Basic Fibroblast Growth Factor Utilizes Both Types of Component Subunits of $G_s$ for Dual Signaling in Human Adipocytes

**STIMULATION OF ADENYLYL CYCLASE VIA $G_s$ AND INHIBITION OF NADPH OXIDASE BY $G_{bg}$**

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Basic fibroblast growth factor (bFGF), a ligand of receptor protein-tyrosine kinases, promoted the dissociation of $G_s$ and had antagonistic stimulatory and inhibitory effects on adenylyl cyclase and NADPH oxidase in human fat cell plasma membranes. The bFGF-induced activation of adenylyl cyclase was blocked by COOH-terminal anti-$G_{bg}$, indicating that it was mediated by $G_{bg}$. The inhibitory action of bFGF was mimicked by exogenously supplied G$_{bg}$-subunits and was reversed by anti-$G_{bg}$, or bARK-CT, a COOH-terminal $\beta$-adrenergic receptor kinase fragment that specifically binds free G$_{bg}$, indicating that it was transduced by G$_{bg}$ complexes. The bFGF-induced inhibition of NADPH-dependent H$_2$O$_2$ generation was also reversed by peptide 100–119, an inhibitor of $G_s$ activation by ligand-occupied $\beta$-adrenergic receptors, indicating that the G$_{bg}$ complexes mediating the inhibitory action of the growth factor are derived from G$_s$. The findings suggest a direct, non-kinase-dependent, coupling of bFGF receptor(s) to $G_s$ and provide the first example of a ligand of receptor protein-tyrosine kinases that is capable of utilizing both types of component subunits of a single heterotrimeric G protein for dual signaling in a single cell type.

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**EXPERIMENTAL PROCEDURES**

**Materials**

Antibodies directed against the C terminus of $G_{bg}$ (RM/1), common anti-$G_s$ (SW/1), and anti-$G_{bg}$, were from PerkinElmer Life Sciences, Bad Homburg, Germany (RM/1 and SW/1), and Upstate Biotechnology Inc., Lake Placid, NY, respectively. Recombinant $G_{bg}$, $G_s$, and protein A-agarose were from Amersham Pharmacia Biotech-Buchler, Braunschweig, Germany. Insulin, GTP$\gamma$S, GDP$\beta$S, and GTP were from Roche Molecular Biochemicals, Mannheim, Germany. Isoproterenol and rbFGF were from Sigma AG, München, Germany. Human albumin and luminol were obtained from Behring Werke, Marburg, Germany. The GST fusion protein containing a carboxyl-terminal fragment (residues 546–670) of the $\beta$-adrenergic receptor kinase ($\beta$ARK ICT-GST) and the corresponding GST protein were a gift of Dr. R. Lefkowitz, Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC (16).

**Methods**

Subjects, Preparation of Fat Cells, and Fat Cell Ghosts—Experimental details have been described in detail elsewhere (5, 9–12). Briefly, adipose tissue was from nondiabetic subjects undergoing elective abdominal or cosmetic breast surgery. The specimens were cut into small pieces, and fat cells were isolated by the method of Rodbell (17) in a HEPES-buffered Krebs-Henseleit solution, pH 7.4, containing 20 mM HEPES, 10 mM NaHCO$_3$, 5 mM glucose, 20 g/liter albumin, and 1 mg/ml collagenase (CLS, Worthington). After 30 min, fat cells were washed and re-suspended in 10 volumes of an ice-cold lysis medium containing 20 mM MES, pH 6.0, 2 mM MgCl$_2$, 1 mM CaCl$_2$, 5 mM KCl, and 100 mg/liter soybean trypsin inhibitor. Mechanical shaking completed cell binding to their receptors (1–3, 7). These consist of a family of high affinity receptor tyrosine kinases (FGF receptors 1–4) displaying overlapping affinities for the various FGFs (1–3, 7). Alternative splicing generates isoforms of receptors 1–3 that exhibit unique binding characteristics (1–3). In addition, an unrelated cysteine-rich transmembrane protein of unknown function has also been identified as a high affinity receptor for FGFs (8, 9).

Mature human adipocytes and 3T3 L1-preadipocytes contain a plasma membrane-bound H$_2$O$_2$ generating system that is under antagonistic control by various hormones, growth factors, and cytokines, including ligands of receptor protein-tyrosine kinases, such as insulin and various isoforms of PDGF and FGF (6, 10–14).

Recent work revealed that the stimulatory effect of insulin on NADPH-dependent H$_2$O$_2$ generation is transduced by a G protein ($G_{bg}$), whereas the inhibitory action of the $\beta$-adrenergic agonist, isoproterenol, which signals through a prototypical $G_s$-coupled receptor, is mediated by G$_{bg}$ derived from G$_s$ (13, 14). In this work we examined whether the inhibitory effect of bFGF, another ligand of tyrosine kinase receptors, on NADPH-dependent H$_2$O$_2$ generation is also transmitted by a G protein.

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The fibroblast growth factors (FGFs)$^1$ are a family of heparin-binding growth factors and oncogenes with at least 20 members (1–3). Basic fibroblast growth factor (bFGF, FGF-2) or a related heparin-binding peptide is produced by white and brown adipocytes (4, 5). The growth factor stimulates the proliferation of preadipocytes and inhibits their conversion to adipocytes. The expression of bFGF is decreased appreciably during adipose differentiation and is increased in obesity (4).

The members of the fibroblast growth factor family interact with cell surface low affinity heparan sulfate that facilitates...
Assay of NADPH oxidase activity. The activation step was carried out in 30 mM 5 min. Thereafter, increasing concentrations of GTPyS were added and incubations were continued for another 20 min. Membranes were washed and assayed for NADPH-dependent \( \mathrm{H}_2\mathrm{O}_2 \) generation as described under “Experimental Procedures.” Values are mean ± S.D. of three separate experiments.

**B.** Dose-response curve for bFGF in the presence of 50 \( \mu \mathrm{M} \) GTPyS. Plasma membranes were incubated with 5 mM insulin and increasing concentrations of bFGF in the presence of 50 \( \mu \mathrm{M} \) GTPyS for 25 min, and assayed for NADPH-dependent \( \mathrm{H}_2\mathrm{O}_2 \) generation, as described under “Experimental Procedures.” Values are mean ± S.D. of three experiments carried out with different membrane preparations.

**RESULTS**

Fig. 1A shows the effects of 0.1 mM bFGF on NADPH-dependent \( \mathrm{H}_2\mathrm{O}_2 \) generation in the presence of increasing concentrations of GTPyS in membranes from cells that had been pretreated with 10 nM insulin. Similar to early observations with the turkey erythrocyte adenyl cyclase, where activation by GMP/PNP) appeared to be totally dependent on hormone because the hormone-independent rate of activation was slow, GTPyS alone had no measurable effect under the conditions used (21). The inhibitory action of bFGF became apparent at concentrations of GTPyS exceeding 5 \( \mu \mathrm{M} \) and was half-maximal at approximately 10 \( \mu \mathrm{M} \) GTPyS. At a maximal concentration of GTPyS (50 \( \mu \mathrm{M} \)), NADPH-dependent \( \mathrm{H}_2\mathrm{O}_2 \) generation was reduced by approximately two-thirds in the presence of 0.1 mM bFGF.

Fig. 1B shows a concentration-response curve for bFGF in the presence of a maximal concentration of GTPyS (50 \( \mu \mathrm{M} \)). The bFGF-induced inhibition of NADPH-dependent \( \mathrm{H}_2\mathrm{O}_2 \) generation was half-maximal at about 1 \( \mu \mathrm{g} \) and was maximal at approximately 30 \( \mu \mathrm{g} \). Recent work showed that the effects of another inhibitor of NADPH-dependent \( \mathrm{H}_2\mathrm{O}_2 \) generation, the \( \beta \)-adrenergic agonist isoproterenol, are transduced by \( \beta \) subunits derived from \( \mathrm{G}_s \) (13, 14). Therefore, the possibility was explored that the effects of bFGF were also mediated by \( \mathrm{G}_s \). Indeed, the inhibitory action of bFGF was dose-dependently reversed by an antibody directed against an internal sequence of \( \beta_2 \)-subunits which had no influence on basal or insulin-stimulated rates of NADPH-dependent \( \mathrm{H}_2\mathrm{O}_2 \) generation (Fig. 2A).

Maximal effects were quantified by densitometric analysis. The amount of \( \mathrm{G}_s \) was normalized to the amount of \( \mathrm{G}_s \), immunoprecipitated for each condition.

**Fig. 1** Synergistic inhibition of insulin-stimulated rates of NADPH-dependent \( \mathrm{H}_2\mathrm{O}_2 \) generation by bFGF and GTPyS. A. effects of increasing concentrations of GTPyS on insulin-stimulated rates of NADPH-dependent \( \mathrm{H}_2\mathrm{O}_2 \) generation in the presence of 0.3 mM bFGF. Plasma membranes from adipocytes that had been pretreated with 10 nM insulin for 10 min at 37 °C were incubated in the presence of 0.3 mM bFGF for 5 min. Thereafter, increasing concentrations of GTPyS were added and incubations were continued for another 20 min. Membranes were washed and assayed for NADPH-dependent \( \mathrm{H}_2\mathrm{O}_2 \) generation as described under “Experimental Procedures.” Values are mean ± S.D. of three separate experiments. B. dose-response curve for bFGF in the presence of 50 \( \mu \mathrm{M} \) GTPyS. Plasma membranes were incubated with 5 mM insulin and increasing concentrations of bFGF in the presence of 50 \( \mu \mathrm{M} \) GTPyS for 25 min, and assayed for NADPH-dependent \( \mathrm{H}_2\mathrm{O}_2 \) generation, as described under “Experimental Procedures.” Values are mean ± S.D. of three experiments carried out with different membrane preparations.

**Dual Signaling of bFGF via \( \mathrm{G}_s \)**

**Assay of NADPH oxidase activity.** The activation step was carried out in 30 mM 5 min. Thereafter, increasing concentrations of GTPyS were added and incubations were continued for another 20 min. Membranes were washed and assayed for NADPH-dependent \( \mathrm{H}_2\mathrm{O}_2 \) generation as described under “Experimental Procedures.” Values are mean ± S.D. of three separate experiments. **B.** Dose-response curve for bFGF in the presence of 50 \( \mu \mathrm{M} \) GTPyS. Plasma membranes were incubated with 5 mM insulin and increasing concentrations of bFGF in the presence of 50 \( \mu \mathrm{M} \) GTPyS for 25 min, and assayed for NADPH-dependent \( \mathrm{H}_2\mathrm{O}_2 \) generation, as described under “Experimental Procedures.” Values are mean ± S.D. of three experiments carried out with different membrane preparations.

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observed at an antibody dilution of 1:100 in the presence of 0.3 nM bFGF (Fig. 2A).

The inhibitory effect of bFGF could also be reversed by 10 μM βARK-1-CT, a fragment of the β-adrenergic kinase that specifically binds free Gβγ (16). Reversal of the bFGF-induced suppression of NADPH-dependent H₂O₂ generation was half-maximal at 0.5 μM βARK-1-CT, maximal effects were observed at 10 μM (Fig. 2B). The GST protein alone failed to reverse the inhibitory effects of isoproterenol and bFGF (not shown).

Fig. 3 shows the effects of aFGF, bFGF, and insulin on Gs subunit composition. In contrast to other heterotrimeric G proteins, Gs does not dissociate in the presence of GTPγS alone under the conditions used (18, 19). Plasma membranes were therefore treated with 50 μM GTPγS in the absence or presence of 0.3 nM bFGF, 3 nM aFGF, or 5 nM insulin. Dissociation of Gs into its component subunits was monitored by an immunoblot technique utilizing antibody RM/1, which does not discriminate between heterotrimeric and monomeric Gαs. Fig. 3 demonstrates that the amount of Gβ recovered in RM/1 immunoprecipitates was selectively reduced in membranes that had been pretreated with bFGF, while aFGF and insulin failed to influence the subunit composition of immunoprecipitated Gs. In the absence of GTPγS, bFGF had no influence on the subunit composition of Gs. Thus, the growth factor caused a guanine nucleotide triphosphate-dependent dissociation of Gs, which is characteristic for a receptor-mediated activation.

The latter finding suggested that bFGF may also stimulate adenyl cyclase, which was in fact the case. Fig. 4 shows the effects of bFGF and isoproterenol on cAMP production and NADPH-dependent H₂O₂ generation in human fat cell plasma membranes. bFGF caused a concentration-dependent increase of cAMP accumulation that became apparent at picomolar concentrations, suggesting that it was transmitted by discrete ligand-specific receptors (Fig. 4A). Compared with the β-adrenergic agonist isoproterenol, which elicited a 6-fold increase in cAMP production, bFGF was able to induce only a modest (3-fold) activation of adenyl cyclase in fat cell plasma membranes (Fig. 4A), whereas both compounds were of comparable efficacy in suppressing NADPH-dependent H₂O₂ generation (Fig. 4B). The differential ability of isoproterenol and bFGF to increase cAMP became even more apparent in intact cells, where a bFGF-induced increase in cAMP was only detectable in the presence of a phosphodiesterase inhibitor (isobutylmethylxanthine). On the average, cellular cAMP levels were doubled by 10 nM bFGF under these conditions, a figure that contrasts to a more than 100-fold elevation seen in the presence of 1 μM isoproterenol, consistent with recent observations in NIH 3T3 cells (22).

Fig. 5 shows the effects of a COOH-terminal decapeptide (peptide 385–395), a sequence comprising residues 100–119 within the α-helical domain of Gαs (peptide 100–119), and a homologous sequence of Gαs which is mediated via Gβδ and the coimmunoprecipitated Gβ. The amount of Gβδ was normalized to the amount of Gαs immunoprecipitated for each condition. Results are the mean ± S.D. of three separate experiments.

**DISCUSSION**

In this report it is shown that bFGF, a ligand of receptor protein-tyrosine kinases, stimulates adenyl cyclase and inhibits NADPH-dependent H₂O₂ generation in human fat cell plasma membranes. The mechanisms by which bFGF elicited its antagonistic stimulatory and inhibitory effects on adenyl cyclase and NADPH oxidase were confined to the plasma membrane, independent of second messengers, and operated in the absence of ATP, indicating that established pathways of signal
transduction, including the tyrosine kinase activity of bFGF receptor(s) were not involved. Along with the observation that bFGF promoted dissociation of Gs, these findings suggested that FGF receptor(s) are directly coupled to Gs via a non-tyrosine kinase-dependent mechanism. Indeed, human adipocytes express at least two members of the FGF receptor family, e.g. FGFR1 and FGFR2. Both isoforms co-eluted with Gaα and Gβ upon affinity chromatography on Sepharose coupled to antibodies directed against their carboxyl-terminal sequences and hence may both contribute to bFGF-signaling via Gs.

Antibodies directed against the COOH termini of Gaα subunits and peptides corresponding to their target sequences are thought to block receptor recognition and activation, and are therefore widely used in functional studies aimed at assessing receptor/G protein coupling (23–26). The stimulatory effect of bFGF on adenyl cyclase could be blocked by COOH-terminal anti-Gαs and a peptide corresponding to its antigenic site, indicating that it was in fact transmitted by Gαs.

The bFGF-induced inhibition of NADPH-dependent H2O2 generation also required the participation of a G protein. In contrast to its stimulatory effects on adenyl cyclase, the inhibitory action of bFGF on NADPH oxidase activity was not influenced by COOH-terminal anti-Gαs. A possible explanation for this unexpected finding would be that activation of Gαb by bFGF receptors may not yield enough, or inappropriately composed, βγ subunits to account for inhibition of NADPH-dependent H2O2 generation, as has been proposed for the regulation of type II adenyl cyclase (27, 28). However, the effects of COOH-terminal Gαα antibodies on Gβγ-mediated responses are variable and may depend on receptors, G proteins, and cell types studied (13, 14, 23–26). Therefore, the lacking effects of COOH-terminal anti-Gαs do not permit conclusions to be made about the origin of the Gβγ complexes transmitting the inhibitory action of bFGF. Indeed, we have recently shown that Gs is capable of providing enough appropriately composed Gβγ subunits to account for the inhibition of NADPH-dependent H2O2 generation seen in the presence of isoproterenol (14).

However, bFGF was considerably less efficacious than isoproterenol in activating adenyl cyclase, although both compounds were of comparable efficacy in inhibiting of NADPH-dependent H2O2 generation. It is possible that the differential effect of bFGF on both systems is merely an apparent one, because the adenyl cyclase assays contained GTP, whereas NADPH oxidase activity was determined in the presence of GTPγS.

However, the differential ability of bFGF and isoproterenol to act as stimulators of adenyl cyclase occurred under identical conditions and was even more pronounced in intact cells, indicating that it was physiologically relevant. A possible explanation, among others, for this difference would be that ac-

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**Fig. 4.** Antagonistic effects of bFGF on adenyl cyclase and NADPH oxidase activation in human fat cell plasma membranes. A, plasma membranes were assayed for adenyl cyclase activity in the presence of increasing concentrations of bFGF (■) or of isoproterenol (▲), as described under "Experimental Procedures." B, plasma membranes were incubated with 5 nM insulin and 50 μM GTPγS in the presence of 5 μM isoproterenol or 0.3 nM bFGF for 25 min, and then assayed for NADPH oxidase activity, as described under "Experimental Procedures." Values are mean ± S.D. of four separate experiments for each condition.

**Fig. 5.** Reversal of the antagonistic effects of bFGF on adenyl cyclase and NADPH oxidase activities by a peptide corresponding to residues 100–119 of Gαs (peptide 100–119). Plasma membranes were preincubated for 45 min at 4 °C in the absence or presence of 2.5 μM peptide 100–119 (Gs), 2.5 μM of a homologous peptide (residues 90–109) derived from Gαs (Gs*), or 2.5 μM of a carboxyl-terminal sequence (residues 385–394) of Gαb (Gbg), respectively. A, effects of different peptides on adenyl cyclase activity. After pretreatment with different peptides, membranes were assayed for adenyl cyclase activity in the presence of 10 nM bFGF. Untreated membranes served as control. B, selective reversal of the bFGF-induced inhibition of NADPH-dependent H2O2 generation by peptide 100–119. After preincubation in the absence or presence of different peptides, the membranes were incubated with 5 nM insulin and 50 μM GTPγS in the absence or presence of 0.3 nM bFGF for 25 min and then assayed for NADPH oxidase activity. Values are mean ± S.D. of four separate experiments for each condition.

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2 H. I. Krieger-Brauer, unpublished results.
tivated β-adrenergic receptors recruit more Gs for signaling than the receptor(s) for bFGF. It therefore appeared possible that βγ complexes derived from G proteins other than Gs participated in bFGF receptor signaling. Indeed, it has been reported that bFGF receptor(s) may also couple to pertussis toxin-sensitive G proteins in certain cell types (29, 30).

To obtain definitive information about the origin of the Gβγ complexes transmitting the inhibitory effects of bFGF, we utilized a peptide comprising residues 100–119 of Goαs (K-20). Recent work showed that this peptide antagonized the agonistic effects of the β-adrenergic agonist isoproterenol on adenylyl cyclase and NADPH oxidase that are transmitted by Goαs and Gβγs, respectively (13, 14). The present studies revealed that peptide 100–119 reversed the effects of bFGF on NADPH-dependent H2O2 generation and cAMP production as well. The peptide did not influence the stimulatory effect of insulin, which is mediated by Goαs. In addition, a homologous peptide derived from Goa had no influence on the antagonistic effects of bFGF on cAMP production and H2O2 generation, indicating that peptide 100–119 is specific for Goαs and does not block the interactions of receptors with other G proteins. Thus, the latter finding strongly supported the view that the βγ subunits mediating the bFGF-induced inhibition of NADPH-dependent H2O2 generation were exclusively derived from Goαs.

G proteins are typically coupled to heptahelical receptors. However, it is becoming increasingly clear that G proteins may be responsible for transmitting signals of other types of receptors as well, including receptor kinases. Aside from the stimulatory effect of insulin on NADPH-dependent H2O2 generation, which is mediated by Goαs (12), these include activation of cardiac adenylyl cyclase by epidermal growth factor through Goαs and induction of fibroblast transformation by TGFβ1 via Goαs, respectively (31, 32). The receptors of some growth factors, such as insulin and epidermal growth factor, may couple to multiple G proteins (12, 33, 34). Intriguingly, at least one ligand of a receptor protein-tyrosine kinase, insulin-like growth factor-1, appears to be capable of utilizing Ga as well as Gβγ subunits for signaling, albeit in different cells (35–37). However, bFGF is the first example of a ligand of receptor protein-tyrosine kinases that has been shown to be capable of signaling through both types of G protein subunits of a single class of heterotrimeric G proteins in a single cell type.

As pointed out recently (14), the mechanisms by which Goαs (which mediates the stimulatory effects of insulin) and Gβγs modulate the activity of the human fat cell oxidase remain to be defined. Current knowledge suggests that activation of heterotrimeric G proteins by ligand-receptor complexes is achieved by exchange of GTP for GDP on the α-subunit, and this is thought to facilitate dissociation into α and βγ subunits (38, 39). G protein-sensitive effectors are then directly regulated by GTP-ligated α-subunits, βγ subunits, or both (38, 39). Consistently, the mechanisms by which insulin and bFGF modulated NADPH-dependent H2O2 generation were confined to the plasma membrane and were independent of soluble second messengers, making it likely that activated Goαs and Gβγs acted upon NADPH oxidase directly. However, indirect mechanisms of action involving intermediate membrane-associated effectors or association of Goαs and Gβγs to yield the inactive G2 heterotrimer cannot definitively be ruled out (40–42).

The generation of H2O2 and other reactive oxygen species seems to be a common signaling event for hormones and cytokines that regulate cell growth and differentiation (40–44). Consistently, previous work from this laboratory revealed that growth factors that stimulated NADPH-dependent H2O2 generation promoted adipogenesis in 3T3 L1-preadipocytes, whereas inhibitory ligands, such as bFGF, were antiadipogenic (6). Intriguingly, it has been reported that inhibition of adipogenesis may be mediated via a Gα pathway that does not involve cAMP (45). The finding that the signal of bFGF, an inhibitor of adipogenesis, is transduced by βγ subunits that are derived from Ga, is consistent with these latter observations and provides further evidence in support of the concept that the H2O2 produced in response to hormones, growth factors, and cytokines may be important in the regulation of adipocyte differentiation and maintenance of the differentiated state.

The physiological role of the bFGF-induced stimulation of cAMP production, if any, is currently unknown. In intact cells, a measurable elevation of cAMP levels by bFGF was only observed in the simultaneous presence of a phosphodiesterase inhibitor. In addition, preliminary evidence suggests that bFGF has no lipolytic activity in human adipocytes. An interesting possibility that will be addressed in future studies is raised by observations in hamster fibroblasts showing that bFGF alone has no effects on cAMP levels but may be capable of potentiating the effects of other stimulators of adenylyl cyclase via activation of Gs (46).

In conclusion, the present findings show that bFGF, a ligand of receptor protein-tyrosine kinases, is capable of utilizing Ga as well as Gβγs for dual signaling in a single cell type via a non-kinase-dependent mechanism, and suggest a physical interaction of one or more members of the FGF receptor family with Gs. These results confirm and extend recent observations demonstrating that the human fat cell oxidase is under antagonistic control by Ga and Gβγ subunits derived from different G proteins and provide further evidence in support of the concept that this membrane-bound redox system represents an universal effector system for hormones, growth factors, and cytokines linking ligand binding to cell surface receptors to changes in the intracellular redox equilibrium.

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