Engagement of Gab1 and Gab2 in Erythropoietin Signaling*

Amittha Wickrema‡‡, Shahab Uddin‡, Arun Sharma‡, Fei Chen‡, Yazan Alsayed‡, Sarfraz Ahmad‡, Stephen T. Sawyer‡, Gerald Krystal‡, Taolin Yi**, Keigo Nishada‡‡, Masahiko Hibi‡‡, Toshio Hirano‡‡, and Leonidas C. Platanias‡‡

From the ‡Section of Hematology-Oncology, University of Illinois at Chicago and West Side Veterans Affairs Medical Center, Chicago, Illinois 60607, the Department of Pharmacology, Medical College of Virginia, Richmond, Virginia 23298, the †Terry Fox Laboratory, British Columbia Cancer Research Centre, Vancouver, British Columbia V5Z 1L3, Canada, the **Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, Ohio 44195, and the δδDivision of Molecular Oncology, Biomedical Research Center, Osaka University Graduate School of Medicine, Osaka 565-0871, Japan

Several signaling cascades are activated during engagement of the erythropoietin receptor to mediate the biological effects of erythropoietin. The members of the insulin receptor substrate (IRS) family of proteins play a central role in signaling for various growth factor receptors and cytokines by acting as docking proteins for the SH2 domains of signaling elements, linking cytokine receptors to diverse downstream pathways. In the present study we provide evidence that the recently cloned IRS-related proteins, Gab1 and Gab2, of the Gab family of proteins, are rapidly phosphorylated on tyrosine during erythropoietin treatment of erythropoietin-responsive cells and provide docking sites for the engagement of the SHP2 phosphatase and the p85 subunit of the phosphatidylinositol 3'-kinase. Furthermore, our data show that Gab1 is the primary IRS-related protein activated by erythropoietin in primary erythroid progenitor cells. In studies to identify the erythropoietin receptor domains required for activation of Gab proteins, we found that tyrosines 425 and 367 in the cytoplasmic domain of the erythropoietin receptor are required for the phosphorylation of Gab2. Taken together, our data demonstrate that Gab proteins are engaged in erythropoietin signaling to mediate downstream activation of the SHP2 and phosphatidylinositol 3'-kinase pathways and possibly participate in the generation of the erythropoietin-induced mitogenic responses.

Erythropoietin (EPO)† is a glycoprotein hormone that acts as the principal regulator of erythroid development. EPO plays a critical role in the regulation of cell survival and proliferation of erythroid progenitors that are at the burst-forming unit erythroid (BFU-E) and colony-forming unit-erythroid (CFU-E) stages of development (1–4). Previous studies have established that binding of EPO to its receptor results in activation of multiple signaling pathways including the Jak/Stat, Src/Grb2/Ras, and the PI 3'-kinase signaling cascades (5–22), suggesting that multiple pathways are activated and operate to mediate the effects of EPO on target cells.

The members of the insulin receptor substrate (IRS) family of SH2 docking proteins participate in signaling cascades activated by various cytokines in hematopoietic cells (23–31). These proteins have multiple tyrosine phosphorylation sites that act as docking sites for the SH2 domains of various signaling proteins. These allow IRS proteins to function as multistate docking proteins and link cytokine receptors to multiple signaling pathways. Recently, two new members of the IRS family have been identified and their cDNAs cloned, Gab1 and Gab2. Gab1 shares amino acid and structural homology with IRS-1 and contains multiple tyrosines that are putative binding sites for several SH2-containing downstream signaling molecules (31). Recent studies have demonstrated that Gab1 is involved in signaling via the epidermal growth factor (31), insulin (31), nerve growth factor (32), IL-3, IL-6/gp130 (32), TPO, T-cell, and B-cell receptors (33). Gab2 is also a recently cloned member of this family that shares common features with Gab1 and IRS-1 (34, 35) and has been shown to participate in IL-2, IL-3, IL-6/gp130, TPO, SCF, CSF-1, BCR, and TCR signaling (34, 35). Both Gab1 and Gab2 contain multiple sites for tyrosine phosphorylation and motifs for binding of SH2 and SH3 containing proteins. In addition, both Gab1 and Gab2 contain a c-Met binding domain and are related to the Drosophila homologue Daughter of Sevenless (DOS). The greatest amino acid sequence identity within the Gab/DOS proteins are found in their pleckstrin homology domains (34, 35). Although IRS proteins have similar structure, they can be distinguished from Gab/DOS proteins by their pleckstrin homology domains and other sequence elements. Therefore, it is likely that, in addition to mediating the activation of common pathways, Gab proteins have also distinct signaling and biological functions as compared with other IRS proteins.

In the current study we determined whether members of the Gab family of proteins are involved in EPO signaling in the EPO-dependent cell lines HCD57, DA3ER, and in highly purified human primary erythroid progenitors. Our data demonstrate that EPO induces strong tyrosine phosphorylation of Gab1 and Gab2, and that both Gab1 and Gab2 interact with the p85 subunit of the PI 3'-kinase and the SHP2 phosphatase, suggesting that their function is required for the engagement of these proteins by the EPO receptor. In addition, our data show

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‡ To whom correspondence and reprint requests should be addressed: Section of Hematology-Oncology, University of Illinois at Chicago, 800 South Ashland Ave., Chicago, IL 60607. Tel.: 312-413-9280; Fax: 312-413-7963; E-mail: Awickrem@uic.edu.

† The abbreviations used are: EPO, erythropoietin; Gab1, Grb2-associated binder-1; Gab2, Grb2-associated binder-2; IRS, insulin receptor substrate; PI 3'-kinase, phosphatidylinositol 3'-kinase; PAG, polyacrylamide gel electrophoresis; IL, interleukin; RT-PCR, reverse transcriptase-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SH2, Src homology domain 2; BFU-E, burst forming unit erythroid; CFU-E, colony forming unit erythroid.
that the proteins are differentially expressed in various cell lines, while only Gab1 is expressed in primary hematopoietic progenitors. In studies with DA3 cells expressing different EPO receptor mutants we identify tyrosines 367 and 425 in the EPO receptor, as the sites required for the engagement of the Gab pathway in EPO signaling.

MATERIALS AND METHODS

Cell Culture and Reagents—HCD57 cells were cultured in Iscove’s modified Dulbecco’s medium (BioWhittaker Inc., Walkerville, MD), 25% fetal calf serum, 10 μg/ml gentamicin, 1 unit of EPO/ml at 37 °C in a 5% CO2 environment. HCD57 and DA3ER cell lines were cultured in RPMI media, 10% fetal calf serum, 10 μg/ml gentamicin, 4 units of EPO/ml at 37 °C in a 5% CO2 environment as described previously (18). All other cell lines were maintained in RPMI media, 10% fetal calf serum, 10 μg/ml gentamicin at 37 °C in a 5% CO2 environment.

Human BFU-E were purified from peripheral blood obtained from normal volunteers. Informed consent was obtained from the volunteers after approval from the Institutional Review Board at the University of Illinois at Chicago. The purified BFU-E cells were cultured for 7 or 8 days to obtain a highly enriched population of erythroid progenitors that were at the CFU-E stage of differentiation as described previously (4–6). Briefly, 300 to 400 μl of whole blood was separated over Ficoll-Hypaque (1.077 g/ml) to obtain mononuclear cells. Platelets were depleted by centrifugation over a 10% bovine serum albumin gradient, followed by adherent cell depletion in polystyrene tissue culture flasks. The cell population was enriched for BFU-E by negative selection with MACS® Antibody-coated paramagnetic microbeads (Miltenyi Biotec, Inc., Auburn, CA) according to the manufacturer’s instructions. One microgram of total RNA was used for cDNA synthesis using random hexamers for the EPO-dependent myeloid cell line DA3ER Gab1 expression and cell lines and in primary hematopoietic progenitors although in these cells and its phosphorylation status did not change after EPO treatment (Fig. 1 E). Thus, in these cells, Gab1 and Gab2, but not IRS-1 or IRS-2, appear to be the principal IRS proteins activated by EPO, strongly suggesting that their phosphorylation is critical for the generation of EPO-induced signals.

In studies to determine the kinetics of tyrosine phosphorylation of Gab1, we found that its activation was rapid and transient, reaching a maximum level of phosphorylation at 15 min, with the signal diminishing after 30 min of EPO treatment. In addition, the Gab1-associated 72-kDa tyrosylphosphoprotein followed a similar time course of phosphorylation (Fig. 2 A). We then sought to determine the expression pattern of Gab1 and Gab2 proteins in various hematopoietic cell lines and primary hematopoietic cells (CD34+ Lin-) by RT-PCR. These cell lines represented all the major hematopoietic lineages. The primary cells that were utilized in the study consisted of highly purified human early hematopoietic stem cell progenitors and highly purified human erythroid progenitors that are at the CFU-E stage of differentiation. Fig. 3 shows the expression pattern of Gab1, Gab2, and GAPDH mRNA for each of the cell types utilized in the study. Gab1 is clearly expressed in all the cell lines and in primary hematopoietic progenitors although in the EPO-dependent myeloid cell line DA3ER Gab1 expression was absent. On the other hand, Gab2 mRNA expression was limited to HCD57, DA3ER, and TF-1 cells. We were unable to detect the expression of Gab2 transcripts in early human hematopoietic stem cell progenitors and committed human erythroid progenitors at the CFU-E stage of differentiation. The bands obtained for Gab1 (316bp) and Gab2(512bp) were specific for cDNA (+RT) as no bands were detected in the absence of RT.
of reverse transcriptase (RT) during the cDNA synthesis step (Fig. 3). Control PCR amplification with primers to GAPDH confirmed that the isolated RNA could be PCR amplified and the quantity of RNA used was similar for all the tissues. Furthermore, DNA sequencing of all positive reactions confirmed the amplification of Gab1 and Gab2 transcripts from these cell types (data not shown).

Since RT-PCR experiments indicated that Gab1 was the only Gab transcript that was detected in primary erythroid progenitors, we sought to determine whether Gab1 is phosphorylated in response to EPO treatment. Fig. 4 shows that Gab1 is phosphorylated in an EPO-dependent manner and that such tyrosine phosphorylation is rapid, reaching a maximum level of phosphorylation at 15 min, with the signal diminishing after 30 min of EPO treatment. A second unidentified protein that closely migrated with Gab1 was co-immunoprecipitated with the anti-Gab1 antibody and was also phosphorylated by EPO (Fig. 4). This was in addition to the Gab1-associated 72-kDa tyrosylphosphoprotein that was seen in the EPO-dependent cell lines (Fig. 4). Greater than 90% of the cells studied were CD71/glycophorin A positive as determined by flow cytometry (Fig. 4, bottom panel), indicating that they were purified progenitors committed to the erythroid lineage.

We next sought to determine whether the p85 regulatory subunit of the PI 3'-kinase interacts with Gab1 and/or Gab2 during EPO stimulation. Fig. 5A shows an experiment in which lysates from HCD57 cells were immunoprecipitated with an anti-Gab1 antibody, followed by SDS-PAGE analysis and immunoblotting with an anti-phosphotyrosine antibody. Association of p85 was detectable before EPO stimulation and increased after EPO stimulation in an EPO-dependent manner, strongly suggesting that the tyrosine-phosphorylated form of Gab1 provides docking sites for the binding of the SH2 domains of PI 3'-kinase during EPO stimulation. However, as there was some constitutive association of p85 with Gab1, it is possible that the SH3 domain of the PI 3'-kinase or other p85-interacting proteins can associate with Gab1 and this needs to be determined in future studies. To determine whether the serine kinase activity of the PI 3'-kinase is induced during its interaction with Gab1, in vitro kinase assays were performed on anti-Gab1 immunoprecipi-
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Fig. 3. Expression pattern of Gab1 and Gab2 genes in hematopoietic cells. RT-PCR analysis was performed utilizing Gab1- and Gab2-specific primers on a series of hematopoietic cell lines and primary hematopoietic cells as indicated. The cell lines were erythroid (HCD57, HEL, TF-1, and K562), myeloid (KG1a and DA3ER), B-cell (DAUDI), and T-cell (Jurkat) origin. The primary hematopoietic cells consisted of early uncommitted hematopoietic cell progenitors (CD34+ Lin-) and erythroid progenitors at the CFU-E stage of differentiation (days 7, 9, and 11 of culture). As positive controls, PCR-specific primers were used for the amplification of the GAPDH gene from the cDNA obtained for each cell type. As a negative control, PCR amplification was performed using specific primers for Gab1 and Gab2 from the negative RT reactions from each cell type. Molecular size markers (M) were included in the gel to confirm the amplified transcript size, bp, base pairs.

Time(min) 5 0 5 15 30 45
IP: anti-Gab1 - + + + + +
IP: NRS + - - - - -

GAPDH/RT
- 142 bp

Blot: anti-PTyr

Gab1/1+RT
- 316 bp

Gab1/-RT
- 512 bp

Gab2/1+RT
- 142 bp

Fig. 4. EPO induces tyrosine phosphorylation of Gab1 in human primary erythroid progenitors. Top panel, human primary erythroid progenitors were stimulated with EPO for different times as indicated. Cell lysates were immunoprecipitated with either an anti-Gab1 antibody or nonimmune rabbit serum (NRS) and immunoprecipitated proteins were analyzed by SDS-PAGE and immunoblotted with anti-phosphotyrosine (PTyr) antibody. Bottom panel, flow cytometric analysis of erythroid progenitors (day 8 of culture) for the surface markers glycophorin A and CD71. Primary erythroid progenitors were stained with phycoerythrin-conjugated anti-glycophorin A antibody and fluorescein-conjugated anti-CD71 antibody and analyzed by flow cytometry along with the isotype controls for each of the antibodies.

followed by SDS-PAGE analysis and immunoblotting with an antibody against the p85 regulatory subunit of the PI 3'-kinase. There was some baseline interaction between Gab2 and p85 detected. Such an interaction increased significantly after treatment with Epo, suggesting that the tyrosine-phosphorylated form of Gab2 also binds the SH2 domains of P85 during Epo stimulation. Thus both members of the Gab family of proteins provide docking sites for the engagement of PI 3'-kinase in EPO-induced signaling.

We subsequently sought to identify the 72-kDa protein that was phosphorylated and co-immunoprecipitated with Gab1 and Gab2 during EPO stimulation. As the relative molecular mass (Mr) of this protein was similar to the Mr of the EPO receptor and the Mr of the SHP2 phosphatase, we performed studies to determine if the EPO receptor or the SHP2 phosphatase can be detected in association with Gab1 during EPO stimulation. HCD57 cells were stimulated with EPO for different times and cell lysates were immunoprecipitated with antibodies against Gab1, followed by SDS-PAGE analysis and immunoblotting with anti-phosphotyrosine (Fig. 6A). The 72-kDa protein co-migrated with the EPO receptor, but it was not recognized by an antibody specific for the EPOR (Fig. 6A, middle panel). However, when anti-SHP2 immunoblots were performed, we found that the protein was recognized by an anti-SHP2 monoclonal antibody (Fig. 6A, bottom panel), establishing that it corresponds, at least in part, to the SHP2 phosphatase. Thus, in addition to binding the p85 regulatory subunit of the PI 3'-kinase, Gab1 interacts with the SHP2 phosphatase, strongly suggesting that its docking function recruits SHP2 in EPO signaling. Similarly, when anti-SHP2 immunoblots were performed on anti-Gab2 immunoprecipitates from EPO-treated cells, we found that SHP2 associates with Gab2 in an EPO-dependent manner, suggesting that Gab2 also provides a binding site for SHP2 (Fig. 6B).

To identify the domains in the EPO receptor that are required for EPO-dependent phosphorylation of Gab proteins, we determined Gab2 phosphorylation in a series of DA3 cell line transfectants, expressing different mutations of the EPO re-
ceptor. Each of these mutants lacked one phosphotyrosine residue due to the substitution of phenylalanine in place of tyrosine, with the exception of the ERH mutant, which contained only the most proximal tyrosine residue to the cytoplasm and lacked seven of the eight tyrosine residues within the cytoplasmic domain of the erythropoietin receptor. Fig. 7 shows phosphotyrosine immunoblots using five such mutant clones of DA3ER cells. Lysates from EPO-stimulated cells of the different transfectants were immunoprecipitated with Gab2 and Jak2 antibodies. The cells expressing EPO receptors with mutated tyrosines 367 and 425 (Y367F and Y425F) showed no phosphorylation of Gab2 in response to EPO, although there was strong activation of Jak2, suggesting that these phosphotyrosine residues are required for the activation of Gab2. Similarly, no phosphorylation of Gab2 was seen in cells expressing the ERH mutant, in which seven of the eight tyrosines including tyrosines 367 and 425 were not present. However, in Y453F and Y467F expressing cells, there was phosphorylation of Gab2, indicating that these phosphotyrosine residues are not required for its activation. Thus tyrosines 367 and 425 in the EPO receptor are required for engagement of Gab2 in EPO signaling.

**DISCUSSION**

Gab1 and Gab2 are recently cloned members of the Gab family of proteins which are related to the IRS proteins (31). Many of the motifs present in IRS-1 are conserved in Gab1, including three tyrosines in YXXM motifs that provide docking sites for the p85 regulatory subunit of the PI 3'-kinase and a tyrosine in a YXDL motif that binds the SH2 domain of the SHP2 phosphatase. Recent studies have demonstrated that Gab1 and Gab2 are activated and engaged in signaling via the insulin, epidermal growth factor, nerve growth factor, IL-6, and other cytokine receptors (31–35), suggesting that the protein plays an important role in the generation of biological responses for various cytokines and growth factors.

Extensive previous studies have demonstrated that erythropoietin activates multiple signaling cascades to generate mitogenic and differentiation signals, including the PI 3'-kinase pathway and a pathway regulated by the SHP2 phosphatase (18, 20–22, 38). It has been also demonstrated that activation of the PI 3'-kinase plays an important role in the induction of EPO-mediated cell proliferation (6, 21, 39) and that the catalytic activity of SHP2 plays a significant role in EPO signal transduction (18, 38). The present study implicates Gab proteins as the principal IRS proteins activated by the erythropoietin receptor. Previous studies using the UT-7 multipotential human leukemia cell line have established that IRS-2 is also tyrosine phosphorylated during EPO treatment and associates with the p85 regulatory subunit of the PI 3'-kinase, suggesting that this protein is also involved in erythropoietin signaling (30). In our studies, using HCD57 cells and primary human erythroid progenitors, we failed to detect EPO-induced phosphorylation of IRS-1 or IRS-2. The lack of detection of EPO-dependent tyrosine phosphorylation of IRS-1 or IRS-2, in combination with the results of this study, suggests that the Gab proteins are the major IRS proteins activated by the erythropoietin receptor in the UT-7 cell line.
sine phosphorylation of IRS-2 in HCDC57 cells may be due to the heavy baseline tyrosine phosphorylation of the protein. However, only Gab proteins and not IRS-2, were found to associate with the SHP2 phosphatase during treatment of cells with EPO (data not shown), indicating that SHP2 requires Gab proteins and not IRS-2 for its engagement in erythropoietin signaling. These findings are consistent with previous studies performed in UT-7 cells, in which no association between IRS-2 and SHP2 was detected (30). Our data also showed that Gab proteins bind the p85 subunit of the PI 3'-kinase. Previous studies have shown that the p85 subunit of the PI 3'-kinase binds directly to EPO receptor (9). Thus our findings strongly suggest and provide evidence that an alternative pathway for the activation of PI 3'-kinase by EPO involves Gab proteins.

The examination of the expression pattern of Gab proteins in various hematopoietic cells revealed that Gab1 is expressed ubiquitously, whereas expression of Gab2 is selective. Among the cell lines utilized in the study, only the EPO-dependent erythroid cell line HCDC57 and TF-1 showed expression of both Gab1 and Gab2. Since Gab1 and Gab2 contain similar structural characteristics (potential phosphorylation sites and their relative positions are conserved in addition to preservation of the pleckstrin homology domain) it would be interesting to determine whether the function of these two proteins are redundant in these cells or they mediate separate biological functions. It is conceivable that in many of the hematopoietic cells, the functional roles of Gab1 and Gab2 are interchangeable. Highly purified primary erythroid progenitors and early uncommitted hematopoietic cells showed expression of only Gab1, indicating that at least in erythroid lineage cells, Gab1 transcripts are continuously expressed throughout differentiation.

Upon EPO stimulation both Gab1 and Gab2 are tyrosine phosphorylated and form complexes with SHP2 and the p85 subunit of the PI 3'-kinase. Our data using DA3ER mutants lacking individual tyrosine residues (Y425F and Y367F) within the cytoplasmic domain of the EPO receptor indicate that both tyrosines 425 and 367 are critical for the phosphorylation of Gab2 by EPO. This is in contrast to a previous study in which it was shown that the tyrosine residues of the cytoplasmic domain of gp130 are not necessary for the activation of Gab proteins (33). Also, the same study utilized a gp130 receptor mutant expressed in 293T cells in which the SHP2-binding site of gp130 was mutated to phenylalanine. When gp130 is stimulated in these cells there is no association of SHP2 with Gab1 although Gab1 is phosphorylated. Taken together with our data, the results of these studies indicate the existence of different mechanisms for phosphorylation of Gab proteins based on the ligand activating them. Our results suggest that in EPO signal transduction, at least two tyrosine residues within the cytoplasmic domain of the EPO receptor are needed to phosphorylate Gab proteins. Furthermore, our finding, that Gab2 is not phosphorylated in cells expressing EPO receptors lacking tyrosine 425 raise the possibility that the Gab pathway mediates EPO-dependent mitogenic signals. A previous study (40) has demonstrated that this tyrosine in the cytoplasmic domain of the EPO receptor promotes cell growth in response to EPO, and in cells expressing EPO receptors lacking this tyrosine residue, the mitogenic effects of EPO are diminished. As this residue is also required for engagement of the SHP2 phosphatase (38, 40), it is tempting to hypothesize that Gab proteins are the primary pathway for the activation of SHP2, which mediates mitogenic effects, in EPO signaling. Although further work is required to precisely define the role of Gab1 and Gab2 in the regulation of the biological effects of EPO, our data strongly suggest that these proteins, and not the related IRS-1 and IRS-2, are the principal docking proteins in EPO signaling. It is likely that, in addition to SHP2 and p85 proteins, Gab1 and Gab2 provide docking sites for other known and unknown proteins activated by the EPO receptor, which remain to be identified in future studies.

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