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G Protein γ Subunits Coimmunoprecipitated with Antibodies against α Subunits: Identification of Major Isoforms in Cultured Cells by Silver Stain and Immunoblotting with Conventional Transfer Procedure[1]

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The $\beta\gamma$ subunits of G proteins were coimmunoprecipitated with antibodies against various α subunits, and analyzed by silver stain and immunoblotting with conventional transfer procedure and membrane-blocking buffer containing 2% BSA. Multiple isoforms of γ were coimmunoprecipitated with no significant difference in form or ratio among the antibodies against α subunits used, suggesting antibodies against any α subunit could coimmunoprecipitate all forms of γ. Therefore, this method was applicable to analyze γ subunits in various cells, especially to clarify what forms of γ subunits are major components. The major isoforms were: γ_5 in C6, NG108-15, HeLa, HEK293, and F9 cells; γ_{12} in Swiss 3T3 and BRL-3A cells; and γ_3 in PC12 cells. In addition to most γ subunits identified, unidentified γ subunits were present in PC12, NG108-15, and BRL-3A cells. Furthermore, the method was applied to examine changes of isoforms of γ during differentiation of HL-60 cells. Undifferentiated cells mainly contained γ_5, but retinoic acid treatment of cells replaced most γ_5 with γ_2. Thus, this method is useful to determine the major isoforms which seem to be the more important in cells.

Key words: differentiation, G protein γ subunit, HL-60 cells, immunoblotting, immunoprecipitation.

Heterotrimeric G proteins, which are involved in signal transduction from cell-surface receptors to intracellular effectors, are composed of α, β, and γ subunits, the latter two being tightly associated under physiological conditions (*1, 2*). Receptor stimulation promotes the dissociation of G protein into a separate α subunit and a $\beta\gamma$ dimer. This suggests simultaneous regulation of multiple cellular responses by G proteins, since both components independently regulate intracellular effectors (*1, 2*).

Analyses of purified proteins and cloned cDNAs have revealed the existence of multiple forms of β and γ in addition to many isoforms of α. At the amino acid level, 5 mammalian β subunits exhibit strong conservation (*3*), while 11 mammalian γ subunits show considerable divergence (*4*). Consequently, functional differences among various forms of $\beta\gamma$ complex have been attributed to the γ rather than β (*5-7*). In their tissue distribution, γ subunits show more variation than β subunits. Among the 5 isoforms of β (β_1-β_5), β_1-β_4 subunits are widely distributed with the exception of a brain specific isoform of β_5 (*3*). By contrast, γ_1 and γ_c are specifically expressed in retinal rods and cones, respectively (*8-10*). The γ_8 subunit is expressed only in olfactory and vomeronasal neuroepithelia (*11*), whereas γ_3 and γ_4 are localized only in the brain (*12-17*). By contrast, γ_2, γ_5, γ_7, γ_{10}, γ_{11}, and γ_{12} are distributed in a variety of tissues (*4, 12-16*).

To determine what forms of γ subunit are present in tissues or cells, extracts are usually examined by immunoblotting. Since most cells and tissues contain multiple forms of γ subunit, the major isoforms seem to be the more important. However, because reactivities of antibodies against γ subunits are considerably different, immunoblotting does not clarify what forms are major components without the use of purified γ subunits as standards, which are not always available. In addition, we cannot rule out the possibility of the existence of novel γ subunits as major isoforms. In the present study, $\beta\gamma$ subunits were coimmunoprecipitated from extracts of cells with antibodies against various α subunits, because antibodies against β subunit were not useful for immunoprecipitation. Analyses of immunoprecipitates by silver stain and immunoblotting indicated coprecipitation of all the γ subunits with the antibodies against α subunits. Using this method, we showed the major γ subunit in various cells and differentiated HL-60 cells.

With respect to detection of γ subunits by immunoblotting, it has been reported that the conventional transfer procedure was not successful, and a high temperature

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; G protein, heterotrimeric guanine nucleotide-binding protein; RA, retinoic acid.

transfer procedure enhanced the sensitivity of immunodetection (*18*). However, we have been able to detect γ subunits with high sensitivity by conventional transfer methods (*4, 15*). Therefore, we show here sensitive detection of γ subunits by immunoblotting with membrane-blocking buffer containing BSA instead of nonfat dry milk.

MATERIALS AND METHODS

Antibodies—The peptide KNNLKDCGLF, corresponding to residues Lys^{345}-Phe^{354} of $G_{11/2}\alpha$, was conjugated to keyhole limpet hemocyanin with glutaraldehyde and injected into rabbits. Antisera were purified using an antigen-coupled Sepharose column, and purified antibodies were referred to as antibodies against $G_{11/2}\alpha$. Antibodies against $G_o\alpha$ and β subunits were previously generated with purified proteins (*19, 20*). The antibodies against $G_{q/11}\alpha$ and $G_s\alpha$ ($G_{s/olf}\alpha$) were purchased from Santa Cruz Biotechnology. The peptide *N*-acetyl-SATNNIAQARKC, corresponding to residues Ser^2-Lys^{12} of γ_7, appended with cysteine for coupling purposes, was synthesized. Antisera against $\gamma_{7(N)}$ were raised in rabbits by the injection of this peptide conjugated to keyhole limpet hemocyanin and purified using an antigen-coupled Sepharose column. Antibodies against $\gamma_{7(N)}$ reacted mainly with γ_7 but also weakly with γ_2, γ_3, and γ_{12} (data not shown). Antibodies against other γ subunits were previously generated with the individual peptides corresponding to N- or C-terminal amino acid sequences (*4, 15, 21*).

Cell Culture and Differentiation Induction—Mouse Swiss 3T3 fibroblasts were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum. Mouse teratocarcinoma F9, obtained from Health Science Research Resource Bank, rat C6 glioma, human leukemia HL-60, human embryonal kidney (HEK) 293, and HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), and rat PC12 pheochromocytoma cells were cultured in DMEM supplemented with 5% FBS and 10% horse serum. Mouse neuroblastoma×rat glioma hybrid NG108-15 cells were grown in DMEM containing 5% FBS and hypoxanthine/aminopterin/thymidine, and rat liver BRL-3A cells were cultured in Ham's F12 medium with 10% FBS. HL-60 cells were differentiated into granulocytes by treatment for 5 days with 1.3% dimethyl sulfoxide (DMSO), 1 mM all-*trans* retinoic acid (RA), or 0.1 mM dibutyryl cAMP. The percentage of differentiated cells was monitored by nitro-blue tetrazolium reduction for granulocytes (*22, 23*). Preparations in which more than 90% of cells were differentiated were used for experiments.

Immunoprecipitation—All cells were washed with cold phosphate-buffered saline and lysed in Buffer A (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl, 1% Triton X-100, 0.5% sodium cholate, 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml trypsin inhibitor). The cell lysates were centrifuged at 4°C at 100,000×*g* for 20 min, and supernatant fractions (cell extracts) were used for immunoprecipitation. The cerebral cortex of rat was homogenized in 9 volumes of buffer A, centrifuged at 4°C at 100,000×*g* for 1 h, and the supernatant thus obtained was referred to as the brain extract. For immunoprecipitation, cell and brain extracts were incubated with antibodies against various α subunits at 4°C for 1 h, and the immunocomplexes were recovered by further incubation with 20 μl of Protein A-Sepharose (1 mg protein A/ml of gel) for 1 h. The immunoprecipitates were washed three times with buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 100 mM NaCl, 0.2 mM PMSF, and 1 μg/ml trypsin inhibitor, and an aliquot was subjected to SDS-PAGE.

SDS-PAGE and Immunoblotting—Tricine/SDS-PAGE (16.5% acrylamide) was performed by the method of Schägger and von Jagow (*24*), and SDS-PAGE was conducted according to the method of Laemmli (*25*). Proteins were transferred electrophoretically from each SDS-polyacrylamide gel to a nitrocellulose sheet (Protran BA85, Schleicher & Schuell) in ice-cold transfer buffer containing 25 mM Tris-192 mM glycine (pH 8.3) and 20% (v/v) methanol (*26*) at 15 V/cm for 2 h. The nitrocellulose sheet was incubated in blocking buffer [10 mM sodium phosphate buffer, pH 7.0, 100 mM NaCl, 2% BSA (Fr. V, Bayer), 0.5% protease-treated gelatin, and 0.1% NaN_3] for 2 h at room temperature, then with primary antibodies in TBS (20 mM Tris-HCl, pH 7.5, 0.15 M NaCl, and 0.01% Nonidet P-40) for 1-2 h (*15*). The sheet was washed with TBS, then incubated with peroxidase-conjugated antibodies raised in goat against rabbit IgG in TBS for 1 h. The washed sheet was soaked in chemiluminescence reagent (Renaissance; DuPont NEN) for 1 min, then exposed to RX-H film (Fuji) for 0.5-2 min (*15*).

RESULTS

For detection of γ subunits by immunoblotting, we used conventional transfer procedures at low temperature and blocking buffer containing 2% BSA (*4, 15*). As shown in Fig. 1, all γ subunits tested could be sensitively detected by this method. However, these γ subunits were hardly detectable when the same nitrocellulose blots were incubated in the blocking buffer containing 5% nonfat dry milk, which was frequently used for detection of γ subunits (*18*) (Fig. 1). In contrast, β subunit could be detected by use of either blocking buffer, indicating nonfat milk was inappropriate for detection of γ subunits by immunoblotting.

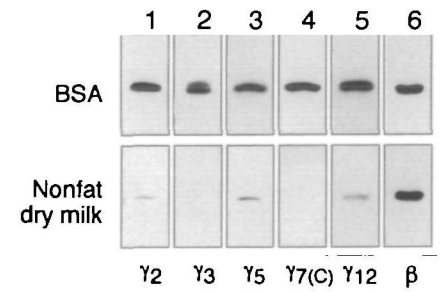

Fig. 1. **Reactivities of various γ and β subunits by immunoblotting after incubation with blocking buffer containing BSA or nonfat dry milk.** Purified $\beta\gamma_2$ (50 ng, lane 1), $\beta\gamma_3$ (10 ng, lanes 2 and 6), $\beta\gamma_5$ (20 ng, lane 3), $\beta\gamma_7$ (10 ng, lane 4), and $\beta\gamma_{12}$ (10 ng, lane 5) were subjected to Tricine/SDS-PAGE (lanes 1-5) or SDS-PAGE (lane 6), then the proteins were transferred to nitrocellulose sheets. The nitrocellulose blots were incubated in blocking buffer containing 2% BSA for 2 h (upper panel) or TBS containing 5% nonfat dry milk (Difco skim milk) for 1 h (lower panel), then incubated with antibodies against γ_2 (lane 1), γ_3 (lane 2), γ_5 (lane 3), $\gamma_{7(C)}$ (lane 4), γ_{12} (lane 5), or β (lane 6). Immunostaining was carried out as described in "MATERIALS AND METHODS."

Antibodies against α subunits of $G_{i1/2}$, G_o, $G_{q/11}$, and G_s specifically immunoprecipitated the respective α subunits in extracts of rat brain (data not shown). When immunoprecipitation was performed in the presence of 1% sodium cholate, $\beta\gamma$ subunits were not coimmunoprecipitated with α subunits, but all of the above antibodies coimmunoprecipitated $\beta\gamma$ subunits in extracts of rat brain in the presence of Triton X-100. In the combination of 1% Triton X-100 and 0.5% sodium cholate, more G proteins were solubilized than in Triton X-100 alone, and $\beta\gamma$ subunits could be coimmunoprecipitated by antibodies against α subunits. Therefore, we first examined the selectivity in α-γ subunit interactions in these conditions. Several isoforms of γ were coimmunoprecipitated with antibodies against α subunits, with no significant difference among the antibodies in the forms of γ subunits coprecipitated or their ratio (Fig. 2). Besides brain extracts, similar results were obtained from extracts of Swiss 3T3 and C6 cells with antibodies against $G_{i1/2}\alpha$, $G_{q/11}\alpha$, and $G_s\alpha$ (data not shown), suggesting random association of α with $\beta\gamma$ in the tissue and cell extracts. When purified $\beta\gamma_2$ subunits were added to extracts of Swiss 3T3 cells, which did not contain a detectable amount of γ_2, the exogenous γ_2 as well as endogenous γ subunits was coimmunoprecipitated with antibodies against $G_{i1/2}\alpha$, suggesting a rapid exchange of $\beta\gamma$ subunits among α subunits in extracts. It was unlikely that lack of selectivity in α-γ subunit interaction was due to the non-specific binding of large amounts of $\beta\gamma$ to protein A-Sepharose beads, because antibodies against γ subunits with the protein A-Sepharose specifically immunoprecipitated the respective γ subunits from the extracts of rat brain and the cells. This approach has the advantage that antibodies against any α subunit could coimmunoprecipitate all forms of γ subunit. To coimmunoprecipitate γ subunits, antibodies against β seemed to be better than antibodies against α subunits. However, our antibodies against β did not precipitate β subunit, probably because they failed to recognize the assembled $\beta\gamma$, as described by Rehm and Ploegh (*27*).

To elucidate the major forms of γ subunit in the various types of cells, γ subunits were coimmunoprecipitated from extracts of PC12, Swiss 3T3, and C6 cells with antibodies against α subunits that were abundant in individual cells, and the immunoprecipitates were analyzed by silver stain after Tricine/SDS-PAGE. In addition to major bands of G protein subunits, minor proteins with higher molecular weights than those of γ subunits were coprecipitated (data not shown). Several bands of γ were obtained from all cell extracts (Fig. 3A), and most of these were identified by immunoblotting with antibodies against γ subunits (Fig. 3B). Antibodies against γ_2, γ_3, γ_5, γ_{10}, γ_{11}, and γ_{12} specifically reacted with the respective γ subunits, but antibodies against $\gamma_{7(C)}$ reacted at least with γ_2, γ_3, γ_7, and γ_{12}. In PC12 cells, two bands were visualized by silver stain, with a dense band of γ_3. The other band (unidentified γ, γ_x), migrated between γ_2 and γ_7, and reacted only with antibodies against $\gamma_{7(C)}$, indicating it was a γ subunit but not γ_2,

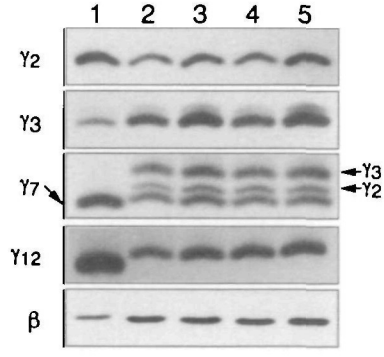

Fig. 2. **Coimmunoprecipitation of $\beta\gamma$ subunits in rat brain extracts with antibodies against various α subunits of G proteins.** Rat brain extracts were immunoprecipitated with antibodies against $G_{i1/2}\alpha$ (lane 2), $G_o\alpha$ (lane 3), $G_{q/11}\alpha$ (lane 4), and $G_s\alpha$ (lane 5). The immunoprecipitates and the standards (lane 1) were subjected to Tricine/SDS-PAGE for analyses of the γ subunits or SDS-PAGE for analyses of the β subunits, and immunoblotted with antibodies against various γ and β subunits. The standards (from top to bottom) were purified bovine $\beta\gamma_2$ (10 ng), $\beta\gamma_3$ (2 ng), $\beta\gamma_7$ (2 ng), $\beta\gamma_{12}$ (2 ng), and $\beta\gamma_{12}$ (5 ng). Since the total amount of $\beta\gamma$ coimmunoprecipitated differed among the antibodies used, the amount of sample applied to electrophoresis was adjusted to give similar densities of bands of β. Because antibodies against $\gamma_{7(C)}$, which reacted with γ_2, γ_3, γ_7, and γ_{12}, were used to detect γ_7, staining of γ_2 and γ_3 as well as γ_7 was observed (middle panel). The rat γ_7 and γ_{12} showed slightly slower migration than bovine γ_7 and γ_{12} of standard proteins, probably due to species differences (*4*). In addition, γ_3 often gave two bands on Tricine/SDS-polyacrylamide gels, though the reason for this is not clear (*15*).

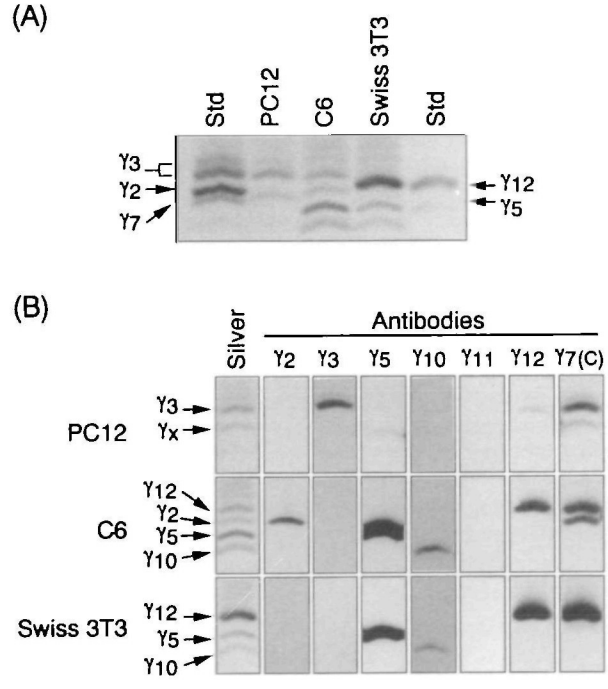

Fig. 3. ***Analyses of γ subunits coimmunoprecipitated with antibodies against various α subunits from extracts of PC12, C6, and Swiss 3T3 cells.*** The extract of PC12 cells was immunoprecipitated with antibodies against $G_o\alpha$, while extracts of C6 and Swiss 3T3 cells were immunoprecipitated with antibodies against $G_{i1/2}\alpha$. The immunoprecipitates were subjected to Tricine/SDS-PAGE and stained with silver (A) or immunoblotted with antibodies against various γ subunits (B). In (A), immunoprecipitates with antibodies against $G_o\alpha$ from rat brain were used for standards of γ_2, γ_3, and γ_7 (lane at the left end) and a mixture of purified $\beta\gamma_5$ and $\beta\gamma_{12}$ (0.1 μg each) was applied to the lane on the right. In (B), immunoblots with antibodies against γ_2, γ_3, γ_5, γ_{10}, γ_{11}, γ_{12}, and $\gamma_{7(C)}$ are shown together with the results with silver stain obtained in (A). The names of γ subunits on the left indicate identified isoforms, with an unidentified form of γ_x.

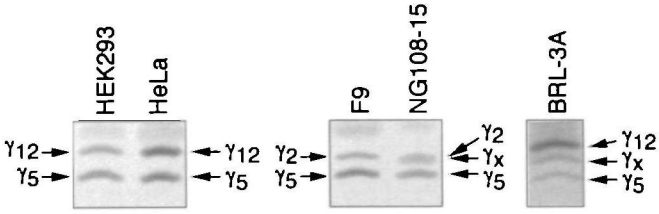

Fig. 4. **Major γ subunits in various cultured cells coimmunoprecipitated with antibodies against various α.** Extracts of HEK293, HeLa, and F9 cells were immunoprecipitated with antibodies against $G_{i1/2}\alpha$, while extracts of NG108-15 and BRL-3A cells were immunoprecipitated with antibodies against $G_o\alpha$ and $G_{q/11}\alpha$, respectively. The immunoprecipitates were subjected to Tricine/SDS-PAGE and stained with silver. The names of γ subunits indicate identified isoforms, with an unidentified form of γ_x.

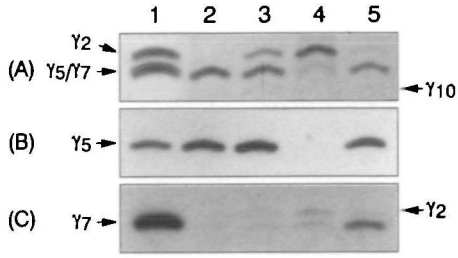

Fig. 5. **Changes of isoforms of γ subunit during differentiation of HL-60 cells induced by various agents.** The immunoprecipitates with antibodies against $G_{i1/2}\alpha$ from undifferentiated cells (lane 2) and cells treated with DMSO (lane 3), RA (lane 4), or dibutyryl cAMP (lane 5) were subjected to Tricine/SDS-PAGE and stained with silver (A), or immunoblotted with antibodies against γ_5 (B) and $\gamma_{7(N)}$ (C). In (A), the standards (lane 1) were a mixture of purified $\beta\gamma_2$, $\beta\gamma_5$, and $\beta\gamma_7$ (0.1 μg each), and γ_5 and γ_7 comigrated under these conditions. In (B) and (C), the standards (lane 1) were $\beta\gamma_5$ (5 ng) and $\beta\gamma_7$ (2.5 ng), respectively.

γ_3, γ_5, γ_7, γ_{10}, γ_{11}, or γ_{12} (Fig. 3). In C6 cells, four bands were observed by silver stain, and identified as γ_2, γ_5, γ_{10}, and γ_{12} by immunoblot, while Swiss 3T3 cells contained a large amount of γ_{12} and less γ_5 and γ_{10} (Fig. 3).

Using this method, the major γ subunits in other cultured cells derived from various tissues were stained by silver and identified by immunoblotting (Fig. 4). The major γ subunits in HEK293, HeLa, and BRL-3A cells were γ_5 and γ_{12}, while F9 and NG108-15 cells contained γ_2 and γ_5 as major γ subunits. In addition, an unidentified γ, whose mobility on a polyacrylamide gel and reactivity with antibodies against various γ subunits were very similar to γ_x found in PC12 cells, was present in NG108-15 and BRL-3A cells.

To study changes of isoforms of γ during cell differentiation, HL-60 cells were treated with various reagents that induced their differentiation into granulocytes and immunoprecipitated with antibodies against $G_{i1/2}\alpha$ (Fig. 5). Untreated HL-60 cells contained only γ_5, with no other γ subunits detectable. Differentiation of HL-60 cells into granulocytes induced the expression of γ_2 (Fig. 5A), which was identified by immunoblotting (data not shown). In particular, RA treatment of cells replaced most γ_5 with γ_2 in HL-60 cells. The γ_{10} subunit was induced only by DMSO treatment. Although γ_5 and γ_7 comigrated under these conditions (Fig. 5A), immunoblotting with antibodies against γ_5 and γ_7 could distinguish them (Fig. 5, B and C). Limited amounts of γ_7 were induced during differentiation by all reagents, with the highest level in cells treated with dibutyryl cAMP.

DISCUSSION

Previous studies have indicated selectivity in α-γ interaction: most showed less association between $\beta\gamma_1$ and α subunits than other $\beta\gamma$ and α subunits *in vitro* (5-7). By ion-exchange chromatography of G protein mixture from bovine brain, it was shown that isolated $G_o\alpha$ isoforms had distinct γ subunit compositions (28). In the present study, however, selective interaction of α and γ was not observed by immunoprecipitation, probably due to the random association in each subunit during incubation of extracts. Although the selectivity in α-γ interaction could not be studied by immunoprecipitation methods, these methods were useful for analyzing the forms of γ subunits in tissues or cells.

Major γ subunits of various cells were visualized with silver stain after immunoprecipitation of cell extracts with antibodies against α subunits, and identified by immunoblotting. In most cells tested, γ_5 and γ_{12} or γ_5 and γ_2 were major γ, while a brain-specific γ, γ_3, was abundant only in PC12 cells. The γ_{10} subunit was also detectable by silver stain in some cell lines, but γ_7 and γ_{11} were not major components in any cells. Unidentified γ_x subunits, which were not γ_2, γ_3, γ_5, γ_7, γ_{10}, γ_{11}, or γ_{12}, were present in PC12, NG108-15, and BRL-3A cells, and they seemed to be identical judging from their reactivities with antibodies against γ subunits and mobility on a Tricine/SDS-polyacrylamide gel. Although we did not examine the existence of tissue-specific γ subunits γ_1, γ_4, γ_8, and γ_c, it is unlikely that γ_x is γ_1, γ_8, or γ_c because of their limited localization. By contrast, it is likely that brain-specific γ_4 may exist in neuronal cells such as PC12 and NG108-15 cells, but the following observations suggest that γ_x is not γ_4. (i) The mobility of γ_x and γ_3 on polyacrylamide gel differed considerably, while that of γ_3 and γ_4 should be very similar because of their close molecular weights. (ii) Since the C-terminal amino acid sequences of γ_2 and γ_4 corresponding to the region of γ_7 used for production of antibodies against $\gamma_{7(C)}$ were identical, γ_4 should react with these antibodies as well as γ_2. However, the reactivity of γ_x with antibodies against $\gamma_{7(C)}$ was much lower than that of γ_2 in C6 cells, in contrast to the similar densities of these two bands stained by silver (Fig. 3). Whatever the case, the amino acid sequence of γ_x must be analyzed to prove that γ_x is a novel γ subunit.

Differentiation of HL-60 cells into granulocytes by treatment for 5 days with DMSO, RA, or dibutyryl cAMP induced the expression of γ_2 and γ_7, which did not occur in undifferentiated cells. In particular, the dominant isoform γ_5 in untreated cells was replaced with γ_2 in RA-treated cells. In contrast, Iiri *et al.* (22) reported that DMSO treatment of HL-60 cells for 5 days did not induce the expression of γ_2, but treatment of cells with DMSO (5 days) plus RA (during the last 24 h of treatment, DMSO/RA) or RA alone (2 days) did. The reason for the discrepancy observed in DMSO-treated cells is not clear, but may be due to the reactivity of antibodies against γ_2: the sensitivity of their antibodies may have been too low to detect relative-

ly small amounts of γ_2. Actually, the reactivity of our antibodies against γ_2 was lowest among antibodies against various γ subunits, and we sometimes did not detect the γ_2 subunit in the extracts. These results indicate the importance of examining γ subunits by the present methods in addition to immunoblotting.

Iiri *et al.* (*22*) reported that trimeric G_{12} purified from DMSO/RA-treated HL-60 cells stimulated $\beta\gamma$-sensitive phospholipase C in extracts of HL-60 cells to a much greater extent and at lower concentrations than G_{12} purified from DMSO-treated cells, suggesting that RA-induced expression of γ_2 altered the function of G_{12}. When we examined γ subunits in DMSO/RA-treated cells, more γ_2 was expressed than in DMSO-treated cells, but much less than in RA-treated cells (data not shown). Taken together with the present results, the difference in G_{12} from DMSO- and DMSO/RA-treated cells seems to be the ratio of γ_2 and γ_5. However, it has also been reported that purified phospholipase Cβ2 and Cβ3, which were detected in HL-60 cells, were stimulated by both $\beta\gamma_2$ and $\beta\gamma_5$ (*7*). It is possible that an unknown isoform of phospholipase C, which is more sensitive to $\beta\gamma_2$, may be present in HL-60 cells.

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