Role of the Grb2-Associated Binder 1/SHP-2 Interaction in Cell Growth and Transformation

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ABSTRACT

Grb2-associated binder 1 (Gab1) is a docking protein that is tyrosine phosphorylated following the activation of multiple cytokine receptors and receptor tyrosine kinases. Its function then is to recruit and activate multiple signaling molecules. In our previous work, we showed that Gab1 enhances cell growth and induces the transformed phenotype in NIH3T3 cells downstream of the epidermal growth factor (EGF) receptor. In this report, we analyze how it produces these effects. Because SHP-2 is the major binding partner of Gab1, we mutated its binding site in the Gab1 cDNA (Gab1/ΔSHP-2). This construct was stably overexpressed in NIH3T3 cells (3T3-Gab1/ΔSHP-2) and in the wild-type Gab1 cDNA (3T3-Gab1) or an empty expression vector (3T3-CTR). Our findings show that after EGF stimulation, Gab1/ΔSHP-2 has a higher level of tyrosine phosphorylation at early time points than Gab1. Gab1/ΔSHP-2 recruits more phosphatidylinositol 3′-kinase than Gab1 after EGF triggering, which accounts for a higher and more sustained AKT activation in 3T3-Gab1/ΔSHP-2 cells relative to 3T3-Gab1 fibroblasts. Moreover, 3T3-Gab1/ΔSHP-2 cells demonstrate a higher level of extracellular-regulated kinase 1 activation at early time points of EGF stimulation. However, there was an unexpected decrease in c-fos promoter induction in 3T3-Gab1/ΔSHP-2 cells when compared with 3T3-Gab1 cells. Additionally, the 3T3-Gab1/ΔSHP-2 cells show a reversion of the transformed phenotype, including fewer morphologic changes, an increase in stress fiber cytoskeletal organization, and a decrease in cell proliferation and anchorage independent growth. These results reveal that the Gab1/SHP-2 interaction is essential for cell growth and transformation but that this must occur through a novel pathway that is independent of extracellular-regulated kinase or AKT. On the basis of its role in growth and transformation, the Gab1/SHP-2 interaction may become an attractive target for the pharmacologic intervention of malignant cell growth.

INTRODUCTION

It is now well documented that receptor tyrosine kinases play important roles in the regulation of cell proliferation, differentiation, motility, and survival. In particular, the epidermal growth factor (EGF) receptor has been shown to have a significant role in several solid human tumors, including those of the brain, breast, colon, and ovary (1). On its activation by ligand, the receptor undergoes autophosphorylation, thus creating binding sites for Src homology 2 (SH2) domain-containing proteins, including the adapter protein SHC, and Grb2. The binding of Grb2 to phosphorylated EGF receptor results in the conversion of Ras-GDP into the GTP form. Once activated, Ras stimulates a kinase cascade that leads to the phosphorylation of extracellular-regulated protein kinase (ERK). Activated ERK translocates to the nucleus and phosphorylates transcription factors such as Elk and Myc, which in turn activate a diverse set of genes. Besides the Ras/mitogen-activated protein kinase (MAPK) pathway, the EGF receptor also can activate other SH2-containing proteins, such as

phosphatidylinositol 3′-kinase (PI3k) or SHP-2. The role of the activation of the MAPK (1, 5–8) and PI3k pathways (9–12) in EGF receptor-mediated tumorigenesis has been well documented. Yet unlike other receptors, the EGF receptor does not strongly bind these proteins. Activation instead occurs through the recruitment of the SH2 domains of PI3k and SHP-2 to the docking proteins Grb2-associated binder 1 (Gab1) (2, 3) or Gab2 (4).

Gab1 is a member of a well-defined family of scaffolding adapter proteins that are characterized by a similar structural organization with an overall ~40–50% homology. Other members of this family include mammalian Gab2, Gab3, *Drosophila* daughter of sevenless, and *Caenorhabditis elegans* Soc1. Gab1 has a pleckstrin homology domain at its NH_2 terminus that binds to phosphatidylinositol 3,4,5-triphosphate and a unique MET binding domain. It also has 16 potential tyrosine phosphorylation sites for the recruitment of SH2 domain-containing proteins and 47 predicted serine/threonine phosphorylation sites. Gab1 becomes tyrosine phosphorylated after stimulation with different growth factors and cytokines, such as EGF, insulin, hepatocyte growth factor, platelet-derived growth factor, and nerve growth factor, after the engagement of B- and T-cell receptors (2, 13, 14–18), or on H_2O_2 triggering (19). After phosphorylation, it recruits proteins with SH2 domains, including Grb2, phospholipase Cγ, PI3k, SHC, SHP-2, and Crk (2, 14, 20). We and others have shown that Gab1 is essential for several cellular processes. Gab1 overexpression in NIH3T3 fibroblasts enhances cell growth and promotes transformation (2). Overexpression of Gab1 in PC12 cells prevents apoptosis induced by serum starvation (14, 21), and it promotes tubulogenesis in epithelial cells (18). Gab1-deficient mice die *in utero* (at E12.5–E17.5) with developmental defects in the heart, placenta, liver, skin, and muscle and phenotypes that resemble those presented by mice deficient in EGF receptor, platelet-derived growth factor receptor, MET, and gp130 (22, 23). Gab1 activates PI3k after the addition of different growth factors (2, 14, 24). Through overexpression studies and by using Gab1$^{-/-}$ mouse embryo fibroblasts (MEFs), some groups have shown a role for Gab1 in ERK activation downstream of EGF receptor (23, 25, 26), whereas other studies show only modest ERK activation in cells overexpressing Gab1 (2). Gab1$^{-/-}$ MEFs also show decreased ERK activity after hepatocyte growth factor and platelet-derived growth factor stimulation (23). Gab1 activates c-Jun N-terminal kinase after hepatocyte growth factor (27) or EGF (3) triggering, and by using Gab1$^{-/-}$ MEFs, we have shown that it is required for H_2O_2-induced c-Jun N-terminal kinase activity (19). Because Gab1 is downstream of the EGF receptor and activates signaling pathways that have been shown to be involved in cell growth and transformation, investigating the role of Gab1 in these biological effects would help us to understand the process of pathogenesis and reveal possible therapeutic implications.

We have reported previously that Gab1 enhances cell growth and induces tumorigenesis in NIH3T3 fibroblasts downstream of the EGF receptor (2). To understand further how Gab1 produces these effects, we focused on its interaction with the tyrosine phosphatase SHP-2 because it is the major intracellular binding partner of Gab1 after EGF stimulation. We mutated a critical SHP-2 binding site in the Gab1 cDNA (Gab1/ΔSHP-2) and overexpressed this construct (Gab1/ΔSHP-2). This protein was expressed stably in NIH3T3 cells (3T3-Gab1/ΔSHP-2), wild-type

Gab1 cDNA (3T3-Gab1), or in an empty expression vector (3T3-CTR). Our findings show that the Gab1/ΔSHP-2 construct has a higher level of tyrosine phosphorylation at early time points on EGF stimulation compared with wild-type Gab1. Gab1/ΔSHP-2 also recruits more PI3k than Gab1, which accounts for a higher and more sustained AKT activation in cells overexpressing Gab1/ΔSHP-2 relative to 3T3-Gab1 fibroblasts. 3T3-Gab1/ΔSHP-2 cells also show a higher ERK1 activation at early time points of EGF stimulation. Despite the activation of these key growth-promoting molecules, we noted a decrease in the induction of the fos promoter when compared with 3T3-Gab1 cells. Additional investigation showed that the Gab1/SHP-2 interaction is responsible for the growth advantage conferred by Gab1 overexpression and the transformed phenotype, cytoskeleton organization, and anchorage independent growth. Our results reveal that the Gab1/SHP-2 interaction is important for cell growth and transformation and suggest that there exist ERK and AKT independent pathways that can mediate these phenotypes.

MATERIALS AND METHODS

Materials. EGF was purchased from Invitrogen (Carlsbad, CA), and LY 294002 from Calbiochem (Darmstadt, Germany). The anti-SHP-2, anti-Shc, and anti-Crk antibodies were from BD Biosciences PharMingen (San Diego, CA). The antiphosphotyrosine and anti-PI3k antibodies were from Upstate Biotechnology, Inc. (Lake Placid, NY). The antibodies against ERK1 and ERK2 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-Gab1 antibody was raised in rabbits by immunization with a glutathione *S*-transferase-Gab1 fusion protein as described previously (2). The anti-AKT, phospho-AKT, and phospho-ERK (phospho-p42/44) were from Cell Signaling Technology, Inc. (Beverly, MA), and the antihemagglutinin (anti-HA) was from Covance Inc. (Princeton, NJ). The antirabbit and antimouse antibodies, enhanced chemiluminescence reagent, and protein G-Sepharose were from Amersham Pharmacia Biotech (Piscataway, NJ). Texas Red-X phalloidin was purchased from Molecular Probes (Eugene, OR), and the dual-luciferase reporter assay system was from Promega (Madison, WI).

Cell Lines, Cell Cultures, and Transfections. Cell cultures were grown in DMEM supplemented with 5% calf serum, 100 units/ml of penicillin, 100 μg/ml of kanamycin, and 100 μg/ml of streptomycin. Constructs contained an HA tag at the amino-terminal end of Gab1, and two artificial BHI-*Eco*RI sites were engineered to insert this fragment into the *Xho*I site in the vector. The generation of Gab1/ΔSHP-2 (a Gab1 cDNA with a Y628F substitution) was performed as described elsewhere (2, 28). For stable transfections of the Gab1 wild-type cDNA and the Gab1 cDNA lacking the binding site for SHP-2 (Gab1/ΔSHP-2), transfection was performed using the pLXSN retroviral expression vector, which contains a neomycin-resistant cassette (a gift from Dr. M. Park). Phoenix cells (gift from G. Nolan) were transfected with 20 μg of plasmid DNA in a 10-cm-diameter dish. Cells were refed 15 h after transfection, and Polybrene (8 μg/ml)-supplemented virus-containing supernatant was transferred to NIH3T3 cells 48 h after transfection. Fibroblasts were refed after an overnight infection period. Selection was started 48 h after infection by using 500 μg/ml of Geneticin (Life Technologies, Inc., Rockville, MD), and stable pools of cells were obtained after 3 weeks (19).

Immunoprecipitation and Western Blot Analysis. Cells were serum starved for 24 h and stimulated in DMEM containing 100 ng/ml of EGF. Cells then were washed in ice-cold PBS and lysed using a buffer containing 10 mM Na$_2$HPO$_4$, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.2% sodium azide, 0.004% sodium fluoride, 1 mM NaVO$_4$, 25 mM glycerophosphoric acid, 100 μg/ml phenylmethanesulfonyl fluoride, and 10 μg/ml each aprotinin and leupeptin, pH 7.35. Lysates were clarified by centrifugation at 12,000 × g for 10 min at 4°C, or in the case of those used for anti-phospho-ERK or phospho-AKT Western blot analyses, they were sonicated for 15 s. Protein concentrations were determined using the Bio-Rad DC protein assay (Hercules, CA). Cell lysates were combined with antibody bound previously to 30 μl of 50% slurry of protein G-Sepharose overnight at 4°C. Immunoprecipitates were washed three times with the same lysis buffer and resuspended in sample buffer. Immunocomplexes and whole lysates were resolved on 4–20% Novex Tris-Glycine gels (Invitrogen) and transferred to

nitrocellulose membranes (S&S, Keene, NH). The membranes were blocked in Tris-buffered saline/5% Blotto [100 mM Tris (pH 7.5), 0.9% NaCl, and 0.1% Tween 20 with 5% nonfat dry milk] and incubated with the different antibodies (19). Proteins were detected by using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech). The quantification of the Western signals was done by densitometry using ImageQuant software (Amersham Pharmacia Biotech).

Fos-Luciferase Assays. NIH3T3 cells were seeded in duplicate in 35-mm dishes at a density of 60–80%. The next day the cells were cotransfected with 1 μg of the empty vector or an expression vector containing the Gab1 wild-type cDNA or Gab1 cDNA lacking the binding site for SHP-2 (Gab1/ΔSHP-2) together with the Fos-Luc target plasmid (gift from Dr. M. Park; 1 μg) composed of nucleotides −356 to +109 relative to the start site of the c-fos promoter cloned upstream of the luciferase gene, 40 ng of pRL-TK renilla as a marker for transfection efficiency, and 8 μl/dish of Fugene-6 (Roche, Basel, Switzerland; Ref. 29). Twenty-four h after transfection, the cells were serum starved for 15 h and then stimulated with 100 ng/ml of EGF for 9 h. Cells were lysed in the passive lysis buffer provided in the dual-luciferase reporter assay system, and luciferase activity then was detected in a luminometer following the manufacturer's protocol. Results were corrected by renilla luciferase as a reporter of transfection efficiency (30).

Proliferation Assay. Stable cell lines were seeded in triplicate in 35-mm dishes at a density of 50,000 cells/dish with complete media. The next day (day 0) the media were changed to 0.5% calf serum containing media with or without 100 ng/ml of EGF. Cells were fed on days 3, 6, and 9 and counted on days 0, 4, and 10 using a hemocytometer (2).

Immunofluorescence. Cells were plated on glass coverslips, grown to ~70% confluency, fixed with 3.7% paraformaldehyde/PBS solution, and permeabilized with 0.1% Triton X-100/PBS, and nonspecific sites were blocked by incubation with PBS containing 1% BSA for 30 min. Coverslips were incubated with 200 units/ml of Texas Red-X phalloidin in 1% BSA/PBS for 1 h, rinsed in PBS, mounted onto microscope slides, and examined using a confocal microscope (Zeiss Axiovert 200M, Oberkochen, Germany; Ref. 31).

Soft Agar Assay. Two thousand cells were suspended in 1 ml of medium containing 0.3% agarose (low melting; Sigma Chemical Co., St. Louis, MO) and 10% calf serum and seeded over a 2-ml 0.6% agarose layer in 35-mm dishes. Cells were fed weekly with 1 ml of suspension medium. After 3 weeks, the number of colonies larger than 60 μm was counted (2).

RESULTS

Differential Pattern of Tyrosine Phosphorylation of Gab1 and Gab1/ΔSHP-2 after EGF Stimulation. We and others have reported that Gab1 becomes tyrosine phosphorylated after EGF addition, resulting in the recruitment of several SH2-containing proteins (2, 25). Among these proteins, SHP-2 is of interest because it shows the greatest degree of association with Gab1 of any protein examined. For these reasons, we investigated the role of this interaction in EGF signaling. We mutated the site in Gab1 responsible of this binding (Y628F) and introduced this cDNA (Gab1/ΔSHP-2), the cDNA of wild-type Gab1, and an empty vector in NIH3T3 fibroblasts using a retroviral expression vector containing an HA tag (2, 28, 32). After neomycin selection, stable pools were generated and characterized. Cell lysates were run on an SDS-PAGE and subjected to Western blot analysis with anti-Gab1 antibody (Fig. 1*A*). The cell lines showed similar levels of transfected Gab1, although we noted that the amounts of Gab1/ΔSHP-2 were consistently slightly lower than those of wild-type Gab1. Because SHP-2 is a tyrosine phosphatase, we investigated the levels of tyrosine phosphorylation of Gab1 and Gab1/ΔSHP-2 after EGF stimulation. The cells were stimulated with 100 ng/ml of EGF for the indicated periods of time and lysed, and immunoprecipitations with anti-HA antibody were performed. This was followed by Western blot analysis with an antiphosphotyrosine antibody (Fig. 1*B*, *top*). Gab1 and Gab1/ΔSHP-2 became tyrosine phosphorylated within 5 min, which was maintained for 10 min and then decreased after 30 min. It is interesting to note that at the earlier time points, Gab1/
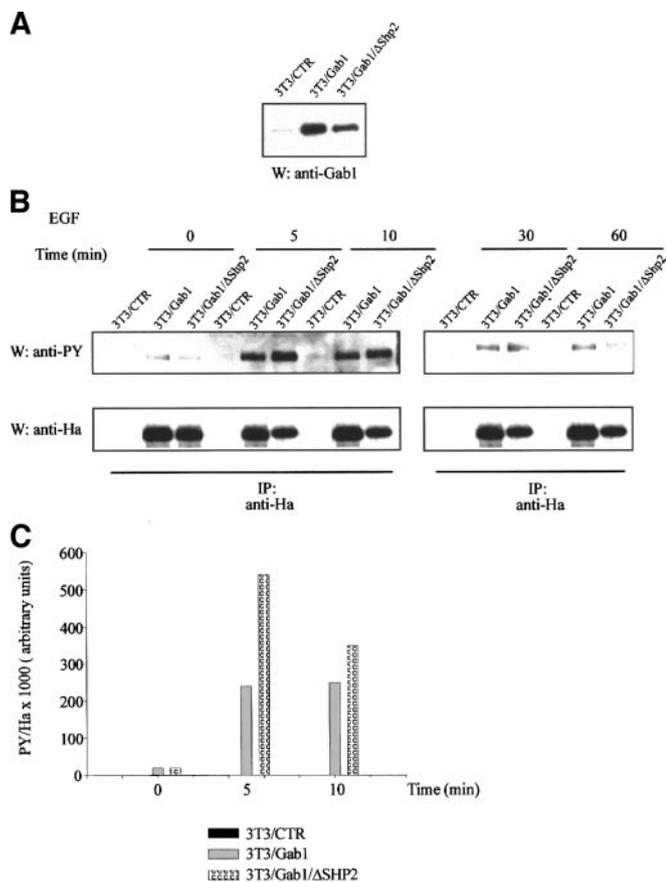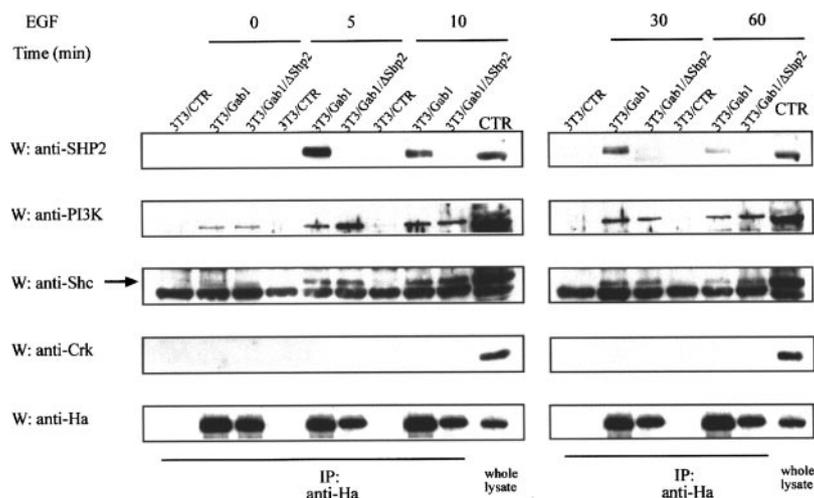
A

W: anti-Gab1

B

C

Fig. 1. Differential pattern of tyrosine phosphorylation of Grb2-associated binder 1 (*Gab1*) and Gab1/ΔSHP-2 after epidermal growth factor (*EGF*) stimulation. *A,* 3T3-CTR, 3T3-Gab1, and 3T3-Gab1/ΔShp-2 fibroblasts were lysed and run on SDS-PAGE, and the resulting Western blot was incubated with anti-Gab1 antibody. *B,* the same cells as in *A* were stimulated with 100 ng/ml of EGF for the times indicated in the figure. Lysates were subjected to immunoprecipitation with antihemagglutinin (*anti-HA*) antibody and run on SDS-PAGE, and the Western blot was incubated with antiphosphotyrosine antibody (*top*). The same membrane was stripped and reincubated with anti-HA antibody (*bottom*). *C,* the assay from *B* was quantified by densitometer analysis, and the results are reported using the arbitrary units assigned by the densitometer (PY/HA ratio $\times 10^3$). Experiments were repeated three times; data from one representative assay are shown. *IP,* immunoprecipitation; *W,* Western blot analysis.

ΔSHP-2 showed higher levels of phosphorylation than Gab1 as indicated by greater intensity with the antiphosphotyrosine antibody and slower mobility on SDS-PAGE. Western blot analysis with anti-HA showed similar amounts of HA in all of the lanes (Fig. 1B, *bottom*).

These results indicate that Gab1 may be a target for dephosphorylation by SHP-2 as suggested previously (28, 33–36).

Gab1/ΔSHP-2 Recruits More PI3k Than Gab1 after EGF Triggering. We next sought to determine whether this difference in phosphorylation altered the binding for different SH2 domain-containing proteins. The cells were stimulated as in Fig. 1, and after immunoprecipitation with anti-HA antibody, the resulting Western blot was incubated with the different antibodies as depicted in Fig. 2. As expected, Gab1/ΔSHP-2 did not recruit SHP-2, whereas Gab1 bound to SHP-2 as early as 5 min and maintained this complex during all of the time points examined. Western blot analysis with anti-SHC antibody revealed a similar pattern of binding of this molecule to either Gab1 or Gab1/ΔSHP-2 after EGF stimulation. At 5 min, we surprisingly detected a greater association with PI3k in 3T3-Gab1/ΔSHP-2 compared with 3T3-Gab1 cells, indicating that SHP-2 may regulate negatively the activation of PI3k after EGF stimulation. We were unable to detect recruitment of Crk to either Gab1 or Gab1/ΔSHP-2 after EGF addition, whereas binding has been noted after stimulation with other growth factors (20, 27).

3T3-Gab1/ΔSHP-2 Fibroblasts Have a Higher and More Sustained AKT Activation Than 3T3-Gab1 Cells after EGF Addition. Gab1 has been shown to play a direct role in AKT activation after stimulation by different growth factors or cellular stresses via its interaction with PI3k (19, 21, 37). Because we noted a greater degree of PI3k association with Gab1/ΔSHP-2, we investigated the effect on AKT activation. 3T3-CTR, 3T3-Gab1, and 3T3-Gab1/ΔSHP-2 were stimulated with EGF for up to 1 h. Cell lysates were run on an SDS-PAGE and subjected to Western blot analysis with anti-phospho-AKT antibody. As shown in Fig. 3, *A* and *C*, 3T3-Gab1 fibroblasts had a lower and less-sustained AKT activation than 3T3-CTR or 3T3-Gab1/ΔSHP-2 cells. Western blot analysis with anti-AKT antibody revealed similar levels of AKT expression in the three cell lines. To study whether AKT activation was downstream of PI3k, the cells were preincubated with LY 294002 (a PI3k-specific inhibitor) for 30 min before EGF stimulation. Western blot analysis with anti-phospho-AKT showed an abolishment of AKT activation compared with cells treated with DMSO as a vehicle control (Fig. 3E). These results additionally suggest that SHP-2 down-regulates PI3k pathway after EGF stimulation.

3T3-Gab1/ΔSHP-2 Fibroblasts Have Higher ERK1 Activation after EGF Stimulation. There are contradictory studies about the role of Gab1 in ERK activation (2, 23, 25). We have shown previously that NIH3T3 fibroblasts overexpressing Gab1 induce only a transient

Fig. 2. Grb2-associated binder 1 (*Gab1*)/ΔSHP-2 recruits more phosphatidylinositol 3′-kinase (*PI3k*) than Gab1 after epidermal growth factor (*EGF*) triggering. 3T3-CTR, 3T3-Gab1, and 3T3-Gab1/ΔSHP-2 fibroblasts were treated with 100 ng/ml of EGF for the times indicated in the figure. After immunoprecipitation (*IP*) with antihemagglutinin (*anti-HA*) antibody, lysates were run on a gel, and Western blot analysis (*W*) was performed using specific antibodies against the different molecules (anti-SHP-2, anti-PI3k, anti-SHC, anti-Crk, and anti-HA) as indicated. Whole lysate was run as a control for the antibodies (*CTR*). The experiments were done three times, and data from one representative assay are shown.
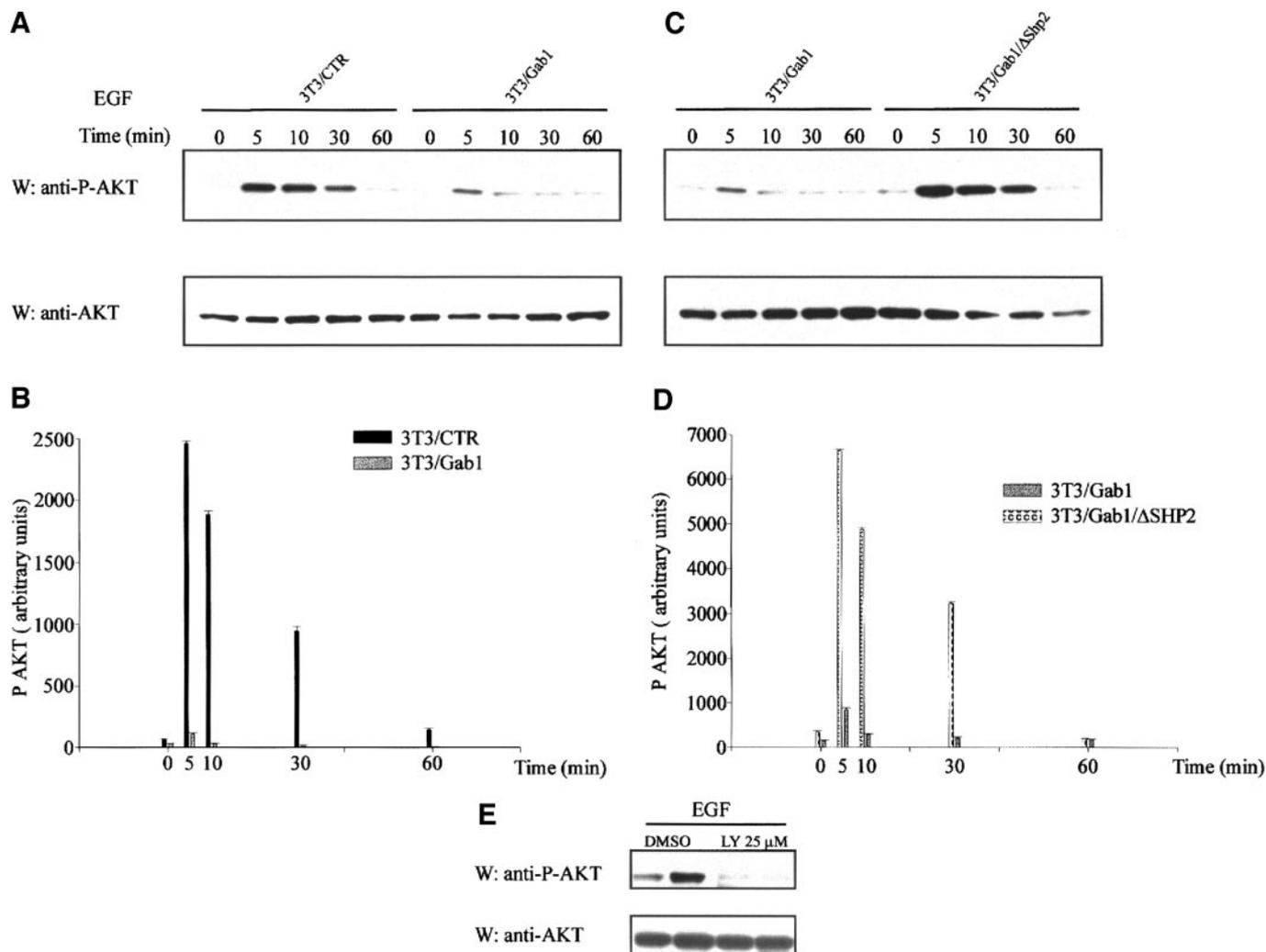
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Fig. 3. 3T3-Gab1/ΔSHP-2 fibroblasts have a higher and more sustained AKT activation than 3T3-Gab1 cells after epidermal growth factor (*EGF*) addition. 3T3-CTR, 3T3-Gab1, and 3T3-Gab1/ΔSHP-2 cells were stimulated with 100 ng/ml of EGF for up to 1 h. Lysates were run on a gel and incubated with anti-phospho-AKT antibody (*A* and *C, top*). The same membranes were stripped and reincubated with anti-AKT antibody (*A* and *C, bottom*). *A,* comparison of phospho-AKT Western blot analysis between 3T3-CTR and 3T3-Gab1 cells. *B,* quantification of *A* by densitometry analysis using ImageQuant software. The results are reported using the units assigned by the densitometer (mean ± SD; $n = 2$). *C,* comparison of phospho-AKT Western blot analysis between 3T3-Gab1 and 3T3-Gab1/ΔSHP-2 cells. *D,* quantification of *C* done as in *B. E,* cells were preincubated with LY 294002 or DMSO as a the vehicle control for 30 min and then stimulated with EGF for 5 min. Lysates were run on an SDS-PAGE and subjected to Western blot analysis with anti-phospho-AKT antibody (*top*). The membrane was stripped and reincubated with anti-AKT (*bottom*). Experiments were repeated three times; data from two representative assays are shown. *W,* Western blot analysis.

ERK activation compared with control cells after EGF addition (2). Several studies implicate SHP-2 in up-regulating ERK activation. 3T3-CTR, 3T3-Gab1, and 3T3-Gab1/ΔSHP-2 cells were stimulated with EGF for the indicated times as shown in Fig. 4. The cells were lysed, run on an SDS-PAGE, and incubated with anti-phospho-ERK (Phospho p42/p44) antibody. Consistent with our previous studies, quantification of that signal showed that cells overexpressing Gab1 had a lower level of ERK activation, especially of the p44 form (ERK1), compared with control cells. However, 3T3-Gab1/ΔSHP-2 fibroblasts presented a higher ERK activation at early time points than Gab1 cells after EGF stimulation, mainly for the p44 form (ERK1). Considering these results, it appears that Gab1 does not have a strong role in ERK activation, and in this context, SHP-2 may act more to repress than promote ERK1 activation. Western blot analysis with anti-ERK (p42/44) showed similar levels of these proteins in the three cell lines.

3T3-Gab1/ΔSHP-2 Fibroblasts Show Lower c-fos Promoter Activation Than 3T3-Gab1 Cells. Receptor tyrosine kinases use different pathways to evoke the transcription of immediate-early genes, such as c-fos (38). Previous studies have shown that c-fos is critical

for cell growth and transformation (30, 39). It has been shown that SHP-2 is required for c-fos expression, acting, at least in part, to control the activity of the transcription factor Elk1. Therefore, we assessed the role of Gab1/SHP-2 interaction in EGF-induced c-fos promoter activity. Transient transfections were performed in NIH3T3 cells either with a construct containing a control empty expression vector or an expression vector containing Gab1 cDNA or Gab1/ΔSHP-2 together with a vector with the Fos-Luc target plasmid and pRL-TK renilla as a marker for transfection efficiency. As described in Fig. 5, overexpression of Gab1 did not increase EGF-induced fos luciferase activity compared with control cells, although we detected a slight increase in basal conditions. However, 3T3-Gab1/ΔSHP-2 cells revealed a decrease of this activity under basal and EGF stimulation conditions in relation to 3T3-CTR or 3T3-Gab1 cells. Thus, the Gab1/SHP-2 interaction is important for c-fos induction, and this may result in a decrease in transformation-related parameters.

3T3-Gab1/ΔSHP-2 Cells Have a Lower Growth Rate Than 3T3-Gab1 and 3T3-CTR Fibroblasts. We and others have shown through overexpression and knockout experiments that Gab1 enhances cell growth (2, 22, 23). To explore the role of the Gab1/SHP-2 interaction
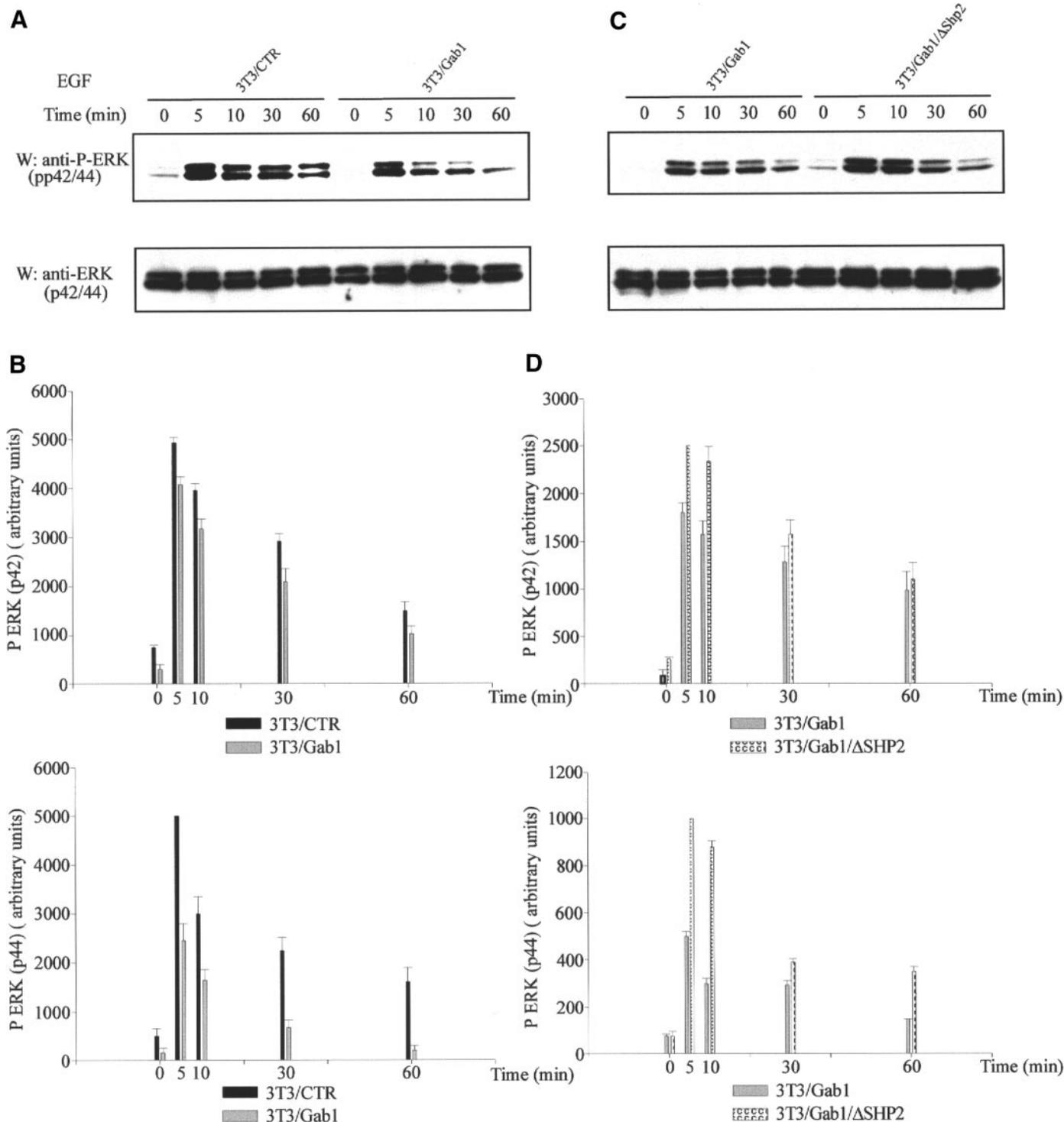
Fig. 4. 3T3-Gab1/ΔSHP-2 fibroblasts have higher extracellular-regulated protein kinase 1 (*ERK1*) activation at early time points than 3T3-Gab1 cells after epidermal growth factor (*EGF*) stimulation. 3T3-CTR, 3T3-Gab1, and 3T3-Gab1/ΔSHP-2 cells were stimulated with 100 ng/ml of EGF for up to 1 h. Lysates were run on a gel and incubated with anti-phospho-ERK antibody (phospho p42/44; *A* and *C, top*). The same membranes were stripped and reincubated with anti-ERK1 (p44) and ERK2 (p42) antibodies (*A* and *C, bottom*). *A,* comparison of phospho-ERK Western blot analysis between 3T3-CTR and 3T3-Gab1 cells. *B,* quantification of *A* by densitometry analysis using ImageQuant software, and the results are reported using the units assigned by the densitometer (mean ± SD; *n* = 2). *C,* comparison of phospho-ERK Western blot analysis between 3T3-Gab1 and 3T3-Gab1/ΔSHP-2 cells. *D,* quantification of *C* done as in *B*. Experiments were repeated three times; data from two representative assays are shown. *W,* Western blot analysis.

in this aspect, we examined the growth rate of fibroblasts in tissue culture. We seeded in triplicate the 3T3-CTR, 3T3-Gab1, and 3T3-Gab1/ΔSHP-2 cells in media containing 0.5% calf serum with or without 100 ng/ml of EGF and followed the for up to 10 days. Fig. 6 shows that 3T3-Gab1 cells had an enhanced growth rate and achieved a high cell density in 0.5% serum-containing media in relation to control cells, and EGF addition did not show any enhancement in these cells. Contrarily, 3T3-Gab1/ΔSHP-2 fibroblasts showed a lower growth rate than 3T3-Gab1 and 3T3-CTR cells under either low serum conditions or after EGF stimulation. Despite the higher levels of ERK and AKT activation in 3T3-Gab1/ΔSHP-2, it appears that other aspects of the Gab1/SHP-2 interaction are more critical for cell growth.
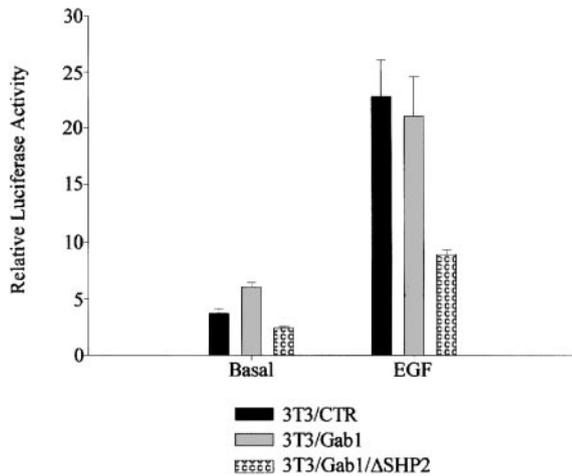
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we observed a key phenotypic difference. As depicted in Fig. 7A, control cells presented a normal refractility and a typical fibroblastic flat appearance. In contrast, 3T3-Gab1 fibroblasts were more refractile, with an elongated, spindle shape that is classic of the transformed phenotype (Fig. 7B). Conversely, 3T3-Gab1/ΔSHP-2 cells were not refractile and more flattened and had a similar morphology to control cells (Fig. 7C). Cell shape is maintained by cellular components such as the actin-based cytoskeleton (40). Therefore, we examined whether these components are affected by overexpression of the different constructs. Immunofluorescence analysis with Texas Red-X phalloidin revealed that 3T3-Gab1 cells do not possess well-defined stress fibers (Fig. 7E) compared with 3T3-CTR cells (Fig. 7D) and also showed increased peripheral actin staining and lamellipodia, which correlates with highly motile cells. Interestingly, 3T3-Gab1/ΔSHP-2 fibroblasts (Fig. 7F) had a marked increase in the number of actin stress fibers in relation to 3T3-Gab1 or 3T3-CTR cells, suggesting that these cells are less motile than control cells.

Soft Agar Colony Formation by Gab1 Overexpression in NIH3T3 Fibroblasts Depends on the GAB1/SHP-2 Interaction. We have shown previously that overexpression of Gab1 in NIH3T3 fibroblasts induces soft agar colony formation after the addition of EGF (2). The phenotypic differences between 3T3-Gab1 and 3T3-Gab1/ΔSHP-2 fibroblasts prompted us to study the anchorage independent growth of these cells. 3T3-CTR, 3T3-Gab1, and 3T3-Gab1/ΔSHP-2-overexpressing fibroblasts were seeded in 35-mm dishes in triplicate in complete media. The cells were followed for 3 weeks, and colonies larger than ~60 μm were counted. Gab1 cells were capable of forming large and numerous colonies in soft agar, whereas Gab1/ΔSHP-2 fibroblasts were not able to form colonies (Fig. 8A). Similar

Fig. 5. 3T3-Gab1/ΔSHP-2 fibroblasts show lower c-fos promoter activation than 3T3-Gab1 and 3T3-Gab1 cells. NIH3T3 cells were seeded in duplicate in 35-mm dishes, and the next day the cells were cotransfected with an empty vector or an expression vector containing the Grb2-associated binder 1 (*Gab1*) wild-type cDNA or the Gab1 cDNA lacking the binding site for SHP-2 together with the Fos-Luc target plasmid and pRL-TK renilla plasmid as a marker for transfection efficiency. Twenty-four h after transfection, the cells were serum starved for 15 h and then stimulated with 100 ng/ml of epidermal growth factor (*EGF*) for 9 h. Cells were lysed, and then luciferase activity was detected in a luminometer following the manufacturer's protocol. Results were corrected by renilla luciferase (mean ± SD; $n = 2$). Experiments were done in duplicate in two separate experiments; data from one representative assay are shown.
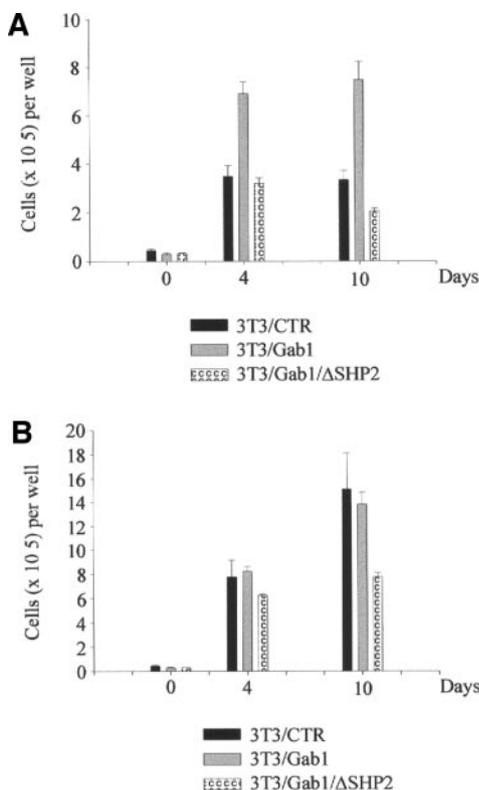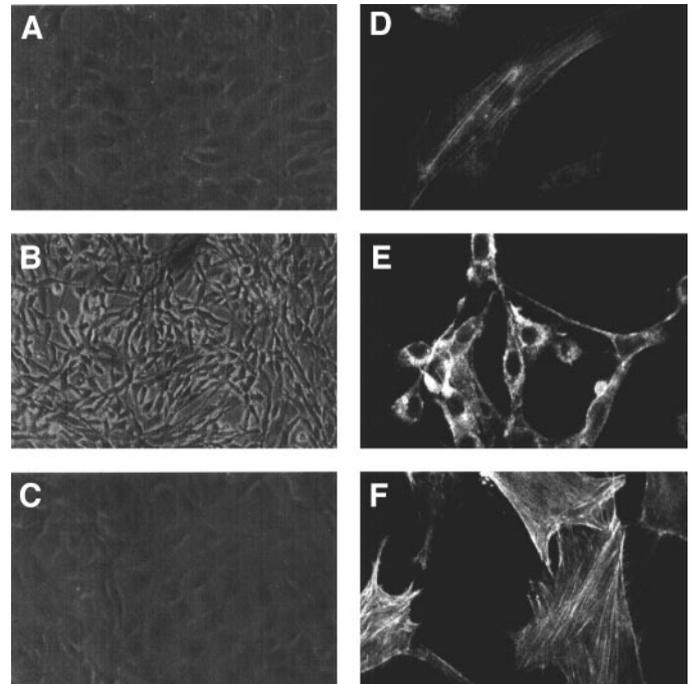

Fig. 6. 3T3-Gab1/ΔSHP-2 cells have a lower growth rate than 3T3-Gab1 and 3T3-CTR fibroblasts. 3T3-CTR, 3T3-Gab1, and 3T3-Gab1/ΔSHP-2 fibroblasts were seeded in triplicate in complete media. The next day the media was changed into either 0.5% calf serum (*A*) or 0.5% calf serum containing 100 ng/ml of epidermal growth factor (*B*) and followed for 10 days. Cells were counted with a hemocytometer (mean ± SD; $n = 3$). The experiments were done in triplicate; data from one representative assay are shown.

Different Morphology and Actin Stress Fibers Organization in 3T3-Gab1 and 3T3-Gab1/ΔSHP-2 Fibroblasts. A hallmark of transformed cells is an altered morphology. After the establishment of the cells overexpressing Gab1 or Gab1/ΔSHP-2 or control cells,

Fig. 7. Different morphology and actin stress fiber organization in 3T3-Gab1 and 3T3-Gab1/ΔSHP-2 fibroblasts. 3T3-CTR (*A*), 3T3-Gab1 (*B*), and 3T3-Gab1/ΔSHP-2 (*C*) fibroblasts were seeded in complete media and cultured for 3 days. The cells were examined with a phase-contrast microscope. The experiments were done three times; data from one representative assay are shown. 3T3-CTR (*D*), 3T3-Gab1 (*E*), and 3T3-Gab1/ΔSHP-2 (*F*) fibroblasts were plated on glass coverslips, grown to ~70% confluency, fixed with 3.7% paraformaldehyde/PBS solution, and permeabilized with 0.1% Triton X-100/PBS. Coverslips were incubated with Texas Red-X phalloidin. Microscope slides were examined using a confocal microscope. The experiments were done in duplicate; data from one representative experiment are shown.

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Fig. 8. Soft agar colony formation by Grb2-associated binder 1 (*Gab1*) overexpression in NIH3T3 fibroblasts depends on the Gab1/SHP-2 interaction. *A,* 3T3-CTR, 3T3-Gab1, and 3T3-Gab1/ΔSHP-2 fibroblasts were suspended in 1 ml of medium containing 0.3% agarose and 10% calf serum and seeded over a 2-ml 0.6% agarose layer in 35-mm dishes. Cells were fed weekly with 1 ml of suspension medium. After 3 weeks, the number of colonies larger than 60 μm was counted (mean $\pm$ SD; $n = 3$). The experiments were done in triplicate; data from one representative experiment are shown. Colonies were photographed using a NIKON 6000 camera (Tokyo, Japan): 3T3-CTR (*B*), 3T3-Gab1 (*C*), and 3T3-Gab1/ΔSHP-2 (*D*) fibroblasts.
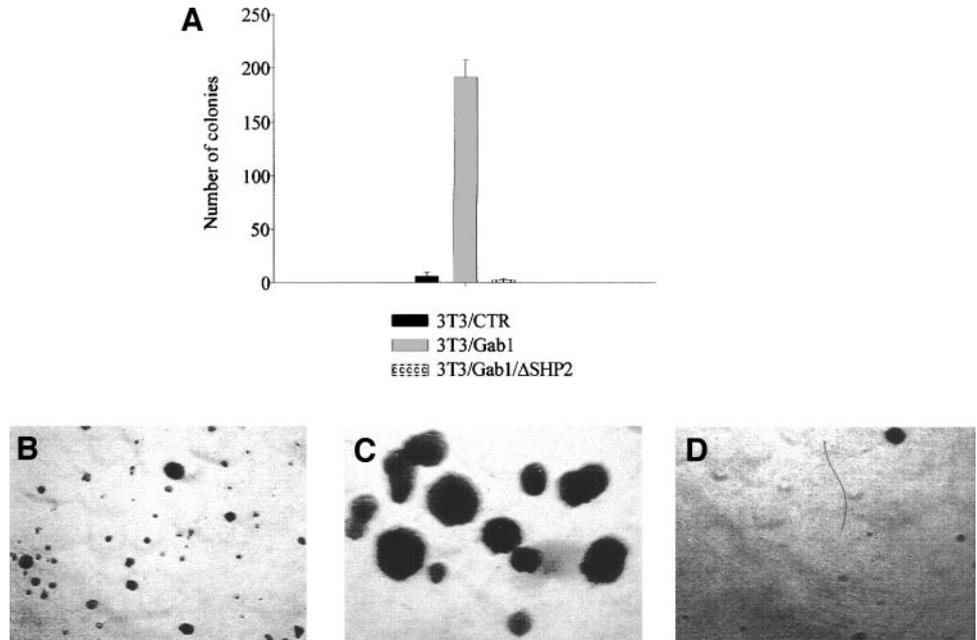
results were observed after EGF addition (data not shown). Pictures of the morphology of these colonies are shown in Fig. 8, *B–D*. As with the other transformation-related parameters studied, the Gab1/SHP-2 interaction appears to be critical for anchorage independent growth.

DISCUSSION

Gab1 has a critical role in signal transduction because it can integrate the signals from a diverse set of upstream receptors and in turn activate a variety of downstream pathways. Several studies have documented that it is important for cell growth and can induce tumorigenesis. Gab1 is the direct activator of PI3k for several ligands, including nerve growth factor and EGF. A considerable number of studies have documented a role for PI3k in cell growth and transformation. Our work thus far has not shown a definitive role for the Gab1/PI3k interaction with respect to transformation (data not shown). However, in this report we have discovered that the Gab1/SHP-2 interaction is key for several transformation-related phenotypes.

First, we studied the downstream signals that depend on the Gab1/SHP-2 interaction. We detected a higher level of tyrosine phosphorylation in Gab1/ΔSHP-2 compared with wild-type Gab1 at earlier time points of EGF stimulation, suggesting that Gab1 is a substrate of this tyrosine phosphatase. This result is consistent with previous reports that demonstrated through *in vitro* experiments or via overexpression of a catalytically inactive SHP-2 that the level of Gab1 tyrosine phosphorylation increased in relation to cells with a wild-type SHP-2 (33). In contrast, other studies were not able to detect a difference in Gab1 tyrosine phosphorylation in SHP-2-deficient fibroblasts when compared with wild-type fibroblasts, probably because SHP-2 may dephosphorylate specific sites in Gab1 that are difficult to detect with the levels of Gab1 present in the cells used (41, 42). Additionally, the increase in Gab1/ΔSHP-2 tyrosine phosphorylation might be explained by the observation made by Hayman *et al.* (36), who detected EGF receptor as a substrate of SHP-2. Mutation of the binding site for SHP-2 in the highly homologous Gab2 also similarly reveals a higher level of tyrosine phosphorylation than the wild-type molecule after EGF stimulation in primary hepatocytes (43).

Having established that Gab1 and Gab1/ΔSHP-2 molecules have a different pattern of tyrosine phosphorylation after EGF stimulation, we investigated whether this would have any effect on the recruitment of SH2 domain-containing proteins. As expected, Gab1/ΔSHP-2 was unable to bind to SHP-2, whereas Gab1 formed a complex that correlated with Gab1 tyrosine phosphorylation status, as reported previously (41). There were no differences in the recruitment of SHC to the Gab1 molecules, and we were not able to detect binding to Crk, although these proteins bind after stimulation by other growth factors, indicating the specificity of Gab1 to transmit downstream signals (20, 27). We detected a higher binding of PI3k to Gab1/ΔSHP-2. This finding is in agreement with the work of Neel *et al.* (42), who described a higher binding of PI3k to Gab1/ΔSHP-2 than to Gab1 in 293 cells and also a higher recruitment of PI3k to Gab1 in MEFs deficient in SHP-2 than in wild-type MEFs after EGF addition. These results suggest that SHP-2 probably dephosphorylates the binding site in Gab1 for PI3k, supported also by *in vitro* experiments in which SHP-2 was able to dephosphorylate a peptide that contained a predicted site for PI3k binding (34).

To assess the downstream consequences of this enhanced PI3k/Gab1/ΔSHP-2 complex, we measured AKT activation using an antibody specific for the phosphorylated form of AKT. 3T3-Gab1/ΔSHP-2 cells have a higher and sustained AKT activation than 3T3-Gab1 cells. By using LY 294002, we were able to confirm that AKT activation was downstream of PI3k. Taken together, these data show that the enhanced association of PI3k to Gab1/ΔSHP-2 after EGF stimulation in these cells results in increased activity of the PI3k/AKT pathway. The role of SHP-2 to decrease Gab1/PI3k interaction and down-regulation of AKT activity is specific for EGF because this does not occur after hepatocyte growth factor stimulation (28). In a similar fashion, SHP-2 may dephosphorylate the binding sites for PI3k in Gab2 after EGF triggering, resulting in a decrease of Gab2/PI3k association and a reduction of AKT activation (43).

We next examined the effect of Gab1/SHP-2 interaction on MAPK activation. As we have reported previously (2), we did not see an increase in MAPK activation in cells overexpressing Gab1 compared with the control cells. Of note, we detected a decrease in the activation of the p44 isoform in 3T3-Gab1 cells relative to control cells. Inter-

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estingly, 3T3-Gab1/ΔSHP-2 fibroblasts demonstrate an increase in the activation of MAPK, particularly ERK1, at earlier time points of EGF stimulation compared with 3T3-Gab1 fibroblasts. These results stand in contrast with other reports, which showed a positive role for Gab1 in MAPK activation after EGF stimulation (3, 25, 26). These differences may be explained by the use of different cell lines that may contain different levels of EGF receptors and also systems in which the study of MAPK activation has been done by overexpression of ERK2, with less attention paid to ERK1. Our results do not contradict those shown by Hirano *et al.* (23), who, by using MEF$^{-/-}$ Gab1, were able to show a decrease in MAPK in these cells compared with wild-type MEFs after EGF stimulation. In contrast to our system, Gab1/SHP-2 interaction has been shown to be responsible for a sustained MAPK activation in MET signaling (28), whereas it does not play any function in growth hormone stimulation (31).

c-fos is an immediate-early gene whose transcription is regulated by the Ras/Raf/MEK/MAPK cascade, and it is required for the transcription of many genes important for cell growth, differentiation, and transformation (44–46). We analyzed the role of Gab1 in c-fos promoter activation on EGF stimulation. Although Gab1 overexpression did not increase c-fos induction relative to control cells, 3T3-Gab1/ΔSHP-2 fibroblasts demonstrate a decrease in basal and after-EGF stimulation compared with either control or 3T3-Gab1 cells. This striking result suggests that Gab1/SHP-2 complex uncouples MAPK activation and c-fos expression, acting probably through the activation of a pathway parallel to MAPK. Additionally, on platelet-derived growth factor stimulation, the Gab1/SHP-2 interaction has been shown to play a positive role in the activation of ELK1, a component of the ternary complex factor that binds to the serum response element within the c-fos promoter (47). Nevertheless, this Gab1/SHP-2 binding does not have any effect on the induction of the fos promoter after growth hormone triggering, which underscores the importance of specificity in the different systems (31).

Despite the increase in AKT and ERK activation, 3T3-Gab1/ΔSHP-2 fibroblasts showed a lower rate of cell proliferation in basal and after-EGF stimulation conditions relative to 3T3-Gab1 or control cells. These results are in agreement with the observations made by Khavari *et al.* (26) in epidermal cells. There also were profound morphologic differences between 3T3-Gab1 and 3T3-Gab1/ΔSHP-2 cells. The 3T3-Gab1 cells are spindle shaped, highly refractile, and appear transformed, whereas the 3T3-Gab1/ΔSHP-2 fibroblasts are flat, with a lack of birefringence, and have a normal fibroblastic appearance. This correlates with a different cytoskeletal organization characterized by the lack of stress fibers in 3T3-Gab1 cells contrasted by an increase of these structures in 3T3-Gab1/ΔSHP-2 fibroblasts. This cytoskeleton organization in 3T3-Gab1/ΔSHP-2 cells is reminiscent of that found in Rat-1 cells overexpressing a catalytically inactive SHP-2 (48), supporting the concept that Gab1-SHP-2 interaction promotes the initiation of the phosphatase activity of SHP-2 (34).

These differences in morphology and actin cytoskeleton organization led us to examine anchorage independent growth. 3T3-Gab1 cells formed colonies even in the absence of EGF. Our previous report showed that colonies were able to form only after EGF addition, and this difference may be explained by clonal differences and/or by higher levels of Gab1 expression in our current retrovirus transfected pools (2). In this study, we found that 3T3-Gab1/ΔSHP-2 fibroblasts were incapable of forming colonies in soft agar. The Gab1/SHP-2 interaction also has been shown to play a positive role in tumorigenesis induced by ErbB2 receptor (49), and the Gab1/Crk complex also has been described to play a role in tumorigenesis downstream of Tpr-Met (27). In contrast, another study implicates a pleckstrin homology domain-truncated form of Gab1 in the promotion of an experimental model of cancer progression and that a wild-type Gab1

inhibits EGF-induced soft agar colony formation in preneoplastic Syrian hamster cells (50). We cannot readily explain these results, although the preponderance of data would suggest a positive role for Gab1 in the promotion of tumorigenesis.

Our results are consistent with a model proposed by other authors (3, 42), in which Gab1 is recruited on EGF stimulation to the EGF receptor and initiates a positive feedback loop where initial Gab1 tyrosine phosphorylation leads to p85 binding, PI3k activation, and phosphatidylinositol 3,4,5-trisphosphate production, which then allows additional recruitment and tyrosine phosphorylation of Gab1 through the binding of phosphatidylinositol 3,4,5-trisphosphate to the Gab1 pleckstrin homology domain (3). SHP-2, by dephosphorylating p85 binding sites on Gab1, regulates the Gab1/PI3k positive feedback loop and ultimately controls the kinetics of PI3k activation. The reduction of PI3k activity would reduce the formation of phosphatidylinositol 3,4,5-trisphosphate products and the stability of Gab1 in the membrane (42). At the same time, Gab1 is recruited indirectly to the EGF receptor through its binding to Grb2. We showed previously that Gab1 competes with SOS for binding to Grb2 (2). This observation, together with a diminution of the duration of Gab1 in the membrane regulated by SHP-2, may explain the down-regulation of MAPK pathway.

Our results reveal that the Gab1/SHP-2 interaction is related almost exclusively to the transformation properties induced by Gab1. Moreover, other signals must emanate as a result of the Gab1/SHP-2 interaction that leads to c-fos induction and the more complex phenotypes of increased cell growth, morphologic alterations, and transformation. A practical implication of this work is that the Gab1 molecule is an attractive target for therapy, and the discovery of the critical nature of the Gab1/SHP-2 interaction immediately suggests that strategies designed to interfere with this association may meet with the greatest success.

ACKNOWLEDGMENTS

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Role of the Grb2-Associated Binder 1/SHP-2 Interaction in Cell Growth and Transformation

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