The Constitutively Active Mutant G_{α13} Transforms Mouse Fibroblast Cells Deficient in Insulin-like Growth Factor-I Receptor*

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Jun-Li Liu‡, Vicky A. Blakesley, J. Silvio Gutkind§, and Derek LeRoith¶

From the Section on Cellular and Molecular Physiology, Diabetes Branch, NIDDK and the Laboratory of Cellular Development and Oncology, NIDR, National Institutes of Health, Bethesda, Maryland 20892

Insulin-like growth factor-I (IGF-I) receptor plays an important role in normal cell cycle progression and tumour growth, and it is thought to be essential for cellular transformation. To test this hypothesis, we stably transfected a GTPase-deficient mutant human G_{α13}, which is highly oncogenic when overexpressed in vitro, into R- fibroblasts derived from IGF-I receptor-deficient mice. Northern blots of multiple clones revealed the expression of a 1.8-kilobase pair mutant G_{α13} transcript in transfected cells, in addition to the 6-kilobase pair endogenous mRNA. The transfection resulted in a doubling of the expression of G_{α13} protein in these cells as assessed by Western blot analysis. The transforming ability of the mutant G_{α13} was tested using the soft agar assay. Nontransfected R- cells cultured with 10% fetal bovine serum failed to form colonies after 3 weeks. Most of the mutant G_{α13}-expressing clones formed significant numbers of colonies (11-50 colonies/1000 cells plated). Overexpression of the IGF-I receptor enabled R- cells to form colonies (27 colonies), and co-transfection of the mutant G_{α13} caused a further increase in colony formation (117-153 colonies) in three of five clones analyzed. Apparently G_{α13} works through pathways other than mitogen-activated protein kinase and c-Jun N-terminal kinase in transforming R- cells, because their activities were not significantly altered by the mutant G_{α13} expression. These results demonstrate that G_{α13} can induce cellular transformation through pathways apparently independent of the IGF-I receptor and that activation of the IGF-I receptor signaling pathways, although not essential for the transforming phenotype, enhances the effect of other pathways.

The insulin-like growth factor-I (IGF-I) receptor, a transmembrane heterotetrameric tyrosine kinase, is expressed by most normal and transformed cells (1). Upon ligand binding, IGF-I receptor undergoes autophosphorylation on intracellular tyrosine residues and activation of its intrinsic tyrosine kinase. Mice without the IGF-I receptor gene invariably die of respiratory failure at birth and exhibit severe intrauterine growth retardation, general organ hypoplasia, and delayed ossification, demonstrating the essential role of the IGF-I receptor in normal growth and development (1-3). Overexpression of the IGF-I receptor can transform mouse fibroblasts (4). The presence of the IGF-I receptor was found to be necessary for proteins such as SV40 large T antigen, Ha-Ras, EWS/FLI-1 (a fusion protein produced by Ewing's family of tumors), v-Src, and bovine papilloma virus to transform target cells (5-8). Furthermore, decreasing the number of IGF-I receptors by antisense RNA technology causes a reversal of the transforming phenotype, including arrest of in vivo tumor growth (7), suggesting that the IGF-I receptor is also essential for oncogene-induced cellular transformation.

Heterotrimeric (α, β, and γ subunits) guanine nucleotide-binding proteins (G proteins) couple to hundreds of different receptors in mammals and are central to the signaling processes of multicellular organisms (9). The α subunits of G proteins belong to a large group of GTPases, which are classified into four sub-families based on amino acid homology: G_{αs}, G_{αi/o}, G_{αq/11}, and G_{α12/13} (10). Stimulatory (G_{s}) and inhibitory (G_{i}) G protein subtypes have been implicated in the regulation of adenyl cyclase and the gating of certain ion channels (11). The G_{i} family of proteins couple to the phospholipase C pathway (12). The ubiquitously expressed G_{α12/13} proteins are involved in mitogenesis and transformation probably through activation of c-Jun N-terminal kinase (JNK) and activation or inhibition of mitogen-activated protein kinase (MAPK) pathways (13-18). Overexpression of wild type G_{α12/13} and the expression of GTPase-deficient active mutants of G_{α12/13} transform fibroblast cell lines (15, 18-22).

To test the hypothesis that IGF-I receptor is essential for cellular transformation, we stably transfected a GTPase-deficient mutant human G_{α13} (Q226L) into R- fibroblasts derived from IGF-I receptor-deficient mice. These cells cannot be transformed by SV40 large T antigen and other oncogenes (5-8). In contrast, we found that activated human G_{α13} (hG_{α13}) is able to transform R- cells independent of the IGF-I receptor and that overexpression of both G_{α13} and IGF-I receptor caused synergistic enhancement in cellular transformation.

**EXPERIMENTAL PROCEDURES**

**Mutant Human G_{α13}, Expression Vector—**A GTPase-deficient mutation was introduced into hG_{α13}, cDNA (Ref. 14; GenBank accession number L22075) by polymerase chain reactions. The codon corresponding to glutamine (Q) at residue 226 was mutated to express leucine (L) and thereby generated a new restriction site Bsu361. The mutant cDNA (BgII/EcoRI) was then subcloned into the vector pcDNAIIIHA (Invitrogen, Carlsbad, CA). The resulting construct pcDNAIIIHAHAG13QL(A) (276) contains the mutant hG_{α13} cDNA (1.5 kb) downstream of a HA tag (derived from influenza protein hemagglutinin) sequence, driven by the human cytomegalovirus promoter, and genes whose products confer resistance to neomycin and ampicillin.

JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; kb, kilobase(s); DMEM, Dulbecco’s modified Essential medium; FBS, fetal bovine serum; PAGE, polyacrylamide gel electrophoresis; SAPK, stress-activated protein kinase; EGF, epidermal growth factor.
Cell Culture and Transfection—R- cells are fibroblasts prepared from IGF-I receptor-deficient mice (5, 23). W6+ cells are R- cells overexpressing IGF-I receptors (5, 23). All cells have been cultured in complete medium consists of Dulbecco’s modified Essential medium (DMEM; Life Technologies, Inc.) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and penicillin/streptomycin/fungizone (Biofluids, Rockville, MD). R- cells were transfected using electroporation (6). Geneticin (G418 sulfate, 567 mg/ml, Life Technologies, Inc.)-resistant colonies were selected, expanded, and tested for expression of the mutant hG1313 expression.

W6+ cells were transfected using a calcium phosphate mammalian transfection kit (Stratagene, La Jolla, CA) (24) with G1313 and pZeosSV (Invitrogen). Transfected cells were selected with complete medium containing zeocin (150 µg/ml, Invitrogen) for 3 weeks.

Northern Blot Analysis—The expression of the mutant hG1313 was evaluated by Northern blot analysis (25). The specific bands of expected sizes were illustrated, together with ethidium bromide staining of ribosomal RNAs, demonstrating equal loading of total RNA on the gel.

Western Blot Analysis—The expression of the G1313 protein was further confirmed by Western blot analysis using standard procedures (26) and rabbit anti-G13 antisera (1:1000, Calbiochem, San Diego, CA), which is specific to an 11-amino acid (LHDNLKQLMMQ)-C-terminal region of hG13. The level of MAPK activity was determined by using phospho-specific MAPK and p44/42 MAPK antibodies (New England Biolabs, Beverly, MA). Rabbit polyclonal Crk-L(C20) and mouse monoclonal JNK1 (F3) antibodies (Santa Cruz Biotech, Santa Cruz, CA) were also employed in Western blots.

Colony Formation in Semisolid Media—For assays of anchorage-independent growth, suspensions of 1000 cells in 1 ml of DMEM with 10% FBS, 20 mM HEPES, pH 7.5, and 0.2% agarose were laid onto 1.5 ml of 10% FBS, 20 mM HEPES, pH 7.5, and 0.2% agarose. New colonies of R2 cells were transfected and analyzed for the expression of the mutant hG1313 using Northern and Western blot analysis (Fig. 3). Abundant hG1313 mRNA is expressed in all clones except untransfected R2 cells. Middle panel, ethidium bromide staining of RNA species (28 and 18 S) of the RNA gel, showing equal loading of total RNA. Lower panel, Western blot analysis of total cell lysate (50 µg of protein) with anti-G13 antibody. A 40-kDa band is present in all cells, and the density of it is doubled following G1313 transfection (densitometric analysis, data not shown). The same blot was subsequently stripped and rebotted with an unrelated Crk-L antibody, which demonstrates equal loading of total protein (data not shown).

RESULTS

Characterization of the Cell Lines—Three cell lines were used in transformation studies. Previously, IGF-I receptor gene-deficient R- cells have been demonstrated to not express IGF-I receptor using immunoprecipitation, IGF-I-cross-linking, and Northern blot analysis, in contrast to wild-type mouse fibroblasts (5, 6). Indeed, R- cells demonstrate a total lack of binding to 125I-IGF-I as determined by Scatchard analysis (data not shown). W6+ cells that overexpress IGF-I receptors demonstrate a level of IGF-I receptor expression (730,000 receptors/cell, Kd of 0.16 nM) comparable with that of NWTb3 (550,000 receptors/cell, Kd of 0.55 nM), which are NIH 3T3 fibroblasts overexpressing the IGF-I receptors (30).

Expression of Human Mutant G1313 in IGF-I Receptor-deficient R- Cells—To study the role of the IGF-I receptor in G1313-induced cellular transformation, we transfected a constitutive active mutant hG1313 into R- cells. All G418 resistance-transfected R- cells express a 1.8-kb ribosome hybridized to an antisense hG13 riboprobe (Fig. 1, upper panel). This band is absent in untransfected cells, despite equal amounts of total RNA loaded as shown by staining of the RNA bands (middle panel). A 4-kb endogenous mouse G13 mRNA has been observed in all clones (data not shown). At the protein level, G1313 (wild type and mutant forms) are present mainly as a 40-kDa band (lower panel), which is increased 2-fold upon transformation in all the clones selected (clones 5–26), as analyzed by densitometry.

G1313 Is Capable of Transforming IGF-I Receptor-deficient R- Cells—G1313 transfection caused no morphological change in R- cells. The effect of the constitutively active mutant hG1313 on cellular transformation was tested using the soft agar assay (Table I). In three experiments each with triplicate dishes, 5 of 6 mutant hG1313-expressing clones (except 12) formed significant numbers of colonies (11–50 colonies/1000 cells plated, p < 0.05) as compared with untransfected R- cells, which formed only 1–3 tiny colonies as shown in Fig. 2. Cells expressing mutant hG1313 protein formed either significant number albeit small colonies (clones 17 and 20) or many colonies comparable in size with the ones seen in NWTb3 control cells (clones 7 and 26) (Table I and Fig. 2). This result suggests that hG1313 can transform R- cells in the absence of the IGF-I receptor.

Expression of Human Mutant G1313 in IGF-I Receptor-overexpressing W6+ Cells—To determine if overexpression of IGF-I receptor can further enhance the transforming ability of hG1313, W6+ cells were transfected and analyzed for the expression of hG1313 using Northern and Western blot analysis (Fig. 3). Abundant hG1313 mRNA is expressed in all clones transfected with the construct. In transfected W6+ cells, hG1313 mRNA is present in two molecular forms (2.2 and 1.8 kb, upper panel); the reason for these two bands is under investigation. Mutant hG1313 expression is undetectable in untransfected R- and W6+ cells. At the protein level, G1313 is present in two molecular forms as well (44 and 40 kDa, lower panel), co-migrating with the positive control sample (lane C). Both forms of G1313 are increased at least 2-fold in clones 3, 12, and 19 but to a lesser degree in 18 and 21.

IGF-I Receptor and G1313 Synergize in Cellular Transformation...
**Table I**

The effect of Gα13 expression in R− and W6+ cells on cell growth in the soft agar assay

| Clones       | n | Colonies (Mean ± SE) |
|--------------|---|----------------------|
| NWTb3        | 9 | 93 ± 18              |
| R−           | 9 | 2 ± 0.2              |
| R−Gα13−5     | 9 | 11 ± 3*              |
| R−Gα13−7     | 9 | 50 ± 8*              |
| R−Gα13−12    | 9 | 6 ± 2                |
| R−Gα13−17    | 9 | 12 ± 5*              |
| R−Gα13−20    | 9 | 35 ± 13*             |
| R−Gα13−26    | 9 | 16 ± 2*              |
| W6+          | 3 | 27 ± 2*              |
| W6-Gα13−13   | 3 | 20 ± 2               |
| W6-Gα13−12   | 3 | 15 ± 2               |
| W6-Gα13−18   | 3 | 145 ± 38*            |
| W6-Gα13−19   | 3 | 117 ± 9*             |
| W6-Gα13−21   | 3 | 153 ± 16*            |

*p < 0.05 versus untransfected R− or W6+ cells.

Discussion

In this study, we have confirmed that IGF-I receptor-deficient R− fibroblasts are incapable of forming colonies in soft agar and that active mutant Gα13 is highly oncogenic. The unique finding is that Gα13 is able to transform fibroblasts independent of IGF-I receptor.

The role of Gα12/13, a novel class of Gα proteins, in signal transduction is under intense investigation. The Gα12/13 proteins are known to be coupled to two receptors: thyrotropin receptors in the thyroid gland, which stimulate adenyl cyclase and phospholipase C pathways (31), and the thrombin receptor in platelets, which stimulates platelet aggregation (32). It is relatively clear that constitutively active Gα12/13 activates Ras, which recruits the small G protein Rac, and leads to activation of JNK/stress-activated protein kinase (SAPK) and enhanced transcriptional activity of c-Jun (14, 16). The effect of Gα12/13 on MAPK activity is not yet fully elucidated. Although the Gα12/13 proteins do not activate MAPK directly, there have been reports that these activated G proteins can enhance epidermal growth factor (EGF)-induced MAPK activation in Rat-1 cells (18) or inhibit EGF-stimulated extracellular signal-regulated kinase expression in COS-7 cells (17). In Swiss 3T3 cells, Gα12/13 regulate Rh-dependent responses in actin polymerization (28). Constitutively active mutants of Gα12/13 have also been known to activate the Na+/H+ exchanger (22, 33) and to potentiate serum-stimulated phospholipase A2 activity but do not stimulate inositol phospholipid hydrolysis (19).

Recent reports by Baserga and co-workers (5, 7, 8, 23, 34–36) have demonstrated that the IGF-I receptor is required for the establishment and maintenance of the transformed phenotype in vivo and in vitro. Indeed, oncogenes including SV40 T antigen and Ha-Ras and overexpressed EGF receptors all failed to transform R− cells (5, 8, 23, 34, 35); the resistance of R− cells...
to transformation can be abolished by expressing IGF-I receptors (5, 23). Furthermore, a decrease in the number of IGF-I receptors causes a reversal of the transformed phenotype (7). C6 rat glioblastoma cells failed to grow into tumors in syngeneic rats when antisense IGF-I receptor mRNA is expressed (36). Our finding that R– cells can be transformed by G$_{a13}$ in contrast to other oncogenes suggests that IGF-I receptor is not required for all the transformation processes. Nevertheless, G$_{a13}$ is not the only signaling protein capable of transforming R– cells. When transfected with constitutively active Ras or Ras plus the SV40 T antigen, R– cells were partially transformed (23). Most recently, Valentini et al. (37) demonstrated that oncprotein v-Src can also bypass the requirement for a functional IGF-I receptor in transforming mouse fibroblasts. However, c-Src was unable to do so. Taken together, the results of these studies suggested that G$_{a13}$ acts independently of the IGF-I receptor in cellular transformation.

Another interesting observation from this study is that G$_{a13}$ and the overexpressed IGF-I receptor act in concert in transforming R– cells. This is a very similar scenario with the reported synergism between G$_{a13}$ and c-Raf-1 in transforming NIH 3T3 cells (38). In that case, the synergism seemed to occur between two parallel pathways: MAPK activated by c-Raf-1 and JNK/SAPK activated by G$_{a13}$. The IGF-I receptor is known to signal through the Ras-Raf-MAPK cascade of serine/threonine and tyrosine kinases. Therefore, our observation of similar synergism may well be explained by cooperation between a G$_{a13}$-activated pathway and overexpressed IGF-I receptor-induced MAPK activation. Analysis of MAPK and JNK activities upon serum stimulation revealed no change caused by mutant G$_{a13}$ expression, although in some clones their basal activities were elevated. Because the cell transformation was studied using 10% serum and the increase in basal kinase activity does not correlate with transforming ability of each clone, we propose that these two pathways are not apparently involved in G$_{a13}$-induced cellular transformation. Investigation is underway to explore the specific targets activated by G$_{a13}$ in R– cells.

In summary, we have found that 1) IGF-I receptor-deficient fibroblasts did not form colonies in soft agar assay; 2) oncogenically active mutant hG$_{a13}$ was able to transform the R– cells; 3) overexpression of the IGF-I receptor also enabled R– cells to form colonies; 4) the effects on transformation of hG$_{a13}$ and the overexpressed IGF-I receptor were synergistic; and 5) G$_{a13}$-induced cellular transformation does not apparently work through MAPK or JNK pathways. Therefore, G$_{a13}$ induces cellular transformation through pathways apparently independent of the IGF-I receptor. Furthermore, whereas IGF-I receptor is not essential for the transforming phenotype, it can enhance the effect of G$_{a13}$-activated pathways.

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