Activation of the Mitogen-activated Protein Kinases Erk1/2 by Erythropoietin receptor via a $G_i$ protein beta gamma-subunit-initiated pathway.

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SUMMARY

We have recently shown that a heterotrimeric $G_i$ protein is coupled to the erythropoietin (Epo) receptor. The $G_i$ protein constitutively associates, in its heterotrimeric form, with the intracellular domain of Epo receptor (EpoR). Following Epo stimulation $G_i$ is released from the receptor and activated. In the present study we have investigated the functional role of the heterotrimeric $G_i$ protein bound to EpoR. In CHO cells expressing EpoR, the $G_i$ inhibitor pertussis toxin blocked mitogen activated protein kinases Erk1/2 (MAPK) activation induced by Epo. Epo-dependent MAPK activation was also sensitive to the $G_{\beta\gamma}$ competitive inhibitor $\beta$ARK1-ct, to the Ras dominant negative mutant RasN17 and to the PI3K inhibitor LY 294002. A region of seven amino acids (469-475) in the C-terminal end of EpoR, was shown to be required for $G_i$ binding to EpoR in vivo. Deletion of this region in EpoR abolished both MAPK and PI3K activation in response to Epo. We conclude that in CHO cells, Epo activates MAPK via a novel pathway dependant on $G_i$ association to EpoR, $G_{\beta\gamma}$ subunit, Ras and PI3K. The tyrosine kinase Jak2 also contributes to this new pathway more likely downstream of $\beta\gamma$ and upstream of Ras and PI3K. This pathway is similar to the best characterized pathway used by seven transmembrane receptors coupled to $G_i$ to activate MAPK and may cooperate with other described Epo-dependent MAPK activation pathways in hematopoietic cells.
INTRODUCTION

The survival, proliferation and differentiation of erythroid progenitor cells is regulated by erythropoietin (Epo)\(^1\) (1). The binding of Epo to its receptor (EpoR), a member of the cytokine receptor superfamily, initiates a signal cascade which includes activation of the Jak2 tyrosine kinase, phosphorylation of the EpoR (2-4), activation of PI3K (5-7), STAT5 (8) and stimulation of the Ras/Raf/MAPK pathway (9-13). The EpoR recruits several SH2-containing proteins through its intracellular phosphorylated tyrosines as well as adaptor molecules ((14) for review). We previously developed an expression cloning strategy to characterize new proteins implicated in EpoR signaling. The strategy was based on the reactivity of such components with antibodies produced against proteins reactive with anti-phosphotyrosine (PY) antibodies, isolated from the Epo-stimulated hematopoietic cell line UT7. This led us to isolate a cDNA encoding G\(_{\beta 2}\), the \(\beta 2\) subunit of the heterotrimeric GTP-binding proteins, and to demonstrate that an heterotrimeric G\(_i\) protein is constitutively associated with EpoR in hematopoietic cell lines as well as in normal erythroid progenitor cells. The G\(_i\)-EpoR interaction occurs through the C-terminal end of the cytoplasmic domain of the receptor. Following Epo activation the G\(_i\) protein is activated and released from the receptor, most likely with the concomitant dissociation of G\(_i\)\(\alpha\) and \(\beta\gamma\) subunits (15).

Defined signal functions of G\(_i\) include inhibition of adenylyl cyclase, regulation of PLC\(\beta\) and activation of K+ channels (16). G proteins such as G\(_q\) and G\(_i\) also regulate cell growth and differentiation through the stimulation of a large number of complex cascades leading to the activation of mitogen activated protein kinases (MAPKs). Agonist stimulation of seven transmembrane-spanning G protein-coupled receptor (GPCR) leads to the exchange of GDP for GTP on the \(\alpha\) subunit of the heterotrimeric G protein and the subsequent dissociation of the \(\alpha\)-GTP and \(\beta\gamma\) subunits. Although the main functional properties of G proteins were initially thought to be essentially determined by the identity of the \(\alpha\) subunit, \(\beta\gamma\) complexes also regulate a number of effectors. The mechanisms of the Erk1/2 MAPK activation by GPCR has been explored in readily transfectable cell lines, such as COS-7 cells. The best characterized and probably the main pathway used by GPCR to activate MAPK via the pertussis toxin (PT)-sensitive G\(_i\) protein depends on the release of...
free $G_{\beta\gamma}$ subunits acting on a Ras-dependent pathway (17-19). MAPK activation is initiated by the $G_{\beta\gamma}$-mediated tyrosine phosphorylation of Shc and proceeds through a Shc/Grb2/Sos pathway common to both GPCR and tyrosine kinase receptors (19). PI3K activity also participates in $G_{\beta\gamma}$-mediated MAPK activation upstream of Ras (20-22) or by stimulating MEK phosphorylation (23). $G_i$ may also regulate MAPK activation through $\alpha$ subunits. Indeed activating mutations have been identified in the $G_{\alpha_{2i}}$ gene, referred to as $gip2$ (24,25) and MAPK is constitutively activated in $gip2$ transformed fibroblasts (24).

The MAPKs Erk1/2 play important role in Epo-induced proliferation, differentiation and apoptosis (26-28). In hematopoietic cells Epo activates the Ras/Raf/MEK/MAPK pathway by recruitment of Grb2, either directly or indirectly via adaptor molecules such as Shc (29,30) or SHP-2 (31,32). This interaction allows the guanine nucleotide release factor Sos, constitutively bound to Grb2, to convert Ras to an active GTP-bound form. Furthermore C3G, an other guanine nucleotide exchange factor, through its interaction with CrkL, has also been found participating in Epo-mediated MAPK signaling pathway via Ras (33) and Rac (34). PI3K and PKC have also been implicated in Raf/MAPK activation by Epo (35,36).

Our demonstration that heterotrimeric $G_i$ proteins associate with EpoR and that receptor activation leads to the activation and dissociation of $G_i$ from the receptor led us to investigate what are the downstream targets of $G_i$. We considered the possibility that Epo may activate MAPK via the $G_i$ protein bound to EpoR like seven transmembrane receptors. In this study, we characterized a new Epo-activated MAPK pathway dependent on $G_i$. This pathway, similar to the best characterized MAPK pathway used by GPCR coupled to $G_i$, is the main pathway for Epo-dependent MAPK activation in CHO cells. It proceeds through the $\beta\gamma$ subunit of the $G_i$ bound to EpoR and involves the activation of Jak2, Ras, PI3K, and MEK. Our data also demonstrate that $G_i$ plays a crucial role for Epo-induced PI3K activation in CHO cells.
EXPERIMENTAL PROCEDURES

Materials

Purified recombinant human Epo (specific activity 120,000 units/mg) was a kind gift of Dr M. Brandt (Roche Molecular Biochemicals). Rabbit polyclonal antibodies against Gβ (M-14), Akt 1/2 (H-136) and Erk (C-16) were purchased from Santa Cruz Biotechnology. Anti-Jak2 antibodies (06-255) were supplied from Upstate Biotechnology. Polyclonal antibodies specific to the phosphoforms of Erk (thr202/tyr204) and Akt (ser473) were supplied from Cell Signaling Technology. Anti-phosphotyrosine (anti-pTyr) monoclonal antibodies 4G10 and PY72 were produced from hybridoma cell lines kindly provided by B. Drucker (Portland, Or) and B. Sefton (La Jolla, Ca). Anti-MalE antiserum and anti-EpoR antiserum against a fusion protein between glutathione S-transferase and the cytoplasmic portion of the human EpoR were described previously (37). Anti-HA peptide monoclonal antibody was purchased from Roche. Peroxidase-conjugated anti-rabbit antibodies were purchased from Amersham and Cell Signaling Technology. MEK1 inhibitor PD98059 was supplied from New England Biolabs, PI3K inhibitor LY 294002 and PMA from Sigma, pertussis toxin from Alexis and protease inhibitors from Roche. Amylose resin was supplied from Biolabs. The minigene encoding the carboxyl terminus of bovine βARK1 (βARK-ct) (38) was kindly provided by R. Jockers at Institut Cochin, Paris, France. The expression plasmid pcDNA3/HA-RasN17 was a gift from A. Eychène at Institut Curie (Orsay, France) and the pXM/EpoR-W282R from J. Ihle (Menphis, USA).

Plasmid constructs

Different constructs of murine EpoR cytoplasmic regions (L433-P459, G458-S483, G458-P470, E465-P477) were obtained by polymerase chain reaction (PCR) and subcloned, in frame with the MalE protein.

The murine EpoR mutant -41 was described previously (15). To generate the ΔV-L EpoR mutant lacking residues V469 to L475 within the intracellular domain, mutation was introduced into the full-length EpoR by PCR. The PCR product was digested with HindIII/XbaI and subcloned into
the same restriction sites of a modified pCDNA3 expression vector (15). The fidelity of all constructs was confirmed by sequencing.

**Transfections**

Stable transfectants of FDCP-1 myeloid cell line expressing the wild-type and mutated forms of EpoR were obtained by electroporation, as previously described (8). CHO cells were transfected with expression plasmids coding for full-length EpoR, ΔV-L or W282R EpoR mutants using Lipofectamine Plus™ Reagent (Life technologies) according to the manufacturer’s instruction, selected with 20 µg/ml puromycin and cloned by limiting dilution. EpoR expression was determined using immunoblot analysis of cellular extracts and ¹²⁵I-Epo binding on whole cells. Cell lines expressing a similar number of ¹²⁵I-Epo binding sites of either wild type or mutated receptors were selected for the study. Transient transfections of CHO cells were carried out by the same methodology, when 80% confluence was reached, using 1 µg of plasmid/well (10 cm²) and 24 h post-transfection the cells were harvested and used in experiments as described. Lipofectamine transfection of CHO cells consistently resulted in transfection efficiencies greater than 60% (data not shown).

**Cell culture and treatments**

The human erythroleukemic cell line UT7 was maintained in α MEM supplemented with 5% fetal bovine serum, penicillin, streptomycin, 2 mM L-glutamine and 2 units/ml Epo. FDCP-1 myeloid cells expressing EpoR were grown in α MEM supplemented with 5% fetal bovine serum and 2 units/ml Epo. The cells were starved of Epo by replacing Epo with 3% WEHI conditioned medium as a source of IL-3 one to 2 days prior to use in the experiments. CHO cells were grown in Dulbecco’s MEM/nut mix F-12 (HAM) medium supplemented with 7 % fetal bovine serum. Before stimulation, cells were washed and serum starved 3 to 4 h, or overnight when indicated, by incubation in HAM medium in the presence of 0.4% bovine serum albumin. The cells were stimulated with 10 units/ml Epo or 10 ng/ml PMA. In some experiments PD98059 (30 µM) or LY
294002 (50 µM) were added 30 or 15 min respectively prior to stimulation, pertussis toxin (50 ng/ml) and PMA (0.6µg/ml) were added 16 h prior to stimulation.

Immunoprecipitations and MalE pulldown assays

Cells (1x10^7) were washed in PBS containing 50 µM Na3VO4 and lysed on ice in 150 mM NaCl, 50 mM Tris-HCL (pH 7.5), 1% brij 98, 1 mM Na3VO4, 2 mM EGTA, 10 % glycerol and proteases inhibitors (lysis buffer). Insoluble material was removed by centrifugation at 15000g for 10 min at 4°C. For immunoprecipitation, lysates were incubated with anti-EpoR antiseraum 1/250 for 1 h at 4°C. Protein G-Sepharose (Pharmacia) was added and the incubation was pursued for 1 h at 4°C with rotation. Samples were washed three times with lysis buffer and two times with 150 mM NaCl, 50 mM Tris-HCL (pH 7.5), 0.1% brij 98, 1 mM Na3VO4. For MalE fusion protein pulldown assays, lysates were incubated with 5µg of MalE fusion protein prebound to 20 µl of amylose resin beads for 2 h at 4°C with rotation. Then, beads were collected and processed as described above for immunoprecipitation. Proteins were eluted by boiling in sodium dodecyl sulfate (SDS) sample buffer, resolved on SDS polyacrylamide gel electrophoresis (PAGE) and subjected to immunoblotting as described previously (3,39).

Determination of MAPK and Akt activation

Erk and Akt activation were studied using phospho-specific antibodies. Cells lysates were prepared as described above using lysis buffer containing 1% NP40. The protein content was normalized by the micro BCA assay (Pierce) and equal amounts of proteins (10 to 50 µg) were separated by 10 % SDS-PAGE. Proteins were transferred to nitrocellulose membranes and analyzed in immunoblot.

ADP-ribosylation Assay

To detect Gαi its ADP-ribosylation was followed by measuring incorporation of 32P ADP-ribose in the presence of pertussis toxin in vitro as previously described (15).
RESULTS

Identification of the sequence in the EpoR required for $G_i$ binding.

In a previous report we demonstrated that constitutive association between heterotrimeric $G_i$ protein and the EpoR occurred through the C-terminal end of the receptor. (15). To characterize more precisely the EpoR domain involved in $G_i$ binding, we first generated recombinant fusion proteins between MalE and various EpoR sequences contained in this region (Fig. 1A). Fusion proteins were incubated with UT7 cell lysates and bound proteins were analyzed by immunoblotting with anti-$G_\beta$ antibodies (Fig. 1B). Recombinant fusion protein containing the EpoR amino acids 458 to 483 allowed $G_\beta$ binding in vitro, in agreement with previous data obtained in vivo using EpoR mutants (15). A similar binding was detected with the MalE-EpoR fusion peptide 465-477, while $G_\beta$ did not bind to EpoR peptides 433-459 and 458-470. Western blotting with anti-MalE antibody showed that the amounts of recombinant proteins bound to amylose resin used in the pulldown assays were similar. These results indicate that amino acids 465 to 477 are sufficient to bind $G_\beta$ in vitro. This region does not include tyrosine residues, which fits well with the constitutive binding of $G_i$ to EpoR (15).

To evaluate the role of the identified sequence (465-477) in vivo we wished to delete this sequence within the EpoR. To avoid perturbation of receptor structure and particularly of Y464 and Y479 located in the vicinity of $G_i$ binding region we constructed an EpoR mutant deleting a shorter region encompassing amino acids 469 to 475 ($\Delta V$-L). The EpoR expressed in FDCP-1 cells was immunoprecipitated from cell lysates and $G_\alpha_i$ associated with EpoR was detected by in vitro ADP-ribosylation assay (Fig. 1C). In cells transfected with full-length mouse EpoR, $G_\alpha_i$ was coprecipitated with anti-EpoR antibodies. However, $G_\alpha_i$ was not precipitated with EpoR when mutants -41(15) or $\Delta V$-L were expressed. The absence of $G_i$ binding to the EpoR in cells expressing the $\Delta V$-L mutant is unlikely to be due to a general signaling defect of the mutated receptor since the receptor kept the ability to bind the tyrosine kinase Jak2 (Fig. 1C). These results indicate that the sequence $V_{469}PDSEPL_{475}$ located in the C-terminal domain of the EpoR is necessary for $G_i$/EpoR association in vivo. This region does not bind any known EpoR effector.
Epo stimulates a $G_i$-dependent Erk1/2 activation pathway in CHO cells expressing the EpoR.

A number of receptors that couple to heterotrimeric $G$ proteins, including $G_i$-coupled receptors, have been shown to stimulate MAPK activation (17,18,38). Therefore we explored the possibility that $G_i$ may participate in MAPK activation following Epo stimulation. The sensitivity of MAPK activation to the $G_i$ inhibitor, pertussis toxin, was first examined in hematopoietic cell lines expressing an endogenous or exogenous EpoR, as well as in normal human erythroid progenitor cells. A weak and inconstant inhibition of Epo-induced MAPK phosphorylation was observed with these cells (data not shown). We previously observed that Epo induced a stronger $G$ protein activation in cell membranes isolated from epithelial CHO cells expressing EpoR than in UT7 hematopoietic cell line (15). This led us to hypothesis that a $G_i$-dependent Epo-activated MAPK pathway may be more easily detected in these cells.

The role of $G_i$ in the activation of Erk1/2 by Epo was investigated in CHO cells stably transfected with the full length EpoR cDNA (CHO-ER WT). Cells were stimulated with Epo for various times and the lysates were subjected to immunoblotting with phospho-specific anti-Erk antibodies as shown in Fig. 2. Epo induced a transient Erk1/2 activation. Preincubation of the cells with 50 ng/ml pertussis toxin overnight inhibited completely Epo-induced Erk activation. Total Erk content was unchanged under all conditions as shown by immunoblotting with anti-Erk antibodies recognizing both the phosphrylated and the unphosphorylated forms of Erk 1 and 2. Toxin treatment had no effect on cell viability (data not shown). These results establish a role for a pertussis toxin-sensitive $G$ protein in Epo-induced Erk activation and show that a $G_i$-dependent pathway is the main pathway used by Epo to activate MAPK in CHO cells.

To ascertain the functional significance of the $G_i$-EpoR interaction on MAPK activation we studied the effect of disrupting it. When stably expressed in CHO cells the EpoR $\Delta V$-L mutant, which is unable to associate with $G_i$ (Fig. 1C), could not activate Erk following Epo stimulation (Fig. 3A). In contrast PMA treatment led to MAPK activation, showing that these cells retained the ability to activate Erk. To evaluate if the $\Delta V$-L deletion would perturb early events of EpoR activation, Jak2 was immunoprecipitated and subjected to analysis using anti-phosphotyrosine antibodies. Fig. 3B illustrates that the EpoR $\Delta V$-L mutant had the ability to associate with the Jak2 tyrosine kinase
and that both proteins became phosphorylated after Epo stimulation. Indeed, when Jak2 was immunoprecipitated, the phosphorylated forms of Jak2 and of the EpoR were detected in immune complexes isolated from cells expressing EpoR WT as well as EpoR ΔV-L. Thus, the ΔV-L deletion has no general effect on Epo signal transduction. Tyrosines located in the vicinity of the $V_{469-L_{475}}$ region constitute potential binding sites for components implicated in Epo-dependent MAPK activation such as CrkL (40) and Grb2 (32) for Y460 and Y 464 respectively. Therefore, we wanted to determine whether the ΔV-L deletion did not alter the phosphorylation of these residues and their ability to bind their effectors. Following Epo activation, phosphorylated EpoR ΔV-L bound GST-CrkL and GST-Grb2 as efficiently as phosphorylated EPOR WT did (data not shown). This strongly suggests that the absence of MAPK activation in EpoR ΔV-L is due to a defect in $G_\iota$ binding and not to a perturbation of the phosphorylation of proximal tyrosine residues.

Altogether, these data show that the EpoR region that couples $G_\iota$ to EpoR is required for Epo-induced Erk activation in CHO cells and confirm the results obtained with pertussis toxin treatment.

**Epo-mediated MAPK activation in CHO-ER cells is dependent on $G_{\beta\gamma}$**

Given the sensitivity of Epo action to pertussis toxin, we first examined whether Epo-mediated Erk1/2 activation may be secondary to inhibition of adenylyl cyclase by $G_\iota$. Indeed, in some cells, a rise in cAMP has been shown to inhibit the Ras/MAPK pathway at the level of Ras/Raf interaction (41). Therefore a $G_\iota$-mediated fall in cAMP level may relieve Raf inhibition by PKA and positively regulate MAPK. Pretreatment of CHO-ER WT cells with the cell-permeable cAMP derivative dibutyryl-cAMP, which mimics the effect of cAMP, failed to prevent Epo-induced MAPK activation (data not shown). This suggests that MAPK activation does not proceed through $\alpha_i$-mediated inhibition of adenylyl cyclase.

$\beta\gamma$ subunits derived from PT-sensitive heterotrimeric G proteins were shown to mediate Ras-dependent MAPK activation (17,18,42). MAPK activation through $G_i$-coupled receptors such as the $\alpha_{2A}$ adrenergic receptor and the lysophosphatidic acid receptor is sensitive to inhibition by the C-terminal fragment of the $\beta$-adrenergic receptor kinase ($\beta$ARK1-ct). $\beta$ARK1-ct interacts with free $\beta\gamma$ and acts as a competitive inhibitor of $G_{\beta\gamma}$-mediated signals, but does not affect $G_\iota$-
mediated signaling, allowing to distinguish between αi and βγ pathways (38). To determine whether the PT-sensitive phosphorylation of Erk1/2 stimulated by Epo was mediated by Gβγ, we studied the effect of Epo on CHO-ER WT cells transiently transfected with the βARK1-ct peptide. As shown in Fig. 4A phosphorylation of endogenous Erk1/2 following Epo stimulation was significantly reduced compared with cells transfected with control plasmid, although total Erk amount was not modified. We conclude that Epo-induced MAPK activation in CHO-ER cells occurs through the release of βγ subunits of heterotrimeric Gi protein.

Epo-mediated MAPK activation in CHO-ER cells is dependent on JAK2, Ras and MEK.

To determine whether Epo-induced JAK2 tyrosine kinase activation was required for MAPK activation, the EpoR mutant (W282R) defective in JAK2 binding and kinase activation (43) was stably expressed in CHO cells. In cells expressing the EpoR W282R mutant, Epo-induced MAPK activation was lost (Fig. 4C). Thus JAK2 activation is required for MAPK activation in these cells. The requirement for MEK kinase, an upstream activator of MAPK, was also evaluated using the MEK inhibitor PD98059. As shown in Fig. 4B, Epo-induced MAPK activation was abolished when the cells were pre-treated with the MEK inhibitor before Epo stimulation.

PKC is activated by Epo and may be implicated in MAPK activation by Epo (36) and also by Gi coupled to classical GPCR (44). We examined whether PKC might contribute to Epo-induced MAPK activation in CHO-ER WT cells. Down-regulation of PKC induced by prolonged exposure of cells to PMA inhibited MAPK activation in response to PMA but not to Epo (Fig. 5B). Therefore, Epo-stimulated MAPK activation is independent of a PMA-sensitive PKC. The best characterized pathway used by Gi-coupled receptors to activate MAPK requires p21ras activity (45,46). To investigate the role of p21ras in Epo-mediated MAPK activation in CHO-ER WT cells, the effects of the dominant negative mutant RasN17 on EpoR-stimulated MAPK activation was assessed. As shown in Fig. 5A, RasN17 expression inhibited Epo-stimulated phosphorylation of Erk. In contrast RasN17 expression did not alter the level of Epo-stimulated Jak2 tyrosine phosphorylation (Fig. 8A). Taken together these results suggest that, in CHO cells expressing EpoR, Epo acts through Jak2 to activate Ras and Erk1/2 MAPK.
PI3K has been implicated in the βγ-dependent activation of MAPK by G_\text{l}-coupled receptors (21,22). Therefore, we next examined whether PI3K also contributed to Epo-induced MAPK activation in CHO-ER WT cells. Pre-treatment of the cells with LY 294002, a PI3K inhibitor, blocked Epo-stimulated activation of the PI3K, as detected by antibodies specific to the phosphoform of the PI3K effector Akt (Fig. 6A). LY 294002 also strongly decreased the activation of MAPK by Epo without modification of the total Erk content (Fig. 6A). These data show that PI3K contributes to Epo-dependent MAPK activation in CHO-ER WT cells. Since MAPK activation is essentially G_\text{i} dependent in these cells, they also suggest that PI3K is a component of the G_\text{i}-dependent Erk1/2 pathway. To determine whether G_\text{i} is required for PI3K activation, Epo-induced Akt phosphorylation was compared in CHO-ER WT expressing the full-length receptor or the ΔV-L mutant defective in G_\text{i} interaction and MAPK activation. As shown in Fig. 7A the deletion mutant had lost the ability to activate Akt in response to Epo. As expected, expression of the βγ competitor βARK1-ct also inhibited Akt phosphorylation by EpoR WT (Fig. 7B).

The requirement for Ras and Jak2 in PI3K activation was then evaluated. Expression of RasN17 in CHO-ER WT prevented Akt activation following Epo stimulation (Fig. 8A). Furthermore the EpoR mutant defective in Jak2 binding and activation (W282R) failed to activate Akt (Fig. 8B), showing that Jak2 tyrosine kinase and Ras are upstream of PI3K activation. Taken together, these data demonstrate that the βγ subunit of G_\text{i} plays a crucial role in Epo-mediated PI3K activation and that PI3K is a component of the Epo-activated G_\text{i}-dependent MAPK activation pathway downstream of Jak2 and Ras.
DISCUSSION

In this study we showed that Epo can activate the Erk1/2 MAPK pathway via a new G\textsubscript{i}-dependent pathway sensitive to pertussis toxin. The activation of MAPK by EpoR is mediated by the \(\beta\gamma\) subunits and not the \(\alpha\) subunit of G\textsubscript{i}. Indeed, MAPK phosphorylation in response to Epo was inhibited by the coexpressed \(\beta\gamma\) competitor \(\beta\text{ARK1}\) polypeptide. The marked inhibition observed with these agents suggests that the \(\beta\gamma\)-pathway is the main pathway for Epo-mediated MAPK activation in CHO cells expressing the EpoR. Classical G protein-coupled receptors, (17,18,42,47) and some tyrosine kinase receptors, such as the receptors for IGF1 and FGF (48,49), activate Erk1/2 through mechanisms involving \(\beta\gamma\) subunits derived from pertussis toxin-sensitive G proteins in neuronal cells, epithelial cells and fibroblasts. To our knowledge our study represents the first demonstration of a functional role of G\textsubscript{\beta\gamma} subunits in cytokine receptor signaling. Signaling from G\textsubscript{i} to MAPK more often involves \(\beta\gamma\) subunits of heterotrimeric G proteins acting on a Ras-dependent pathway (42,45). Likewise, Epo-induced MAPK activation is consistent with this general mechanism since it is dependent on Ras as shown by its inhibition by a dominant negative mutant of Ras (N17 Ras).

Our data also demonstrate that PI3K activation by Epo is dependent on G\textsubscript{i} \(\beta\gamma\) subunits and is required for Erk activation downstream of Ras in CHO cells. Indeed: (i) Epo-induced Akt phosphorylation was inhibited by expression of the \(\beta\text{ARK1-ct}\) or RasN17; (ii) the EpoR mutant defective in G\textsubscript{i} binding lost the ability to phosphorylate both Erk1/2 and the PI3K effector Akt; (iii) the PI3K inhibitor LY294002 inhibited MAPK activation by Epo. Since MAPK activation by Epo is mainly G\textsubscript{i}-dependent in CHO cells, these data also suggest that the \(\beta\gamma\)-dependent PI3K activation by Epo is upstream of MAPK and does not form a separate pathway. Similarly, PI3K was shown to be involved in G\textsubscript{i}-dependent MAPK activation by PDGF in airway smooth muscle cells (50,51). PI3K has a critical role linking G protein-coupled receptors and G\textsubscript{\beta\gamma} to the MAPK signaling pathway. Different mechanisms could be implicated in this process. Previous reports have implicated PI3K\(\alpha\) or \(\beta\) (21,52) and PI3K\(\gamma\) (22) in the activation of MAPK by classical G\textsubscript{i}-coupled receptors upstream of Sos and Ras activation (21,22). PI3K\(\gamma\) could represent the link between \(\beta\gamma\) and a
downstream tyrosine kinase required for Ras-dependent MAPK activation (22). However, this mechanism does not apply here since we show that PI3K is downstream of Ras. PI3K could also contribute to the G_i-initiated MAPK pathway without Ras requirement (23).

Several candidate tyrosine kinases have been proposed as intermediates between βγ and Ras/MAPK activation including c-Src family kinases (53), Syk (54) and Pyk2 (55). Alternatively, G_i can activate MAPK through transactivation of the EGF tyrosine kinase receptor (56). Activated c-Src or EGF receptor mediate Ras/MAPK activation through tyrosine phosphorylation of Shc and its subsequent association with Grb2 (53,56). An essential component for Gi-dependent Erk activation by EpoR is the tyrosine kinase Jak2. Indeed CHO cells expressing the W282R EpoR mutant defective in Jak2 binding and activation have lost the ability to activate MAPK. Epo-induced PI3K activation was also inhibited in these cells suggesting that PI3K is located downstream of Jak2 in this pathway. Ras is probably positioned downstream of Jak2, since RasN17 expression had no effect on Jak2 phosphorylation. Jak2 contribution to MAPK activation is independent of Gi binding to EpoR and subsequent Gi dissociation from the receptor. Indeed the ΔV-L mutation in EpoR, which disrupts Gi binding to EpoR, did not alter Jak2 phosphorylation by Epo and its binding to the receptor. Furthermore in 32D cells, we previously showed that Epo-induced Gi dissociation from the EpoR is not affected when the Jak2-deficient EpoR mutant is expressed (15). Therefore Jak2 is more likely acting downstream of βγ release and upstream of Ras and PI3K. In addition to Jak2, the tyrosine kinases Syk and Lyn have been implicated in EpoR activation (39,57) but their contribution to Gi-dependent MAPK activation remains to be determined.

Four documented pathways have been implicated in Epo-mediated regulation of Erk cascade in various hematopoietic cell lines or erythroid progenitors expressing the EpoR. Three of them, the Shc/Grb2/Sos pathway (11), the Grb2/Sos pathway (32) and the CrkL/C3G pathway (33) lead to MAPK activation via Ras. The fourth one involves PI3K and might require an atypical PKC (36). All these pathways are initiated through effector binding to phosphorylated tyrosines located in the intracellular domain of the receptor. Particularly, tyrosines 460 and 479 have been shown to be involved in CrkL/C3G and in PI3K-dependent Erk activation by the EpoR respectively (36,40) and tyrosine 464 has a consensus Grb2 binding motif (32). In CHO cells, MAPK activation by Epo is unlikely to occur through PI3K binding to tyrosine 479 since PI3K activation is inhibited
by the βγ competitor βARK-ct. The region of the EpoR required for G\textsubscript{i} binding in vivo and G\textsubscript{i}-dependent MAPK phosphorylation by Epo (amino acids 469-475) does not include tyrosines. Thus G\textsubscript{i}-dependent Erk activation may involve a different mechanism. The possibility that the absence of Erk activation in the ΔV-L EpoR, deleted of the G\textsubscript{i}-binding domain resulted from a conformational change affecting the nearby tyrosines is highly unlikely. Indeed Epo-induced CrkL and Grb2 interaction is preserved in the ΔV-L mutant. Furthermore pertussis toxin and βARK-ct also inhibit Epo-induced MAPK activation.

In hematopoietic cells, G\textsubscript{i} binds to the EpoR through the same EpoR domain required for G\textsubscript{i}-dependent Erk activation in CHO cells and G\textsubscript{i} is activated and dissociates from the receptor following Epo stimulation (15). However, in these cells, pertussis toxin has little effect on Epo-dependent Erk activation. As described above, there are several different pathways leading to MAPK activation by Epo in hematopoietic cells. The Gi/PI3K/MAPK pathway may be only one of them, explaining the relative inefficiency of the toxin in such cells. The relative contribution of the different pathways leading to MAPK activation by the EpoR and their interconnections remain poorly documented and need to be explored further. In contrast with hematopoietic cells, the G\textsubscript{i}-dependent pathway is the main pathway involved in Epo-dependent MAPK activation in CHO epithelial cells. Epo-induced EpoR tyrosine phosphorylation is lower in CHO than in hematopoietic cells\textsuperscript{2}. CHO cells may mainly use the G\textsubscript{i}-dependent pathway because of the inefficiency of MAPK pathways initiated by phosphorylated tyrosines of the receptor.

In erythroid precursors G\textsubscript{i} regulates an Epo-modulated Ca\textsuperscript{2+} channel (58,59). This regulation is mediated by G\textsubscript{ai2} and tyrosine 460 in EpoR is critical for Epo-stimulated Ca\textsuperscript{2+} influx in CHO and in Ba/F3 hematopoietic cells (60). Tyrosine 460 is not required for G\textsubscript{i} interaction with the EpoR in vitro and G\textsubscript{i}-dependent Erk activation involves the βγ subunits of G\textsubscript{i}. These data suggest that different G\textsubscript{i}-dependent pathways lead to calcium (through α\textsubscript{i2}) and MAPK (through βγ) activation by EpoR. Epo function in non-hematopoietic cells is not unique to epithelial cells. Indeed, myoblasts, neurals cells and endothelial cells have been shown to express EpoR (61-63). Epo has anti-apoptotic function in neurals cells and in brain (64,65), is implicated in brain development and neurogenesis (66,67). Epo has also been implicated in angiogenesis (68) and may regulate muscle cell proliferation and differentiation (62). Although the intracellular pathways activated by Epo in non
hematopoietic cells are still poorly documented, Epo was shown to activate MAPK and Jak2 in neural and in muscle cells (62,65,69). Further investigations would allow to determine the function and the contribution of the G-dependent pathway to MAPK activation pathway in hematopoietic and non-hematopoietic cells.
REFERENCES

G\textsubscript{i}-mediated MAPK activation by Erythropoietin


FOOTNOTES

1 The abbreviations used are: Epo, erythropoietin; EpoR, erythropoietin receptor; MAPK, mitogen activated protein kinase; Erk, extracellular signal-regulated kinase; PI3K, phosphoinositide 3-kinase; GPCR, seven transmembrane-spanning G protein-coupled receptor; EGF, epidermal growth factor; IGF-I, insulin-like growth factor-I; FGF, fibroblast growth factor; GTP, guanosine triphosphate; G protein, heterotrimeric GTP-binding protein; PT, pertussis toxin; MalE, maltose binding protein; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

2 P. Mayeux, unpublished data.
ACKNOWLEDGEMENTS

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**FIGURE LEGENDS**

**Figure 1** : Characterisation of EpoR domain coupling to Gᵢ

A, schematic representation of EpoR mutants and peptides used in this study. Top : truncation (-41) and deletion (ΔV₄₆₉-L₄₇₅) EpoR mutants expressed into cells. Bottom : EpoR peptides used as MalE fusion proteins for *in vitro* binding studies. B, association of EpoR intracellular domain with Gβ *in vitro*. Lysates from UT7 cells were incubated with immobilized MalE-EpoR fusion proteins. Bound proteins were resolved by SDS-PAGE, transferred to nitrocellulose membrane and analyzed by anti-Gβ and anti-MalE immunoblotting. C, FDCP-1 cells expressing no EpoR (-), wild type EpoR (WT) or the indicated EpoR mutants were lysed with 1% brij 98 and immunoprecipitation was performed with anti-EpoR antiserum. Immunoprecipitates were subjected to ADP-ribosylation as described under "Experimental Procedures". The proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane. Gαᵢ was visualized by autoradiography and Jak2 was identified by immunoblotting.

**Figure 2** : Pertussis toxin treatment inhibits the phosphorylation of endogenous MAPK following stimulation of EpoR with Epo.

CHO-ER cells expressing WT EpoR were preincubated overnight at 37°C in the absence (PT-) or presence (PT+) of 50 ng/ml pertussis toxin. Subsequently the cells were stimulated with 10 U/ml Epo for the indicated time and lysed. The soluble fraction was separated by SDS-PAGE and transferred to nitrocellulose membrane. Determination of MAPK activity was assessed by immunoblotting with phospho-specific anti-Erk antibodies (anti-pErk). The same blot was also probed with antibodies to Erk1/2 (anti-Erk) to confirm that equal amounts of enzyme were present in each lane.

**Figure 3** : MAPK phosphorylation requires the integrity of Gᵢ binding domain of the EpoR.

CHO-ER cells expressing WT EpoR or mutant ΔV-L EpoR were serum depleted and stimulated with 10 U/ml Epo or 10 ng/ml PMA for the indicated time (A) or for the indicated time with Epo (B) and lysed. A, phosphorylated Erk and total Erk were detected by immunoblotting as in
Fig. 2. B, Jak2 was immunoprecipitated and phosphorylated Jak2 and EpoR were identified by immunoblotting with anti-phosphotyrosine antibodies.

**Figure 4**: The phosphorylation of endogenous Erk-1/2 following stimulation of EpoR with Epo is dependent on βγ, MEK and Jak2.

CHO-ER WT cells were transfected with minigene encoding the carboxyl terminus of bovine βARK1 (βARK-ct) or empty vector. 24 h later the cells were serum starved for 4 h (A). CHO-ER WT cells were serum starved and preincubated (+) or not (-) with 30mM PD98059 for 30 min (B). CHO-ER cells expressing WT EpoR or mutant W282R EpoR were serum depleted (C). Subsequently the cells were stimulated with 10 U/ml Epo for 5 min and lysed. The soluble fraction was analyzed by anti-phospho-Erk and anti-Erk immunoblotting.

**Figure 5**: Contribution of Ras and not PKC to Epo-dependent MAPK activation.

CHO-ER WT cells were transfected with various amounts of HA-RasN17 as indicated. Total plasmid DNA amount was kept constant by adding pCDNA empty vector. 24 h later the cells were serum starved for 4 h and stimulated with Epo(+) or not (-) for 5 min (A). CHO-ER WT cells were serum depleted and preincubated or not for 16 h with 0.6µg/ml PMA followed by stimulation with 10U/ml Epo or 10ng/ml PMA for 5 min (B). Erk activation was assessed by immunoblotting with phosphospecific anti-Erk antibodies. The same blot was also probed with anti-Erk antibodies.

**Figure 6**: PI3K is necessary for MAPK phosphorylation.

Inhibition of MAPK activation with PI3K inhibitor. CHO-ER WT cells were serum starved and preincubated (+) or not (-) with 50µM LY 294002 for 15 min. Subsequently the cells were stimulated with 10 U/ml Epo for the indicated time and lysed. Erk activation was assessed by immunoblotting with phosphospecific anti-Erk antibodies (anti-pErk) and PI3K activation by immunoblotting with phosphospecific anti-Akt antibodies (anti-pAkt). The same blot was probed with antibodies to Erk1/2 (anti-Erk) and Akt (anti-Akt) to confirm that equal amounts of enzymes were present in each lane.

**Figure 7**: PI3K activation requires the integrity of the Gᵢ₁ binding domain in EpoR and βγ.

A, PI3K activation requires the integrity of Gᵢ₁ binding domain of the EpoR. CHO-ER cells expressing WT EpoR or mutant ΔV-L EpoR were serum depleted and stimulated with Epo for the
indicated time. B, PI3K activation is dependent on βγ. CHO-ER WT cells were transfected with minigene encoding the carboxyl terminus of bovine βARK1 (βARK-ct) or empty vector. 24 h later the cells were serum starved for 4 h, stimulated with 10 U/ml Epo for 5 min and lysed. Phosphorylated Akt and total Akt present in cell lysates were detected by immunoblotting.

Figure 8: PI3K activation is dependent on Jak2 and Ras and Jak2 activation is independent on Ras.

A, Ras is required for PI3K activation but not for Jak2 activation. CHO-ER cells WT were transfected with 0.75 µg of HA-RasN17 or empty vector. 24 hours later the cells were serum starved for 4 h, stimulated with Epo (+) or not (-) for 5 min and lysed. Jak2 was immunoprecipitated and phosphorylated Jak2 was identified by immunoblotting with anti-phosphotyrosine. Total Jak2 in immunoprecipitates was also determined by anti-Jak2 antibodies (top). Phosphorylated Akt, total Akt, phosphorylated Erk, total Erk and HA-RasN17 present in cell lysates were visualized by immunoblotting as indicated (bottom). B, Jak2 is required for PI3K activation. CHO-ER cells expressing WT EpoR or EpoR mutant W282R were serum depleted, stimulated with Epo (+) or not (-) for 5 min and lysed. Phosphorylated and total Akt, phosphorylated EpoR, phosphorylated and total Erk present in cell lysates were analyzed by immunoblotting.
Fig. 1
Fig. 2
Fig. 3

A

EpoR | WT | ΔV-L
---|---|---
Epo | 0 5 15 | 0 5 15 | 0 5 15
PMA | 0 5 15 | 0 5 15 | 0 5 15

Time (min)

blot

anti-pErk

anti-Erk

B

EpoR | WT | ΔV-L
---|---|---
Epo | - + | - + | - +

blot

anti-pTyr blot

Jak2

EpoR

Fig. 3
Fig. 4

Panel A: cDNA C and βARK-ct blots with Epo and PD 98059 treatment. 0, 5, 15, 60 time points for anti-pErk and anti-Erk.

Panel B: EpoR WT and W282R blots with PD 98059 treatment. 0, 5, 15, 60 time points for anti-pErk.

Panel C: Additional blots for anti-Erk.
### A

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blot

anti-pErk

anti-Erk

anti-pAkt

anti-Akt

Fig. 6
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### Figure 7

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Epo
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Fig. 8
Activation of the mitogen-activated protein kinases Erk1/2 by Erythropoietin receptor via a Gi protein beta gamma-subunit-initiated pathway
Christine Guillard, Stany Chrétien, Anne-Sophie Pelus, Françoise Porteu, Odile Muller, Patrick Mayeux and Véronique Duprez

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