Coupling of Heterotrimeric G\textsubscript{i} Proteins to the Erythropoietin Receptor*  

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To identify new proteins involved in erythropoietin (Epo) signal transduction, we purified the entire set of proteins reactive with anti-phosphotyrosine antibodies from Epo-stimulated UT7 cells. Antiserum generated against these proteins were used to screen a λEXLox expression library. One of the isolated cDNAs encodes G\textsubscript{\beta\textsubscript{2}}, the \( \beta \) subunit of heterotrimeric GTP-binding proteins. G\( \beta \) and G\( \alpha \) coprecipitated with the Epo receptor (EpoR) in extracts from human and murine cell lines and from normal human erythroid progenitor cells. In addition, in vitro G\( \beta \) associated with a fusion protein containing the intracellular domain of the EpoR. Using EpoR mutants, we found that the distal part of the EpoR (between amino acids 459–479) was required for G\( \beta \) binding. Epo activation of these cells induced the release of the G\( \alpha \) protein from the EpoR. Moreover in isolated cell membranes, Epo treatment inhibited ADP-ribosylation of G\( \alpha \), and increased the binding of GTP. Our results show that heterotrimeric G\( \alpha \) proteins associate with the C-terminal end of the EpoR. Receptor activation leads to the activation and dissociation of G\( \alpha \) from the receptor, suggesting a functional role of G\( \alpha \) protein in Epo signal transduction.

Activation of the EpoR\( ^{2} \) elicits multiple intracellular signals that ultimately lead to cell division and differentiation of erythroid progenitor and precursor cells. One primary signaling event following receptor activation is the phosphorylation of certain cellular proteins on tyrosine residues (1, 2). Interaction of Epo with its receptor results in the activation of the cytoplasmic tyrosine kinase JAK2 and in the phosphorylation of the intracellular domain of EpoR (3–5). Tyrosine phosphorylated residues on EpoR then constitute binding sites for other intracellular proteins. Several tyrosine kinase substrates phosphorylated upon Epo activation have been identified including STAT5 transcription factor (9), SHP-2 tyrosine phosphatase (10), Src (6), phospholipase C-\( \gamma \) (12), Vav (13), c-Cbl (14), IRS-2 (15), GAB-1 (16), and CrkL (17). Yet the relations between all the components involved in Epo signaling as well as the identification of their respective targets are only partially elucidated, and EpoR may activate other additional signal transduction pathways.

In an attempt to characterize substrates for epidermal growth factor (EGF) receptor, Fazioli et al. (18) developed an expression cloning strategy for cDNAs encoding EGF receptor substrates. The approach relied on batch purification of an entire set of putative substrates, achieved by immunoaffinity chromatography using anti-phosphotyrosine antibodies (19, 20). Antiserum generated against the entire pool of purified proteins were subsequently used for the screening of cDNAs expression libraries. We applied this methodology to identify new cDNAs encoding signaling proteins involved in Epo activation, either tyrosine phosphorylated proteins or proteins bound to these proteins. In the present work we report that one of these cDNAs encodes G\( \beta \)\( \gamma \), the \( \beta \) subunit of heterotrimeric GTP-binding proteins, or G proteins.

G proteins traditionally associate with G protein-coupled receptors (GPCRs) that contain seven membrane-spanning domains. G proteins function as intermediaries that couple cell surface receptors to intracellular effectors. Heterotrimeric G proteins are made of three polypeptides: an \( \alpha \) subunit that binds and hydrolyzes GTP, and \( \beta \)\( \gamma \) subunits that form a functional monomer. Receptor activation induces the exchange of GDP for GTP on the G\( \alpha \)\( \gamma \) subunit. Once GTP is bound, the \( \alpha \) subunit dissociates both from the receptor and from \( \beta \gamma \). The free \( \alpha \) and \( \beta \gamma \) subunits each activate target effectors. However, a number of single-spanning transmembrane receptors such as receptors for EGF (21), insulin and insulin-like growth factor (IGF-1 and IGF-II (22–25), fibroblast growth factor (26), and T lymphocyte receptors (27, 28) have been reported to activate G proteins. In some cases a physical association, in addition to a functional coupling, has also been demonstrated between a single-spanning membrane receptor and G proteins (29–32).

Heterotrimeric G proteins could be important intermediates in the signal transduction of hematopoietic cytokines. Changes in the expression level and GTPase activity of G\( \alpha \)\( \omega \), a member of the G\( \alpha \) family of G proteins uniquely expressed in hematopoietic cells, may modulate cellular proliferation or differentiation in T lymphocytes and in MB-02 erythroleukemia cells (33, 34). Pertussis toxin (PT) modifies the response to several hematopoietic growth factors. PT catalyzes the ADP-ribosylation of the G\( \alpha \) family of G proteins and uncouples G proteins from surface receptors. PT inhibits the signal transduction and/or proliferation induced by interleukin (IL)-1, IL-3, granulocyte-
macrophage colony-stimulating factor (GM-CSF) and colony-stimulating factor-1 (CSF-1) (35-38) in hematopoietic cells. Expression of a dominant negative mutant of Go alpha2 also inhibits cell proliferation in response to CSF-1 in BAC 1.2F5 macrophage cell line (39). In erythroid precursor cells a pertussis toxin-sensitive G protein identified as Go alpha2 is required for the regulation of voltage-independent calcium channels by Epo (40, 41). The increase in [Ca2+]i, appears to be a stage of differentiation specific and restricted to differentiating erythroblasts (42).

In the present study we demonstrate the constitutive association of Gi with the EpoR in hematopoietic cell lines as well as in erythroid progenitors. We show that the C-terminal region of EpoR is required for Gi protein binding. In addition, Epo activates G protein in cell membranes and induces the release of Gi bound to the EpoR in hematopoietic cells. Thus, EpoR appears to be physically and functionally coupled to G proteins.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—Anti-phosphotyrosine (anti-Tyr(P)) monoclonal antibodies 4G10 and PY2 were produced from hybridoma cell lines provided by D. Drucker (Mount Sinai School of Medicine, New York, NY) and W. Lane (Jolla, CA), and were affinity purified by chromatography on phosphotyramine. Anti-EpoR antisemur used for immunoprecipitation was produced against a fusion protein between glutathione S-transferase and the cytoplasmic portion of human EpoR. Peroxidase-conjugated anti-rabbit antibodies were purchased from Amersham Pharmacia Biotech. Antibodies specific for human Epo used for immunoblotting were purchased from Santa Cruz (ser-695) and anti-JAK2 antisemur from Upstate Biotechnology Inc. (catalog number 06-255). The fusion protein between the maltose-binding protein (MalE) and the cytoplasmic region of EpoR was described previously (43). Purified recombinant human Epo (specific activity, 120,000 units/mg) was a gift of Dr. M. Brandt (Roche Molecular Biochemicals). Pertussis toxin was purchased from Alexis, ATP was from Amersham Pharmacia Biotech, and GTP and GTP-S were from Sigma.

**DNA Constructs and Expression Vectors**—The murine EpoR mutant F1-Y58 that contains a deletion between Glu 377 and Tyr 431 was described previously (9). A panel of EpoR deletion mutants was produced as described previously (9). A panel of EpoR deletion mutants was produced by variable-length PCR-amplified fragments. In mutants −41, −24, −20, and −5, stop codons were inserted just after codons 442, 459, 463, and 475, respectively (see Fig. 6). All receptor constructs were subcloned into a modified pCDNA3 expression vector where the cyto-megalovirus promoter was changed to Rous sarcoma virus and the neomycin resistance gene was replaced by puromycin. The fidelity of all plasmids was confirmed by sequencing. Nucleotide and protein data bases were screened with the NCBI program.

**EXoXo Library Screening**—Absorbed IgG were used to screen a commercial (Novagen RD Systems Europe) 16-day-old murine embryonic DNA library in AE exoXo vector, according to the manufacturer’s instructions. Screening conditions were chosen to get an optimal signal-to-noise ratio and no reactivity with preimmune serum in immunoblotting. This serum was also tested for its ability to precipitate proteins known to be tyrosine phosphorylated following Epo stimulation and was shown to contain anti-Shc antibodies (data not shown). IgG were purified and antibodies reacting with bacterial proteins were removed by absorption on column of bacterial proteins cross-linked to Sepharose beads (Hi Trap® NHS-activated Sepharose from Amersham Pharmacia Biotech).

**Immunoprecipitation, MalE Precipitations, and Immunoblotting**—Immunoprecipitations, MalE precipitations, and immunoblots were performed as described previously (4, 8). Proteins were immunoblotted with a mixture of 4G10 (0.5 μg/ml) and PY2 (1 μg/ml) to detect tyrosine phosphorylated proteins, with anti-Ga6 antibodies (1 μg/ml), anti-EpoR antibodies (1 μg/ml), or anti-JAK2 antisemur (1:1000). Bound antibodies were detected by incubation with horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence system (Amersham Pharmacia Biotech).

**Preparation of Cell Membranes**—After washing UT77 cells in phosphate-buffered saline, the cells were suspended in hypotonic buffer (10 mM Tris-HCl, pH 7.4, 10 mM KCl, 2 mM EDTA) in the presence of protease inhibitors and homogenized with a Dounce pestle. After addition of 0.25 M sucrose, nuclei and unbroken cells were separated by centrifugation at 375 × g, for 45 min. The resulting membrane pellet was stored at −80°C in 25 mM Hepes, pH 7.5, 150 mM NaCl, 5 mM EGTA, 10% glycerol or resuspended in appropriate buffer and used immediately for [35S]GTPγS binding. CHO-ER membranes were prepared as described previously (50).
ADP-ribosylation Assay—ADP-ribosyltransferase activity was measured by following the incorporation of [32P]-ADP-ribose (51). To assess the activity present in immunoprecipitates, immune complexes bound to protein G-Sepharose were washed in 50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 1 mM ATP, and 0.1 mM GTP. Immunoprecipitated proteins were suspended in 50 μl of the same buffer containing 2 μCi of adenylyl [32P]NAD (PerkinElmer Life Sciences; 800 Ci/mmol) and 7 μg/ml activated pertussis toxin. The toxin (17 μg/ml) was preactivated immediately before use for 1 h at room temperature in 50 mM Tris-HCl, pH 7.5, containing 62.5 mM dithiothreitol. ADP-ribosyltransferase was carried out at 37 °C for 1 h, and the reaction was stopped by adding SDS sample buffer. Samples were boiled for 5 min, and the proteins were separated on a 10% SDS-polyacrylamide gel followed by transfer to nitrocellulose and autoradiography.

To assess the effect of Epo on pertussis toxin-induced ADP-ribosylation, in cell membranes, membranes (50 μg) were initially incubated in 35 μl of buffer A (25 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 100 μM GTP, 10% glycerol) with or without Epo for 10 min at 37 °C. Other additions or deletions are as noted under “Results” and in the figure legends. Then membranes were combined with 35 μl of buffer B (100 mM Tris-HCl, pH 7.5, 2 mM EDTA, 2 mM ATP, 0.2 mM GTP). After addition of adenylate [32P]NAD and activated pertussis toxin in 1× buffer B, ADP-ribosylation was performed as described above.

GTpS Binding Assay—The [35S]GTPS binding was measured as described (52) with slight modifications. Membranes (30 μg) were suspended in an assay volume of 50 μl of buffer containing 20 mM Hepes, pH 7.4, 10 mM MgCl2, 100 mM NaCl, 10 μM GDP. Following a preincubation of membranes in the presence or absence of Epo for 15 min at 30 °C, the assay was initiated by adding [35S]GTPS (New England Nuclear, 1250 Ci/mmol) to yield a final concentration of 0.3 nM. Nonspecific binding was measured in the presence of 100 μM unlabeled GTP. After 60 min at 30 °C, 0.5 ml of ice-cold buffer containing 20 mM Hepes, pH 7.4, 20 mM MgCl2, 100 mM NaCl, 100 μM GTP was added. Bound and free [35S]GTPS were separated by filtration over glass fiber filters and three washings in the same buffer without GTP.

RESULTS

Screening of a Bacterial Expression Library with Antibodies Specific for Anti-Tyr(P) Reactive Fractions of Epo-stimulated Cells—To purify proteins that become tyrosine phosphorylated upon Epo stimulation and their associated proteins, our initial concern was to get a high level of tyrosine phosphorylation. UT7 cells were selected for their high surface EpoR expression (~7000 receptors/cell) and their ability to proliferate in response to Epo. An optimal system was obtained by stimulating the cells with 10 units/ml Epo for 10 min at 37 °C. Cell lysates were loaded on anti-phosphotyrosine column and anti-Tyr(P) reactive fractions were eluted with 40 mM phenyl phosphate. Proteins before purification (lane 1) in the column flow-through (lane 2) and in phenyl phosphate eluted proteins (lanes 3–5) were separated by SDS-PAGE and transferred to nitrocellulose membrane. Tyrosine phosphorylated proteins extracted from 2 × 106 cells were detected by immunoblotting with anti-Tyr(P) antibodies. B, Gβ detection in anti-Tyr(P) reactive fractions. Starved UT7 cells were stimulated (+) or not (−) with 10 units/ml Epo for 10 min at 37 °C. Cell lysates were loaded on anti-phosphotyrosine column and anti-Tyr(P) reactive fractions were pooled. Purified proteins (PP, 5 × 105 cell equivalents), or total cell lysates (Lysate, 2 × 105 cell equivalents) were analyzed by SDS-PAGE and immunoblotting with anti-Gβ antibodies.

Fig. 1. Gβ detection in anti-Tyr(P) affinity purified proteins from Epo-stimulated cells. A, immunofinity chromatography. Epo- and serum-starved UT7 cells were stimulated with 10 units/ml Epo for 10 min at 37 °C. Cell lysates were loaded on anti-phosphotyrosine (pTyr) column as described under “Experimental Procedures.” Proteins bound to the column were eluted with 40 mM phenyl phosphate. Proteins before purification (lane 1) in the column flow-through (lane 2) and in phenyl phosphate eluted proteins (lanes 3–5) were separated by SDS-PAGE and transferred to nitrocellulose membrane. Tyrosine phosphorylated proteins extracted from 2 × 106 cells were detected by immunoblotting with anti-Tyr(P) antibodies. B, Gβ detection in anti-Tyr(P) reactive fractions. Starved UT7 cells were stimulated (+) or not (−) with 10 units/ml Epo for 10 min at 37 °C. Cell lysates were loaded on anti-phosphotyrosine column and anti-Tyr(P) reactive fractions were pooled. Purified proteins (PP, 5 × 105 cell equivalents), or total cell lysates (Lysate, 2 × 105 cell equivalents) were analyzed by SDS-PAGE and immunoblotting with anti-Gβ antibodies.

A

Anti-pTyr blot

1 2 3 4 5

kDa

158

116

97

66

56

43

B

Lysate PP

Epo + +

− −

kDa 36

Anti-Gβ blot

Gβ Subunit Associates with EpoR—When Gβ was immunoprecipitated from Epo-stimulated UT7 cells, we never detected a tyrosine phosphorylated form of the protein by immunoblotting with anti-Tyr(P) antibodies. This suggests that Gβ is not phosphorylated upon Epo activation. Because Gβ was present in anti-Tyr(P) reactive fractions (Fig. 1B), we hypothesized that Gβ may have been copurified because of its association with another tyrosine-phosphorylated protein upon Epo stimulation. We then wanted to determine whether Gβ was associated with the EpoR, one of the highly phosphorylated proteins upon Epo activation (4). UT7 cells were incubated or not with Epo at 37 °C for various times, and after cell solubilization, the EpoR was precipitated. As shown in Fig. 2A, immunoblotting with anti-Gβ antibodies revealed the presence of Gβ when cell ly-
EpoR fusion protein was used. We conclude that Gb3Lysates from UT7 cells (1.5 the EpoR, assuming that Gi/ formed where the G proteins were also associated to the EpoR. Several forms of Gb3 were identified in UT7 cells by immunoblotting including Gi, Gs, and Gq. Our initial attempt to detect Gb3 in vitro, incorporation of [32P]ADP-ribose in the presence of pre-immune serum. The amount of Gb3 was measured. Fig. 3 illustrates that Gb3 was coprecipitated with EpoR both in vivo and in vitro and that the interaction between Gb3 and EpoR occurs through the intracellular region of EpoR.

Gb3 Subunit Associates with EpoR—G proteins are associated to seven transmembrane receptors in an heterotrimeric form where the α subunit is associated to βγ. Because EpoR is not a "classical" G protein-coupled receptor, we investigated whether only Gb3 binds to the EpoR or whether the α chain of Gb3 proteins was also associated to the EpoR. Several forms of Ga were identified in UT7 cell lysates, using a recombinant fusion protein between MalE and the cytoplasmic region of EpoR or a control MalE protein bound to amylose resin (Fig. 2B). When bound proteins were analyzed by immunoblotting with anti-Gb3 antibodies, Gb3 was detected only when MalE/EpoR fusion protein was used. We conclude that Gb3 binds to EpoR both in vivo and in vitro and that the interaction between Gb3 and EpoR occurs through the intracellular region of EpoR.

Gbα Subunit Associates with EpoR—G proteins are associated to seven transmembrane receptors in an heterotrimeric form where the α subunit is associated to βγ. Because EpoR is not a "classical" G protein-coupled receptor, we investigated whether only Gb3 binds to the EpoR or whether the α chain of Gb3 proteins was also associated to the EpoR. Several forms of Ga were identified in UT7 cell lysates, using a recombinant fusion protein between MalE and the cytoplasmic region of EpoR or a control MalE protein bound to amylose resin (Fig. 2B). When bound proteins were analyzed by immunoblotting with anti-Gb3 antibodies, Gb3 was detected only when MalE/EpoR fusion protein was used. We conclude that Gb3 binds to EpoR both in vivo and in vitro and that the interaction between Gb3 and EpoR occurs through the intracellular region of EpoR.

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Gprotein-coupled receptors

Gi Coupling to Epo Receptor

Fig. 4. Gαi association with EpoR in EpoR transfected cells and in erythroid progenitors. A, M07E cells expressing murine EpoR (M07E-ER) or not (M07E) were stimulated with 10 units/ml Epo for 50 min. Cell lysates (1 x 10^7 cell equivalents) were immunoprecipitated with anti-EpoR antiserum. B, erythroid progenitors were deprived of growth factor and stimulated (+) or not (−) with Epo for the indicated time. After cell solubilization the EpoR was precipitated, and Gαi was detected in the precipitates by in vitro ADP-ribosylation followed by autoradiography.

shown to overexpress an abnormal EpoR caused by a deletion of the 96 C-terminal amino acids, together with a minor expression of full-length EpoR (47, 56). TF-1 cells were solubilized, and the Gαi protein coprecipitated with EpoR was detected by in vitro ADP-ribosylation.

To examine this possibility in greater detail we next studied the association of Gαi with wild-type murine EpoR or C-terminal deletion mutants (Fig. 6A) expressed in FDCP-1 cells. In FDCP-1 cells transfected with the murine EpoR deletion mutants −41 and −24, no Gαi was found associated with EpoR, and a weak binding was detected in mutant −20 (Fig. 6B). The absence of binding was not due to a decrease in EpoR surface expression because FDCP-1 cells transfected with wild-type

Fig. 6. G protein binding to the C-terminal end of EpoR intracytoplasmic region. FDCP-1 cells or FDCP-1 cells expressing murine EpoR or deletion mutants of EpoR (1 x 10^7 cell equivalents) were starved of Epo and stimulated with 10 units/ml Epo for 50 min. Cell lysates (1 x 10^7 cell equivalents) were immunoprecipitated with anti-EpoR antiserum or preimmune serum and Gαi coprecipitated with EpoR was detected by in vitro ADP-ribosylation (Fig. 7). Epo induced a rapid decrease in the amount of Gαi bound to the EpoR. About 50% of the binding was lost after 10 min of incubation, and then the amount of binding decreased more slowly. We conclude that heterotrimeric G protein binding to the EpoR is necessary for heterotrimeric G protein binding to the EpoR. It cannot be excluded that amino acids 432–458, also present in F1-Y58 mutant, contribute to the binding.

Epo Induces the Release of G Protein from the EpoR—A decrease in G protein association with EpoR following Epo addition was constantly observed in the different hematopoietic cells studied. The kinetic of G protein binding to EpoR was analyzed in UT7 cells stimulated or not with Epo for various times at 37 °C. The amount of Gαi coprecipitated with EpoR in the soluble fraction was determined by measuring the 32P incorporation in the 41-kDa protein following in vitro ADP-ribosylation. Fig. 7. Epo induced a rapid decrease in the amount of Gαi bound to the EpoR. About 50% of the binding was lost after 10 min of incubation, and then the amount of binding decreased more slowly. We conclude that heterotrimeric G protein binding to EpoR is necessary for heterotrimeric G protein binding to the EpoR. It cannot be excluded that amino acids 432–458, also present in F1-Y58 mutant, contribute to the binding.

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were subjected to immunoprecipitation with anti-EpoR antibodies. The immunoprecipitates were subjected to in vitro ADP-ribosylation. The proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane. Gα<sub>i</sub> was visualized by autoradiography and the amount of <sup>35</sup>P present in the bands was quantified with a PhosphorImager. Data are the means ± S.E. of three individual experiments.

![Graph](image)

**Fig. 7.** Decrease of G<sub>i</sub> coprecipitated with EpoR after Epo stimulation. UT7 cells were deprived of growth factor and stimulated with 10 units/ml Epo for the indicated time. Cell lysates were immunoprecipitated with anti-EpoR antibodies. The immunoprecipitates were subjected to a 10 units/ml Epo for the indicated time. Cell lysates were immunoprecipitated with anti-EpoR antibodies. The immunoprecipitates were subjected to in vitro ADP-ribosylation. The proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane. G<sub>α</sub> was visualized by autoradiography and the amount of <sup>35</sup>P present in the bands was quantified with a PhosphorImager. Data are the means ± S.E. of three individual experiments.

![Graph](image)

**Fig. 8.** Decrease of G<sub>i</sub> coprecipitated with EpoR in 32D cells expressing EpoR mutant defective in Jak2 activation. 32D cells expressing murine EpoR or EpoR mutant W282R (1 × 10<sup>7</sup> cell equivalents) were starved of Epo and stimulated for 30 min with 10 units/ml Epo. Analysis of G<sub>α</sub> coprecipitated with EpoR was followed by in vitro ADP-ribosylation. WT, wild type.

![Graph](image)

**Fig. 9.** Effect of Epo on pertussis toxin-induced ADP-ribosylation and on [γ<sup>35</sup>S]GTP<sub>γ</sub>S binding to membranes. A, inhibition of pertussis toxin-induced ADP-ribosylation by Epo. UT7 cell membranes were preincubated with 10 units/ml Epo or 100 μM GTP<sub>γ</sub>S for 10 min in the presence of increasing concentrations of Mg<sup>2+</sup> using Mg<sup>2+</sup>-EDTA buffer before ADP-ribosylation by pertussis toxin. The proteins were separated by SDS-PAGE, and G<sub>α</sub> was visualized by autoradiography. B, increase in [γ<sup>35</sup>S]GTP<sub>γ</sub>S binding by Epo. UT7 or CHO-ER membranes were pretreated or not with Epo for 15 min, and [γ<sup>35</sup>S]GTP<sub>γ</sub>S binding was determined as described under "Experimental Procedures." Data are the means ± S.E. of duplicate samples and are expressed as percentages of values from untreated cell membranes.

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DISCUSSION

In the present investigation, we provide the first demonstration of the physical association between the heterotrimeric G protein of the G<sub>i</sub> family and the erythropoietin receptor, both in hematopoietic cell lines and in human normal erythroid progenitor cells. The G<sub>i</sub> protein associated with the EpoR is more likely in a heterotrimeric conformation. Indeed both G<sub>β</sub> and G<sub>α</sub><sub>γ</sub><sub>i</sub>, identified as a 41-kDa ADP-ribosylated band, coprecipitated with the EpoR. By utilizing natural and engineered truncated EpoR mutants, we showed that the intracellular C-terminal end of the EpoR is required for G protein association. The association between the G protein and EpoR is constitutive. Classical G protein-coupled receptors are believed to associate with G proteins following ligand activation (58), although they may in some cases be preassociated. Actually, the association between a G<sub>i</sub> protein and a single-spanning transmembrane receptor has previously been reported. A peptide corresponding to the intracytoplasmic sequence of β<sub>1</sub>,4-galactosyltransferase, the sperm receptor for the mouse egg, bound a heterotrimeric G protein that contained the G<sub>i</sub> subunit (30). G<sub>α</sub> was found to copurify with the β chain of insulin receptor isolated from adipocyte plasma membranes (31). A transient association between G<sub>α</sub><sub>i</sub> and the EGF receptor also occurred in rat hepatocytes after ligand activation (29). G<sub>α</sub><sub>γ</sub> and G<sub>β</sub> were recently shown to constitutively bind the IGF-I receptor (60). A region of 18 amino acids in the C terminus of EpoR is necessary for G<sub>i</sub> binding to EpoR. A 14-residue sequence in the IGF-II receptor, with several basic residues, presents structural similarity with the terminal portion of the third cytoplasmic loop of most G-coupled receptors. This sequence activates and directly interacts with G<sub>i</sub> proteins (61, 62), but the EpoR cytoplasmic domain does not contain such a G protein-binding
motif. Another possibility is that the interaction between G_i and EpoR occurs through an adaptor protein bound to the EpoR. The tyrosine kinase Jak2 and the docking protein IRS2 constitute a binding site for the EpoR membrane proximal region (5, 15). Because G protein binding to EpoR involves the C-terminal end of the receptor, these proteins are unlikely to play a role in adaptation between G protein and EpoR, and another component whose nature is unknown could be required. The ability of the EpoR mutant W282R to bind G_i in the absence of Jak2 binding also excludes Jak2 as a potential adaptor.

Epo induces the release of G_i from the EpoR. Hallak and co-workers (60) have recently reported that heterotrimeric G_i is constitutively associated with IGF-I receptor and that IGF-I also induces the release of the Gbg subunit from the IGF-I receptor. Jak2 tyrosine kinase is probably not required for G_i protein release from the EpoR because this process still occurs in 32D cells that express the EpoR mutant W282R defective in Jak2 activation. It cannot be excluded that other tyrosine kinases involved in Epo activation such as Lyn, Syk, or c-Fes (6–8) are required for G_i release. Although Jak2 plays a pivotal role in EpoR signaling (63, 64), Gi protein release from the receptor in hematopoietic cells and in 32D cells expressing wild-type and mutant EpoR, Dr. E. Goldwasser for CHO-ER cell line, and Dr. Catherine Lacombe for MOTE-ER and TF-1-ER cell lines. We are indebted to Dr. Sylvie Gisselbrecht for continuous support and for critically reviewing the manuscript. We gratefully acknowledge the expert technical assistance of Christelle Chardes, Jean-Marc Freysinnish and Odile Muller.

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