like staining pattern was not detectable in myotubes able in myotubes (Fig. 5B). Staining using AS/7, which recognizes $G\alpha_{i1}$ and $G\alpha_{i2}$, showed that these

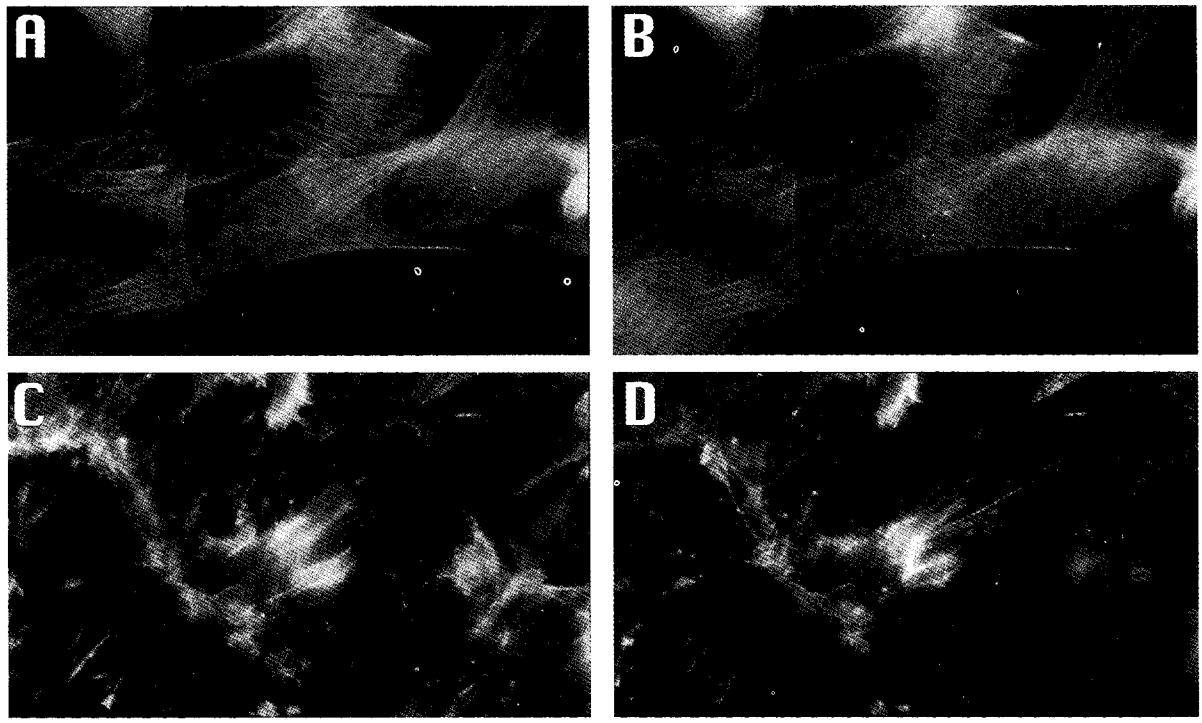

Fig. 4. Colocalization of Gαq and actin cytoskeleton in L8E63 cells and effects of cytochalasin D treatment. Cells cultured for 3 days were incubated 90 min at 37℃ with 1.5 μM cytochalasin D (C and D) or with vehicle (A and B). Cells were then fixed, permeabilized, and stained by double-labeling with anti-Gαq antiserum(A and C) followed by rhodamine-conjugated phalloidin (B and D). In A and C, second antibody was a FITC-conjugated anti-rabbit IgG.

proteins were distributed in structures resembling Golgi apparatus and perinuclear region in myoblasts (Fig. 5C). However, the bright staining pattern was less apparent in myotubes, and most of the staining pattern except for the perinuclear region disappeared (Fig. 5D). Immunoreactivity with EC2, specific for $G\alpha_{i3}$ and $G\alpha_o$, revealed that these G proteins localized to nuclear membranes and this staining pattern was retained in myotubes (Fig. 5E and F).

Discussion

We have demonstrated that Gαq protein is closely associated with actin filaments in myoblasts. Whether this association is a direct interaction of Gαq with actin filaments or an indirect interaction via actin binding proteins remains to be clarified. However, the preservation of association even in a situation where F-actin filaments were disorganized by treatment with 1.5 μM cytochalasin D, suggest that binding of Gαq protein to F-actin is tight. A similar observation was obtained in WRK_1 cell where the association of Gαq protein with F-actin was preserved even after cytochalasin D treatment (Ibarrondo et al., 1995). Fig. 2 shows that there is a striking difference in cellular localization of Gαq between myoblasts and myotubes. The anti-Gαq immunoreactive structures resembling F-actin filament can be observed clearly in mononucleated myoblasts but not in multinucleated myotubes. However, the immunoblot experiment revealed that such a difference does not mean the decrease in the expression level of Gαq protein in myotubes (Fig. 3). These results suggest that the disappearence of the staining pattern of Gαq may be due to the translocation of Gαq during myogenesis. The significance of this change in subcellular localization of Gαq during myogenesis remains unclear. The differentiation of skeletal muscle *in vivo* and *in vitro* is accompanied by dramatic morphological changes leading to the fusion of mononucleated myoblasts into multinucleated myotubes. As cells differentiate, their signaling characteristics change. Stimulation of G protein-coupled receptors activates multiple signalling cascades, including tyrosine kinases, phospholipase C, and ras pathway (Yang et al., 1994; Moolenaar, 1995). The cytoskeleton may provide the site where these signal transducing molecules, including G proteins, assemble and interact with each other, resulting in cross- talk of signalling pathways (Coulter and Rodbell, 1992; Luna and Hitt, 1992). Alternatively, the cytoskeleton may be directly involved in the G protein-mediated signal transducing events. It was reported that Gq protein was involved in actin polymerization in Swiss 3T3 cells (Craig and Johnson, 1996). Phospholipase C-β is an effector of Gq protein. There is a variety of regulatory peptide factors and hormones that lead to the activation of phospholipase C-β (Lee and Rhee, 1995). It has

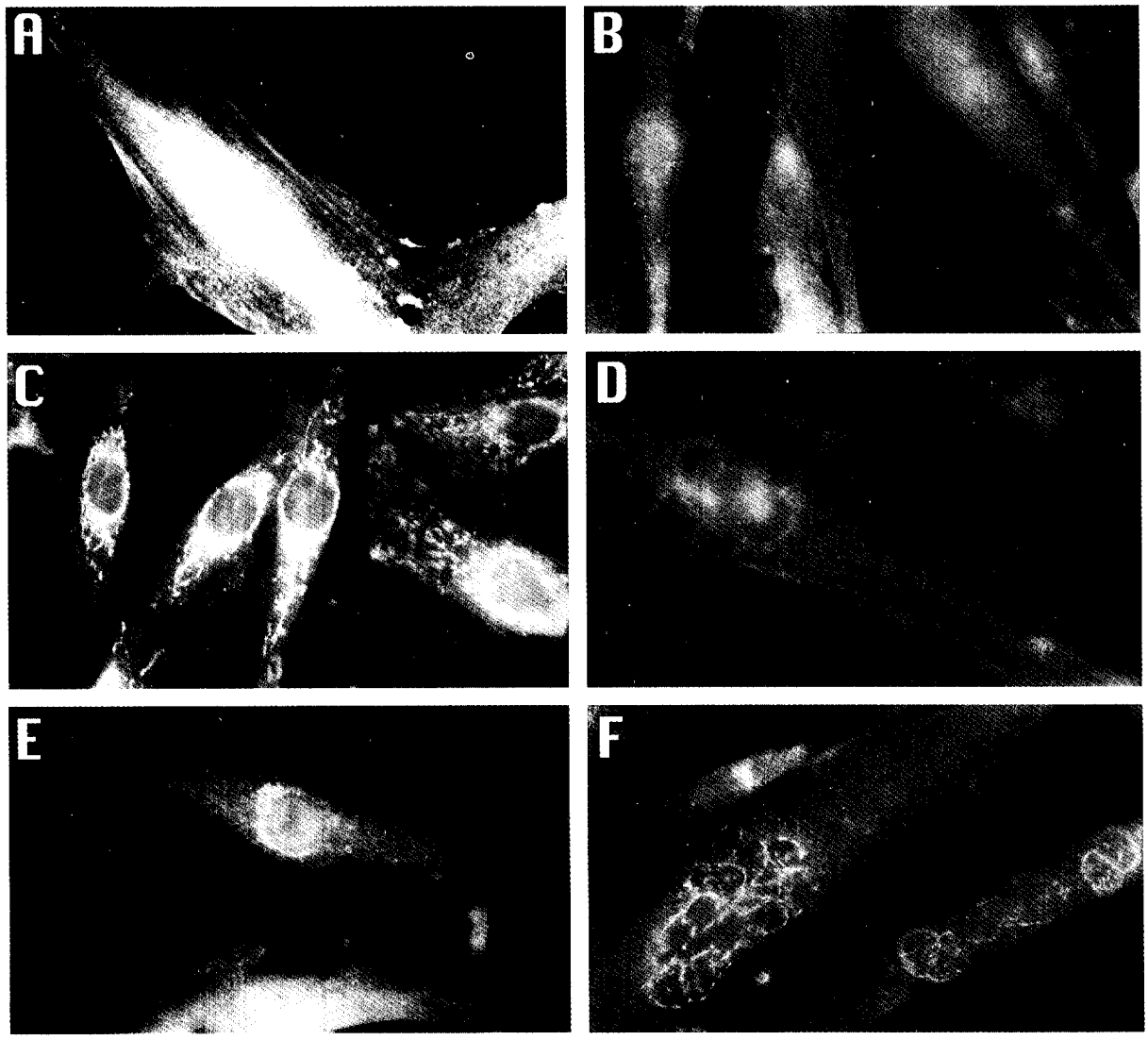

Fig. 5. Distribution of several heterotrimeric G proteins in L8E63 myogenic cell lines. Cells were fixed, permeabilized, and immunostained with RM1 (rabbit anti-$G\alpha_s$ and -$G\alpha_{olf}$, A and B), AS/7 (rabbit anti-$G\alpha_{i1}$ and -$G\alpha_{i2}$, C and D), or EC2 (rabbit anti-$G\alpha_{i3}$ and -$G\alpha_o$, E and F). Second antibody was a FITC-conjugated anti-rabbit IgG. A, C, E; 3 day after culture. B, D, F; 8 days after culture.

been reported that PIP_2, membrane phospholipid which is hydrolyzed upon phospholipase C activation, interacts specifically with the actin-capping protein profilin. This association disrupts the profilin actin complex, thereby increasing the monomeric actin available for polymerization (Goldschmidt-Clermont et al., 1990). Therefore, stimulation of phospholipase C-β activity by Gq protein may influence on the actin cytoskeletal organization. This modulation may be more critical in the actively proliferating myoblasts which need more dynamic changes in actin cytoskeletons than in differentiated nonproliferating myotubes. However, the similar level of Gαq expression in myotubes also indicates that this G protein may have different functions in nonproliferating myotubes. Immunocytochemical experiments using specific antibodies directed against several G proteins indicate that the subcellular localization of $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_o$ differ from those obtained with Gαq (Fig. 5). These results suggest that different signaling pathways mediated by a specific G protein may exist locally within the cell. By confining a specific G protein to local regions of the cell, their ability to transduce signals could be more effective.

Acknowledgements

We thank Han SJ and Hong SH in the Institute for Molecular Biology and Genetics, Seoul National University for the help in raising antibodies against Gαq. This work was supported by Non-directed Research Grant from the Korea Science and Engineering Foundation.

References

Birnbaumer L (1992) Receptor to effector signaling through G proteins: roles for βγ dimers as well as α subunits. *Cell* 71: 1069-1072.

Blank JL, Ross AH, and Exton JH (1991) Purification and characterization of two G-proteins that activate the β1 isozyme of phosphoinositide-specific phospholipase C. Identification as members of the Gq class. *J Biol Chem* 266: 18206-18216.

Bourguignon LYW, Walker G, and Huang HS (1990) Interactions between a lymphoma membrane-associated GTP-binding protein and the cytoskeleton during receptor patching and capping. *J Immunol* 144: 2242-2252.

Calson KE, Woolkalis MJ, Newhouse MG, and Manning DR (1986) Fractionation of the β subunit common to guanine nucleotide-binding regulatory proteins with the cytoskeleton. *Mol Pharmacol* 30: 463-468.

Coulter S and Rodbell M (1992) Heterotrimeric G proteins in synaptoneurosom membranes are crosslinked by p-phenylenedimaleimide, yielding structures comparable in size to crosslinked tubulin and F-actin. *Proc Natl Acad Sci USA* 89: 5842-5846.

Craig SW and Johnson RP (1996) Assembly of focal adhesions: progress, paradigms, and portents. *Curr Opin Cell Biol* 8: 74-85.

Goldschmidt-Clermont PJ, Machesky LM, Baldassare JJ, and Pollard TD (1990) The actin-binding protein profilin binds to PIP_2 and inhibits its hydrolysis by phospholipase C. *Science* 247: 1575-1578.

Graeser D and Neubig RR (1993) Compartmentation of receptors and guanine nucleotide-binding proteins in NG108-15 cells: lack of cross-talk in agonist binding among the α 2-adrenergic, muscarinic, and opiate receptors. *Mol Pharmacol* 43: 434-443.

Ibarrondo J, Joubert D, Dufour MN, Cohen-Solal A, Homburger V, Jard S, and Guilon G (1995) Close association of the α subunits of Gq and G11 G proteins with actin filaments in WRK1 cells: Relation to G protein-mediated phospholipase C activation. *Proc Natl Acad Sci USA* 92: 8413-8417.

Jhon DY, Lee H-H, Park D, Lee C-W, Lee K-H, Yoo OJ, and Rhee SG (1993) Cloning, sequencing, purification, and Gq-dependent activation of phospholipase C-β3. *J Biol Chem* 286: 6654-6651.

Lee SB and Rhee SG (1995) Significance of PIP2 hydrolysis and regulation of phospholipase C isozymes. *Curr Opin Cell Biol* 7: 183-189.

Leiber D, Jasper JR, Alousi AA, Martin J, Bernstein D, and Insel PA (1993) Alteration in Gs-mediated signal transduction in S49 lymphoma cells treated with inhibitors of microtubules. *J Biol Chem* 268: 3833-3837.

Luna EJ and Hitt AL (1992) Cytoskeleton-plasma membrane interactions. *Science* 258: 955-964.

Moolenaar WH (1995) Lysophosphatidic acid signaling. *Curr Opin Cell Biol* 7: 203-210.

Nakano T, Hanasaki K, and Arita H (1989) Possible involvement of cytoskeleton in collagen-stimulated activation of phospholipases in human platelets. *J Biol Chem* 264: 5400-5406.

Neer EJ (1995) Heterotrimeric G proteins: Organizers of transmembrane signals. *Cell* 80: 249-257

Painter RG, Zahler-Bentz K, and Dukes RE (1987) Regulation of the affinity state of the N-formylated peptide receptor of neutrophils: role of GTP-binding proteins and the cytoskeleton. *J Cell Biol* 105: 2959-2971.

Rhee SG and Choi KD (1992) Regulation of inositol phospholipid-specific phospholipase C isozymes. *J Biol Chem* 267: 12393-12396

Sarndahal, E, Bokoch GM, Stendahl O, and Andersson T (1993) Stimulus-induced dissociation of α subunits of heterotrimeric GTP-binding proteins from the cytoskeleton of human neutrophils. *Proc Natl Acad Sci USA* 90: 6552-6556.

Simon MI, Strathmann MP, and Gautam N (1991) Diversity of G proteins in signal transduction. *Science* 252: 802-808.

Smrcka AV, Hepler JR, Brown KO, and Sternweis PC (1991) Regulation of polyphosphoinositide-specific phospholipase C activity by purified Gq. *Science* 251: 804-807

Vaziri C and Downes CP (1992) Association of a receptor and G protein-regulated phospholipase C with the cytoskeleton. *J Biol Chem* 267: 22973-22981.

Wilson BS, Komoru M, and Farquhar MG (1994) Cellular variations in heterotrimeric G protein localization and expression in rat pituitary. *Endocrinology* 134: 233-244.

Yang LJ, Rhee SG, and Williamson JR (1994) Epidermal growth factor-induced activation and translation of phospholipase C-γ1 to the cytoskeleton in rat hepatocytes. *J Biol Chem* 269: 7156-7162.

[Received January 9, 1997; accepted February 17, 1997]