

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)¹ is an enzyme

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¹ 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, Src 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.

that phosphorylates the D-3 position of the inositol ring of PI to produce three novel phosphoinositides: phosphatidylinositol 3-monophosphate, phosphatidylinositol 3,4-bisphosphate, 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 (NRHER5), 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^{cb1} 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 hemopoietic 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]methylthymidine, [¹²⁵I]-labeled goat anti-rabbit antibody, and [¹²⁵I]-labeled goat anti-mouse antibody were from ICN

Biomedicals Canada Ltd. (Mississauga, Ontario, Canada). [γ - 32 P]ATP was purchased from PerkinElmer Life Sciences. Protein A-Sepharose was from Amersham Pharmacia Biotech. The Tyr(P) (PY99), EGFR (for immunoblotting), SHP-2, and Grb2 antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The PI3-kinase p85 and Gab2 (for immunoblotting) antibodies were from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-Gab2 antibodies (for immunoprecipitation studies) were generous gifts of Dr. Gen-Sheng Feng (Burnham Institute, La Jolla, CA) (22) and Dr. Haihua Gu (Harvard Medical School, Boston, MA) (24). The anti-EGFR antibody (for immunoprecipitation studies) was kindly provided by Dr. John J. M. Bergeron (McGill University, Montreal). The pShuttle-CMV and pAdEasy-2 vectors were generously provided by Dr. Bert Vogelstein (Howard Hughes Medical Institute, Baltimore, MD). All other reagents were obtained from Sigma and were of the highest grade available.

Preparation of a Dominant Negative p85 Mutant—Previous work has shown that the p85 deletion mutant lacking the p110 binding site works as a dominant negative regulator for wild-type p85 in competing for upstream docking proteins (25). The mutant p85(Δ 478–513) was constructed as described previously (25). Briefly, the 478–513 coding region, corresponding to the p110 binding domain, was deleted from the wild-type p85 (a generous gift from Dr. Thomas Franke, Columbia University, NY) using PCR. Two PCR fragments (P1 and P2) were generated. P1 encompassed nucleotides 1–1431 and had a *Bgl*III site introduced at the 5'-end. P2 encompassed nucleotides 1542–2172 and had 30 bases complementary to the 3'-end of P1 product introduced at the 5'-end. After purification, the two PCR fragments were mixed, denatured, and reannealed. Using primers at each end, the mutated p85 was then reamplified by PCR and subcloned into pShuttle-CMV vector digested by *Bgl*III and *Eco*RV. The pShuttle-CMV- Δ p85 plasmid was then mixed with pAdEasy-1 vector to prepare recombinant adenovirus as described previously (26).

Cell Culture—Primary hepatocytes, isolated from 160–180-g male Harlan Sprague-Dawley rats (Charles River, St. Constant, 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 $9.6\text{-cm}^2 \times 6$ -well plates (Corning Costar Corp., Cambridge, MA) or seeding 5×10^6 cells onto 78-cm 2 culture 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 NaHCO $_3$, 500 IU/ml penicillin, and 500 μ g/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 1.25 μ g/ml Fungizone, 0.4 mM ornithine, 2.25 μ g/ml L-lactic acid, 2.5×10^{-8} M selenium, and 1×10^{-8} M ethanolamine. In some experiments, treatment with adenovirus was performed after cell attachment. Cells were infected with stocks of either recombinant (Δ p85) or wild-type (Δ E1/ Δ E3) adenovirus for 4 h at 37 °C. After viral exposure, wild type and Δ p85-infected cells were serum-starved for 20 h in serum-free medium.

[3 H]Thymidine Incorporation Assay—After viral exposure, wild type and Δ p85-infected cells were serum-starved for 20 h in serum-free medium, and then 100 nM insulin or EGF and [3 H]thymidine (5 μ Ci/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 3 H.

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 MgCl $_2$, 1 mM EGTA, 200 μ M sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 10% glycerol, and 1% Triton X-100). Cell lysates were clarified by centrifugation at $10,000 \times g$ for 20 min at 4 °C, and protein concentrations in the resulting supernatants were determined using the Bio-Rad protein assay (27).

PI3-kinase Activity Assay—Lysates (500 μ g of protein) from EGF-treated (100 nM EGF for 1 min) or nontreated cells were immunoprecipitated in the presence of protein A-Sepharose, using different antibodies as indicated in the figure legends (Figs. 5 and 6). Immunoprecipitates were extensively washed, and the protein A-Sepharose pellets were resuspended in 50 μ l of kinase assay buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.5 mM EGTA) containing 0.5 mg/ml L- α -phosphatidylinositol (Avanti Polar Lipids, Inc., Alabaster,

AL) and assayed for PI3-kinase activity as described previously (11).

Immunoprecipitation and Immunoblotting—Before immunoprecipitation, lysates (1 mg of protein) from EGF-treated (100 nM EGF for 1 min) or nontreated cells were precleared using rabbit IgG (Sigma) in the presence of protein A-Sepharose for 1 h at 4 °C. After centrifugation, the resulting supernatants were incubated for 2 h at 4 °C with 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). For immunoblotting, membranes were probed with the indicated first antibody for 90 min followed by a 1-h incubation with 125 I- or horseradish peroxidase-labeled goat anti-rabbit IgG except for the use of anti-Tyr(P), where the second antibody was 125 I- 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 μ g of protein) from EGF-treated cells (100 nM EGF for 1 min) were precleared 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-p85 antibody 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 μ g of protein) from EGF-treated (100 nM EGF for 1 min) or nontreated cells were immunoprecipitated with anti-p85 antibody after preclearing with rabbit IgG. Immunoprecipitated proteins were then detected by immunoblot with anti-Tyr(P) antibody.

RESULTS

Dominant Negative PI3-kinase (Δ p85) 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 adenovirus 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 adenovirus by measuring Δ p85 expression using a p85 antibody (Fig. 1A, right). The average expression of p85 was increased 9.3 ± 2.7 -fold (mean \pm S.E.) in cells infected with Δ p85 recombinant adenovirus compared with cells infected only with the wild-type adenovirus. The effect of Δ p85 expression was, in accordance with our study using the PI3-kinase pharmaceutical inhibitors (11), to inhibit completely basal as well as insulin- and EGF-stimulated DNA synthesis (Fig. 1B). This confirmed the critical role for PI3-kinase in mediating 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 (Fig. 2A, solid circles) was measured as well as the amount of p85

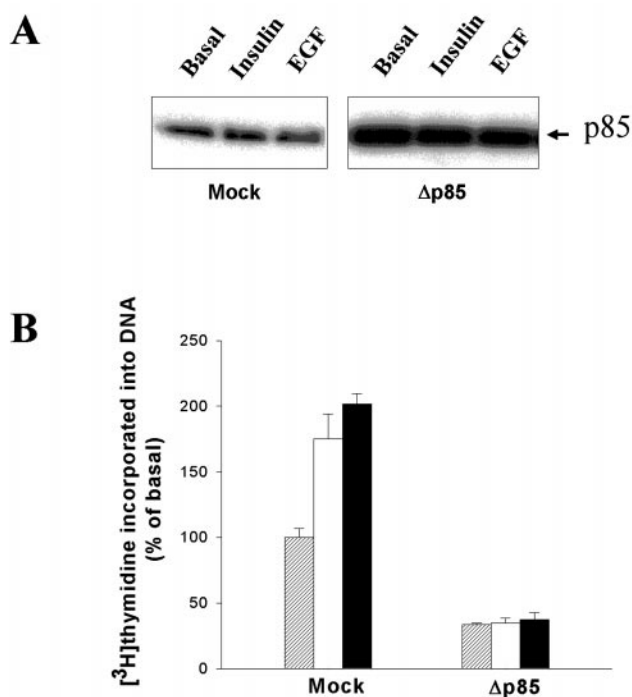


FIG. 1. Dominant negative PI3-kinase (Δ p85) blocks insulin- and EGF-induced DNA synthesis in primary rat hepatocytes. *A*, cell lysates, prepared from wild type (*Mock*) or Adeno- Δ p85-infected primary rat hepatocytes, were subjected to SDS-PAGE (7.5% gel) followed by immunoblot analysis with an anti-p85 (α p85) antibody. *B*, hepatocytes were infected with either wild type or adeno- Δ p85 for 3 h and then starved for 24 h before a 20-h incubation in serum-free medium containing 5 μ Ci 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 \pm 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 (a minor band at 46-kDa was also seen) as indicated in Fig. 2B. The time course of p85 association with these PY proteins was assessed (Fig. 2C). Our results show that the tyrosine phosphorylation of each band reached a maximum level at 30 s and then declined to basal level. Only the tyrosine phosphorylation of the 105-kDa protein (Fig. 2C, *middle*) followed the same pattern as total EGF-induced PI3-kinase activity (anti-Tyr(P) (α PY) 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.

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 PY52 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 phosphoryl-

ation of p105 best correlated with the time course of EGF-induced PI-3 kinase activation (Fig. 2C), we examined in detail the EGF-induced complex formed by p105 and p85 in primary rat hepatocytes. In agreement with an earlier report (34), we found that the association of SHP-2 with p85 increased 4–5-fold after EGF stimulation (Fig. 3A, *top and bottom*, compare *lanes 3 and 4*). Interestingly, we also found that SHP-2 associated with EGFR (*top panel, lanes 5 and 6*) but not with ErbB3 (*top panel, lanes 1 and 2*). Since SHP-2 associated with p85, we determined if it was part of the complex formed by p85 and p105 by assessing which EGF-induced PY proteins are present in SHP-2 immunoprecipitates. Following EGF treatment (Fig. 3B, *lane 4*) four PY proteins were detected in SHP-2 immunoprecipitates with molecular masses of ~180, 105, 67, and 52 kDa. It is clear that SHP-2 associates with the EGFR (Fig. 3A, *lanes 5 and 6*). The band detected at 180 kDa in the SHP-2 immunoprecipitates (Fig. 3B, *lane 4*) thus corresponds to PY-EGFR. The 67-kDa protein corresponds to PY-SHP-2 (data not shown), which was not readily detected in p85 immunoprecipitates (Fig. 3B, *lane 2*) due to the smaller quantity of SHP-2 present in the latter than in SHP-2 immunoprecipitates (Fig. 3A, compare *top, lanes 7 and 8*, with *bottom, lanes 3 and 4*). A band migrating at 105 kDa, as in anti-p85 immunoprecipitates, is observed in both control and EGF-stimulated hepatocytes (compare *lane 2* with *lanes 3 and 4*). Since SHP-2 associates with Shc 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 anti-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 nonimmunodepleted cells (compare *Basal* and *SN lanes*). It is of interest to note that the intensities of the 180-kDa (ErbB3) 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 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-SHP-2, and the 52- and 46-kDa proteins as PY-Shc (data not shown). A PY protein, migrating at 105 kDa, was also detected in Grb2 immunoprecipitates of lysate from EGF-stim-

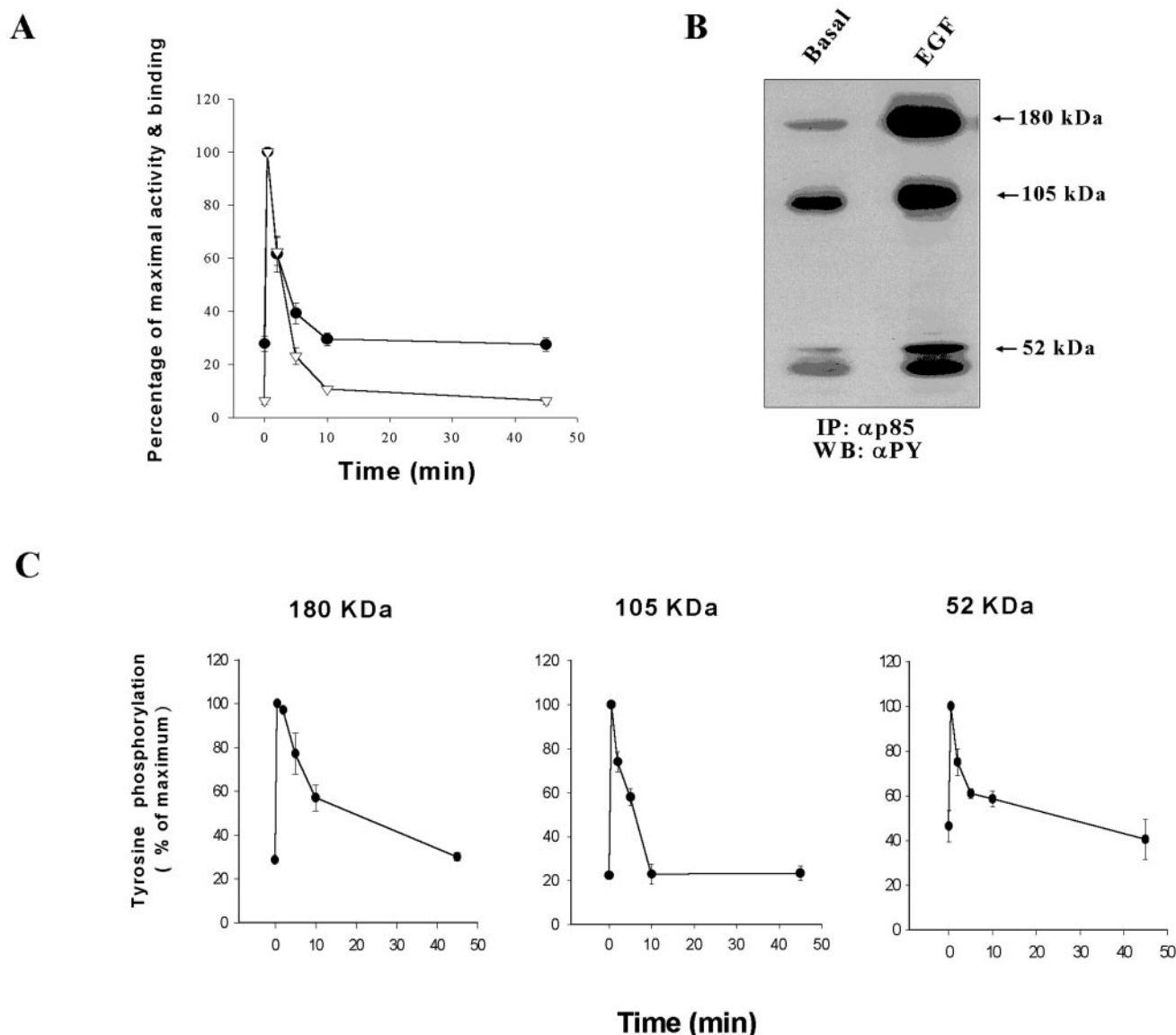


FIG. 2. EGF-stimulated PI3-kinase activity correlates with the association of p85 with tyrosine-phosphorylated proteins. *A*, serum-deprived hepatocytes were treated with 100 nM EGF for the indicated times. Cell lysates were immunoprecipitated with an anti-Tyr(P) (α PY) antibody. The immunoprecipitated proteins were analyzed for PI3-kinase activity (solid circles) or for p85 content using immunoblot analysis with α p85 antibody (open triangles). Results are expressed as percentage of maximum values obtained for each analysis (mean \pm half the range, two separate experiments). *B*, hepatocytes were treated with or without 100 nM EGF for 1 min. Cell lysates were immunoprecipitated with α p85, resolved by 7.5% SDS-PAGE, and subjected to immunoblotting with α Tyr(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 \pm S.E., three separate experiments).

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

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 (α ErbB3), α p85, anti-EGFR (α EGFR), or anti-SHP-2 (α SHP-2), were resolved on 7.5% SDS-PAGE and immunoblotted with α SHP-2 (*top*) or α p85 (*bottom*). *B*, proteins, immunoprecipitated with α p85 or α SHP-2 and resolved on 7.5% SDS-PAGE were subjected to immunoblotting with α Tyr(P). *C*, EGF-treated cell lysates were preadsorbed on protein A-Sepharose beads with α SHP-2. The pellet (PT) and supernatant (SN) were prepared by centrifugation, and the latter was subjected to immunoprecipitation with α SHP-2. The pellet and the SN immunoprecipitate were resolved on 7.5% SDS-PAGE and subjected to immunoblotting with α SHP-2 (*top*). The immunodepleted SN and lysates of cells with and without EGF were immunoprecipitated with α p85, run on 7.5% SDS-PAGE, and immunoblotted with α Tyr(P) (*bottom*).

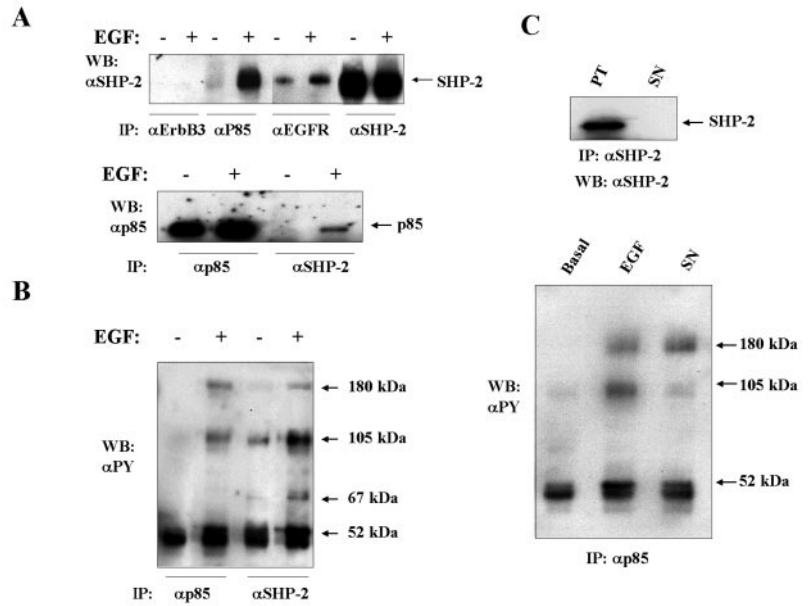


FIG. 4. Grb2 is part of a complex formed by p85, PY105, and SHP-2. Hepatocytes were treated with (+) or without (-) 100 nM EGF for 1 min. *A*, proteins immunoprecipitated with anti-Grb2 (α Grb2) were resolved on 10% SDS-PAGE and immunoblotted with α SHP-2 (*top panel*), α p85 (*middle panel*), or α Grb2 (*bottom panel*). *B*, proteins immunoprecipitated with α p85 or α Grb2 were resolved on 7.5% SDS-PAGE and immunoblotted with α Tyr(P). *C*, EGF-treated cell lysates were preadsorbed on protein A-Sepharose beads with α Grb2. The pellet (PT) and supernatant (SN) were prepared by centrifugation, and the latter was subjected to immunoprecipitation with α Grb2. The pellet and the supernatant immunoprecipitate were resolved on 10% SDS-PAGE and subjected to immunoblotting with α Grb2 (*top panel*). The immunodepleted SN and lysates of cells with or without EGF were immunoprecipitated with α p85, run on 7.5% SDS-PAGE, and immunoblotted with α Tyr(P) (*middle panel*), and the stripped membranes were reblotted with α SHP-2 or α p85 (*bottom panel*).

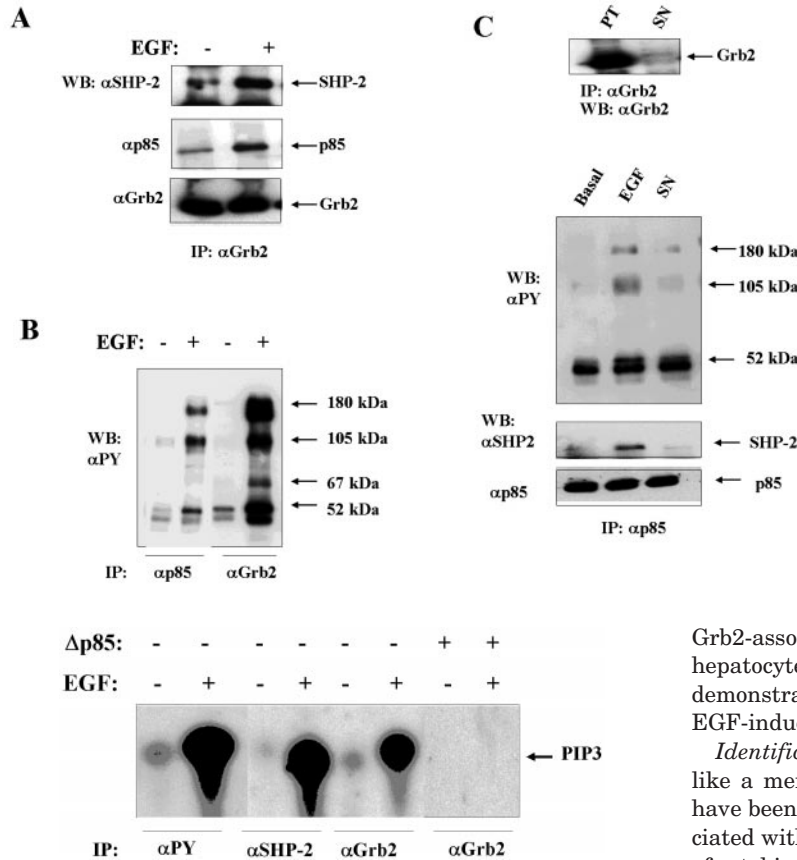


FIG. 5. A complex of p105 with p85, SHP-2, and Grb2 is the major species responsible for EGF-induced PI3-kinase activation. Hepatocytes were infected with (+) or without (-) adeno- Δ p85 for 3 h, serum-deprived for 48 h, and then treated with (+) or without (-) 100 nM EGF for 1 min. Cell lysate proteins were immunoprecipitated with α SHP-2, α Grb2, or α Tyr(P) and analyzed for PI3-kinase activity as described under "Experimental Procedures." The arrow indicates the location of the reaction product, phosphatidylinositol-3-phosphate (PIP3).

which EGF-induced PI3-kinase activity is concentrated. To establish the significance of the p105 complex for EGF-induced DNA synthesis, we measured Grb2-associated PI3-kinase activity in lysates from cells infected with Δ p85. As seen in Fig. 5,

Grb2-associated PI3-kinase activity was totally abolished in hepatocytes overexpressing Δ p85. 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

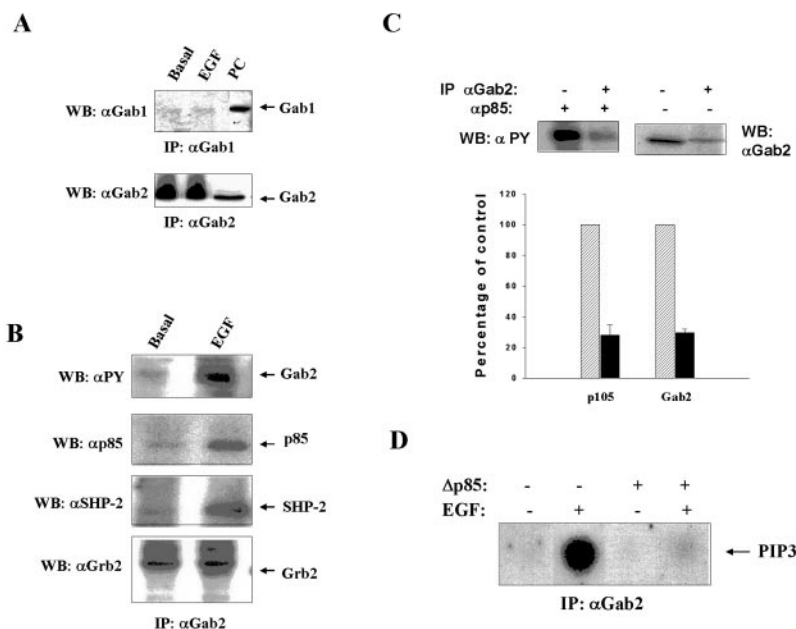


FIG. 6. Identification of Gab2 as the PY105 protein. *A*, hepatocytes were treated with (+) or without (-) 100 nM EGF for 1 min. Proteins, immunoprecipitated with anti-Gab1 (α Gab1) or anti-Gab2 (α Gab2), were resolved on 7.5% SDS-PAGE and subjected to immunoblotting with α Gab1 (*top*) or α Gab2 (*bottom*). Lysate (20 μ g of protein) from nonstimulated A431 or HeLa cells was a positive control (PC) for Gab1 or Gab2, respectively. *B*, proteins, immunoprecipitated with α Gab2, were resolved on 10% SDS-PAGE and immunoblotted with α Tyr(P) (*first panel*), α p85 (*second panel*), α SHP-2 (*third panel*), or α Grb2 (*fourth panel*). *C*, lysates from EGF-treated cells were incubated with α Gab2 (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*) α p85. The supernatants from this latter incubation along with the original supernatants were subjected to 7.5% SDS-PAGE followed by immunoblotting with α Tyr(P) (*upper left panel*) or α Gab2 (*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 α Gab2. The data are expressed as a percentage of control values obtained for each analysis (mean \pm S.E., four separate experiments). *D*, hepatocytes were infected with (+) or without (-) adeno- Δ p85 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 α Gab2 and analyzed for PI3-kinase activity as described under "Experimental Procedures."

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 Δ p85 (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).

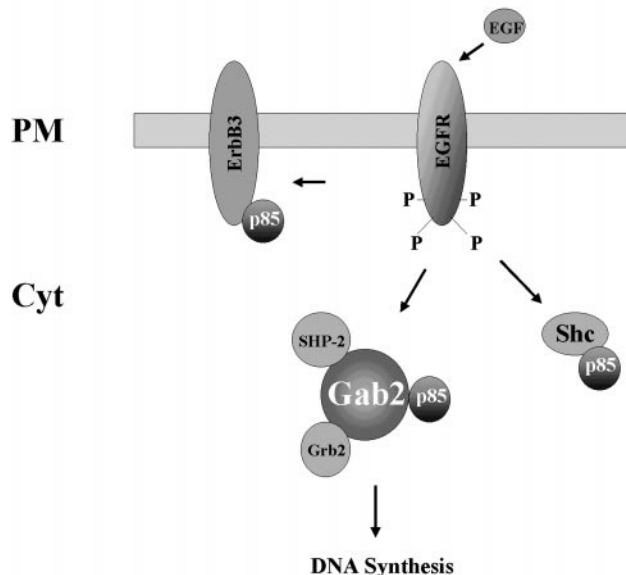


FIG. 7. Scheme depicting PY protein complexes formed in rat hepatocytes following EGF treatment. *PM*, plasma membrane; *Cyt*, cytosol.

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,

wortmannin 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 consensus PI 3-kinase, SHP-2, Grb2, and Crk tyrosine binding sites (23, 24). The 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). However, the direct association of Grb2 and p85 in rat hepatocytes still needs to be established. Although SHP-2 was observed in anti-p85 immunoprecipitates in various cells, no direct association of SHP-2 and p85 has been reported. Rather, this association was probably mediated via a docking protein(s) (48), such as Gab1 (49) and Gab2 (24). Indeed, on the basis of its similarity to Gab1 and homology with other docking molecules such as the IRSs (50), it is likely that Gab2 works as a platform for the signaling complex consisting of p85, SHP-2, and Grb2. Our data also showed that dominant negative p85 fully inhibited both Grb2 and Gab2-associated PI3-kinase as well as the EGF-induced DNA synthesis. Taken together, these results argue for a critical role for the Gab2 protein in EGF-induced PI3-kinase activation and DNA synthesis in primary rat hepatocytes.

Several reports have shown that, in hematopoietic cells, different stimuli (*viz.* interleukin-2, interleukin-3, and M-colony-stimulating factor) promote the association of PY proteins (95–110 kDa) with SHP-2, p85, and Grb2 (34, 48, 51–54). These reports are consistent with the possibility that the 95–110-kDa protein(s) is Gab2, which associates with p85, Grb2, and SHP-2 to play an important role in cytokine- and growth factor-regulated cell proliferation and differentiation.

The function of ErbB3/p85-containing complex is unclear. Analysis of PI3-kinase activity in ErbB3 immunoprecipitates shows that it is less than 10% of the total EGF-induced PI3-kinase activity present in the Tyr(P) immunoprecipitates (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.

REFERENCES

- Auger, K. R., Serunian, L. A., Soltoff, S. P., Libby, P., and Cantley, L. C. (1989) *Cell* **57**, 167–175
- Whitman, M., Downes, C. P., Keeler, M., Keller, T., and Cantley, L. (1988) *Nature* **332**, 644–646
- Carpenter, C. L., Duckworth, B. C., Auger, K. R., Cohen, B., Schaffhausen, B. S., and Cantley, L. C. (1990) *J. Biol. Chem.* **265**, 19704–19711
- Escobedo, J. A., Navankasattusas, S., Kavanaugh, W. M., Milfay, D., Fried, V. A., and Williams, L. T. (1991) *Cell* **65**, 75–82
- Booker, G. W., Breeze, A. L., Downing, A. K., Panayotou, G., Gout, I., Waterfield, M. D., and Campbell, I. D. (1992) *Nature* **358**, 684–687
- Holt, K. H., Olson, L., Moye-Rowley, W. S., and Pessin, J. E. (1994) *Mol. Cell. Biol.* **14**, 42–49
- Jhun, B. H., Rose, D. W., Seely, B. L., Rameh, L., Cantley, L., Saltiel, A. R., and Olefsky, J. M. (1994) *Mol. Cell. Biol.* **14**, 7466–7475
- Cheatham, B., Vlahos, C. J., Cheatham, L., Wang, L., Blenis, J., and Kahn, C. R. (1994) *Mol. Cell. Biol.* **14**, 4902–4911
- Derossi, D., Williams, E. J., Green, P. J., Dunican, D. J., and Doherty, P. (1998) *Biochem. Biophys. Res. Commun.* **251**, 148–152
- Roche, S., Koegl, M., and Courtneidge, S. A. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 9185–9189
- Band, C. J., Mounier, C., and Posner, B. I. (1999) *Endocrinology* **140**, 5626–5634
- Backer, J. M., Myers Jr., M. G., Shoelson, S. E., Chin, D. J., Sun, X. J., Miralpeix, M., Hu, P., Margolis, B., Skolnik, E. Y., and Schlessinger, J. (1992) *EMBO J.* **11**, 3469–3479
- Hadari, Y. R., Tzahar, E., Nativ, O., Rothenberg, P., Roberts, C. T., LeRoith, D., Jr., Yarden, Y., and Zick, Y. (1992) *J. Biol. Chem.* **267**, 17483–17486
- Tobe, K., Tamemoto, H., Yamauchi, T., Aizawa, S., Yazaki, Y., and Kadowaki, T. (1995) *J. Biol. Chem.* **270**, 5698–5701
- Thompson, D. M., Cochet, C., Chambaz, E. M., and Gill, G. N. (1985) *J. Biol. Chem.* **260**, 8824–8830
- Bjorge, J. D., Chan, T. O., Antczak, M., Kung, H. J., and Fujita, D. J. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 3816–3820
- Kim, H. H., Sieke, S. I., and Koland, J. G. (1994) *J. Biol. Chem.* **269**, 24747–24755
- Soltoff, S. P., Carraway, K. L., III, Prigent, S. A., Gullick, W. G., and Cantley, L. C. (1994) *Mol. Cell. Biol.* **14**, 3550–3558
- Soltoff, S. P., and Cantley, L. C. (1996) *J. Biol. Chem.* **271**, 563–567
- Holgado-Madruga, M., Emler, D. R., Moscatello, D. K., Godwin, A. K., and Wong, A. J. (1996) *Nature* **379**, 8560–8564
- Wickrema, A., Uddin, S., Sharma, A., Chen, F., Alsayed, Y., Ahmad, S., Sawyer, S. T., Krystal, G., Yi, T., Nishada, K., Hibi, M., Hirano, T., and Platanius, L. C. (1999) *J. Biol. Chem.* **274**, 24469–24474
- Zhao, C., Yu, D. H., Shen, R., and Feng, G. S. (1999) *J. Biol. Chem.* **274**, 19649–19654
- Nishida, K., Yoshida, Y., Itoh, M., Fukada, T., Ohtani, T., Shirogane, T., Atsumi, T., Takahashi-Tezuka, M., Ishihara, K., Hibi, M., and Hirano, T. (1999) *Blood* **93**, 1809–1816
- Gu, H., Pratt, J. C., Burakoff, S. J., and Neel, B. G. (1998) *Mol. Cell.* **2**, 729–740
- Dhand, R., Hara, K., Hiles, I., Bax, B., Gout, I., Panayotou, G., Fry, M. J., Yonezawa, K., Kasuga, M., and Waterfield, M. D. (1994) *EMBO J.* **13**, 511–521

26. He, T. C., Zhou, S., da Costa, L. T., Yu, J., Kinzler, K. W., and Vogelstein, B. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 2509–2514
27. Khan, M. N., Baquiran, G., Brule, C., Burgess, J., Foster, B., Bergeron, J. J., and Posner, B. I. (1989) *J. Biol. Chem.* **264**, 12931–12940
28. McLroy, J., Chen, D., Wjasow, C., Michaeli, T., and Backer, J. M. (1997) *Mol. Cell. Biol.* **17**, 248–255
29. Klippel, A., Escobedo, J. A., Hu, Q., and Williams, L. T. (1993) *Mol. Cell. Biol.* **13**, 5560–5566
30. Eder, A. M., Dominguez, L., Franke, T. F., and Ashwell, J. D. (1998) *J. Biol. Chem.* **273**, 28025–28031
31. Carver, R. S., Sliwkowski, M. X., Sitaric, S., and Russell, W. E. (1996) *J. Biol. Chem.* **271**, 13491–13496
32. Ruff-Jamison, S., McGlade, S. J., Pawson, T., Chen, K., and Cohen, S. (1993) *J. Biol. Chem.* **268**, 7610–7612
33. Rozakis-Adcock, M., McGlade, J., Mbamalu, G., Pelicci, G., Daly, R., Li, W., Batzer, A., Thomas, S., Brugge, J., and Pelicci, P. G. (1992) *Nature* **360**, 689–692
34. Takahashi, Y., Akanuma, Y., Yazaki, Y., and Kadowaki, T. (1999) *J. Cell. Physiol.* **178**, 69–75
35. Wong, L., and Johnson, G. B. (1996) *J. Biol. Chem.* **271**, 20981–20984
36. Wang, J., Auger, K. R., Jarvis, L., Shi, Y., and Roberts, T. M. (1995) *J. Biol. Chem.* **270**, 12774–12780
37. Lecoq-Lafon, C., Verdier, F., Fichelson, S., Chretien, S., Gisselbrecht, S., Lacombe, C., and Mayeux, P. (1999) *Blood* **93**, 2578–2585
38. Wymann, M. P., Bulgarelli-Leva, G., Zvelebil, M. J., Pirola, L., Vanhaesebroeck, B., Waterfield, M. D., and Panayotou, G. (1996) *Mol. Cell. Biol.* **16**, 1722–1733
39. Vlahos, C. J., Matter, W. F., Hui, K. Y., and Brown, R. F. (1994) *J. Biol. Chem.* **269**, 5241–5248
40. Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J., and Saltiel, A. R. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 7686–7689
41. Meyers, R., and Cantley, L. C. (1997) *J. Biol. Chem.* **272**, 4384–4390
42. Fantl, W. J., Escobedo, J. A., Martin, G. A., Turck, C. W., del Rosario, M., McCormick, F., and Williams, L. T. (1992) *Cell* **69**, 413–423
43. Kanagasundaram, V., Jaworowski, A., and Hamilton, J. A. (1996) *Biochem. J.* **320**, 69–77
44. Lee, C. C., and Yamada, K. M. (1995) *J. Biol. Chem.* **270**, 507–510
45. Korhonen, J. M., Said, F. A., Wong, A. J., and Kaplan, D. R. (1999) *J. Biol. Chem.* **274**, 37307–37314
46. Laffargue, M., Raynal, P., Yart, A., Peres, C., Wetzker, R., Roche, S., Payrastra, B., and Chap, H. (1999) *J. Biol. Chem.* **274**, 32835–32841
47. Maroun, C. R., Moscatello, D. K., Naujokas, M. A., Holgado-Madruga, M., Wong, A. J., and Park, M. (1999) *J. Biol. Chem.* **274**, 31719–31726
48. Gesbert, F., Guenzi, C., and Bertoglio, J. (1998) *J. Biol. Chem.* **273**, 18273–18281
49. Ingham, R. J., Holgado-Madruga, M., Siu, C., Wong, A. J., and Gold, M. R. (1998) *J. Biol. Chem.* **273**, 30630–30637
50. Ando, A., Yonezawa, K., Gout, I., Nakata, T., Ueda, H., Hara, K., Kitamura, Y., Noda, Y., Takenawa, T., and Hirokawa, N. (1994) *EMBO J.* **13**, 3033–3038
51. Zhang, S., and Broxmeyer, H. E. (1999) *Biochem. Cell Biol.* **254**, 440–445
52. Gadina, M., Sudarshan, C., and O'Shea, J. J. (1999) *J. Immunol.* **162**, 2081–2086
53. Carlberg, K., and Rohrschneider, L. R. (1997) *J. Biol. Chem.* **272**, 15943–15950
54. Craddock, B. L., and Welham, M. J. (1997) *J. Biol. Chem.* **272**, 29281–29289