Association of Heterotrimeric G<sub>i</sub> with the Insulin-like Growth Factor-I Receptor

RELEASE OF G<sub>βγ</sub> SUBUNITS UPON RECEPTOR ACTIVATION

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The insulin-like growth factor-I receptor (IGF-IR) is a key regulator of cell proliferation and survival. Activation of the IGF-IR induces tyrosine autophosphorylation and the binding of a series of adaptor molecules, thereby leading to the activation of MAPK. It has been demonstrated that pertussis toxin, which inactivates the G<sub>i</sub> class of GTP-binding proteins, inhibits IGF-I-mediated activation of MAPK, and a specific role for G<sub>βγ</sub> subunits in IGF-I signaling was shown. In the present study, we have investigated the role of heterotrimeric G<sub>i</sub> in IGF-IR signaling in neuronal cells. Pertussis toxin inhibited IGF-I-induced activation of MAPK in rat cerebellar granule neurons and NG-108 neuronal cells. G<sub>αi</sub> and G<sub>βγ</sub> subunits were associated with IGF-IR immunoprecipitates. Similarly, in IGF-IR-null mouse embryo fibroblasts transfected with the human IGF-IR, G<sub>i</sub> was complexed with the IGF-IR. G<sub>αi</sub> was not associated with the IGF-IR in any cell type. IGF-I induced the release of the G<sub>βγ</sub> subunits from the IGF-IR but had no effect on the association of G<sub>αi</sub>. These results demonstrate an association of heterotrimeric G<sub>i</sub> with the IGF-IR and identify a discrete pool of G<sub>βγ</sub> subunits available for downstream signaling following stimulation with IGF-I.

Many receptors are coupled to heterotrimeric GTP-binding proteins (G-proteins). Prototypic G-protein coupled receptors (GPCRs) contain a seven-membrane spanning region (1). Activated GPCRs bind to G-proteins and induce the release of G<sub>βγ</sub> subunits from G<sub>αi</sub> subunits, which allows for the exchange of GDP for GTP on the G<sub>αi</sub> subunit. Activated G<sub>αi</sub> subunits and G<sub>βγ</sub> heterodimers interact with numerous signaling effectors, including adenyl cyclase, ion channels, protein kinases, and phospholipases (2–4).

In addition to their role in fully differentiated cells, GPCRs have been linked to mitogenesis and development (5–8). A specific role for G<sub>i</sub> in the induction of mitogenesis has been highlighted by the use of pertussis toxin, which inactivates G<sub>i</sub> by ADP-ribosylation of the G<sub>i</sub> subunit. However, G<sub>i</sub> subunits from several classes of G-proteins are not strongly mitogenic. Rather G<sub>βγ</sub> heterodimer subunits activate a series of nonreceptor tyrosine kinases, which in turn activates p21<sup>ras</sup> and extracellular signal-regulated kinases (or MAPK). Thus, G<sub>βγ</sub> subunits serve to bridge intracellular signaling of classical GPCRs and mitogenic tyrosine kinase receptors (RTKs).

G<sub>i</sub> also appears to be involved in the mitogenic actions of RTKs. Pertussis toxin variably inhibits the metabolic actions of insulin, both in vitro and in vivo (9–16), and the insulin receptor may associate with G<sub>i</sub> (17–19). Importantly, mice with targeted knockout of G<sub>i</sub> have defects in insulin signaling (20). EGF-dependent signaling is also impaired by pertussis toxin in rat hepatocytes (21–23) and other cells (24–27).

The insulin-like growth factor-I receptor (IGF-IR), which has strong homology to the insulin receptor, exists as an α<sub>2</sub>β<sub>2</sub>-heterodimer and contains a cytoplasmic tyrosine kinase domain (28, 29). Upon activation by its ligands IGF-I and IGF-II, the IGF-IR undergoes tyrosine autophosphorylation, after which it phosphorylates key signaling molecules and leads to the sequential activation of ras, raf, and MAPK. The role of G<sub>i</sub> in signaling by the IGF-IR is controversial. In fibroblasts, pertussis toxin inhibits the IGF-I-induced opening of a calcium-permeable cation channel (30) and the activation of MAPK (31). In the latter study, Luttrell et al. (31) demonstrated that MAPK activation by IGF-I was also inhibited by G<sub>βγ</sub> subunit binding proteins. Inhibitory effects of pertussis toxin have been observed for other IGF-I-dependent cellular effects (32–35) although not in all cases (36–39).

Because IGF-I exerts profound proliferative and survival effects on many cell types, including neuronal cells, we have investigated the role of G<sub>i</sub> in IGF-I signaling in neuronal cells. We report the specific association of G<sub>i</sub> with the IGF-IR and inhibition of MAPK activation by pertussis toxin. Importantly, IGF-I stimulation induces the release of G<sub>βγ</sub> subunits from the IGF-IR. The findings offer a model wherein G<sub>i</sub> heterotrimeric receptors are constitutively associated with the IGF-IR and identify a discrete pool of G<sub>βγ</sub> subunits available for IGF-I signaling.

EXPERIMENTAL PROCEDURES

Materials— Antibodies against G<sub>αi</sub>, G<sub>αG</sub>, G<sub>βγ</sub>, IGF-IR β domain, and anti-phosphotyrosine (pY99) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-MAPK and anti-phospho-MAPK (E10) antibodies, horseradish peroxidase-conjugated goat anti-rabbit IgG, and horse anti-mouse IgG antibodies and signal-enhanced chemiluminescence reagents were obtained from New England Biolabs (Beverly, MA). Pertussis toxin was purchased from Calbiochem (La Jolla, CA). Human recombinant IGF-I was obtained from Bachem Biosciences, Inc. (King of Prussia, PA). All other chemical and biochemicals were of the highest purity commercially available.

Cell Lines and Culture Conditions— Balb/c3T3 cells were obtained from American Type Culture Collection (Manassas, VA). Mouse embryo fibroblasts with targeted knockout of the IGF-IR (R<sup>−</sup> cells) and R<sup>−</sup> cells that express human IGF-IR (R<sup>+</sup> cells) were a gift from Dr. B. Baserga (Rothman Cancer Center, Thomas Jefferson University). The generation and characterization of R<sup>−</sup> and R<sup>+</sup> cells have been described elsewhere (40, 41). Fibroblasts were passaged in Dulbecco’s modified Eagle’s medium supplemented with 10% normal calf serum and 2 mm glutamine. NG-108 neuroblastoma cells, a gift from Dr. I. Diamond, University of...
California, were cultured in Dulbecco’s modified Eagle’s medium containing 6% (v/v) fetal calf serum and supplemented with 1 μM aminopterin, 100 μM hygromycin B, and 16 μM thymidine. Fibroblasts and NG-108 cells were cultured in serum-free medium for 18 h prior to addition of IGF-I. Where indicated, cells were treated with pertussis toxin for 4 h prior to the addition of IGF-I.

Rat cerebellar granule neurons were prepared from 7-day-old rat pups as reported previously (42, 43). Briefly, cerebella were obtained from postnatal day 7 Harlan Sprague-Dawley rat pups, cross-chopped pups as reported previously (42, 43). The figures are scanned images of blots and are representative of 3-5 experiments.

**RESULTS**

The role of Gi in the activation of p42/p44 MAPK by IGF-I was investigated in Balb/c 3T3 cells, rat cerebellar granule neurons, and NG-108 neuronal cells (Fig. 1). Basal MAPK phosphorylation was reduced by pertussis toxin in Balb/c 3T3 and NG-108 cells but not in cerebellar granule neurons. In agreement with a previous report (31), pertussis toxin markedly inhibited IGF-I-induced MAPK phosphorylation in all cell types. Total MAPK content was unchanged under all conditions. Pertussis treatment had no effect on cell viability. Pertussis toxin inactivates both Gi and Gα, but 3T3 cells lack Gαi. The results indicate that Gi is at least partially required for MAPK activation by IGF-I. Having established a requirement for Gi in IGF-I-induced neuronal MAPK activation, we next examined the relationship between Gαi and the IGF-IR. IGF-IR was immunoprecipitated under non-denaturing conditions and probed for Gαi content by Western blot analysis with anti-Gαi antibody. Fig. 2 illustrates that Gαi is associated with the IGF-IR in both cerebellar granule cells and NG-108 cells. The IGF-IR and Gαi were similarly complexed in Balb/c 3T3 cells (data not shown). Gαs subunits were not detected in IGF-IR immunoprecipitates from any cell type despite copious expression in total cell lysates (not shown).

The IGF-IR/Gαi interaction was completely disrupted when the IGF-IR was immunoprecipitated under denaturing conditions (not shown). IGF-I had no effect on the association of Gαi with the IGF-IR. The IGF-IR was active under these experimental conditions, as manifest by IGF-I-induced tyrosine autophosphorylation within total cell lysates was identical under all conditions. Pertussis treatment had no effect on cell viability. Pertussis toxin inhibited IGF-I-induced MAPK phosphorylation in all cell types. Pertussis treatment had no effect on cell viability. Pertussis toxin inactivates both Gi and Gα, but 3T3 cells lack Gαi. The results indicate that Gi is at least partially required for MAPK activation by IGF-I. Having established a requirement for Gi in IGF-I-induced neuronal MAPK activation, we next examined the relationship between Gαi and the IGF-IR. IGF-IR was immunoprecipitated under non-denaturing conditions and probed for Gαi content by Western blot analysis with anti-Gαi antibody. Fig. 2 illustrates that Gαi is associated with the IGF-IR in both cerebellar granule cells and NG-108 cells. The IGF-IR and Gαi were similarly complexed in Balb/c 3T3 cells (data not shown). Gαs subunits were not detected in IGF-IR immunoprecipitates from any cell type despite copious expression in total cell lysates (not shown).

The IGF-IR/Gαi interaction was completely disrupted when the IGF-IR was immunoprecipitated under denaturing conditions (not shown). IGF-I had no effect on the association of Gαi with the IGF-IR. The IGF-IR was active under these experimental conditions, as manifest by IGF-I-induced tyrosine autophosphorylation of the β domain of the IGF-IR in both cell types (Fig. 2, bottom panel).

The anti-IGF-IR antibody did not cross-react with Gαi. Both proteins were immunoprecipitated by IGF-I-induced MAPK activation in all cell types. Total MAPK content was unchanged under all conditions. Total MAPK content was unchanged under all conditions. Pertussis treatment had no effect on cell viability. Pertussis toxin inactivates both Gi and Gα, but 3T3 cells lack Gαi. The results indicate that Gi is at least partially required for MAPK activation by IGF-I.
expression of IGF-IR or \( \beta \) subunit. We conclude that heterotrimeric Gi is constitutively associated with IGF-IR and responds to IGF-I by release of free \( \beta \gamma \) subunits.

**DISCUSSION**

The use of pertussis toxin and \( \beta \gamma \) subunit binding proteins has provided indirect evidence for an additional role of Gi, in the activation of MAPK by IGF-I (31). In the current study, we extended these findings to neuronal cell types and now provide direct evidence for the constitutive association of heterotrimeric Gi, with the IGF-IR complex. Both \( G_{ai} \) and \( G_{bi} \) subunits co-immunoprecipitate with IGF-IR in cerebellar neurons and NG-108 cells. Importantly, IGF-I induces the release of \( G_{ai} \) subunits from the IGF-IR complex, whereas the association of \( G_{ai} \) with the IGF-IR is unaffected. The current data do not allow us to determine whether \( G_{ai} \) binds directly to the IGF-IR or to another protein in the IGF-IR complex. \( G_{p\gamma} \) subunits exhibit a pleckstrin homology domain which mediates binding to several molecules that are part of the IGF-IR signaling complex including IRS-1 and PI 3-kinase (3). However, these interactions are unlikely to play a role in binding of \( G_{p\gamma} \) subunits to the IGF-IR because IRS-1 and PI 3-kinase are not bound to the IGF-IR in its unactivated state.

RTKs for insulin, IGF-I and EGF, among others, contain SH2 binding domains which anchor adaptor proteins such as IRS-1 and Shc (28, 29). Phosphorylation of these intermediaries leads to the activation of p21ras, MAPK, and transcriptional activation. Both IRS-1 and Shc can independently lead to MAPK activation, although studies in some cell types have suggested that Shc may ultimately be more important for mitogenesis (45, 46). The IGF-IR also activates PI 3-kinase, which has diverse functions, including an anti-apoptotic role mediated by its activation of Akt (47–49). However, the relative contribution of these diverse signaling pathways to functional effects must be studied individually. There is also ample experimental evidence to support a role for the IGF-IR in the maintenance of the transformed phenotype. Notably, R- cells are resistant to transformation in response to several oncogenic influences (50). Transformation is restored upon expression of the IGF-IR. In contrast, Leroith and co-workers have recently demonstrated that a constitutively active mutant of \( G_{i13} \) can transform R- cells (51).

The role of Gi in IGF-I signaling likely varies among different cell types. Pertussis toxin variably inhibits biological actions of IGF-I. In 3T3 cells (30) and chondrocytes (32), pertussis toxin inhibits IGF-I-induced mobilization of intracellular calcium. Anti-G\(_{ai} \) antibodies inhibit the opening of a calcium-permeable cation channel in response to IGF-I (30). Pertussis toxin also inhibits the activation of human neutrophil phagocytosis (35) and blocks IGF-I induced proliferation of myoblasts (33). By contrast, pertussis toxin failed to inhibit several IGF-I-dependent phenomena including GTP[S] binding to rat kidney epithelial cell membranes (36), DNA synthesis in MG-63 osteosarcoma cells (38), and IGF-I-induced human melanoma cell motility (39). We have also noted that pertussis toxin has no effect on the ability of IGF-I to protect cerebellar granule cells from apoptosis induced by removal of growth factors and low extracellular potassium (43, 52). Thus, as is the case for other signaling mediators of the IGF-IR, the specific functional role of \( G_{p\gamma} \) subunits in IGF-I signaling is likely dependent upon cell context.

\( G_{ai} \) may also associate with the insulin receptor. \( G_{ai2} \) and \( G_{ai} \) (but not \( G_{ai1} \) or \( G_{ai3} \)) were identified in purified insulin receptor preparations (14). In a recent study, Sanchez et al. (19) reported that activation of the insulin receptor recruits \( G_{ai} \) to this receptor, in a manner similar to classical GPCRs. However, this differs from our finding that \( G_{ai} \) constitutively associates with the IGF-IR. Pertussis toxin inhibits metabolic and mitogenic actions of insulin in a variety of cell culture models (9–16), and insulin enhances GTP binding to membranes (16, 18). In a recent study of rat hepatoma cell membranes (19), antibodies targeted against \( G_{ai} \) but not \( G_{ai} \) prevented insulin-

\(^2\) H. Hallak, and R. Rubin, unpublished data.
stimulated GTP binding. Similarly, anti-Gr antibodies blocks insulin-induced NADPH-dependent generation of hydrogen peroxide in human adipocyte membranes (14).

The mechanism by which the IGF-IR induces Gbg subunit release is unclear. Direct tyrosine phosphorylation of Gbg by the insulin receptor was suggested in studies of phospholipid vesicles co-internalized with Gbg (17). However, other data are inconsistent with the notion that Gbg is phosphorylated by the insulin receptor (53–56). Thus, a pathway other than tyrosine kinase appears to be involved in the regulation of neuronal MAPK activity, which is a key element in neuronal development and regeneration.

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