RAPID COMMUNICATION

Phosphatidylinositol 3-Kinase Associates, Via Its Src Homology 2 Domains, With the Activated Erythropoietin Receptor

By Jacqueline E. Damen, Alice L.-F. Mui, Lorri Puil, Tony Pawson, and Gerald Krystal

The erythropoietin receptor (EpR) belongs to a family of hematopoietin receptors whose members lack tyrosine kinase activity. Nonetheless, within minutes of binding Ep, a number of cellular proteins become transiently phosphorylated on tyrosine residues. One of these proteins, as we and others have shown previously, is the EpR itself. To identify the remaining protein substrates, we have examined the antiphosphotyrosine immunoprecipitates of lysates from Ba/F3 cells expressing high levels of cell surface EpRs. We now present data showing that, in response to Ep, the 85-Kd regulatory subunit of phosphatidylinositol 3-kinase (PI 3-kinase) becomes immunoprecipitable with antiphosphotyrosine antibodies. This appears to be due, in large part, to the specific association of PI 3-kinase with the tyrosine-phosphorylated EpR, either directly or through a 93- or 70-Kd tyrosine-phosphorylated intermediate. The activity of this EpR associated PI 3-kinase, assessed in anti-EpR immunoprecipitates, is maximal within 2 minutes of incubation with Ep and returns almost to baseline levels by 10 minutes. In vitro studies suggest that the interaction between PI 3-kinase and the activated EpR is mediated by the N- and C-terminal SH2 domains of p85 and tyrosine-phosphorylated motifs on the EpR.

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A 200- +
97.4 -
69 -
46 -
50 -

B 200- +
97.4 -
69 -
46 -
85 -
72 -
70 -

C 200- +
97.4 -
69 -
46 -
85 -

Fig 1. Identification of proteins tyrosine phosphorylated in response to Ep. Equivalent numbers of Ba/F3 C5 cells were incubated for 10 minutes at 37°C with (+) or without (−) Ep and the cells were then lysed and immunoprecipitated with the anti-P-Tyr MoAb, 4G10. (A) Phosphotyrosine-containing proteins were identified by immunoblotting with 4G10. The numbers in bold print refer to the major tyrosine-phosphorylated species. (B) The immunoblot shown in (A) was reprobed with an anti-C-terminal EpR antibody. (C) The same immunoblot was reprobed with an anti–PI 3-kinase antibody. Similar results were obtained in four different experiments.

human Ep was purified from culture supernatants of baby hamster cells expressing an Ep cDNA and was biotinylated as described previously. Streptavidin-agarose beads and protein grade Nonidet P-40 (NP-40) were purchased from Calbiochem (San Diego, CA). Anti–N-terminal and anti–C-terminal EpR antibodies were generated in rabbits by immunizing with a peptide (covalently linked to KLH) corresponding to the predicted N- and C-terminal 15 amino acids of the murine EpR cDNA, respectively. The antibodies were purified from serum by affinity binding to the N- and C-terminal peptides. The glutathione S-transferase (GST) fusion proteins, consisting of the 27-Kd amino-terminal of GST linked to the PI 3-kinase (N) SH2 domain (residues 312 to 444 of bovine brain PI 3-kinase p85), the PI 3-kinase (C) SH2 domain (residues 612 to 722 of the 85-Kd subunit), the GAP (N) SH2 domain (residues 178 to 277 of human GAP), and the PLC-γ1 (N) SH2 domain (residues 547 to 659 of bovine PLC-γ1) were expressed in Escherichia coli in pGEX-2T plasmids (Pharmacia LKB Biotechnology, Inc, Baie d’Urfe, Quebec, Canada) as described previously. After induction with 1 mmol/L isopropylthiogalactopyranoside, the bacteria were lysed by sonication and the fusion proteins recovered from clarified lysates by using glutathione-agarose (Pharmacia) as described. The amount of each GST fusion protein was determined by Coomassie blue staining and cross-linked at 1 mg/mL to CNBr-activated Sepharose 4B beads (Pharmacia). 35P-γ ATP (3,000 Ci/mmol) was obtained from Dupont (Mississauga, Ontario, Canada). The enhanced chemiluminescence (ECL) Western blotting reagents were purchased from Amersham Corp (Arlington Heights, IL). PI, PI-4-P, PI-4, 5-P2, L-α-phosphatidyl-L-serine (PS), and all other reagents were purchased from Sigma (St Louis, MO) unless otherwise indicated.

Cells. The murine IL-3 (mIL-3)-dependent pro-B-cell line, Ba/F3, kindly supplied by Dr A. Miyajima (DNAX Research Institute, Palo Alto, CA), was routinely cultured in RPMI containing 10% heat-inactivated fetal calf serum (FCS) and 3 mmol/L COS-cell–derived mIL-3. These cells were retrovirally infected with a JZen TKneo vector containing the murine EpR, as previously described, and the present studies were performed with one of the high EpR-expressing clones, C5. These cells were routinely maintained in RPMI containing 10% FCS and 0.5 U/mL Ep.

Preparation of cell lysates and immunoprecipitation. Ba/F3 clone C5 cells were incubated overnight in RPMI, 1% FCS at 37°C, resuspended at 2 × 10^6 cells/mL in RPMI containing 0.1% bovine serum albumin (BSA), and incubated with or without 50 U/mL Ep for 10 minutes at 37°C. The cells were then washed and solubilized at 2 × 10^6 cells/mL with 0.5% NP-40 in phosphorylation solubilization buffer (PSB), ie, 50 mmol/L HEPES, pH 7.4, 100 mmol/L NaF, 10 mmol/L Na3VO4, 2 mmol/L EDTA, and 2 mmol/L (NH4)2 Mo containing the protease inhibitors aprogin (100 KIU/mL), leupeptin (40 μg/mL), and phenylmethylsulfonyl fluoride (PMSF; 1 mmol/L). After 1 hour of incubation at 4°C, insoluble material was removed by centrifugation for 10 minutes at 16,000g and the supernatants were gently rocked either for 4 hours with anti–P-Tyr–bound Sepharose beads or for 1 hour with anti–PI 3-kinase antiserum or anti–C-terminal EpR antibody, followed by protein A-Sepharose beads (20 μL; Pharmacia) for an additional 2 hours at 4°C. The beads were then either prepared for PI 3-kinase assays (see below) or washed extensively with PSB containing 0.1% NP-40 and the bound proteins eluted with 100 mmol/L phenylphosphate (for anti–P-Tyr immunoprecipitates) or boiled in sodium dodecyl sulfate (SDS) sample buffer for SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Biotinylated-Eplstreptavidin-agarose purification of the EpR.
Ba/F3 clone C5 cells were incubated overnight in RPMI at 37°C, resuspended in Hanks' Balanced Salt Solution (HBSS) containing 2% FCS and 0.02% NaN₃ (HFN) buffer at 2 × 10⁵ cells/mL, and then stimulated with 50 nmol/L biotinylated Ep (b-Ep) for 10 minutes at 37°C in the absence or presence of a 100-fold excess of unlabeled Ep (to control for nonspecific binding to streptavidin agarose). The cells were then solubilized using a final concentration of 8 mmol/L 3-[(3-cholamidopropyl)-dimethyammonio]-l-propanesulfonate (CHAPS) in HBSS containing 2 mmol/L Na₂VO₃ and 2 mmol/L (NH₄)₂MO. After 1 hour at 4°C, insoluble material was removed by centrifugation for 10 minutes at 16,000g and the supernatants were incubated for 2 hours at 4°C with 10 mg of streptavidin-agarose beads. The beads were exhaustively washed with HFN buffer containing 8 mmol/L CHAPS and bound proteins eluted by boiling for 3 minutes in SDS sample buffer containing 5% β-mercaptoethanol (β-ME) and analyzed by SDS-PAGE.

Western blot analysis. After SDS-PAGE with 7.5% polyacrylamide gels, proteins were electrophoretically transferred onto Immobilon PVDF membranes (Millipore, MA) at 100 V for 90 minutes at 4°C using 10 mmol/L CAPS, pH 10.0, and 10% methanol. Residual binding sites on the membranes were blocked by incubation in Tris-buffered saline (TBS; 10 mmol/L Tris-Cl, pH 8.0, 150 mmol/L NaCl) containing 5% BSA plus 5% skim-milk powder for 1 hour at 23°C. Blots were then washed in TBST (TBS with 0.05% Tween-20) and incubated for 1 hour in TBST with 1.0 μg/mL antibody. Blots were then washed three times for 5 minutes each with TBST and probed with a 1:10,000 dilution of goat antimouse or goat antirabbit horseradish peroxidase conjugate (Jackson Labs, Bar Harbor, ME) for 45 minutes at 23°C. After washing, blots were incubated with enhanced chemiluminescence substrate solution and exposed to Kodak X-Omat film (Eastman Kodak, Rochester, NY) to visualize immunoreactive bands. In some experiments, blots were stripped with 62.5 mmol/L Tris-Cl, pH 6.8, 2% SDS, 100 mmol/L β-ME at 50°C for 30 minutes, reblocked, washed, and reprobed with either anti-PI 3-kinase (p85 subunit) or anti-N-terminal—EpR antibody, using goat antirabbit horseradish peroxidase conjugate (Jackson Labs) at 1:10,000 as second antibody.

In vitro PI 3-kinase assay. Immunoprecipitated protein A beads were washed with the following buffers: (1) PBS; (2) 100 mmol/L Tris-Cl, pH 7.4, 50 mmol/L LiCl; (3) 10 mmol/L Tris-Cl, pH 7.4, 0.1 mol/L NaCl, 1 mmol/L EDTA. The assay was performed directly with the beads in the presence of 25 mmol/L HEPES, pH 7.4, and 10 mmol/L MgCl₂. A mixture of PI, PI-4, 5-P₂, and PS (1:1:2) at a total concentration of 0.2 mg/mL was dispersed by sonication in 25 mmol/L HEPES, pH 7.4, 1 mmol/L EDTA and then added to the reaction mixture in a final volume of 50 μL. The reaction was initiated by adding 10 to 20 μCi of [γ-³²P]-ATP (3,000 Ci/mmol; DuPont-New England Nuclear) and terminated by adding 20 μL of 6 N HCl. The products were extracted into chloroform:methanol (1:1) and separated by thin-layer chromatography (TLC) on oxalate-treated silicone gel plates (Merck, Richmond, British Columbia, Canada) as previously described.³⁴ Unlabeled lipid standards, PI-4-P and PI-4, 5-P₂, were cochromatographed with the samples and visualized by exposure to iodine vapor.

Binding to SH2 domains. NP-40 lysates from C5 cells, incubated in the presence and absence of Ep, were mixed at 4°C with Sepharose beads containing GST fusion proteins (1 mL of lysate with 20 μL of packed beads containing 20 μg of fusion protein) for 3 hours. The beads were then washed four times with PBS, 0.1% NP-40 and bound proteins eluted by boiling with 100 μL of SDS sample buffer for 3 minutes.

RESULTS AND DISCUSSION

As a first step towards identifying the intracellular proteins that are tyrosine phosphorylated in response to Ep,
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Fig 3. p85 is not associated with the inactive EpR. C5 cells were incubated for 2 hours at 4°C with (+) or without (−) b-Ep and then washed, lysed, and added to streptavidin-agarose (lane 1) or shifted up to 37°C for 5 minutes before washing, lysing, and mixing with streptavidin-agarose (lane 2). (A) An anti-C-terminal EpR immunoblot. (B) The blot shown in (A) was reprobed with anti-P-Tyr antibodies. (C) The blot shown in (A) was reprobed with anti-PI 3-kinase antibodies.

anti–P-Tyr immunoprecipitations were performed with lysates from Ba/F3 cells to express high numbers of cell surface murine EpRs.13 These cells, designated Ba/F3 clone C5 and shown previously to proliferate in response to low levels of Ep,13 were treated with or without Ep for 10 minutes at 37°C. As can be seen in Fig 1A, Western analysis of these anti–P-Tyr immunoprecipitates, using anti–P-Tyr antibodies, showed that Ep stimulates the appearance of 6 prominent tyrosine phosphorylated bands at 145, 130, 93, 72, 70, and 56 Kd. Minor, Ep-specific, tyrosine-phosphorylated bands were also seen with molecular masses of 110, 85, and 50 Kd. Reprobing this blot with antibody to the EpR showed, as we13 and others10,11 have shown previously, that the major 72-Kd band is the tyrosine phosphorylated form of the EpR (Fig 1B). (Preclearing of Ep-stimulated C5 cell lysates with anti-EpR antibodies showed that the EpR is the only component of this 72-Kd band [data not shown].) As a start towards identifying the remaining tyrosine-phosphorylated proteins, the blot was reprobed with antibody to the p85 subunit of PI 3-kinase (Fig 1C). This showed that, in response to Ep, the p85 subunit of PI 3-kinase is substantially increased in anti–P-Tyr immunoprecipitates and that it comigrates with the minor 85-Kd band seen in Fig 1A. These experiments suggest that Ep stimulates the tyrosine phosphorylation of the p85 subunit of PI 3-kinase and/or its physical association with a tyrosine-phosphorylated protein in these cells.

Because PI 3-kinase has been shown previously to become physically associated with several activated, tyrosine kinase-containing cell surface receptors20–23 and, more recently, with certain nontyrosine kinase-containing receptors,24,27 we next wanted to determine if p85 was physically associated with the activated EpR. However, for technical reasons, our rabbit anti-EpR antibodies could not be used in conjunction with the rabbit anti-p85 antiserum from UBI (for immunoprecipitation and immunoblots). We therefore incubated C5 cells for 10 minutes at 37°C with or without b-Ep and cell lysates were exposed to streptavidin-agarose to enrich for EpRs (and, potentially, EpR-associated proteins). An anti–P-Tyr Western blot of the streptavidin-agarose-bound material showed, as expected, that b-Ep bound tightly to a major 72-Kd phosphoprotein (Fig 2A, lanes 1 and 2) that, upon reblotting with anti-EpR antibody, was shown to be the EpR (data not shown). Minor tyrosine-phosphorylated bands were also seen at 93, 70, and 56 Kd in this EpR-enriched preparation (Fig 2A, lanes 1 and 2), corresponding, most likely, to the major tyrosine-phosphorylated species seen in Fig 1A. Importantly, reprobing of this blot with anti–PI 3-kinase antibody showed that b-Ep also enriched for the p85 subunit of PI 3-kinase (Fig 2A, lanes 3 and 4). The converse was also performed, ie, lysates from C5 cells, incubated for 10 minutes at 37°C in the presence or absence of Ep, were immunoprecipitated with antibodies to PI 3-kinase. An anti–P-Tyr immunoblot showed that, in the presence of Ep, three bands at 93, 72, and 70 Kd were consistently precipitated with anti–PI 3-kinase (Fig 2B, lanes 1 and 2). If lysates from Ep-stimulated C5 cells were precleared with anti-EpR antibody before immunoprecipitation with anti–PI 3-kinase, the 72-Kd protein was not seen in anti–P-Tyr immunoblots, indicating that this was the tyrosine-phosphorylated EpR (data not shown). Reprobing of lanes 1 and 2 with anti–PI 3-kinase showed equal loading of PI 3-kinase in lanes 3 and 4 and indicated that very little, if any, of total cellular p85 is tyrosine phosphorylated in response to Ep. This is consistent with very recent studies in which IL-4 was shown to dramatically increase PI 3-kinase.
levels at this temperature (Fig 3C). Thus, because PI 3-kinase was not associated with the EpR after incubation of C5 cells with b-Ep at 4°C, it is most likely not associated with the receptor before Ep stimulation and EpR phosphorylation.

To determine whether the 110-Kd catalytic component of PI 3-kinase is also associated with the activated EpR, PI 3-kinase assays were performed with anti-EpR immunoprecipitates. As can be seen from Fig 4A, only when Ep was activity in anti–P-Tyr immunoprecipitates from FDCP-2 cell lysates, but did not stimulate the tyrosine phosphorylation of p85. Thus, after Ep stimulation, p85 is most likely brought down with antiphosphotyrosine antibodies because of its association with the tyrosine-phosphorylated EpR (and/or a 93- or 70-Kd tyrosine-phosphorylated protein).

To ascertain whether p85 is associated with the EpR before ligand binding, C5 cells were incubated with b-Ep for 2 hours at 4°C and then either processed directly for streptavidin-agarose affinity chromatography or shifted up first to 37°C for 5 minutes. We found, using anti-EpR immunoblotting, that shifting up to 37°C resulted in a slight loss in total EpRs (Fig 3A). We also found, upon reblotting with anti–P-Tyr antibodies, that the EpR was significantly tyrosine phosphorylated only at 37°C (Fig 3B). Stripping and re-probing with anti–PI 3-kinase showed that, even though there was less total EpR at 37°C, the p85 subunit of PI 3-kinase was only substantially increased above background activity in anti–P-Tyr immunoprecipitates from FDCP-2 cell lysates, but did not stimulate the tyrosine phosphorylation of p85. Thus, after Ep stimulation, p85 is most likely brought down with antiphosphotyrosine antibodies because of its association with the tyrosine-phosphorylated EpR (and/or a 93- or 70-Kd tyrosine-phosphorylated protein).

To determine whether the 110-Kd catalytic component of PI 3-kinase is also associated with the activated EpR, PI 3-kinase assays were performed with anti-EpR immunoprecipitates. As can be seen from Fig 4A, only when Ep was
added to C5 cells did anti-EpR and anti-P-Tyr immunoprecipitated preparations possess significant PI 3-kinase activity. This finding was consistent with those of our previous study showing that PI 3-kinase is not associated with the EpR until the latter was bound by its ligand (ie, as shown in Fig 3). A kinetic analysis of PI 3-kinase association with the EpR indicated that maximal PI 3-kinase activity was reached by 2 minutes and returned almost to baseline by 10 minutes (Fig 4B).

Lastly, because the SH2 domains of the 85-Kd subunit of PI 3-kinase have been shown recently to bind with high affinity to tyrosine-phosphorylated PDGF receptors,20,23 EGF receptors,26,27 CSF-1 receptors,33 SF receptors,35 and insulin-like growth factor receptors,33 we investigated whether the p85 subunit of PI 3-kinase bound via its SH2 regions to the activated EpR. For these studies, GST fusion proteins, consisting of the amino terminus of GST, linked to either the N- or C-terminal SH2 domains of p85, the N-terminal SH2 domain of GAP, or the N-terminal SH2 domain of PLC-5 were expressed and cross-linked to Sepharose beads.35 NP-40-solubilized lysates from treated C5 cells, incubated in the presence or absence of Ep for 10 minutes at 37°C, were mixed with the various cross-linked beads and, after extensive washing, tightly associated proteins were released by boiling with SDS sample buffer and subjected to Western analysis with anti-P-Tyr antibodies (Fig 5A). The results suggested that both the N- and C-terminal SH2 domains of p85 were capable of binding tightly to three tyrosine-phosphorylated proteins present in lysates from Ep-treated C5 cells; two minor phosphoproteins with molecular masses of 93 and 70 Kd and a major tyrosine-phosphorylated 72-Kd protein. Upon reprobing with anti-EpR antibody, the 72-Kd protein was shown to be the tyrosine-phosphorylated EpR (Fig 5B). Because of the insensitivity of the anti-EpR antibody, it could not be determined if the minor 70-Kd band represented a less phosphorylated form of the EpR or an unrelated protein. The N-terminal SH2 domain of GAP displayed a far more modest affinity for the EpR and the N-terminal SH2 domain of PLC-γ 1 appeared unable to bind this receptor at all under the conditions used. This indicates that both SH2 domains of the p85 subunit of PI 3-kinase have a high affinity for the activated form of the EpR and PI 3-kinase may therefore associate via its SH2 domains directly with the tyrosine-phosphorylated EpR. Alternatively, because the EpR does not possess the YMXM consensus sequence for PI 3-kinase binding that is present in the kinase insert region of the PDGFR family,33 in the IRS1 protein,39,40 and in the polypeptide middle T antigen,35 PI 3-kinase may bind indirectly to the EpR via either the tyrosine-phosphorylated 93- or 70-Kd protein because both are present with the EpR in b-Ep/streptavidin-agarose-enriched preparations (Fig 2A), in PI 3-kinase immunoprecipitates of Ep-stimulated C5 cell lysates (Fig 2B), and in the eluates from p85 N- and C-terminal SH2 Sepharose beads (Fig 5A). A third possibility is that p85 acts as a bridge to bind, via its two SH2 domains, the tyrosine-phosphorylated 93-Kd protein (or 70-Kd protein) to the tyrosine-phosphorylated EpR. Alternatively, because Miura et al41 recently showed that a truncated EpR lacking detectable tyrosine-phosphorylated residues could still function in mitogenic signalling, it is conceivable that an Ep-induced conformational change rather than tyrosine phosphorylation of the receptor is critical for PI 3-kinase binding. To distinguish among these possibilities, we are currently examining the effect of various EpR truncations on PI 3-kinase binding to the EpR and to the 97- and 70-Kd proteins.

Relevant to our data is the very recent finding by Linnekin et al41 that a 97-Kd tyrosine-phosphorylated protein is physically associated with the EpR in EpR-positive Ba/F3 cells and that this protein may be a tyrosine kinase. Also of interest are the recent reports that a pp100 is tyrosine phosphorylated in response to IL-3, granulocyte-macrophage colony-stimulating factor, or Ep in EpR-transfected FDC-P1 cells,42 and that a 97-Kd tyrosine-phosphorylated protein becomes associated with PI 3-kinase after IL-3 stimulation of FDCP-2 cells.43 It remains to be determined whether the 93-Kd protein we observe in C5 cells is related to the 97- and/or 100-Kd proteins reported by these groups.

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