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Measurement of GTPγS binding to specific G proteins in membranes using G-protein antibodies

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We developed a novel method to quantitatively measure GTPγS binding to specific G proteins in crude membranes using G-protein antibodies. The basic strategy was that the materials were initially incubated with [^{35}S]GTPγS at 37°C. After 4°C incubation in the wells of an ELISA plate precoated with G-protein antibodies, the radioactivity of each well was counted. This method, using an anti-G_i antiserum and an anti-G_s antiserum, quantitatively and specifically detected the binding of GTPγS to purified G_{i2} and G_s. In S49 cell membranes, GTPγS binding to immunoreactive G_s was observed in a time-dependent manner that obeyed first-order kinetics, and the rate constant was stimulated ~twofold in response to isoproterenol. The effect of isoproterenol was not observed in *unc* mutant membranes. The present method thus makes it possible to quantitatively measure GTPγS binding to specific G proteins in cell membranes.

G protein; Antibody; GTPγS binding; ELISA plate; G protein activity; Crude membrane

1. INTRODUCTION

The G proteins (guanine nucleotide-binding proteins) comprise a family of signal transducers that modulate the activity of various effectors following the activation of cell surface receptors [1]. The activity of G proteins is regulated by their bound guanine nucleotides. When GDP is bound to them, G proteins are inactive. When they bind GTP, they become active. Upon receptor stimulation the GDP-bound inactive form of G proteins releases GDP and binds GTP. Thus, receptors stimulate the GDP/GTP exchange on G proteins. After stimulation, the bound GTP is spontaneously converted into GDP by the intrinsic GTPase activity of the G proteins themselves, and they thereby return to being inactive. In addition, receptors are thought to act on G proteins by increasing GDP/GTP exchange, not by affecting GTPase activity. Measurement of the binding of GTPγS (guanosine-5′-*o*-(3-thio-triphosphate)), a nonhydrolyzable GTP analog, to G proteins thus plays a key role in the assessment of receptor-induced G-protein stimulation.

It is inferred that receptors selectively recognize G proteins. For example, the β-adrenergic receptor is rather specifically linked to G_s, and M_2 subtypes of muscarinic acetylcholine receptors preferentially couple to G_i and G_o. These findings have been shown mainly by using phospholipid vesicles that reconstitute purified receptors and G proteins. However, it has been difficult to clarify whether receptors are selectively linked to specific classes of G proteins in a native membrane environment. This is because plasma membranes contain a number of G proteins, including trimeric G proteins and low molecular weight G proteins, and nevertheless, we have had no systems available by which we can monitor the activity of a specific G protein after the receptor stimulation of plasma membranes. To examine whether one specific receptor is functionally linked to G proteins in cell membranes, a low K_m GTPase activity of membranes has so far been measured before and after agonist stimulation [2]. It has however been impossible by this method to identify the G protein involved in the receptor signalling pathway. This is also the case with the experiment where GTPγS binding is measured. Even if stimulation by receptor agonists increases the amount of GTPγS binding to membranes, it remains unclear what kind of G protein is involved in that increase. Only cross-linkable GTP analogs were utilized for this purpose [3], but their cross-linkage with G proteins should become more efficient and quantitative. We conducted this study to develop a method to quantitatively assess the amount of GTPγS binding to a specific G protein contained in cell membranes. For this purpose, we have utilized antibodies specific against G proteins.

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2. EXPERIMENTAL

2.1. *Materials*

Trimeric G_{i2} and G_o purified to homogeneity from bovine brain, was kindly provided by Dr. Toshiaki Katada (Tokyo Institute of Technology, Yokohama, Japan) [4]. $G_{\alpha z}$ was purified by the four steps of chromatography using DEAE-Sephacel, Ultrogel AcA 34, heptylamine-Sepharose, and DEAE-Toyopearl from the membranes of Sf9 cells infected with baculovirus encoding $G_{\alpha z}$ cDNA, which was kindly provided by Dr. Toshihide Nukada (University of Tokyo, Tokyo, Japan) [5]. The baculovirus that encodes $G_{\alpha z}$ cDNA was kindly provided by Drs. Koji Ishii and Atsushi Imaizumi (Teijin first Biomedical Research Laboratories, Japan) and Dr. Yoshiharu Matsuura (National Institute for Preventive Medicine and Hygiene, Japan). These purified G proteins were resolved in 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid)/NaOH (pH 7.4), 1 mM EDTA (ethylenediamine tetraacetic acid) and 0.7% CHAPS. AS/7 and RM/1 are antisera against the synthetic peptides KENLKDCGLF and RMHLRQYELL [6,7]. Both AS/7 and RM/1 were obtained from Du Pont-New England Nuclear. The culture of S49 lymphoma cells and *unc* mutant cells kindly provided by Dr. Tatsuya Haga (University of Tokyo, Tokyo, Japan) was previously described [8].

2.2. *Conventional filter assay for GTPγS binding*

GTPγS binding to a purified G protein was assayed in the presence of 10 mM Mg^{2+} and [^{35}S]GTPγS, as described in [9]. In brief, a G protein was incubated in buffer A (10 mM HEPES/ NaOH (pH 7.4), 100 μM EDTA and 10 mM $MgCl_2$) supplemented with 60 nM [^{35}S]GTPγS at 37°C (the G protein preparation used contained 100 μM $MgCl_2$). The reaction was terminated by buffer B (100 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$ and 100 mM NaCl) supplemented with 20 μM GTP. Samples were kept on ice for 2 h, then filtered onto nitrocellulose membranes. After washing the filter, the radioactivity of the filter was counted in a liquid scintillator.

2.3. *Preparation and solubilization of cell membranes*

Cell membranes were prepared as follows. After washing with ice-cold PBS two times, S49 cells were collected and centrifuged at 1500 rpm for 10 min at 0-4°C. The pellet was suspended in a solution containing 20 mM HEPES/NaOH (pH 7.5), 2 mM $MgCl_2$, 1 mM EDTA, 2 mM DTT, and homogenized by passing through a 24-gauge syringe, and centrifuged at 15000 rpm for 5 min at 0–4°C. After the supernatant was centrifuged at 15000 rpm for 30 min at 0–4°C, the pellet was resuspended in buffer C (25 mM Tris-HCl (pH 7.5), 1 mM EDTA and a mixture of 2 mM PMSF, 20 μg/ml aprotinin, and 20 μM leupeptin (PAL) and frozen in liquid nitrogen. For the experiment, these crude membranes were solubilized by the same volume of buffer D (20 mM HEPES/NaOH (pH 7.4), 2 mM EDTA, 240 mM NaCl, 1% CHAPS and 2× PAL) for 60 min at 0–4°C. These were employed by a final tenfold dilution in the experiment using isoproterenol.

2.4. *ELISA plate assay for GTPγS binding*

Purified G proteins or prepared membranes were incubated with 60 nM [^{35}S]GTPγS in buffered A for various periods at 37°C. At the end of the incubation, ten volumes of ice-cold buffer B, supplemented with 20 μM GTP, were added to the samples, and put into wells of an ELISA (enzyme-linked immunosolvent assay) plate that had been coated with antibodies. After 2 h incubation on ice, the solution was discarded and the wells were washed three times with ice-cold PBS containing 0.05% Tween 20. The radioactivity of each well was counted by putting it in liquid scintillator. For the coating of G-protein antibodies, an anti-rabbit immunoglobulin antibody of goat (final 1:1000, affinity purified, TAGO) was initially coated onto the 24-well plates by incubating the antibody in the wells for two hours on ice. After discarding the antibody, the wells were coated with an anti-Gα antibody of rabbit (final 1:1000 by incubating 2 h on ice. For this purpose, a 24-well plate for ELISA (Corning, Cat No. 24106-8), each well of which is detachable, was suitable.

3. RESULTS AND DISCUSSION

The basic strategy of the present assay was to incubate solubilized membranes with radioactive GTPγS at 37°C for various periods and to then reasure the radioactivity that was associated with anti-G_α antibodies that were coated onto ELISA plates by incubating the sample in the plates at 0–4°C. In order to selectively coat ELISA plates with an anti-G_α antibody, they were initially coated with an anti-rabbit immunoglobulin goat antibody, then with a specific anti-G_α antibody. We refer to the present assay as ELISA plate assay.

In the initial experiment, we examined whether the ELISA plate assay using AS/7, could qualitatively measure GTPγS binding to purified G_i. AS/7 is an antiserum against transducin and also recognizes G_i but not other G proteins [6]. In this study, GTPγS binding to 0.5, 1.0 and 2.0 nM of G_i was measured, respectively, by the conventional filter assay and the present ELISA plate assay. As shown in Fig. 1 (left), there was an extremely good correlation between the values of GTPγS binding obtained from both assays. The GTPγS binding to the wells of the ELISA plate precoated with antibodies in the absence of G proteins was below 50 cpm. When GTPγS binding to purified G_o was measured by the ELISA plate assay with AS/7, the amount of radioactivity detected was ≈1/6 of the amount detected by the conventional filter assay (Fig. 1, right). Because the G_o used here contains ≈10–20% of G_i, this result suggests that the labeled G protein detected by the ELISA plate assay is G_i. Therefore, the present assay using AS/7 is assumed to detect GTPγS binding to G_i specifically.

Why is the value of GTPγS binding to G_i measured by the ELISA plate assay considerably less than that measured by the conventional filter assay? The value from the ELISA plate assay represents only part of all GTPγS binding to G_i, since the detection of G_i as AS/7 is shown to be specific [6]. It is remarkable that the ratio of the value from the ELISA plate assay to that of the conventional method is constant. AS/7 strongly recognizes G_{i1} and G_{i2}, and only slightly recognizes G_{i3}. However, we can exclude the possibility that the antibody-nonreactive G_i is G_{i3}, because the G_i protein preparation used in this study is a highly purified G_{i2} (to near homogeneity).

Another possibility is that the amount of the antibody is insufficient for the complete detection of G_{i2}. However, even when the concentration of the antibody used for the coat was increased up to sixfold, the finally detected radioactivity yielded no significant change (data not shown). This was also the case with the incubation period. Even when the period for treatment of the precoated ELISA plates with samples was prolonged, the result was similar. These findings suggest that this possibility is unlikely.

An alternative possibility that AS/7 recognizes a con-

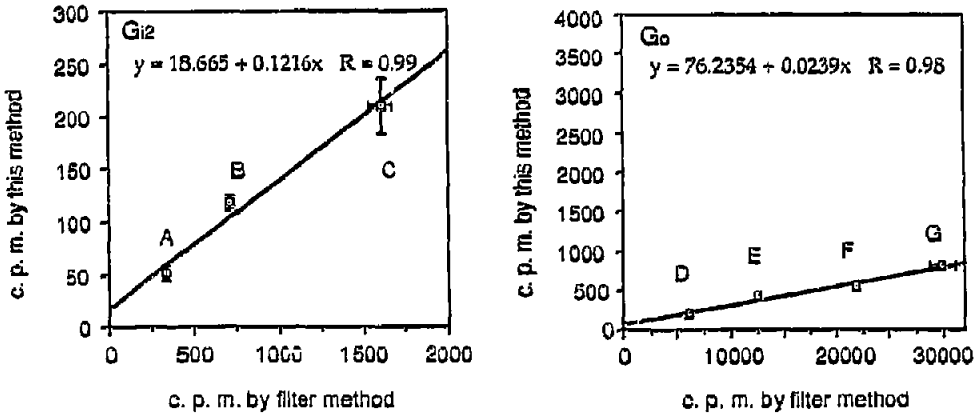

Fig. 1. Correlation between the ELISA plate using AS/7 (an anti-$G_{i\alpha}$ antibody) and the conventional filter assay. 0.5 (A), 1.0 (B), and 2.0 nM (C) of purified G_{i2} (left) and 2.5 (D), 5 (E), 10 (F), and 20 nM (G) of G_o (right) was incubated with radioactive GTPγS for 30 min at 30°C in the presence of 10 mM Mg^{2+}. The filter assay and the ELISA plate assay were carried out using the same samples as described in section 2. The radioactivity counts obtained from both assays were plotted (the horizontal and the longitudinal lines indicate the counts obtained by the filter assay and those by the ELISA plate assay, respectively). Bars indicate the S.E. of the values. The equations represent the regression lines that were obtained from the minimal square method.

stant fraction of G_i in a quantitative fashion is therefore likely. At present, it remains unclear what fraction is represented by this immunoreactive G_i. However, since AS/7 prefers the detection of G_i in immunoblot analysis to that in immunoprecipitation, the fraction of G_i detected by this method may be related to the secondary or the tertiary structure of G_i. Therefore, by altering G_i antibodies, the efficiency for the detection by the present method may be improved. In any event, it is possible to quantitatively assess the overall binding of GTPγS to G_i, based on the relationship of the values between the present assay and the conventional filter assay.

Next we tested the effectiveness of the present assay in measuring the amount of G_s using RM/1, an antiserum specific for $G_{s\alpha}$ [7]. In this experiment, four points of the $G_{s\alpha}$ amount were examined, and again we found an excellent correlation between this ELISA plate assay and the conventional filter assay (Fig. 2, left). In contrast, the radioactivity of GTPγS that bound to purified G_{i2} was scarcely detected by the ELISA plate assay using RM/1 (Fig. 2, right). Thus, by combining the measurement using AS/7 with that of using RM/1, it is possible to differentially measure the GTPγS binding to G_i and G_s in one sample.

Based on these results, we next examined the binding of GTPγS to native S49 lymphoma cell membranes by the ELISA plate method. The crude membranes were first solubilized with CHAPS and then diluted without centrifugation to the level at which CHAPS was finally included at < 0.1%. As shown in Fig. 3, GTPγS was found to bind time-dependently to the ELISA wells that had been precoated with RM/1. This GTPγS binding obeyed the first order kinetics according to the equation, $\ln[B_T-B)/B_T]=-k_{app}t$, where B is the binding at time t and B_T is the total binding observable at an infinite time. The k_{app} signifies the apparent first-order rate constant for GTPγS binding and is equivalent to the slope of the GTPγS binding curve at time 0 and represents the actual GTPγS binding rate. The k_{app} value given from the result presented in Fig. 3 is ≈0.07 min^{-1}, which is comparable to the k_{app} value of purified G_s in the presence of 20 μM Mg^{2+}, suggesting that the

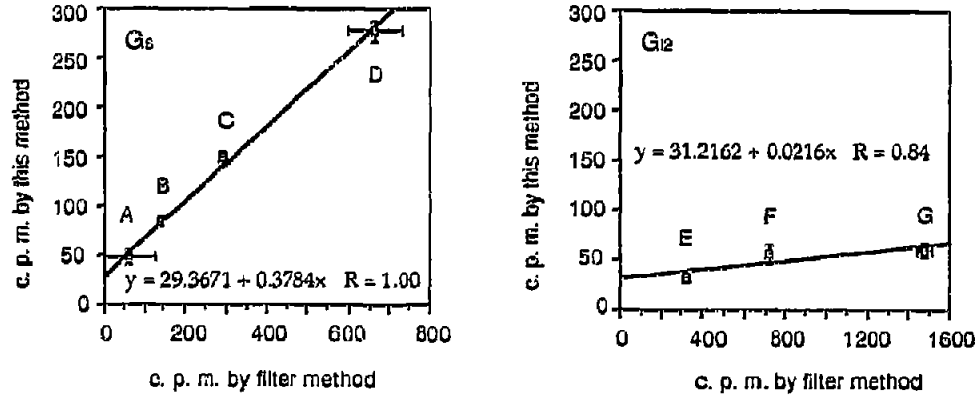

Fig. 2. Correlation between the ELISA plate assay using RM/1 (an anti-$G_{s\alpha}$ antibody) and the conventional filter assay. 0.05 (A), 0.1 (B), 0.2 (C), and 0.4 nM (D) of purified G_s (left) and 0.5 (E), 1.0 (F), and 2.0 nM (G) of purified G_{i2} (left) were incubated with radioactive GTPγS for 30 min at 30°C in the presence of 10 mM Mg^{2+}. The radioactivity counts obtained from the assays were plotted as indicated in the legend of Fig. 1. Bars indicate the S.E. of the values.

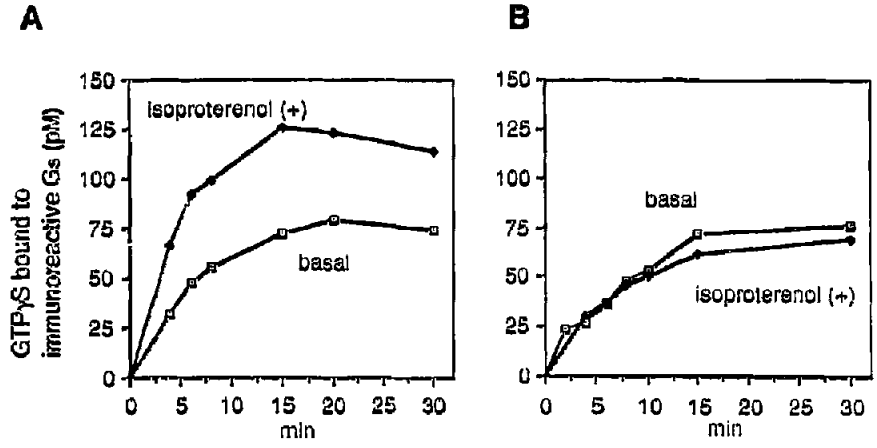

Fig. 3. The binding of GTPγS to immunoreactive G_s of S49 lymphoma cells and *unc* mutant cells in response to isoproterenol. Crude membranes isolated from S49 lymphoma (A) and *unc* mutant cells (B) were incubated with [^{35}S]GTPγS in the absence (▫) or presence (●) of 100 μM (−)isoproterenol for various periods at 37°C. The materials were then put into the RM/1-coated wells of the ELISA plate, and the radioactivity of the well was measured as described in section 2. The non-specific binding of GTPγS to the antibody-coated wells in the absence of membranes was 39.6 ± 1.5 cpm., which was subtracted from the count of the GTPγS that bound to the wells in the presence of membranes.

present assay mainly detects the binding of GTPγS to G_s in native cell membranes.

When the membranes from S49 cells were incubated with 100 μM (−)isoproterenol at 37°C and then subjected to the measurement of GTPγS bound to G_s by the present ELISA plate assay at 4°C, the k_{app} value was found to be increased by ~ twofold. In contrast, when isoproterenol was incubated with the membranes from *unc* mutant cells at 37°C and the samples were subjected to the ELISA plate assay, the binding of GTPγS was not affected. These findings suggest that isoproterenol promotes GTP-binding activity of G_s in wild-type S49 cell membranes, but not in *unc* mutant membranes. It has been well documented that isoproterenol stimulation of the β-adrenergic receptor leads to the activation of G_s in phospholipid vesicles reconstituting the purified β-adrenergic receptor and G_s [10], and that isoproterenol treatment of cells or cell membranes stimulates adenylyl cyclase activity [11]. To the best of our knowledge, however, isoproterenol-induced activation of G_s in crude membranes has not been documented directly. Thus, this is the first record in which G_s activation by isoproterenol was detected in native cell membranes. Furthermore, *unc* mutant cells have a point mutation on $G_{s\alpha}$, by which they become unresponsive to β-adrenergic stimulation. This therefore provides evidence that the increase in GTPγS binding recorded by the present assay is attributed to the increase in G_s activity.

In this study, we have developed a novel method for the differential measurement of GTPγS binding to specific G proteins in cell membranes, by using anti-G protein antibodies. This may provide a useful tool for the measurement of the coupling between cell surface-receptor stimulation and specific classes of G proteins in native cells or cells that express mutant receptors and/or G proteins.

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