Expression of a G Protein Subunit, α₁₁, in Balb/c 3T3 Cells Leads to Agonist-specific Changes in Growth Regulation*

(Received for publication, November 29, 1990)

Zheng Cui†, Mercedes Zubiaur†, Donald B. Bloch†, Thomas Michel‡, J. G. Seidman§, and Eva J. Neer††

From the Departments of †Medicine and ‡Genetics, Harvard Medical School, Boston, Massachusetts 02115

Cellular receptors for many hormones, neurotransmitters, and growth factors are coupled to intracellular effector enzymes or ion channels through a set of heterotrimeric G proteins. In order to determine whether isoforms of G protein α subunits contribute differentially to mitogenic responses, we introduced an α subunit isoform, α₁₁, into Balb/c 3T3 cells that normally lack this subtype. Balb/c 3T3 cells transfected with a plasmid containing cDNA encoding α₁₁ expressed the α₁₁ protein as judged both by the appearance of immunoreactive α₁₁ protein on Western blots and by two-dimensional analysis of the proteins [32P]ADP-ribosylated by pertussis toxin. The amount of α₁₁ expressed is less than the amount of α subunits endogenously present in these cells.

Expression of α₁₁ in the transfected cells slightly blunts stimulation of adenylylcyclase by GTP, guanosine 5'-3-O-(thio)triphosphate, or forskolin, but has no major effect on the ability of thrombin to inhibit the enzyme. In contrast, the expression of α₁₁ has significant effects on cell growth and on the mitogenic response to thrombin. The α₁₁-transfected cells have a doubling time that is twice as long as control cells transfected with the same plasmid without a cDNA insert. Despite their slower growth, thymidine incorporation in response to thrombin is greater in transfected than in control cells. Thrombin-stimulated DNA synthesis is sensitive to inhibition by pertussis toxin and is 5-fold more sensitive to inhibition by pertussis toxin in transfected cells than in control cells. The changes are receptor-specific since the mitogenic response to platelet-derived growth factor is indistinguishable between control and transfected cells. These studies suggest that the α₁₁ subunit composition of the cell may have profound effects on its growth and its response to stimulation through a specific cell surface receptor.

Many membrane receptors for neurotransmitters, hormones, and mitogens are coupled to intracellular effectors and ion channels by a set of heterotrimeric G proteins composed of α, β, and γ subunits. The α subunits bind guanine nucleotides and are substrates for covalent modification by bacterial toxins. One structurally very similar set of G protein α subunits can be covalently modified by Bordetella pertussis toxin. This set includes the two transducins that are localized exclusively in the retina, as well as four proteins (α₁, α₁₂, and α₁₃) that are found in many kinds of cells (reviewed by Neer and Clapham (1988)). The α₁ protein is most abundant in the central nervous system but is not located exclusively there (Neer et al., 1984; Sternweis and Robishaw, 1984; Huff et al., 1985). α₁₂ and α₁₃ have been found in all cells studied to date, whereas α₁₁ appears to have a more limited distribution, being absent in some cells of myelocytic origin, some fibroblast lines, endothelial cells (Kim et al., 1988; Lee et al., 1989), and GH3 cells. The pertussis toxin substrates mediate inhibition of adenylylcyclase, activation of phospholipase C and phospholipase A₂, and modulation of ion channels.

Within the family of nonretinal pertussis toxin substrates, the structural similarity is extremely close. The amino acid sequence of α₁₁ is 85% identical with α₁₂ and 95% identical with α₁₃; α₁ is about 70% identical with the α₁ group (reviewed by Loehr and Simon (1988)). Given such a high degree of structural similarity, one important question is whether the different α subunits in this family preferentially couple particular plasma membrane receptors and effectors or whether the cell uses them interchangeably. If the functions of the subunits are entirely interchangeable, then hormonal responsiveness might be determined only by the total level of α subunits in this family and not by the subtype composition. One argument against the complete interchangeability of the α proteins is the extreme conservation of their structures across species. For example, α₁₁ is 98% identical among humans, rats, mice, and cows (Loehr and Simon, 1988). This conservation argues that even the small difference in sequence must be important in order to have been conserved.

Analysis of the kinetics of GTP binding, hydrolysis, and release shows that there are subtle differences among the three α₁ isoforms (Carty et al., 1990; Linder et al., 1990). Although these differences in kinetics may be important for setting the rate of onset and duration of signal transmission, they do not reveal whether G protein α subunits are specific for particular receptors or effectors. To define the specificity of their interactions with receptors or effectors, different α₁ subtypes have been reconstituted with target proteins in vitro. The results of such reconstitution experiments have shown some specificity but also many examples of complete interchangeability (reviewed by Neer and Clapham, 1988).

We have tested the hypothesis that G protein subunit isoforms contribute differentially to horseradish responses by

---

* This work was supported by National Institutes of Health Grant GM 36295 (to E. J. N.), a grant from the Howard Hughes Medical Institute and National Institutes of Health Grant CA 46361 (to J. G. Z.), a fellowship (to Z. C.) and a Clinician Scientist Award (to T. M.) from the American Heart Association (Massachusetts Affiliate), and a Fulbright-Ministerio de Educacion y Ciencia, Spain Postdoctoral Fellowship (to M. Z.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Cardiovascular Division, Brigham and Women's Hospital, 75 Francis St., Boston, MA 02115.

‡ From the Departments of Medicine and Genetics, Harvard Medical School, Boston, Massachusetts 02115.

§ J. G. Seidman, unpublished data.
determining the consequences of altering a cell’s complement of these proteins. To carry out the study, we needed a cell line with well defined plasma membrane receptors that does not express all three subtypes of α. We found that Balb/c 3T3 cells lack α1 and α2 proteins but express α3 and α3.0. The growth of these cells and of other fibroblast cell lines is regulated by mitogens and growth factors that act through cell surface receptors, some of which are coupled to G proteins (Chen and Buchanan, 1975; reviewed by Rozengurt, 1986; and Pouyssegur, 1990). Treating the cells with pertussis toxin blocks the mitogenic effect of such agonists, including thrombin, bombesin, vasopressin, or bradykinin, but does not affect growth stimulation by PDGF, fibroblast growth factor, or epidermal growth factor (Letterio et al., 1986; Chambard et al., 1987; Murayama and Ui, 1987; Hoshijima et al., 1988). The agonists that act through G proteins, as well as those that act through tyrosine kinase receptors, activate phospholipase C but do so by different mechanisms (Berridge and Irvine, 1984; Hasegawa-Sasaki et al., 1988; Hoshijima et al., 1988; Nanberg and Rozengurt, 1988; Paris et al., 1988; Taylor et al., 1988). However, activation of phospholipase C is not obligatory for their stimulation of cell growth (Zachary et al., 1987; Taylor et al., 1988; Hill et al., 1990). The exact pathway leading to increased cell growth in response to these agents is not known, although it appears to involve elevation of intracellular calcium and/or modulation of arachidonic acid metabolites and Na+/H+ exchange (Pouyssegur et al., 1982; Murayama and Ui, 1985; Raben et al., 1987; Nanberg and Rozengurt, 1988; Handler et al., 1990; also reviewed by Rozengurt, 1986).

In this paper we report the results of studies comparing control Balb/c 3T3 and cells expressing α1 with respect to growth and to stimulation by thrombin and PDGF. We have previously shown that introduction of an additional α subunit (α3) into Y1 adrenal cells slows their growth (Bloch et al., 1989). Expression of α3 in a cell type that normally does not produce this subunit both slows its growth and alters its response to thrombin. Comparison of the thrombin response of the transfected cells to that of control cells suggests that the receptor for this mitogen may couple preferentially to the newly introduced α subunit.

**MATERIALS AND METHODS**

**Cell Culture**

Balb/c 3T3 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, penicillin (50 units/ml), and streptomycin (50 μg/ml) at 37 °C with 5% CO2. Transfected cells were cultured in the same medium with 0.2 mg/ml G418 (GIBCO).

**Construction of the Expression Vector Containing Rat α3.0 cDNA**

The rat α3.0 cDNA was isolated from a rat olfactory neuroepithelium cDNA library by R. Reed and D. Jones (Johns Hopkins University School of Medicine) who kindly provided the clone (Jones and Reed, 1987). The EcoRI fragment of α3.0 cDNA (representing the entire coding region) was inserted into the EcoRI site of the retroviral expression vector, pDOJ (Price et al., 1987; provided by C. Cepko, Harvard Medical School). The orientation of the insertion was confirmed by asymmetric digestion with restriction enzymes HindIII and XbaI. In this vector (pDOJ-α3.0), the transcription of α3.0 cDNA is directed by the Moloney murine leukemia virus long terminal repeat. The vector also contains the neomycin resistance gene under the control of the simian virus 40 early region promoter in mammalian cells, expression of the neomycin resistance gene confers resistance to the antibiotic G418 sulfate.

**Transfection of Balb/c 3T3 Cells**

Balb/c 3T3 cells were transfected with pDOJ-α3.0, and pDOJ control vector by the calcium phosphate precipitation method (Wigler et al., 1978). Five 10-cm dishes of confluent Balb/c 3T3 cells were split 1:15 on the day before transfection. The cells were fed again with complete (10% serum) medium mediating transfection 20 μg of plasmid DNA that had been purified twice by CsCl gradient centrifugation were used for each dish. DNA was precipitated in 0.5 ml of 250 μg CaCl2, 5 mM Tris-OH, 0.5 mM EDTA (pH 7.6) and added to an equal volume of 2 × concentrated HEFES-buffered saline. The mixture was incubated at room temperature for 30 min to allow the DNA to precipitate before being added to 10-cm dishes of cells. The precipitates were left on the cells for 16 h, after which they were washed off with fresh culture medium containing 0.5 mg/ml G418. Control cells were transfected with the pDOJ vector without a cDNA insert and grown in G418.

Control cells and cells transfected with pDOJ-α3.0 were selected in parallel. The cells were grown in 0.5 mg/ml G418, and approximately 40 resistant clones from pDOJ and pDOJ-α3.0 transfection were obtained after 12-14 days in culture. Analysis of the DNA from clones transfected with pDOJ-α3.0, by Southern blotting showed positive bands with an α3 probe. However, none of these clones was positive on Northern analysis of their mRNA. Because of previous experience that Y1 adrenal cells expressing α3 grow slowly (Bloch et al., 1989), we continued the selection to try to isolate slow growing colonies. Four such colonies were isolated after 3–4 weeks (as described below). These more slowly growing colonies were positive for α3, mRNA (data not shown) and protein (data not shown) and were used in the subsequent studies. We did not see multinucleate cells such as were seen in Y1 adrenal cells transfected with α2 (Bloch et al., 1989). To establish that all the functional changes that we observe are due to transfection and not to clonal variations within the Balb/c 3T3 cell line, three control and three transfected lines were characterized.

**Analysis of α3 Isoforms**

Two-dimensional Electrophoresis of a Subunit—Cells from 10-cm culture dishes were collected by scraping them from the dishes. They were washed 3 times with phosphate-buffered saline, and the pellet taken up in a buffer containing 50 mM Tris, pH 7.6, 75 mM sucrose, 6 mM MgCl2, 1 mM EDTA, and 1 mM dithiothreitol. The cells were lysed by freeze/thawing three times and by repeated passage through a 23-gauge needle. To make a crude particulate fraction, the lysate was centrifuged at 10,000 rpm in a SS-34 rotor of a Sorval centrifuge for 20 min. Protein was determined by the method of Lowry (1951), as modified by Bailey (1967), using bovine serum albumin as a standard.

30 μg of either total cellular protein or of the crude particulate fraction was incubated in a total of 40 μl with 5 μM NAD, 3 mM ATP, 12.5 mM isoniadiazid, 10 mM thymidine, 0.1 mM GTP, 50 mM Tris (pH 7.6), 0.5 μCi [32P]NAD, 0.1% Lubrol PX, and 10 μg/ml activated pertussis toxin (List Biochemicals). The toxin was activated in 20 mM dithiothreitol at 30 °C for 15 min. The ADP-ribosylation reaction was carried out at 37 °C for 30 min. For one-dimensional SDS-PAGE, the reaction was stopped by adding 3× concentrated Laemmli sample buffer (Laemmli, 1970). For two-dimensional gel electrophoresis, the reaction was stopped with 5 mM K2CO3 and 0.2% deoxycholate. Two-dimensional gel electrophoresis was carried out as described by O’Farrell (1975). The gel was dried between cellulose membranes and used to expose Kodak XAR film at −70 °C.

Western Blots—50–75 μg of total cell lysate or crude particulate fraction was applied to lanes of an 11% SDS-PAGE gel. The proteins were transferred to nitrocellulose, as described by Towbin et al. (1979). The filters were blocked with 3% bovine serum albumin or with 3% bovine serum albumin and 3% goat serum for 1–2 h at room temperature or overnight at 4 °C. They were incubated with antibody diluted in 3% bovine serum albumin or 3% bovine serum albumin, 3% goat serum overnight at 4 °C. The nitrocellulose was washed twice with 10 mM Tris, 0.9% NaCl, pH 7.4 (TBS), once with TBS plus 0.05% Nonidet P-40 and again with TBS before incubating with 32P-labeled goat anti-rabbit IgG (ICN, Inc.). The radioactive IgG was washed from the membrane as described above, and the membrane was used to expose Kodak XAR film with two screens at −70 °C. The antibodies used were LDL, an antibody raised against peptide corresponding to α1, sequence 159–168 (a kind gift of Dr. Alan Spiegel,
Washington, D. C. (Spiegel et al., 1990); R7, an antibody against bovine brain β; R4, an antibody against brain αs (Huff et al., 1985); AS/7, an antibody raised against a peptide corresponding to the carboxyl terminus of transducin, which recognizes αs1 and αs2 equally (Du Pont-New England Nuclear; NEI, 801, Spiegel, 1990); NE1, 805 (Du Pont-New England Nuclear), an antibody to the carboxy-terminal peptide of αs2 which recognizes αs1 and αs2. To quantitate the amounts of αs1 to αs3 was estimated in two ways that are described below under "Results."

**Adenylylcyclase Assays**

The adenylylcyclase assay was performed as described by Salomon (1979). The standard assay contained 0.5 mM ATP, 1 μCi of [32P]ATP (Amersham Corp.), 50 μM cAMP, 5 mM MgCl₂, 50 mM Tris, pH 7.6, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 10 mM creatine phosphate, 5 units/ml creatine phosphokinase, and 20-30 μg of membrane protein in a total volume of 50 μl. The reaction was carried out at 30 °C for 20 min and stopped with a solution containing 8,000-15,000 cpm of [32P]cAMP to monitor recovery of [32P]cAMP from the columns. Under these conditions, the reaction was proportional to the amount of protein (not shown).

**Analysis of Cell Growth**

Cells were plated in 24-well plates and allowed to grow for 24 h. They were trypsinized to remove them from the dish, stained with trypan blue (final concentration 0.2%), and counted in a hemocytometer.

**[3H]Thymidine Labeling of DNA**

Approximately 2-5 × 10⁵ cells were plated into each well of a 24-well culture dish (Nunc, Inc.) and grown to confluence (2 days) in 2 ml of Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 2 mM L-glutamine, 50 units/ml penicillin, 50 mg/ml streptomycin. The concentration of fetal calf serum was then lowered to 1% for 4 days. Prior to the experiment, the cells were placed in serum-free medium for 24 h to make cells quiescent and to remove them from serum mitogens. The cells were washed once with serum-free medium, then incubated in serum-free medium containing 1 μCi of [3H]thymidine/ml, and the indicated amounts of mitogen and/or pertussis toxin. Bovine α-thrombin was from Sigma. For stimulation through the PDGF receptor, we used recombinant c-sis (R-B homologue). The radioactive labeling was carried out for 36 h after addition of [3H]thymidine. The cells were harvested by trypsinization and resuspended in 0.5 ml of 0.1% bovine serum albumin in phosphate-buffered saline. Pertussis toxin, when it was used, was added 3 h prior to addition of the the mitogen and the [3H]thymidine. Labeling was carried out for 36 h after addition of [3H]thymidine. Labeled cells were treated with trypsin and resuspended in 0.5 ml of phosphate-buffered saline. The cells were transferred to microcentrifuge tubes and precipitated with 5% trichloroacetic acid. The pellets were washed twice with 75% ethanol and dissolved in 0.5 ml of 0.1 M NaOH, 2% Na₂CO₃, and the radioactivity was determined by liquid scintillation counting.

**RESULTS**

**Expression of αs1 Protein by Transfected Cells**—We used two methods to show that the αs1-transfected cells, but not the control cells, expressed αs1 protein. Fig. 1 shows the results of a Western blot probed with antisera that recognize αs1 (Panel A) and the β subunit (Panel B). Panel A shows that cells transfected with pDOJ-αs1 express an immunoreactive protein, with a molecular weight of 41,000, whereas the control cells do not. The same blot as was previously probed with anti-αs1 antibody was reprobed with antibody to the β subunit and is shown in Panel B. There is no detectable difference in the amount of β subunit in the two cell types. All the immunoreactivity was associated with membranes. Fig. 2 shows analysis of membrane proteins from two control and three transfected cell lines with antisera AS/7 (Du Pont-New England Nuclear). This antiserum was raised against a carboxyl terminal peptide of transducin. It cross-reacts equally with αs1 and αs2, which are identical with each other in this region (Spiegel, 1990).

The immunologic studies show that αs1-transfected cells contain a new immunoreactive protein that is absent from control cells, confirming the results described above. Because the antibody recognizes αs1 and αs2 equally, the ratio of immunoreactivity in the two bands should reflect the proportion of αs1 to αs2 in the transfected cells. We used two methods to estimate the ratio of αs1 to αs2. The first was to average the area of the peaks of αs1 and αs2, which is more abundant than this method would tend to overestimate the amount of αs1.

**Fig. 2.** Comparison of levels of αs1 and αs2 in control and transfected cells. 50 μg of crude particulate fraction was loaded onto each lane of the gel. After separation by electrophoresis on an 11% SDS-PAGE gel and transfer to nitrocellulose, the proteins were identified with antibody AS/7 (1:300 dilution). The sequences in αs1 and αs2 recognized by this antibody are identical. Lanes 1 and 2, two different control clones. Lanes 3-5, three different αs1-transfected clones. Bound antibody was detected with 125I-labeled anti-rabbit IgG. The radioautogram was exposed for 66 h without screens.
posed for 16 h with intensifying screens. The results are representative of two similar experiments using other clones of control and transfected cells.

As described under "Materials and Methods," cellular protein were labeled with [3H]-labeled NAD and pertussis toxin-catalyzed ADP-ribosylation of collected as described under "Materials and Methods." 20 cell, transfected cells; Panel B, control cells. Both kinds of cells were labeled and analyzed simultaneously. The radioautographs were exposed for 16 h with intensifying screens. The results are representative of two similar experiments using other clones of control and α1-1-transfected cells.

FIG. 3. Two-dimensional gel electrophoresis of pertussis toxin-catalyzed ADP-ribosylation of α subunits in control and transfected cells. Late log phase Balb/c 3T3 mouse fibroblasts were collected as described under "Materials and Methods." 20 μg of total cellular protein were labeled with [3H]-labeled NAD and pertussis toxin as described under "Materials and Methods." +, +, positive and negative poles for isoelectric focusing, respectively. Panel A, α1-transfected cells; Panel B, control cells. Both kinds of cells were labeled and analyzed simultaneously. The radioautographs were exposed for 16 h with intensifying screens. The results are representative of two similar experiments using other clones of control and α1-1-transfected cells.

minor spot. The α1-1-transfected cells have an additional ADP-ribosylated protein with a more positive isoelectric point. This pertussis toxin-labeled protein has a pI very similar to that which we have found for purified ADP-ribosylated α1, from bovine brain (data not shown). By densitometry, the α1-3 spot was 80 ± 20% (n = 2) of the endogenous α1-2 spot.

Balb/c 3T3 cells were also transfected with pDOJ containing rat α1 cDNA. Although G418-resistant colonies were obtained that were positive on Southern blots using rat α1 cDNA as a probe, none of these produced α1 protein (data not shown).

Adenylyl cyclase Activity in α1-transfected Cells—The α1 proteins were first described as inhibitors of adenylyl cyclase (Murayama and Ui, 1985). Therefore, changes in the complement of α1 subtype might be expected to change the activity of that enzyme. Fig. 4 shows a comparison of GTPγS-stimulated adenylyl cyclase activity in membranes from control and α1-transfected cells. In α1-transfected cells, the level of adenylyl cyclase activity achieved at maximal concentrations of GTPγS is approximately 60% of that in the control cells. In addition, the EC50 for GTPγS apparently increased from 33 ± 4 to 115 ± 15 nM (n = 4). The maximal level of GTP-stimulated activity is also diminished in the α1-transfected cells, although there seems to be no difference in the apparent EC50 (data not shown).

Thrombin inhibits adenylyl cyclase in several cell types (Murayama and Ui, 1985; Magnaldo et al., 1988) and does so both in α1-transfected and control cells (Fig. 5). Although all activities are blunted in the α1-transfected cell, there is no dramatic difference in the ability of thrombin to inhibit adenylyl cyclase, especially when the activities are calculated relative to the basal activity.

Effect of Transfection of α1 on Cell Growth—Cells expressing α1 protein grow more slowly than cells transfected with the pDOJ plasmid without insert. The slowness of growth created some difficulties in the isolation of cells expressing α1 (see "Materials and Methods"). Fig. 6 shows a comparison of growth rates of three independent α1-transfected and control clones. The doubling time is 11 ± 1 (n = 3) h for control cells and 24 ± 1 (n = 3) h for α1-transfected cells.

Stimulation of DNA Synthesis by Thrombin in α1-transfected and Control Cells—Fig. 7 shows the response of quiescent Balb/c 3T3 cells to stimulation by thrombin at the concentrations indicated on the figure. The EC50 for thrombin is 8 nM for both control and α1-transfected cells, although
the response is greater in cells expressing α1 than in controls. Thymidine uptake is increased 2-fold in control cells, but 5-fold in α1-transfected cells. The response of the cells to 1 and 10% serum was measured in the same experiments. The absolute response of control cells and α1-transfected cells was similar, although the increase over unstimulated thymidine incorporation was greater in control than in α1-transfected cells (1% serum, 8- and 6-fold increases, respectively; 10% serum, 15- and 10-fold increases, respectively).

The stimulation of DNA synthesis by thrombin is inhibitable by pertussis toxin (Fig. 8). The α1-transfected cells are more sensitive than the controls to pertussis toxin, with an EC50 of 0.9 ± 0.1 ng/ml (n = 4) compared with 5 ± 0.6 ng/ml (n = 4) for the controls. Furthermore, the inhibition of DNA synthesis is more complete for the α1-transfected than the control cells.

Effect of PDGF (rc-sis) on the Growth of Transfected and Control Cells—Control and α1-transfected cells respond to PDGF (rc-sis) in exactly the same way (Fig. 9). Pertussis toxin inhibits the growth response to PDGF, although it does so at higher concentrations and to a lesser extent than the inhibition of response to thrombin (Fig. 10). The significance of growth inhibition at high concentrations of pertussis toxin is not clear.

**DISCUSSION**

Transfection of BALB c/3T3 cells with pDOJ-α1 results in expression of α1 protein. Immunologic analysis confirmed the presence of α1 protein in α1-transfected cells but not in control cells. One antibody we used recognizes a sequence that is identical in α1 and α2. The ratio of immunoreactive bands allows us to estimate the relative concentration of these two α subunits. By this analysis, the amount of α1 is between 30 and 44% of the amount of α2. There was no detectable change in the amount of β in α1-transfected cells. These calculations are important for the interpretation of subsequent experiments. A massive overproduction of an α subunit could reveal low affinity associations or could significantly shift the equilibrium between α and β subunits by sequestering βγ subunits. Our estimate of the increase in α subunits caused by transfection is an overestimate since we are relating it only to α2, whereas in fact the cell also has αα, αα (by immunoblot, data not shown) and probably other, as yet unidentified, α subunits that might participate in an equilibrium with βγ subunits.

The expression of α1 in the transfected cells was also shown by the appearance of a new radiolabeled spot on two-dimensional gel electrophoresis of pertussis toxin-catalyzed [125I]ADP-ribosylation of α subunits. Densitometry of the radiograms shows that the new α subunit is present in an amount that is approximately 80% of the endogenous α subunit(s). Susceptibility to ADP-ribosylation is a complex process. It requires interaction of α with βγ subunits (Neer et al., 1984) and may reflect the affinity of different α subunits for βγ subunits (Huff et al., 1985), as well as factors intrinsic to the different α structures. ADP-ribosylation of α1 appears to be more efficient than that of α1, since ADP-ribosylation in α1 is 80% of that in α2, whereas by immunologic analysis, the α1 protein is 30% of α2. This observation is consistent with our
findings that the thrombin responses of α₁-transfected cells were more sensitive to pertussis toxin than control (see below).

A major goal of this study was to determine whether a change in the α subunit isofrom composition affected coupling of receptors. To this end, we analyzed the effects of thrombin on adenylylcyclase and on DNA synthesis. Expression of α₁ blunted basa activity, as well as stimulation of adenylylcyclase by GTP·S, GTP, or forskolin, as if the adenylylcyclase inhibitory "tone" was generally higher. However, inhibition of adenylylcyclase by thrombin was not substantially changed. Since the changes were small and generalized, we could not interpret them unambiguously.

In contrast, transfection of the cells with α₁ had a marked effect on thrombin stimulation of DNA synthesis. Thymidine uptake in response to thrombin was greater in transfected than in control cells. The growth response to thrombin was inhibited by pertussis toxin, and the α₁-transfected cells were now 5-fold more sensitive to pertussis toxin than the control cells. Taken together, these results suggest that thrombin receptor may couple preferentially to the newly introduced α₁, simultaneously giving a greater response to thrombin and a greater sensitivity of that response to inhibition by pertussis toxin. We were not able to determine how thrombin affects proliferation. Thrombin responses are blocked by serum, and we were not successful in supporting proliferation of either the control or α₁-transfected cells in 0.5% serum or less.

The effects of transfection are not the same for all agonists that stimulate DNA synthesis in these cells. There was no difference in the dose-response curve to PDGF (rc-sis) between control and α₁-transfected cells nor in the effect of pertussis toxin on PDGF (rc-sis)-stimulated DNA synthesis. Pertussis toxin does cause a partial inhibition of DNA synthesis, but this happens at rather high concentrations of the toxin. Similar effects have been observed in other cells (for example, Taylor et al., 1988), but the significance of this observation is not clear.

Selection of transfectants expressing α₁ was hampered by the fact that the cells expressing α₁ grew more slowly than the controls. A similar decrease in growth rate was seen in Y-1 adrenal cells transfected with α₁ (Bloch et al., 1989). In addition to decreased growth rates, the Y-1 cells became multinucleate. We did not see multinucleate α₁-transfected or control BALB c/3T3 cells. The stimulation of DNA synthesis in quiescent α₁-transfected and control cells by 1 or 10% serum is similar, although the amount relative to the stimulated values is greater in control than in α₁-transfected cells. It is possible that α₁-transfected cells are unable to respond normally to a growth factor in serum. Such a defect would explain their slower growth. However, the α₁-transfected cells are not intrinsically defective in DNA synthesis nor in the ability to go from G₀ to S phase since their response to thrombin is greater than control. We propose, therefore, that steps distal to DNA synthesis are affected by transfection of α₁. Future studies to define the precise step in the cell cycle that is affected may suggest the mechanism by which this effect takes place.

The pathway by which thrombin and other mitogens stimulate cell growth is not yet known. Activation of phospholipase C and initiation of a cascade of intracellular changes in Ca²⁺ and protein kinase C may be involved (reviewed by Rozengurt, 1986 and Pouyssegur, 1990). Thrombin does activate this pathway in 3T3 cells and other fibroblasts (Dicker and Rozengurt, 1980; Carney et al., 1985; Magnaldo et al., 1987; Burch and Axelrod, 1987; Magnaldo et al., 1988; Paris et al., 1988). However, in several instances, receptor activation of phospholipases has been uncoupled from the mitogenic response (Zachary et al., 1987; Taylor et al., 1988; Hill et al., 1990). The modest effects on adenylylcyclase do not suggest a central role of cyclic nucleotide metabolism in mediating the effects of α₁ on cell growth.

These studies suggest that the α subunit composition of the cell can have profound effects on its response to stimulation through cell surface receptors. Further analysis of the specificity of α₁, actions will require transflecting these cells with low levels of other α subunits. However, in the present studies, it is important that α₁ is a quantitatively minor addition to the α subunits already present in Balb/c 3T3 cells. The difference between α₁-transfected and control cells in the sensitivity of thrombin-stimulated DNA synthesis to inhibition by pertussis toxin suggests that the newly introduced α subunit effectively competes with the endogenous proteins for receptor and/or effectors. Although the difference in amino acid sequence between α₁, the major endogenous G protein α₁ subunit in BALB c/3T3 cells, and α₁ is only 12%, that difference appears to have important functional consequences for the growth of intact cells.

Acknowledgments—We are grateful to Paula McColgan for expertly typing the manuscript and to Theresa Sladek for doing the densitometry.

REFERENCES

Bailey, J. L. (1967) Techniques in Protein Chemistry, pp. 340, Elsevier Publishing Co., New York
Berridge, M. J., and Irvine, R. F. (1984) Nature 312, 315-320
Bloch, D. B., Bonventre, J. V., Neer, E. J., and Seidman, J. G. (1989), Mol. Cell. Biol. 9, 5434-5439
Burch, R. M., and Axelrod, J. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 6374-6378
Carney, D., Scott, D., Gordon, E., and Labelle, E. (1985) Cell 42, 473-480
Carty, D. J., Padrell, E., Codina, J., Birnbaumer, L., Hildebrandt, J. D., and Iyengar, R. (1990) J. Biol. Chem. 265, 6268-6273
Chambard, J. C., Paris, D., L'Allemain, G., and Pouyssegur, J. (1987) Nature 326, 800-803
Chen, L. B., and Buchanan, J. M. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 131-135
Dicker, P., and Rozengurt, E. (1980) Nature 287, 607-612
Handler, J. A., Danilowicz, R. M., and Eling, T. E. (1990) J. Biol. Chem. 265, 3669-3673
Hasegawa-Sasaki, H., Lutz, F., and Sasaki, T. (1988) J. Biol. Chem. 263, 12970-12976
Hill, T. D., Dean, N. M., Mardan, L. J., Lau, A., Kanemitsu, M., and Boynton, A. L. (1990) Science 248, 1660–1663
Hoshijima, M., Ueda, T., Hamamori, Y., Ohmori, T., and Takai, Y. (1988) Biochem. Biophys. Res. Commun. 152, 286–293
Huff, R. M., Axton, J. M., and Neer, E. J. (1985) J. Biol. Chem. 260, 10864–10871
Jones, D. T., and Reed, R. R. (1987) J. Biol. Chem. 262, 14241–14249
Kim, S., Ang, S.-L., Bloch, D. B., Bloch, K. D., Kawahara, Y., Tolman, C., Lee, R., Seidman, J. G., and Neer, E. J. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 4153–4157
Laemmli, U. K. (1970) Nature 227, 680–685
Lee, R. T., Brock, T. A., Tolman, C., Bloch, K. D., Seidman, J. G., and Neer, E. J. (1989) FEBS Lett. 249, 139–142
Letterio, J. J., Coughlin, S. R., and Williams, L. T. (1986) Science 234, 1117–1119
Linder, M. E., Ewald, D. A., Miller, R. J., and Gilman, A. G. (1990) J. Biol. Chem. 265, 8243–8251
Lochrie, J., and Simon, M. I. (1988) Biochemistry 27, 4957–4965
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
Magnaldo, I., Talwar, H., Anderson, W. B., and Pouyssegur, J. (1987) FEBS Lett. 210, 6–10
Magnaldo, I., Pouyssegur, J., and Paris, S. (1988) Biochem. J. 253, 711–719
Murayama, T., and Ui, M. (1985) J. Biol. Chem. 260, 7226–7233
Murayama, T., and Ui, M. (1987) J. Biol. Chem. 262, 12463–12467
Neer, E. J., and Rozengurt, E. (1988) EMBO J. 7, 2741–2747
Neer, E. J., and Clapham, D. E. (1988) Nature 333, 129–134
Neer, E. J., Lok, J. M., and Wolf, L. G. (1984) J. Biol. Chem. 259, 14222–14229
O'Farrell, P. H. (1975) J. Biol. Chem. 250, 4097–4021
Paris, S., Chambard, J. C., and Pouyssegur, J. (1988) J. Biol. Chem. 263, 12805–12900
Pouyssegur, J. (1990) in G Proteins (Birnbaumer, L., and Iyengar, R., eds) pp. 555–566, Academic Press, New York
Pouyssegur, J., Chambard, J. C., Franchi, A., Paris, S., and Van Obberghen-Schilling, E. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 3935–3939
Price, J., Turner, D., and Cepko, C. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 156–160
Raben, D. M., Yasuda, K., and Cunningham, D. D. (1987) Biochemistry 269, 2759–2765
Rozengurt, E. (1986) Science 234, 161–166
Salmon, Y. (1979) Adv. Cyclic Nucleotide Res. 10, 35–55
Spiegel, A. M., Simonds, W. F., Jones, T. L., Goldsmith, P. K., and Unson, C. G. (1990) Soc. Gen. Physiol. Ser. 45, 185–195
Sternweis, P., and Robishaw, J. (1984) J. Biol. Chem. 259, 13806–13813
Taylor, C. W., Elakeley, D. M., Corps, A. N., Berridge, M. J., and Brown, K. D. (1988) Biochem. J. 249, 917–920
Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
Wigler, M., Pellicer, A., Silverstein, S., and Axel, R. (1978) Cell 14, 725–731
Zachary, I., Millar, J., Nanberg, E., Higgins, T., and Rozengurt, E. (1987) Biochem. Biophys. Res. Commun. 146, 456–463