Epidermal Growth Factor-induced Phosphatidylinositol 3-Kinase Activation and DNA Synthesis

IDENTIFICATION OF Grb2-ASSOCIATED BINDER 2 AS THE MAJOR MEDIATOR IN RAT HEPATOCYTES*

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In previous work we showed that the phosphatidylinositol 3-kinase (PI3-kinase), not the mitogen-activated protein kinase, pathway is necessary and sufficient to account for insulin- and epidermal growth factor (EGF)-induced DNA synthesis in rat hepatocytes. Here, using a dominant-negative p85, we confirmed the key role of EGF-induced PI3-kinase activation and sought to identify the mechanism by which this is effected. Our results show that EGF activates PI3-kinase with a time course similar to that of the association of p85 with three principal phosphotyrosine proteins (i.e. PY180, PY105, and PY52). We demonstrated that each formed a distinct p85-associated complex. PY180 and PY52 each constituted about 10% of EGF-activated PI3-kinase, whereas PY105 was responsible for 80%. PY105 associated with Grb2 and SHP-2, and although it behaved like Gab1, none of the latter was detected in rat liver. We therefore cloned a cDNA from rat liver, which was found to be 95% homologous to the mouse Grb2-associated binder 2 (Gab2) cDNA sequence. Using a specific Gab2 antibody, we demonstrated its expression in and association with p85, SHP-2, and Grb2 upon EGF treatment of rat hepatocytes. Gab2 accounted for most if not all of the PY105 species, since immunoprecipitation of Gab2 with specific antibodies demonstrated parallel immunodepletion of Gab2 and PY105 from the residual supernatants. We also found that the PI3-kinase activity associated with Gab2 was totally abolished by dominant negative p85. Thus, Gab2 appears to be the principal EGF-induced PY protein recruiting and activating PI3-kinase and mitogenesis.

Phosphatidylinositol 3-kinase (PI3-kinase) is an enzyme that phosphorylates the D-3 position of the inositol ring of PI to produce three novel phosphoinositides: phosphatidylinositol 3-monophosphate, phosphatidylinositol 3,4-biphosphate, and phosphatidylinositol 3,4,5-trisphosphate (1, 2). This enzyme is a heterodimer of a 110-kDa (p110) catalytic subunit and an 85-kDa (p85) regulatory subunit (3). p85 has two SH2 domains that bind to tyrosine-phosphorylated sites and an SH3 domain that binds proline-rich sequences on receptors or docking proteins (4, 5). It has been demonstrated that p110 requires the binding of p85 to achieve full activation (6).

Several studies in various cell lines, suggest that the PI3-kinase pathway is important for both insulin (7, 8) and epidermal growth factor (EGF)-induced mitogenesis (9, 10). In primary rat hepatocytes, we previously demonstrated that the PI3-kinase, and not mitogen-activated protein kinase, pathway is necessary and sufficient to account for both insulin- and EGF-induced DNA synthesis (11).

Several reports show that insulin achieves activation of PI3-kinase through the recruitment of p85 to tyrosine-phosphorylated IRS-1 and IRS-2 (12–14). In the case of EGF, studies in different cell lines have identified several possible mechanisms leading to PI3-kinase activation. Thus, in mouse fibroblast cell line overexpressing human EGFR (NRH1ER5), immunoprecipitates of EGFR were shown to contain PI3-kinase activity (15, 16). In A431 cells and MDA-MB-468 breast cancer cell lines, tyrosine-phosphorylated erbB3, a member of the EGFR family, was implicated in the activation of PI3-kinase upon EGF stimulation (17, 18). In PC12 and A549 cells, p120
cbl was found to associate with both SH2 and SH3 domain of p85, leading to activation of PI3-kinase, upon EGF stimulation (19). Other studies have demonstrated that, in A431 cells, the recently cloned docking protein Grb2-associated binder 1 (Gab1) interacts with the p85 subunit of PI3-kinase following EGF (20). Another member of the Gab family, Gab2, was found to associate with p85 after treatment of hepatoma cells with erythropoietin (21) and interleukin-3 (22, 23).

The aim of this work was to clarify the mechanism of PI3-kinase activation following EGF stimulation of primary rat hepatocytes, a relevant physiological system. By overexpressing a dominant-negative p85, we confirmed the key role of PI3-kinase in EGF-induced DNA-synthesis. We next characterized three different p85-associated complexes generated by EGF treatment and identified Gab2 as a key molecule responsible for EGF-induced PI3-kinase activation in rat hepatocytes.

EXPERIMENTAL PROCEDURES

Materials—Porcine insulin was a gift from Lilly, and mouse EGF was obtained from Collaborative Biomedical Products (Bedford, MA). Collagenase was from Worthington. Cell culture medium and antibiotics were from Life Technologies, Inc. Vitrogen-100 was from Collagen Corp. (Toronto, Canada). [3H]methylythymidine, 125I-labeled goat anti-rabbit antibody, and 125I-labeled goat anti-mouse antibody were from ICN

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§ The abbreviations used are: PI3-kinase, phosphatidylinositol 3-kinase; EGF, epidermal growth factor; EGFR, EGF receptor; Gab1 and Gab2, Grb2-associated binder 1 and 2, respectively; Grb2, growth factor receptor bound 2; SHP-2, Ssrc homology 2 domain-containing protein-tyrosine phosphatase-2; SH2 and SH3, Src homology 2 and 3 domain, respectively; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; IRS, insulin receptor substrate; IP, immunoprecipitation; WB, Western blot.
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Biomedicals Canada Ltd. (Mississauga, Ontario, Canada). (γ-32P)ATP was purchased from PerkinElmer Life Sciences. Protein A-Sepharose was from Amersham Pharmacia Biotech. The Erythro-2 (Ad5) adenovirus was amplified in 293 cells and purified using a cesium chloride gradient method (25). It was kindly provided by Dr. John J. M. Bergeron (McGill University, Montreal, Quebec, Canada) by in situ liver perfusion with collagenase (protocol 4110) approved by McGill, were plated on a collagen matrix (Vitrogen-100). Cultures were prepared by seeding 1 × 10^6 cells onto 7.6-cm^2 dishes (Starstedt Canada, St. Laurent, Quebec, Canada). Cells were bathed for 24 h in seeding medium (Dulbecco’s modified Eagle’s medium/Ham’s F-12 containing 10% fetal bovine serum, 10 mM Hepes, 20 mM NaHCO3, 500 IU/ml penicillin, and 500 μg/ml streptomycin) and then for 48 h in serum-free medium that differed from the seeding medium in that it lacked fetal bovine serum and contained 1% ethanolamine, 2.5 × 10⁻⁹ M ethanolamine. In some experiments, treatment with adenosine was performed after cell attachment. Cells were infected with stocks of either recombinant (AdΔp85) or wild-type (AdΔE1/E23) adenovirus for 4 h at 37 °C. After viral exposure, wild type and Δp85-infected cells were serum-starved for 20 h in serum-free medium.

Tyrosine Phosphorylation Assay—After viral exposure, wild type and Δp85-infected cells were serum-starved for 20 h in serum-free medium, and then 100 nM insulin or EGF and [3H]thymidine (5 μCi/ml) was added to the medium. After an 18-h incubation, cells were rinsed twice with 3 ml of cold phosphate-buffered saline, incubated for 15 min at 4 °C in 10% trichloroacetic acid, solubilized at room temperature in 1 ml of 1 N NaOH, and then transferred to scintillation vials and counted for 3H.

Preparation of Cell Lysates—After treatment with the test agents for the time and concentration indicated in the figure legends, primary rat hepatocytes were rinsed twice with ice-cold phosphate-buffered saline (pH 7.4) and solubilized with lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 1.5 mM MgCl2, 1 mM EDTA, 200 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10% glycerol, and 1% Triton X-100). Cell lysates were centrifuged at 10,000 × g for 20 min at 4 °C, and protein concentrations in the resulting supernatants were determined using the Bio-Rad protein assay (27).

Phosphatase Activity Assay—Lysates (500 μg of protein) from EGF-treated (100 nM EGF for 1 min) or nontreated cells were pretreated with 1 μg/ml of Protein Phosphatase 1 (28) and 1 μg/ml of Protein Phosphatase 2A (29) for 15 min on ice. Immunoprecipitates were extensively washed, and the protein A-Sepharose pellets were resuspended in 50 μl of kinase assay buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.5 mM EDTA) containing 10 μg/ml l-aspartylphosphatidylinositol (Avanti Polar Lipids, Inc., Alabaster, AL) and assayed for PI3-kinase activity as described previously (11).

Immunoprecipitation and Immunoblotting—Before immunoprecipitation, lysates (1 μg of protein) from EGF-treated (100 nM EGF for 1 min) or nontreated cells were preclarified using rabbit IgG (Sigma) in the presence of protein A-Sepharose for 1 h at 4 °C. After centrifugation, the resulting supernatants were incubated with 2 μg/ml of the indicated antibody. Protein A-Sepharose was then added to each sample and incubated for an additional 1 h. The beads were collected by centrifugation, washed three times in lysis buffer, and boiled in Laemmli sample buffer. After separation on SDS-PAGE, immunoprecipitated proteins were transferred to Immobilon-P membranes (Millipore Ltd., Mississauga, Ontario, Canada). Immunoblots were probed with the indicated first antibody for 90 min followed by a 1-h incubation with 125I- or horseradish peroxidase-labeled goat anti-rabbit IgG except for the use of anti-Tyr(P), where the second antibody was 125I- or horseradish peroxidase-labeled goat anti-mouse IgG. Immunoreactive proteins were detected by autoradiography or by the ECL system (Amersham Pharmacia Biotech). Densitometric quantifications of the signals were performed using the Bio-Rad densitometer model GS-700. Immunodepletion Studies—Lysates (500 μg of protein) from EGF-treated cells (100 nM EGF for 1 min) were preclarified using rabbit IgG in the presence of protein A-Sepharose for 1 h at 4 °C. After centrifugation, the resulting supernatants were incubated overnight at 4 °C with the indicated antibody. Protein A-Sepharose was then added to each sample and incubated for an additional 1 h. After centrifugation, the immunodepleted supernatants were equally divided into two fractions. One fraction was immunoprecipitated in the presence of protein A-Sepharose with the antibody used for the immunodepletion. After separation on SDS-PAGE, immunoprecipitated proteins were transferred to Immobilon-P membranes and immunoblotted with the same antibody. The other fraction was immunoprecipitated with anti-phosphotyrosine antibodies in the presence of protein A-Sepharose, run on SDS-PAGE, transferred to membranes, and blotted with anti-Tyr(P) antibody. As control for nonimmunodepleted samples, lysates (150 μg of protein) from EGF-treated (100 nM EGF for 1 min) or nontreated cells were immunoprecipitated with anti-phosphotyrosine antibody after preclarification with rabbit IgG. Immunoprecipitated proteins were then detected by immunoblot with anti-Tyr(P) antibody.

RESULTS

Dominant Negative PI3-kinase (Δp85) Blocks Insulin- and EGF-induced DNA Synthesis in Primary Rat Hepatocytes—PI3-kinase activated by growth factors, including insulin and EGF, has been implicated in DNA synthesis in various cell lines (28). We have previously shown that the phosphatidylinositol 3-kinase pathway regulates DNA synthesis in response to insulin and EGF in primary rat hepatocyte cultures by using PI3-kinase inhibitors, wortmannin and LY294002 (11). To further confirm this result, we used a recombinant adenosine containing a cDNA encoding the p85 regulatory subunit, whose p110 binding region was deleted (25, 29, 30). Infection efficiency was assessed in cells transiently infected with recombinant adenosine by measuring Δp85 expression using a p85 antibody (Fig. 1A, right). The average expression of p85 was increased 9.3 ± 2.7-fold (mean ± S.E.) in cells infected with Δp85 recombinant adenovirus compared to cells infected only with the wild-type adenovirus. The effect of Δp85 expression was, in accordance with our study using the PI3-kinase pharmaceutical inhibitors (11), to induce completely basal as well as insulin- and EGF-stimulated DNA synthesis (Fig. 1B). This confirmed the critical role for PI3-kinase in medicating DNA synthesis in these cells.

Tyrosine-phosphorylated (PY) Proteins Play a Key Role in EGF-induced PI3-kinase Activity—The mechanism by which insulin stimulates PI3-kinase activity is well known (12–14). Since the mechanism for EGF is less clear, we sought to evaluate it in primary rat hepatocytes. Cells were stimulated at different times with 100 nM EGF, and tyrosine-phosphorylated proteins were immunoprecipitated using anti-Tyr(P) antibody (Fig. 2A). After immunoprecipitation, PI3-kinase activity (2A, solid circles) was measured as well as the amount of p85...
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Fig. 1. Dominant negative PI3-kinase (Δp85) blocks insulin- and EGF-induced DNA synthesis in primary rat hepatocytes. A, cell lysates, prepared from wild type (Mock) or Adeno-Δp85-infected primary rat hepatocytes, were subjected to SDS-PAGE (7.5% gel) followed by immunoblot analysis with an anti-p85 (op85) antibody. B, hepatocytes were infected with either wild type or adeno-Δp85 for 3 h and then starved for 24 h before a 20-h incubation in serum-free medium containing 5 and then starved for 24 h before a 20-h incubation in serum-free medium containing 5 μCi of [3H]methylthymidine without (hatched bars) or with 100 nM insulin (empty bars) or EGF (black bars). Incorporation of [3H]thymidine into DNA was determined as described under "Experimental Procedures." Results are expressed as the percentage of basal level (wild-type cells) (mean ± S.E., three separate experiments).

protein (Fig. 2A, open triangles) present in the Tyr(P) pellets. As shown, EGF augmented PI3-kinase activity in Tyr(P) immunoprecipitates and the association of p85 with particular PY proteins. The activation of PI3-kinase activity and the association of p85 with PY proteins were maximum at 30 s and then declined to basal levels by 10 min after EGF. To identify the PY proteins associated with p85, we subjected the immunoprecipitate generated with anti-p85 antibody to SDS-PAGE followed by immunoblotting with anti-Tyr(P) antibody as shown in Fig. 2B. Following EGF stimulation, three major PY protein bands were evident at 180, 105, and 52 kDa (Shc) bands were not affected by the SHP-2 immunodepletion. These results demonstrate that in primary rat hepatocytes, EGF treatment affects the association of p85 with SHP-2 immunoprecipitates after EGF treatment. With anti-Tyr(P) immunoblotting we detected four Tyr(P) bands (Fig. 2B, lane 4), and identified the 180-kDa protein as PY-EGFR, the 67-kDa protein as PY-EGFR, and the 52- and 46-kDa proteins as PY-Grb2 (data not shown). A PY protein, migrating at 105 kDa, was also detected in Grb2 immunoprecipitates of lysate from EGF-stimulated cells (compare lanes 3 and 4). A PY protein, migrating at 105 kDa, was also detected in Grb2 immunoprecipitates of lysate from EGF-stimulated cells (compare lanes 3 and 4). Since SHP-2 associates with She upon EGF treatment of hepatocytes (data not shown), the 52-kDa protein is probably PY-Shc.

The association of SHP-2 with the 105-kDa protein led us to determine whether this protein was the same p105 as that associated with p85. We therefore immunodepleted SHP-2 molecules by preadsorbing lysates from EGF-treated cells with SHP-2 antibody and then tested for the presence of PY-p105 in p85 immunoprecipitates. The efficiency of the SHP-2 immunodepletion was confirmed by demonstrating the full removal of SHP-2 proteins from the supernatant of SHP-2 immunoprecipitates (Fig. 3C, top). Using the immunodepleted SHP-2 supernatants (Fig. 3C, bottom), we showed that after anti-p85 immunoprecipitation and immunoblotting with Tyr(P) antibody, the intensity of the p105 band decreased to essentially the same level as observed in lysates from control immunodepleted cells (compare Basal and SN lanes). It is of interest to note that the intensities of the 180-kDa (ErbB3) and 52-kDa bands were not affected by the SHP-2 immunodepletion. These results demonstrate that in primary rat hepatocytes, EGF treatment affects the association of p85 with SHP-2 and p105.

EGF has been shown to induce the coupling of SHP-2 to Grb2 via the COOH-terminal SH3 domain of Grb2 (35). In addition, a direct association of Grb2 and p85, mediated by the SH3 domains of Grb2 and the proline-rich motifs of p85, has also been reported (36). We therefore evaluated the extent of Grb2 association with SHP-2 and p85. Lysates from control and EGF-stimulated cells were immunoprecipitated with anti-Grb2 antibody and subjected to anti-SHP-2, anti-p85, and anti-Grb2 immunoblotting. Grb2 was shown to associate with SHP-2 (Fig. 4A, top) and p85 (Fig. 4A, middle), and this was increased following EGF stimulation. These results indicated that Grb2 might engage in the complex of p85 with SHP-2 and p105. We thus assessed which PY proteins are present in Grb2 immunoprecipitates after EGF treatment. With anti-Tyr(P) immunoblotting we detected four Tyr(P) bands (Fig. 4B, lane 4), and identified the 180-kDa protein as PY-EGFR, the 67-kDa protein as PY-EGFR, and the 52- and 46-kDa proteins as PY-Grb2 (data not shown). A PY protein, migrating at 105 kDa, was also detected in Grb2 immunoprecipitates of lysate from EGF-stim-

The Association of p105 with p85, SHP-2, and Grb2 following EGF Treatment—Using specific antibodies, we found that the PY180 species was largely accounted for by ErbB3, a member of the EGFR protein family (31), and that PYS2 was accounted for by Shc, an adapter protein tyrosine-phosphorylated in response to EGF (32, 33). Neither of these species associated with p105 (data not shown). Since the time course of tyrosine phosphorylation of the 105-kDa protein (Fig. 2C, middle) followed the same pattern as total EGF-induced PI3-kinase activity (anti-Tyr(P) (αPY) immunoprecipitates, Fig. 2A) by declining to basal levels 10 min after exposure to EGF. These results demonstrate that EGF-induced PI3-kinase activation correlates with the association of p85 with three PY proteins, among which the one migrating at 105 kDa showed the best correlation.

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ulated cells. Immunodepletion of Grb2 from the lysates was performed to determine if the p105 protein present in the anti-p85 immunoprecipitates was the same as that associated with Grb2. As with the SHP-2 study, the efficiency of the immunodepletion was confirmed by immunoblotting the anti-Grb2 supernatants (Fig. 4C, top panel). It can be seen that the level of p105 is barely detectable in anti-p85 immunoprecipitates after Grb2 immunodepletion, whereas the level of the 46–52-kDa band (corresponding to Shc) was unaffected (Fig. 4C, second panel).

To determine if SHP-2 is part of the same complex with Grb2, the membranes used to detect PY proteins after Grb2 immunodepletion were stripped and reblotted with either anti-SHP2 or anti-p85. It can be seen that immunodepletion of Grb2 leads to the disappearance of SHP-2 (Fig. 4C, third panel), indicating association between them. Our results show that EGF stimulation of hepatocytes leads to the formation of a unique complex containing Grb2, p85, SHP-2, and p105.

**EGF-induced PI3-kinase Activity Associated with p105**—We next sought to evaluate the proportion of total EGF-activated PI3-kinase that associated with the p105-containing complex. Hepatocytes were treated with or without 100 nM EGF for 1 min, and the lysates were subjected to immunoprecipitation with the appropriate antibodies to generate the above noted complexes in which PI3-kinase activity was measured (Fig. 5).

Quantitation by scanning densitometry revealed that ErbB3- and Shc-associated PI3-kinase activity represented less than 10% of the PI3-kinase activity in Tyr(P) immunoprecipitates (data not shown). More than 80% of total EGF-induced PI3-kinase activity of rat hepatocytes was found in anti-SHP2 or anti-Grb2 immunoprecipitates, indicating that the complex formed by p105, p85, SHP-2, and Grb2 is the major one in

![Graph](image)

**Fig. 2.** EGF-stimulated PI3-kinase activity correlates with the association of p85 with tyrosine-phosphorylated proteins. A, serum-deprived hepatocytes were treated with 100 nM EGF for the indicated times. Cell lysates were immunoprecipitated with an anti-Tyr(P) (αPY) antibody. The immunoprecipitated proteins were analyzed for PI3-kinase activity (solid circles) or for p85 content using immunoblot analysis with αp85 antibody (open triangles). Results are expressed as percentage of maximum values obtained for each analysis (mean ± half the range, two separate experiments). B, hepatocytes were treated with or without 100 nM EGF for 1 min. Cell lysates were immunoprecipitated with αp85, resolved by 7.5% SDS-PAGE, and subjected to immunoblotting with αTyr(P). The three major bands (PY180, PY105, and PY52) are indicated. C, hepatocytes were treated with 100 nM EGF for the indicated times. Cell lysates were processed as described in A. The graphs represent quantification of the three major bands as described under “Experimental Procedures.” Results are expressed as percentage of maximum tyrosine phosphorylation (mean ± S.E., three separate experiments).
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Fig. 3. SHP-2 is a part of the complex formed by p85 and PY105. Hepatocytes were treated with (+) or without (−) 100 nM EGF for 1 min. A, proteins, immunoprecipitated with anti-ErbB3 (αErbB3), p85, anti-EGFR (αEGFR), or anti-SHP-2 (αSHP-2), were resolved on 7.5% SDS-PAGE and immunoblotted with αSHP-2 (top) or αp85 (bottom). B, proteins, immunoprecipitated with αp85 or αSHP-2 and resolved on 7.5% SDS-PAGE were subjected to immunoblotting with αTyr(P). C, EGF-treated cell lysates were preadsorbed on protein A-Sepharose beads with αSHP-2. The pellet (PT) and supernatant (SV) were prepared by centrifugation, and the latter was subjected to immunoprecipitation with αSHP-2. The pellet and the SN immunoprecipitate were resolved on 7.5% SDS-PAGE and subjected to immunoblotting with αSHP-2 (top). The immunodepleted SN and lysates of cells with or without EGF were immunoprecipitated with αp85, run on 7.5% SDS-PAGE, and immunoblotted with αTyr(P) (bottom).

Grb2-associated PI3-kinase activity was totally abolished in hepatocytes overexpressing Δp85. Taken together, our data demonstrate the important role of p105 as a docking protein for EGF-induced PI3-kinase activation.

Identification of the p105 Protein as Gab2—p105 behaves like a member of the Gab family of adapter proteins, which have been shown to become tyrosine-phosphorylated and associated with p85, SHP-2, and Grb2 in response to several kinds of cytokines or growth factors (20, 24, 37). Using immunoblotting, we showed that Gab1 is not expressed in primary rat hepatocytes (Fig. 6A, top), consistent with previous determinations of Gab1 distribution using Northern blotting to measure mRNA levels (20). Recently, Gab2, a Gab1 isoform, was cloned from human and mouse cDNA libraries (23, 24). Using degenerate oligonucleotides, whose sequences corresponded to conserved regions between mice and humans (one in the pleckstrin homology domain and other in the c-Met binding domain), we performed reverse transcriptase-PCR using rat hepatocyte mRNA and cloned the full-length rat cDNA (GenBank™ accession number AF230367). At the amino acid level, the rat clone exhibited 96.5 and 93% identity with the mouse and human Gab2, respectively. Taken together, our data suggest that Gab2, like Gab1, is a Gab family member that is differentially expressed in hepatocytes.
human Gab2, respectively, indicating that the isolated clone encodes a rat Gab2 protein. As with human and mouse, rat Gab2 has many functional domains, in particular the binding motifs for Grb2, Crk, PI3-kinase, and SHP-2, the pleckstrin homology domain (97 and 95% with mouse and human, respectively), and also a region similar to the c-Met binding domain of Gab1 (23).

By the time we had completed the cloning, a commercial antibody raised against the human Gab2 protein became available. Using this antibody, we showed that Gab2 is indeed expressed in primary rat hepatocytes (Fig. 6A, bottom) and confirmed that EGF stimulation produces PY-Gab2 (Fig. 6B, first panel). We also demonstrated that Gab2 associates with p85 (Fig. 6B, second panel), SHP-2 (Fig. 6B, third panel), and Grb2 (Fig. 6B, fourth panel) in an EGF-dependent manner. Immunodepletion studies confirmed that p105 is Gab2. EGF-treated cell lysates were preadsorbed by Gab2 antibody, and the amount of p105 and Gab2 associated with p85 in the residual supernatants was tested. Both Gab2 and p105 decreased by about 70% after an initial immunoprecipitation with Gab2 (Fig. 6C), indicating that the p105 protein is largely if not completely identical to tyrosine-phosphorylated Gab2. In addition, we found that Gab2-associated PI3-kinase activity was totally abolished by Δp85 (Fig. 6D).

Taken together, our data suggest an important role for PI3-kinase in EGF-induced DNA synthesis in primary rat hepatocytes. Activated PI3-kinase was shown to be largely associated with a complex constituted by Gab2, SHP-2, and Grb2. We also demonstrated the presence of two other complexes in which PI3-kinase associated with ErbB3 and Shc, respectively (Fig. 7).

**Fig. 6. Identification of Gab2 as the PY105 protein.** A, hepatocytes were treated with (+) or without (−) 100 nM EGF for 1 min. Proteins, immunoprecipitated with anti-Gab1 (αGab1) or anti-Gab2 (αGab2), were resolved on 7.5% SDS-PAGE and subjected to immunoblotting with αGab1 (top) or αGab2 (bottom). Lysate (20 μg of protein) from nonstimulated A431 or HeLa cells was a positive control (PC) for Gab1 or Gab2, respectively. B, proteins, immunoprecipitated with αGab2, were resolved on 10% SDS-PAGE and immunoblotted with αTyr(P) (first panel), αp85 (second panel), αSHP-2 (third panel), or αGrb2 (fourth panel). C, lysates from EGF-treated cells were incubated with αGab2 (to effect immunodepletion) or with normal IgG as described under “Experimental Procedures.” The supernatants from these incubations were further incubated with (upper left panel) or without (upper right panel) αp85. The supernatants from this latter incubation along with the original supernatants were subjected to 7.5% SDS-PAGE followed by immunoblotting with αTyr(P) (upper left panel) or αGab2 (upper right panel). The bar graph (bottom panel) represents quantification of the upper bands. Control refers to the bands obtained from supernatants not immunoprecipitated with αGab2. The data are expressed as a percentage of control values obtained for each analysis (mean ± S.E., four separate experiments). D, hepatocytes were infected with (+) or without (−) adeno-Δp85 for 3 h, serum-deprived for 48 h, and then treated with (+) or without (−) 100 nM EGF for 1 min. Cell lysate proteins were immunoprecipitated with αGab2 and analyzed for PI3-kinase activity as described under “Experimental Procedures.”

**Fig. 7. Scheme depicting PY protein complexes formed in rat hepatocytes following EGF treatment.** PM, plasma membrane; Cyt, cytosol.

**DISCUSSION**

Using pharmacologic inhibitors of PI3-kinase and mitogen-activated protein kinase activation, we and others have demonstrated that the former and not latter pathway is both necessary and sufficient for insulin- and EGF-induced DNA synthesis (11, 38–40). However, inhibitors may not always be sufficiently specific to warrant precise conclusions. Hence,
wirtzmann has also been shown to inhibit phosphatidylinositol 4-kinase as well as PI3-kinase (41). Using a dominant negative p85, in which the p110 binding site was deleted, we showed that both insulin- and EGF-induced DNA synthesis are totally abrogated (Fig. 1), confirming the essential role of PI3-kinase in mediating this effect in rat hepatocytes.

Two general mechanisms for the recruitment and activation of PI3-kinase by growth factor receptors have been described in different transformed cell lines. The first involves the direct binding of the p85 regulatory subunit of PI3-kinase to PY receptor tyrosine kinases such as the platelet-derived growth factor (42), colony-stimulating factor-1 (43) and c-Met receptors (44). The second mechanism comprises the recruitment to and activation of p85 by substrates of receptor tyrosine kinases (i.e. PY docking proteins) such as the IRS (12–14) and Gab protein families (20). The mechanism by which PI3-kinase becomes activated upon insulin stimulation has been well characterized (12–14); however, the events consequent to EGF stimulation have been less clearly defined. As noted above, EGF activates PI3-kinase through recruitment to a range of PY proteins in a cell line-specific manner. In this paper, we demonstrate that PY proteins, especially that migrating at 105 kDa, play a key role in EGF-induced PI3-kinase activity in rat hepatocytes, a physiologically relevant system. Further analysis demonstrated that, upon EGF treatment, three distinct p85-associated complexes were formed, with the one containing the PY 105-kDa protein (identified as Gab2; Fig. 6) accounting for a large proportion of the activated PI3-kinase generated by EGF stimulation (Fig. 5).

This study is the first to demonstrate that in rat hepatocytes EGF stimulates the formation of a complex composed of SHP-2, p105, p85, and Grb2, which accounts for over 80% of total EGF-induced PI3-kinase activity (Fig. 5).

Based on our immunodepletion studies (Fig. 6), we conclude that PY105 is largely if not completely accounted for by Gab2. Gab2, a 100-kDa protein, has recently been cloned from mouse and human tissues (22, 23). It shows high homology with Gab1, a previously identified docking protein involved in growth factor signaling (45–47). Gab2 contains an N-terminal pleckstrin homology domain and proline-rich sequences as well as consen-

The function of ErbB3/p85-containing complex is unclear. Analysis of PI3-kinase activity in ErbB3 immune-precipitates shows that it is less than 10% of the total EGF-induced PI3-kinase activity present in the Tyr(P) immune-precipitates (data not shown). Since the association of p85 to ErbB3 does not lead to activation of a significant proportion of the PI3-kinase pool, we suggest that its main role might be to recruit p85 to the plasma membrane, in which compartment it may play a selective role. PY 52- and 46-kDa isoforms of Shc also associate with p85 in response to EGF in primary rat hepatocytes. As with ErbB3, this p85/Shc association, measured in Shc immunoprecipitates, represents less than 10% of the total PI3-kinase activation effected by EGF (data not shown). It also remains to be determined whether this association is direct or is via other proteins. However, we cannot see the association of ErbB3 with Shc, which suggests that p85 formed a complex with Shc distinct from that formed with ErbB3.

In summary, we have demonstrated the critical role played by the recruitment of p85 to PY proteins in mediating EGF-induced PI3-kinase activity. We have identified three distinct p85-associated complexes that form in primary rat hepatocytes in response to EGF (Fig. 7). One complex contained ErbB3 and p85; the second contained p85 and Shc; and the third contained p85, Gab2, SHP-2, and Grb2. The last complex accounted for most EGF-induced PI3-kinase activation in rat hepatocytes. These findings point to a key role for Gab2 in effecting EGF-dependent biological functions such as mitogenesis.

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