Mediation of Growth Factor Induced DNA Synthesis and Calcium Mobilization by \( G_q \) and \( G_{12} \)

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Abstract. A newly identified subclass of the heterotrimeric GTP binding regulatory protein family, \( G_q \), has been found to be expressed in a diverse range of cell types. We investigated the potential role of this protein in growth factor signal transduction pathways and its potential relationship to the function of other \( G_\alpha \) subclasses. Recent biochemical studies have suggested that \( G_q \) regulates the \( \beta I \) isozyme of phospholipase C (PLCb), an effector for some growth factors. By microinjection of inhibitory antibodies specific to distinct \( G_\alpha \) subunits into living cells, we have determined that \( G_{q1} \) transduces bradykinin- and thrombin-stimulated intracellular calcium transients which are likely to be mediated by PLCb. Moreover, we found that \( G_{q2} \) function is required for the mitogenic action of both of these growth factors. These results indicate that both thrombin and bradykinin utilize \( G_q \) to couple to increases in intracellular calcium, and that \( G_q \) is a necessary component of the mitogenic action of these factors. While microinjection of antibodies against \( G_{q2} \) did not abolish calcium transients stimulated by either of these factors, such microinjection prevented DNA synthesis in response to thrombin but not to bradykinin. These data suggest that thrombin-induced mitogenesis requires both \( G_q \) and \( G_{q2} \), whereas bradykinin needs only the former. Thus, different growth factors operating upon the same cell type use overlapping yet distinct sets of \( G_\alpha \) subtypes in mitogenic signal transduction pathways. The direct identification of the coupling of both a pertussis toxin sensitive and insensitive G protein subtype in the mitogenic pathways utilized by thrombin offers an in vivo biochemical clarification of previous results obtained by pharmacologic studies.

Signal transduction mechanisms for growth factor receptors in the plasma membrane have conventionally been classified into two predominant types. One type involves a mechanism in which receptors that span the membrane a single time utilize an intrinsic protein tyrosine kinase activity (Hunter, 1991; Cantley et al., 1991) whereas the other type is for receptors which span the membrane seven times and couple to heterotrimeric \( G \) proteins (Dohman et al., 1991; Gupta et al., 1992). For the latter case, while it is thought that each of these receptors couple to a single subtype of heterotrimeric \( G \) protein, numerous signaling pathways are activated by these receptors; and this suggests that multiple coupling mechanisms may be utilized. Presently, there are 15 genes encoding the \( \alpha \) subunits of heterotrimeric \( G \) proteins (Simon et al., 1991). The best studied subtype is \( G_{\alpha1} \), which activates adenyllyl cyclase, while, a second subclass, \( G_{\alpha2} \), is best known as an inhibitor of this effector (Gilman, 1987). This diversity is thought to underlie the numerous stimuli and effectors that utilize this general type of signal transduction mechanism.

A newly identified subclass of this family, \( G_{\alpha} \), has recently been suggested to couple to PLCb, by biochemical studies (Smrcka et al., 1991; Taylor et al., 1991; Berstein et al., 1992). This new subclass possesses four members, two of which have \( \alpha \) subunits that are 88% homologous, \( G_{\alpha1} \) and \( G_{\alpha2} \). The carboxy termini of \( G_{\alpha1} \) and \( G_{\alpha2} \) are identical, and they lack sites for ADP-ribosylation by pertussis toxin (Strathmann and Simon, 1990). These biochemical findings make \( G_\alpha \) a likely candidate for the activity called \( G_q \), which is responsible for GTP-dependent PIP2 hydrolysis by PLC in a pertussis toxin insensitive manner (Gutowski et al., 1991). This activity leads to the subsequent rise in intracellular calcium (calcium transient). The role of this subclass of \( G \) proteins in living cell systems and its potential role in mitogenesis has not as yet been explored.

Identification of a specific subtype of heterotrimeric \( G \) protein that provides a functional coupling between a specific hormone receptor, its corresponding second messenger effectors, and downstream events has proven to be a complex task. While it is thought that \( G \) proteins transduce signals from numerous hormone receptors to a variety of cellular effectors, only in a few cases is it possible to make a clear assignment of a signal pathway. One approach to establishing functional relationships has been based upon reconstitution of the receptors, \( G \) proteins, and effectors. This method has been proven powerful but can be confounded by the ability of different subtypes to substitute for one another in reconstitution studies (Jones et al., 1990; Strathmann and Simon, 1990).
As an alternative approach to establishing the functional role of particular G proteins in signaling pathways in living cells, we have combined the use of functionally inhibitory antibodies specific to the carboxy-terminal regions of G proteins and needle microinjection into living somatic cells. This approach has been shown to be an effective means to inhibit the function of single classes of G proteins in living cells. It results in a rapid onset of inhibition and is not dependent on the turnover rate of the G protein as is the case for antisense oligonucleotides. The antibodies used for microinjection have been raised to various members of the G protein family through the use of distinct carboxy-terminal peptide sequences as antigens. These sequences are found in the region of each G protein that is thought to interact with an appropriate receptor, and antibodies directed against these sequences have been shown to uncouple G proteins from their respective receptors in vitro (Simonds et al., 1989). By microinjection, these inhibitory antibodies can be introduced into living cells and the resultant phenotype of the cells may be determined. This approach, for example, has been used to identify the role of $G_\alpha_1$ in serum-stimulated mitogenesis of fibroblasts (LaMorte et al., 1992); and $G_\alpha_i$ in the mediation of TSH-induced DNA synthesis in thyroid cells (Meinkoth et al., 1992).

In the present experiments, we have examined the role of $G_\alpha_i$ in the mobilization of intracellular calcium in response to a host of growth factors including PDGF, bradykinin, thrombin, and vasopressin. All of the examined factors appear to stimulate a similar response in cells with respect to an increased formation of the second messenger inositol (1,4,5) trisphosphate and a subsequent increase in intracellular calcium upon interaction with their respective receptors (Berridge and Irvine, 1989). The mechanisms responsible for these responses, however, appear to be quite distinct. PDGF appears to increase phosphatidylinositol hydrolysis through its intrinsic tyrosine kinase activity and subsequent activation of the $\gamma$ isozyme of phospholipase C (Cantley et al., 1991). Bradykinin induced phosphatidylinositol hydrolysis and subsequent mobilization of intracellular calcium is thought to be regulated by the putative G protein, $G_\alpha_q$, which is pertussis toxin insensitive (Vicentini and Villereal, 1984); while for thrombin, it has been reported to be both pertussis toxin sensitive (Chambard et al., 1987) and insensitive (Hung et al., 1992). In support of this, recent biochemical experiments have suggested that the $G_\alpha_i$ subfamily may regulate bradykinin activation of PLC in membranes in situ. (Gutowski et al., 1991). The present studies offer further identification of the molecular connections between these receptors and the subsequent transduction reactions.

Materials and Methods

Culture Cell

Mouse Balb/c3T3 fibroblasts (A31) were obtained from American Type Culture Collection (Rockville, MD) and propagated in DME (Fischer Scientific Co., Pittsburgh, PA) containing 10% calf serum (Colorado Serum Co., Denver, CO) at 37°C in an atmosphere containing 10% CO$_2$ as described by American Type Culture Collection. Rat embryo fibroblasts (REF52) were obtained from Cold Spring Harbor Laboratory (Cold Spring Harbor, NY) and propagated in DME containing 10% FBS (Gemiini Bio-Products Inc., Calabasas, CA). For calcium ratio imaging experiments, both cell types were plated on 25-mm etched coverslips (Bellco Glass, Vineland, NJ) and for DNA synthesis experiments, cells were plated on 12-mm coverslips (Fisher Scientific Co.). Balb/c3T3 cells were rendered quiescent by starvation in DME containing 0.05% calf serum before injection whereas REF52 cells were starved in DME containing 0.05% FCS for 24 h.

Pertussis Toxin Treatment

Quiescent cells were treated with Bordetella pertussis toxin (Sigma) at 25 and 50 ng/ml for 2 h and subsequently transferred to Hepes DME containing 0.05% calf serum for calcium ratio imaging experiments.

Western Analysis

A 60-mm plate of logarithmic Balb/c3T3 and REF52 cells was solubilized in 200 μL of 0.5% NP-40/PBS for 5 min on a rocker at 4°C. The supernatant was transferred to an Eppendorf tube and 200 μL of 5× Laemnni sample buffer was added. The tube was boiled for 15 min and 40 μL of each sample was electrophoresed on a 10% SDS-polyacrylamide gel. The gel was transferred to nitrocellulose and incubated with 5 μg/ml of the affinity-purified QL antisera. A secondary goat anti-rabbit biotinylated antibody (Vector Laboratories Inc., Burlingame, CA) and streptavidin-alkaline phosphatase colorimetric detection system (Bio Rad Laboratories, Hercules, CA) was used.

Microinjection

Cells were microinjected with 10 mg/ml of affinity purified polyclonal antisera in microinjection buffer containing 100 mM KCl and 5 mM NaPO$_4$. In brief, QL antisera was raised to a synthetic decapeptide (QLNLKEX2GLF) which is specific for $G_\alpha_q$ in Balb/c3T3 fibroblasts (Simonds et al., 1989). QL, a control antisera to GPA I, a yeast G protein, was generated to the carboxy terminus (QQNLKKGIG) and has no known homology with any mammalian G proteins (Shenker et al., 1991). Quiescent cells were microinjected with approximately $5 \times 10^{-14}$ L/cell using an automated Eppendorf microinjection system and a Zeiss axiophot microscope (Carl Zeiss, Inc., Thornwood, NY) which introduced $10^5$ molecules of IgG into each cell.

Calcium Ratio Imaging

The methods and design for single cell calcium ratio imaging are described in detail in Tsien and Harootunian (1990). In brief, etched coverslips (Bellco Glass) with a defined injected area were loaded with fura-2/AM (Molecular Probes Inc., Eugene, OR) by incubating cells in a solution of DME containing 200 nM fura2/AM buffered with 20 mM Hepes at room temperature (23°C) for 1 h. Cells were imaged with a Zeiss im35 microscope and imaging system as described previously (Tsien and Harootunian, 1990). Growth factors were applied in a single bolus with a final concentration of 25 ng/ml PDGF (Upstate Biotechnology Inc., Lake Placid, NY), 5 U/ml thrombin (Sigma Chemical Co., St. Louis, MO), 1 μM bradykinin (Calbiochem-Novabiochem Corp., La Jolla, CA), 50 ng/ml bombesin, 50 nM vasopressin, 10 nM and 20 nM insulinlike growth factor II (Upstate Biotechnology Inc.,) and 125 ng/ml insulinlike growth factor I (Upstate Biotechnology, Inc.). While 0.5 U/ml of thrombin elicited calcium transients equivalent to those seen for 5 U/ml, we routinely used the higher dose in these experiments to facilitate a rapid delivery of a single bolus of drug. Approximately 5 min elapsed between growth factor stimulation. After calcium imaging, the cells were fixed in 3.7% formaldehyde/PBS for 5 min and subsequently permeabilized with 0.3% Triton X-100 (Sigma Chemical Co.) for 5 min. The injected antibody was detected with a secondary goat anti-rabbit antibody conjugated to rhodamine (Cappell Laboratories, Malvern, PA) at 5:100 dilution of the stock concentration in 0.5% Nonidet P-40 (Sigma Chemical Co.). Its fluorescence intensity was calculated and assigned arbitrary units in order to quantitate the amount of injected antibody in each cell.

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DNA Synthesis

3 h postmicroinjection, Balb/c3T3 cells were stimulated with either 1 μM bradykinin or 0.5 U/ml thrombin in DME containing 0.1% calf serum. For thrombin, 0.5 U/ml and 5 U/ml were found to both elicit maximal DNA synthesis. At this time bromodeoxyuridine (Amersham Corp., Arlington Heights, IL) was added to the media. Cells were fixed and stained 24 h after stimulation as previously described (La Morte et al., 1992) except that a secondary goat anti-mouse antibody conjugated to rhodamine (Cappell Laboratories) was used. The percentage of antibody-injected cells that were positive for BrdU incorporation was compared to the percentage of uninjected cells that were positive for BrDU incorporation. Background levels of DNA synthesis in DME containing only 0.1% calf serum were determined and subtracted from the percentage of injected and un.injected cells to calculate the percent reduction in DNA synthesis. A paired *t* test analysis ± background was performed.

Results

Calcium Transients in Growth Factor Action

To establish assays for the diverse roles of G proteins in growth factor signaling suitable for the microinjection approach, several mitogens were tested for their ability to mobilize intracellular calcium in Balb/c3T3 fibroblasts. Mobilization of intracellular calcium in living cells was quantitated by single cell ratio imaging using the fluorescent calcium indicator dye, fura-2 (Tsien and Harootunian, 1990). This calcium transient results from the production of inositol (1,4,5)trisphosphate from the enzymatic hydrolysis of PiP2 from PLC (Berridge and Irvine, 1989). Bradykinin, thrombin, PDGF, and bombesin, but not vasopression, IGF-I, or IGF-II elicited transient increases in intracellular calcium in quiescent Balb/c3T3 fibroblasts. To further clarify potential mediators of this response, the sensitivity of the response to the ADP-ribosylating agent Bordetella pertussis toxin was determined. Calcium transients produced by bradykinin, thrombin, or PDGF appeared to be insensitive to *pertussis* toxin, whereas the transient produced by bombesin was reduced by the toxin (data not shown). These pharmacological data suggested that bradykinin, thrombin, and PDGF stimulate calcium transients in a *pertussis* toxin insensitive manner as expected. Additionally, REF 52 fibroblasts were tested and found to respond to vasopressin, bradykinin, and PDGF; and these cells were used in experiments as noted.

The newly identified subclass of G proteins, Gq, possesses a carboxy terminus lacking the cysteine residue that is the site of ADP-ribosylation and as a result is *pertussis* toxin insensitive. To investigate a potential role for Gq, an antibody to the carboxy terminus of the yeast G protein GPAI was determined. Anti Gq,~ antibodies were injected. Previous studies have established the presence of this protein in these cells and an involvement in serum stimulated mitogenesis (La Morte et al., 1992). Cells injected with these antibodies (AS) elicited a normal response to bradykinin but did to subsequent stimulation with PDGF as a control. Those cells that were un.injected as indicated by the arrows were able to produce calcium transients in response to both bradykinin and PDGF. To determine if the block of the calcium transient was a specific effect of anti Gq,~ antibodies to a *pertussis* toxin-sensitive G protein, Gq, were injected. Figure 1 shows the results of such an experiment. Each panel of four images (i.e., A–C) shows a different field of cells injected with one of the antibody preparations, while the same field is shown within each panel (A–C). The top left of each matrix illustrates cells loaded with fura-2 that have not been stimulated with any of the factors. The top right depicts cells stimulated with 1 μM bradykinin and the bottom left depicts the same cells stimulated with 25 ng/ml PDGF. After quantitation of the calcium response to bradykinin or thrombin followed by PDGF, the cells were fixed and stained for the injected antibody to allow for unambiguous identification of the injected cells. The bottom right of each panel shows the stained cells.

As seen in Fig. 2 A, the cells injected with anti Gq,~ (QL) did not show a calcium transient in response to bradykinin but did to subsequent stimulation with PDGF as a control. These cells that were uninjected as indicated by the arrows were able to produce calcium transients in response to both bradykinin and PDGF. To determine if the block of the calcium transient was a specific effect of anti Gq,~ antibodies to a *pertussis* toxin-sensitive G protein, Gq, were injected. Previous studies have established the presence of this protein in these cells and an involvement in serum stimulated mitogenesis (La Morte et al., 1992). Cells injected with these antibodies (AS) elicited a normal response to bradykinin and subsequently to PDGF (Fig. 2 B). As an additional control, the cells were injected with a control antibody, QQ, raised to the carboxy terminus of the yeast G protein GPAI (Fig. 2 C). The QQ-injected cells responded normally to both bradykinin and PDGF stimulation.
the right of each matrix corresponds to an individual cell in the field and is indicated by the open white circle. These data show a virtual quantitative block of the calcium transient induced by bradykinin by anti-Gα11.

To further establish that the block of the calcium transient seen by anti-Gα11 was specific, we preabsorbed the antibody with the antigen peptide to which it was raised. The remaining solution was then microinjected into cells. The preabsorbed antibodies did not inhibit the calcium transients induced by bradykinin (data not shown). The inhibition of bradykinin-induced calcium transients by anti-Gα11 was also observed in REFS2 fibroblasts. As a control, no inhibition was seen in response to PDGF. In response to vasopressin stimulation, anti-Ga12-injected cells exhibited a partial block (data not shown).

Fig. 3 (i.e., A–C) depicts three fields of cells that have been injected with the same series of affinity purified antibodies, but in this case were stimulated with 5 U/ml thrombin and subsequently with PDGF. Fig. 3 A demonstrates that antibodies specific for Ga inhibited calcium mobilization in response to thrombin, whereas antibodies to Gα (Fig. 3 B) and a yeast G protein (Fig. 3 C) had no effect on thrombin's ability to mobilize intracellular calcium, as was found for calcium transients induced by bradykinin.

To compensate for the inherent variability of microinjection, the amounts of injected antibody were quantified at the end of each experiment by fixing and staining with a secondary anti-rabbit antibody. The immunofluorescence intensity integrated over each cell was retrospectively compared with the inhibition of the agonist-induced [Ca2+]i response in that cell (Fig. 4). Throughout this analysis, we found that cells that were either un.injected or injected with the lowest detectable amount of anti-Gα11 behaved similarly; they produced transients in response to both bradykinin and thrombin. Injections of higher levels of anti-Gα11, but not anti-Gα2 nor anti-Gα1, led to an abolition of the calcium transients and showed what appeared to be a threshold relationship for the inhibition.

Mitogenic Regulation by Gα Subunits

These data provided strong support for the notion that Gα is the protein that regulates bradykinin and thrombin receptor coupling to calcium transients in fibroblasts, which most likely represents molecular coupling of those receptors to the β1 isotype of phospholipase C. This enzyme in turn catalyzes the hydrolysis of phosphatidylinositol to produce the second messenger IP3, which leads to the mobilization of calcium from intracellular stores. While there is evidence that PLC activation is necessary for the mitogenic action of serum (Smith et al., 1990), it is not yet known if this is a requirement for these specific growth factors. Accordingly, to examine the role of Gα in bradykinin and thrombin mitogenesis in Balb/c3T3 cells, the effect of Gα and Gα2 antibodies on mitogenesis was assessed. Quiescent Balb/c3T3 fibroblasts were injected with antibodies specific for different Gα subunits and 3 h later were stimulated with bradykinin, thrombin or PDGF. To monitor DNA replication, BrDU was added at the time of mitogen addition and the cells were incubated for a further 2 h. Injection of anti-Gα11 resulted in a 95% reduction of bradykinin-stimulated DNA synthesis compared to unstimulated background levels of DNA synthesis (Table I). Thrombin-stimulated DNA synthesis was also assessed by these procedures. Quiescent cells were again injected with the antibodies specific for Gα11, Gα2, and Gα1; and subsequently stimulated with 0.5 U/ml thrombin. As observed for bradykinin, cells injected with anti-Gα11 showed a significant reduction in DNA synthesis (96% reduction compared to background levels of DNA synthesis) (Table I). Anti-Gα1 injected cells showed equal levels of DNA synthesis, as compared to uninjected cells. In contrast to bradykinin-stimulated mitogenesis, injected anti-Gα2 completely blocked thrombin stimulated DNA synthesis. As a control, neither anti-Gα11, anti-Gα1 (Table I), nor anti-Gα2 as previously published (La Morte et al., 1992) had any effect on PDGF-induced DNA synthesis.

These experiments showed that while both thrombin and bradykinin required Gα for coupling to increased intracellular calcium and for mitogenic responses, thrombin but not bradykinin appeared to require Gα2 in addition to Gα for the mitogenic response in cells. Moreover, PDGF required neither Gα nor Gα2 for these two cellular responses. Considering that Gα2 but not Gα is a pertussis toxin sensitive G protein, these data provide strong evidence for divergent signal transduction pathways emanating from the thrombin receptor in a manner dependent upon two distinct subtypes of G proteins. These experiments may also provide a molecular explanation for the observations that (a) calcium transients induced by thrombin or bradykinin are not sensitive to pertussis toxin (i.e., Gα) while (b) mitogenesis induced by thrombin but not bradykinin is sensitive to pertussis toxin (i.e., Gα2) and that (C) neither response to PDGF appears to be mediated by G proteins.

Discussion

It appears that the function of Gα in the case of both thrombin and bradykinin is, at the least, to provide transduction of the receptors to an increase in phosphoinositide breakdown and intracellular calcium, presumably via the action of PLCβ, and that this transduction is most likely an essential pathway for the mitogenic stimulation by these factors. It is unclear, however, if Gα couples to other pathways or what role Gα2 plays in the mitogenic action of thrombin, and why this is not also necessary for mitogenesis stimulated by

Figure 2. Single cell calcium ratio imaging of bradykinin-stimulated Balb/c3T3 fibroblasts. A–C each depict four different images of three different fields of cells loaded with fura-2/AM that have been injected with anti-Gα (QL) (A); anti-Gα2 (AS) (B); and anti-Gα1 (C). The top left image of each panel illustrates the cells before stimulation. The top right corresponds to the image after bradykinin stimulation. The bottom left image depicts the same cells stimulated with PDGF; and, last, the bottom right image identifies the injected cells after fixation and indirect immunofluorescence. The white arrows indicate the cells in the field that have not been injected. The single cell tracing to the right of each panel (A–C) corresponds to the cell with the white circle.
Figure 3. Single cell calcium ratio imaging of thrombin-stimulated Balb/c3T3 fibroblasts. A–C each depict four different images of three different fields of cells loaded with fura-2/AM that have been injected with anti-
\( \text{G}_{\text{o}} \) (QL) (A); anti-\( \text{G}_{\text{ai2}} \) (AS) (B); and anti-GPA I (C). The top left image of each panel illustrates the cells before stimulation. The top right corresponds to the image after thrombin stimulation. The bottom left image depicts the same cells stimulated with PDGF; and, last, the bottom right image identifies the injected cells after fixation and indirect immunofluorescence. The white arrows indicate the cells in the field that have not been injected. The single cell tracing to the right of each panel (A–C) corresponds to the cell with the white circle.
For thrombin, this could occur by receptor coupling to both Gq and Gαι2. A possible function for Gαι2 in mitogenic pathways might lie in its potential regulation of adenylyl cyclase where it usually acts as an inhibitory agent, or in its activation of phospholipase A2 (Gupta et al., 1990) or in the activation of extracellular signal regulated kinases (ERKs) (Gupta et al., 1992). If these are necessary components of the mitogenic pathways for both of these growth factors, then we might suspect that bradykinin receptors may couple to these pathways as well, either through Gq or some other factor.

Table I. Percent DNA Synthesis

| Background | Antibody | Injected | Uninjected | Percent reduction |
|------------|----------|----------|------------|------------------|
| Bradykinin| QL       | 7% (n = 69) | 33% (n = 225) | 100%             |
|            | QQ       | 21% (n = 70) | 37% (n = 251) | 84%              |
|            | AS       | 20% (n = 69) | 30% (n = 208) | 82%              |
| Thrombin   | QL       | 17% (n = 102) | 31% (n = 247) | 100%             |
|            | QQ       | 8% (n = 102) | 31% (n = 247) | 100%             |
| PDGF       | QL       | 8% (n = 102) | 31% (n = 247) | 100%             |
|            | QQ       | 8% (n = 102) | 31% (n = 247) | 100%             |

Percent reduction of DNA synthesis in microinjected fibroblasts. Briefly, as described in Materials and Methods, random areas of Balb/c3T3 fibroblasts were microinjected with the appropriate G protein antiserum and stimulated with either bradykinin, thrombin, or PDGF. Cells were analyzed using a Zeiss axiophot fluorescent microscope with a x63 oil immersion Achromat objective. Background fluorescence intensity (rhodamine) was integrated over each cell and assigned arbitrary units. Each cell's corresponding maximal change in rhodamine intensity was plotted against the cell's corresponding maximal change in intracellular calcium concentration (μM). Basal calcium levels (n = 101) averaged 122 nM ± 30 nM. The mean increase in intracellular calcium for bradykinin-stimulated cells was as follows: QL (27.5 nM ± 80.6 nM); AS/QQ (718.5 nM ± 335.9 nM). The mean increase in intracellular calcium for thrombin-stimulated cells was as follows: QL (0 nM ± 4.4 nM); AS/QQ (846.0 nM ± 348.4 nM). The average increase in calcium was derived from injected cells with an injection fluorescence intensity >20 (arbitrary units). t-test analysis of QL compared to AS- and QQ-injected cells was significantly different with a p < 0.0001 for both bradykinin- and thrombin-stimulated cells. QQ- and AS-injected cells were not significantly different for both bradykinin- and thrombin-stimulated cells.
as does stimulation by thrombin and bradykinin (Melcho et al., 1992; Kahan et al., 1992), it is conceivable that these growth factors stimulate extracellular signal regulated kinases through pathways shared by ras stimulated events. In preliminary experiments, we have found that the stimulation of mitogenesis by thrombin is blocked by prior microinjection of a mutant form of the ras protein which interferes with endogenous ras function (see Feig and Cooper, 1988). Together with the present results, this suggests that in addition to G$_2$ and C$_4$, this mitogen also requires ras function. This points to a new potential linkage between the important protooncogene ras and heterotrimeric G proteins in cell signaling. The potential requirement for these GTP binding proteins in the mitogenic signaling suggests a complex interdependence of pathways. We are currently exploring this possibility. Mutations of G$_2$ which cause an activation of the GTPase activity of the protein are mitogenic when transfected in fibroblasts (Hermouet et al., 1991). Antibodies that apparently inhibit the function of the endogenous G$_2$ in fibroblasts cause a diminution of serum-stimulated DNA synthesis (LaMorte et al., 1992) and a block of IGF-II-dependent mitogenesis (LaMorte, V. J., unpublished results). These observations provide strong evidence that G$_2$ is a necessary cellular transducer of certain types of mitogens. As known functions of G subunits include the inhibition of adenyllycylase and activation of extracellular signal regulated kinases, this suggests important roles for these pathways in G protein dependent mitogenesis.

The identification of both a pertussis toxin sensitive and insensitive Gs subtype in thrombin mitogenesis as shown here clarifies certain aspects of the complex pharmacologic data concerning this mitogen. The present data would suggest that the target of the toxin used in the pharmacologic studies was G$_{Gz}$, which we show is involved in the mitogenic signaling of thrombin. The insensitivity of thrombin induced phosphoinositol hydrolysis to pertussis toxin (Hung et al., 1992) would, on the other hand, suggest a role for some other Gs subtype in this transduction mechanism. The present results identify this as G$_4$. These results demonstrate that a single mitogen type can require functional coupling to two distinct subtypes of G proteins, and offer further support for the idea that growth factor receptors alter diverse biochemical pathways within the cell by multiple linkages. The possible extent of the multitude of these linkages to Gi2 and Gq, this mitogen also requires Gi$_2$ antibodies. J. R. Lefkowitz. 1991. Model systems for the study of seven-transmembrane-segment receptors. Annu. Rev. Biochem. 60:653–688.

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