A novel role for Gab2 in bFGF-mediated cell survival during retinoic acid–induced neuronal differentiation

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Gab proteins amplify and integrate signals stimulated by many growth factors. In culture and animals, retinoic acid (RA) induces neuronal differentiation. We show that Gab2 expression is detected in neurons in three models of neuronal differentiation: embryonic carcinoma (EC) stem cells, embryonic stem cells, and primary neural stem cells (NSCs). RA treatment induces apoptosis, countered by basic FGF (bFGF). In EC cells, Gab2 silencing results in hypersensitivity to RA-induced apoptosis and abrogates the protection by bFGF. Gab2 suppression reduces bFGF-dependent activation of AKT but not ERK, and constitutively active AKT, but not constitutively active MEK1, reverses the hypersensitization. Thus, Gab2-mediated AKT activation is required for bFGF's protection. Moreover, Gab2 silencing impairs the differentiation of EC cells to neurons. Similarly, in NSCs, Gab2 suppression reduces bFGF-dependent proliferation as well as neuronal survival and production upon differentiation. Our findings provide the first evidence that Gab2 is an important player in neural differentiation, partly by acting downstream of bFGF to mediate survival through phosphoinositide 3 kinase–AKT.

Introduction

FGFs are peptide growth factors playing critical roles in various cellular processes including cell proliferation, differentiation, and survival. Among these, basic FGF (bFGF) is required for normal cortex development (Dono et al., 1998). FGFs mediate their actions through four different cell surface receptors, the FGF receptors (FGFRs; Reuss and von Bohlen und Halbach, 2003). Ligand binding induces FGFR dimerization and autophosphorylation on multiple tyrosine residues in its cytoplasmic domain. The phosphorylated tyrosines serve as docking sites for SH2 domain–containing effectors including PLCγ and Crk. A major downstream mediator is the PTB domain–containing protein FRS2, which binds to FGFR and the NGF receptor in a phosphotyrosine (PY)–independent manner. Several tyrosine residues in the COOH terminus of FRS2 are phosphorylated by activated FGFR or NGF receptors and serve to recruit the adaptor protein Grb2 (Kouhara et al., 1997). FRS2 also regulates the phosphoinositide 3 kinase (PI3K)–AKT pathway through its interaction with Gab1, with Grb2 as an intermediate (Ong et al., 2001).

Gab1 belongs to an adaptor family consisting of Gab1, Gab2, and Gab3 in mammals, the *Drosophila melanogaster* orthologue Daughter of Sevenless (DOS), and the *Caenorhabditis elegans* orthologue Suppressor of Clear (Soc 1). These proteins share a highly conserved NH$_2$-terminal pleckstrin homology (PH) domain, a central proline-rich domain, and multiple tyrosine residues that are recognized by SH2 domain–containing proteins. Gab proteins function downstream of multiple receptors, such as the receptors for EGF, hepatocyte growth factor, and FGF (Gu and Neel, 2003). Gab1 is expressed ubiquitously, whereas Gab2 and Gab3 expression are more restricted. Although the three mammalian Gabs share significant homology, they are not functionally redundant. Gab1−/− mice are embryonically lethal due to developmental defects in the heart, placenta, and skin (Itoh et al., 2000). Gab2−/− mice are viable but show specific defects in allergic responses (Gu et al., 2001). Finally, Gab3−/− mice are reportedly normal (Seiffert et al., 2003). Gab1 directly associates with c-Met, Grb2, SHP2, and the p85 subunit of PI3K (Gu and Neel, 2003) and is important for cell survival (Holgado-Madruga et al., 1997). Gab2 was cloned as an SHP2 interacting protein and binds to many of the same proteins as Gab1. It functions in many cellular processes, including differentiation, migration (W.M. Yu et al., 2002), transformation (Sattler et al., 2002), and phagocytosis (Gu et al., 2003). For Gab1 and Gab2, the SHP2–Gab interaction is thought to regulate the ERK pathway, whereas the p85 binding sites are important for AKT activation.

Supplemental Material can be found at:
http://www.jcb.org/cgi/content/full/jcb.200505061/DC1

Retinoic acid (RA) plays a fundamental role in the development of the central nervous system, stimulating neurite outgrowth and migration of the neural crest (Maden and Holder, 1992). During the differentiation process, several FGFs and FGFRs are expressed. bFGF is highly expressed in the nervous system and has multiple roles. It is a differentiation factor in the hippocampus and has neurotrophic activities, supporting the survival and growth of cultured neurons and neural stem cells (NSCs; Gage, 2000; Reuss and von Bohlen und Halbach, 2003). NSCs are found in the hippocampus and subventricular zone (SVZ) of fetal and adult brain and are more committed cells with limited self-renewal capabilities and with the potential to differentiate into neurons, astrocytes, and oligodendrocytes (Gage, 2000). P19 embryonic carcinoma (EC) cells are pluripotent stem cells similar to embryonic stem (ES) cells. They are induced by RA to differentiate into neuron- and glia-like cells (McBurney et al., 1982). RA treatment also induces apoptosis in EC cells. bFGF was reported to suppress RA-associated apoptosis, possibly via the PI3K pathway (Miho et al., 1999). However, the mechanism for bFGF-mediated activation of the PI3K pathway in EC or ES cells and the interaction between bFGF and RA during neuronal differentiation remain to be clarified.

We show that Gab2 expression markedly increases during RA-induced differentiation of EC and ES cells along the neural lineage. Gab2 expression is high in NSCs and persists in neurons after differentiation. Further, Gab2, acting through the PI3K–AKT pathway, plays a key role in bFGF-mediated cell survival in EC cells, inhibiting the apoptosis that accompanies RA-induced neural differentiation. Gab2 gene silencing by small hairpin RNA (shRNA) vectors not only reduces the number of surviving cells after RA treatment but also diminishes the percentage and quality of the neurons that emerge. Lastly, lentiviral transduction of Gab2 shRNA into NSCs markedly inhibits the ability of bFGF to support neurosphere formation and also impairs neuronal survival during differentiation. Together, these results demonstrate for the first time that Gab2 may be an important component of neural differentiation.

Results

Gab2 is part of the FGF signaling pathway

We examined the expression levels of mammalian Gab members in P19 cells by semiquantitative RT-PCR (Fig. 1 A). All three Gab proteins were present, with Gab3 being expressed at the highest levels, whereas Gab1 levels were the lowest. We initially focused on Gab2, which has been shown to function as an adaptor protein downstream of numerous receptors but not the FGFR. After stimulation with bFGF, Gab2 showed increased tyrosine phosphorylation, accompanied by coimmunoprecipitation with the p85 subunit of PI3K (Fig. 1 B). Gab2 contains a PH domain, which in Gab1 is known to bind to phosphatidylinositol-3,4,5-P_3 (PIP_3) on the membrane (Maroun et al., 1999). Cells were transiently transfected with HA-Gab2. In starved cells, Gab2 was diffusely localized in the cytoplasm, but in response to bFGF, a subpopulation of Gab2 was recruited to the plasma membrane (Fig. 1 C). These results show that Gab2 is a component of the bFGF pathway.
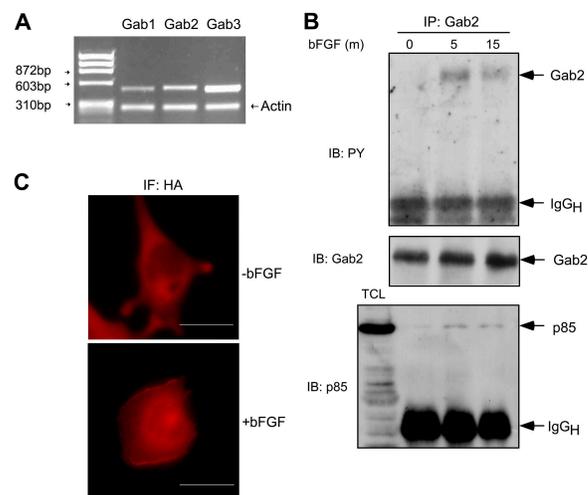

Figure 1. **Gab2 functions downstream of bFGF.** (A) Expression levels of Gab1, 2, and 3 in P19 cells were estimated by semi-quantitative RT-PCR. β-Actin levels were used as a control. (B) Gab2 immunoprecipitates from cells treated as indicated were analyzed sequentially by α-PY and α-Gab2 blotting. TCL, total cell lysate. (C) P19 cells were transfected with HA-Gab2 and starved overnight as in B. FGF-stimulated (15 min) and unstimulated cells were stained with α-HA antibody. Bars, 10 μm.

Gab2 is essential for bFGF-mediated cell survival during RA-induced cell death

P19 cells undergo neuronal differentiation in response to RA. RA also induces apoptosis, which is rescued by exogenous bFGF (Miho et al., 1999). To determine if bFGF acts through Gab family proteins to counteract RA-induced cell death, we constructed shRNA vectors to specifically silence Gab2 and Gab3 expression in P19 cells. The 3′-UTRs of Gab2 and Gab3 were selected as targets to enable subsequent reconstitution with Gab mutants. As the 3′-UTR sequence of Gab2 is not available, we used 3′-RACE to amplify it from 32D mouse myeloid cells. Transient transfection assays demonstrated that the Gab2 and Gab3 shRNA constructs were specific and efficient as they suppressed 70–90% of the appropriate reporter activity (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200505061/DC1).

We next examined the ability of the Gab2 and Gab3 shRNA vectors to suppress endogenous gene expression. Semiquantitative RT-PCR showed that Gab2 shRNA specifically silenced endogenous Gab2 expression but did not affect Gab1 or Gab3 expression in transient knockdown experiments (Fig. 2 A). Similar specificity was demonstrated for the Gab3 shRNA. Neither Gab2 nor Gab3 shRNA suppressed β-actin levels. We next determined if knockdown of Gab2 and Gab3 affected the viability of P19 cells when treated with RA. Consistent with a role for Gab2 in survival signaling, Gab2 silencing sensitized P19 cells to RA-induced cell death (Fig. 2 B). Only 30% of the Gab2 shRNA-transfected cells survived upon RA treatment, compared with 75% of cells transfected with either control or Gab3 shRNA. bFGF increased the survival of cells transfected with control or Gab3 shRNA but not Gab2 shRNA. As transfection with Gab3 shRNA did not increase cell death in response to RA, Gab2 may play a unique role in mediating cell
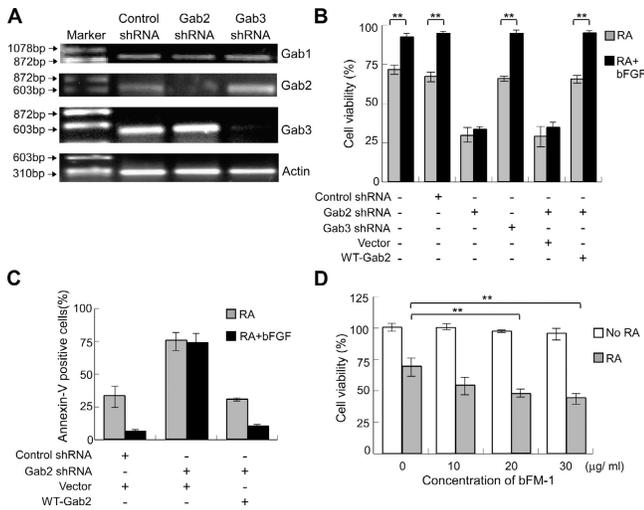
Figure 2. **bFGF-mediated cell survival is Gab2 dependent.** (A) P19 cells were transiently transfected with shRNA vectors. Expression levels of Gab1, 2, and 3 were determined by semi-quantitative RT-PCR. (B) P19 cells, transfected with the indicated plasmids, were treated with RA ± bFGF for 48 h. Apoptotic and viable cells were counted using a hemocytometer and Trypan blue staining, and viability was calculated as a percentage of surviving to total cells. Shown is the mean ± SD ($n = 7$ for all conditions except for the untransfected cells [$n = 5$]; **, $P < 0.0005$). (C) Cells were treated as in B and apoptotic cells were detected by FITC-labeled Annexin-V staining. Total cell number and the number of Annexin-V–positive cells were obtained from five different fields. Greater than 400 cells were counted for each condition. Shown is the mean ± SD, based on five fields. (D) P19 cells were preincubated with different concentrations of bFM-1 for 1 h and treated as described in B. Shown is the mean ± SD ($n = 4$; **, $P < 0.001$).

Figure 3. **Gab2 can be stably silenced by shRNA transfection.** (A) Shown are α-Gab2 immunoblots for parental P19 cells, a stable clone transfected with control shRNA, and three stable clones transfected with Gab2 shRNA. (B) Stable clones described in A were seeded in α-MEM/10% FBS. Cell numbers were counted for 4 d as described in Fig. 2 B. This is a representative experiment out of three. (C) Parental P19 cells and their stable derivatives expressing Gab2 shRNA were transiently transfected with vector or WT Gab2 and treated as described in Fig. 2 B. Apoptotic and surviving cells were counted as described in Fig. 2 B. Shown is the percentage of surviving to total cells (mean ± SD; $n = 4$; **, $P < 0.001$). (D) Stable control and Gab2-silenced cells were preincubated with 20 μg/ml bFM-1 for 1 h and treated as described in Fig. 2 D. Shown is the percentage of surviving to total cells (mean ± SD; $n = 4$; **, $P < 0.001$).

survival. In support of this hypothesis, reconstitution of Gab2 shRNA-transfected cells with wild-type (WT) Gab2 reversed the hypersensitivity to RA-induced cell death and restored the ability of bFGF to promote survival. We confirmed that cell death occurred via apoptosis by Annexin-V (Fig. 2 C) and Hoechst (not depicted) staining. In agreement with Fig. 2 B, there were more Annexin-V–positive cells and fragmented nuclei in Gab2 shRNA-transfected cells after RA treatment compared with control shRNA-transfected cells. Because Gab2 knockdown had such a significant effect, we did not investigate the role of Gab1, which is expressed at much lower levels.

In the presence of FBS alone, RA had a more modest effect on the viability of parental and control cells (Fig. 2 B, first and third columns). We reasoned that bFGF may be present in the conditioned medium because a previous study showed that media conditioned by P19 cells contain multiple heparin-binding growth factor activities (Heath et al., 1989). To test this possibility, we used either a neutralizing antibody to bFGF (bFM-1) or a specific kinase inhibitor of FGFR, PD173074 (Bansal et al., 2003; Nilsson and Skinner, 2004). Neither significantly affected the viability of parental P19 cells in the absence of RA, but in the presence of RA, bFM-1 (Fig. 2 D) or PD173074 (not depicted) could further reduce cell viability in a dose-dependent manner. These results are consistent with the presence of bFGF in the conditioned medium and with the ability of endogenous bFGF to protect against RA-induced cell death. FGF/ FGFR inhibition was deleterious only when RA was present,

suggesting that bFGF's survival function is particularly important during neuronal differentiation.

Stable Gab2 knockdown in P19 cells was achieved by cotransfection with Gab2 shRNA and a vector containing the puromycin resistance gene. Three puromycin-resistant clones showed greatly reduced Gab2 expression compared with parental or control shRNA-transfected cell lines (Fig. 3 A). When grown in complete medium, cells expressing Gab2 shRNA showed modest growth reduction compared with control cells (Fig. 3 B). In agreement with the transient transfection findings, cells with stable Gab2 silencing also showed hypersensitivity to RA-induced cell death and were not rescued by exogenous bFGF (Fig. 3 C). Moreover, reconstitution of stable knockdown cells with WT Gab2 reversed the hypersensitivity to RA-induced cell death and restored the protective effect of bFGF. Similar to
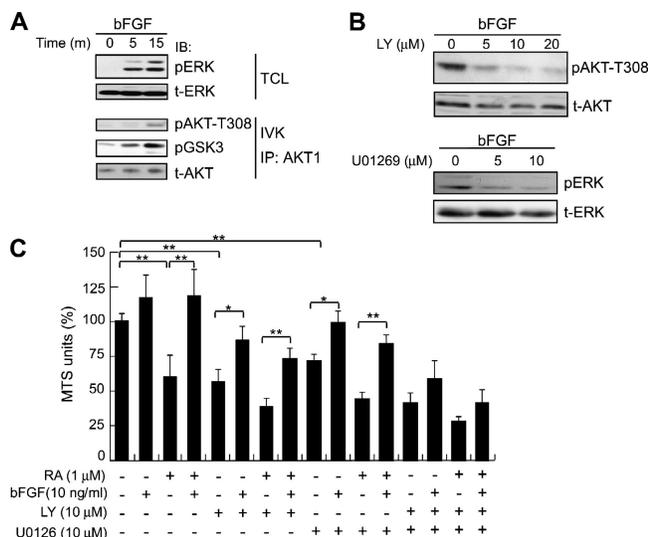
Figure 4. **AKT and ERK pathways modulate bFGF-mediated survival.** (A) 40 μg of total cell lysates were probed with α-pERK and reprobed with α-total ERK antibodies (top). An in vitro AKT kinase assay was performed to measure AKT activity (bottom). The top part of the membrane was probed with α-pAKT, stripped, and reprobed with α-total AKT antibodies. The bottom part of the membrane was probed with α-pGSK3 antibody to detect phosphorylation of the substrate in the kinase assay, a GSK3 fusion protein. (B) Starved P19 cells were pretreated with different concentrations of LY or U0126 for 1 h and stimulated with 10 ng/ml bFGF for 15 min. AKT and ERK activation was detected with α-pAKT and α-pERK antibodies, respectively. (C) P19 cells were treated as shown. MTS absorbance (A$_{490}$) units are plotted as a percentage of that measured in untreated cells and shown as the mean ± SD (n = 4; *, P < 0.05; **, P < 0.005).

the effect of bFGF/FGFR inhibition on parental cells (Fig. 2 D), bFM-1 (Fig. 3 D) or PD173074 (not depicted) could further reduce the viability of control shRNA-transfected cells in the presence of RA but without exogenous bFGF. Consistent with Gab2 being a downstream target of bFGF/FGFR, ablation of Gab2 expression eliminated any additional effect of bFGF/FGFR inhibition on cell survival (Fig. 3 D). Together, these results indicate that bFGF, present in the conditioned medium, is a key survival factor for P19 cells, protecting them from significant cell death induced by RA, and that bFGF/FGFR works through Gab2 to mediate survival. Because exogenous bFGF conferred additional protection, the bFGF levels in the conditioned medium were at submaximal levels.

Gab2 regulates bFGF-mediated cell survival mainly through the AKT pathway

To determine which bFGF-mediated signaling pathway uses Gab2, we first demonstrated, using an α-phosphorylated ERK antibody and an in vitro AKT kinase assay, that both the ERK and AKT pathways were activated upon bFGF stimulation (Fig. 4 A). Next, we used the PI3K inhibitor LY294002 (LY) and the MEK1 inhibitor U0126 to assess the importance of PI3K and ERK in mediating cell survival. Dose-dependent titration of the inhibitors showed that 10 μM LY or U0126 was sufficient to block bFGF-mediated AKT or ERK activation (Fig. 4 B). Therefore, we used 10 μM of either inhibitor in an MTS cell viability assay (Fig. 4 C). RA reduced cell viability by ~40%, and viability was fully restored by bFGF. When

used alone, LY or U0126 reduced survival by 40 and 25%, respectively. Addition of RA, in combination with either LY or U0126, further decreased cell viability to 40 and 50% of untreated cells, respectively. bFGF had a marginal protective effect when both pathways were inhibited simultaneously but this was not statistically significant. These findings indicate that the PI3K and ERK pathways are required for bFGF-mediated cell survival during RA-induced cell death.

Because PI3K and ERK activities were important for P19 survival, and Gab2 silencing markedly reduced bFGF-dependent survival, we tested if Gab2 can function downstream of bFGF to mediate PI3K–AKT and ERK activation. Control, Gab2, or Gab3 shRNA was transiently cotransfected with FLAG-AKT into P19 cells. Compared with control or Gab3 shRNA-transfected cells, bFGF-induced AKT activity was reduced by ~80% in Gab2-silenced cells (Fig. 5 A). We further validated that the reduction in AKT activity was specifically due to Gab2 silencing by cotransfection with Gab2 shRNA and WT Gab2 (Fig. 5 B). Maximal AKT activation requires phosphorylation at both T308 and S473. In addition to a reduction in AKT T308 phosphorylation, AKT phosphorylation at S473 was almost abolished, implying that Gab2 is essential for S473 phosphorylation. WT Gab2 reconstitution in Gab2-silenced cells greatly enhanced AKT phosphorylation on both T308 and S473 (Fig. 5 B). These results confirm a specific effect of Gab2 silencing on bFGF-induced Akt activation.

Next, we determined if Gab2 suppression affected bFGF-mediated ERK activation. Myc- or HA-tagged ERK was cotransfected with the indicated shRNA constructs (Fig. 5, C and D). In contrast to AKT, bFGF-induced ERK activation was not affected by Gab2 silencing. Moreover, reconstitution with WT Gab2 did not increase ERK activation, suggesting that bFGF-provoked ERK activation is Gab2 independent. Together, our results imply that Gab2-dependent bFGF-mediated cell survival occurs via the PI3K–AKT pathway. Consistently, constitutively active AKT (E40K) and to a much lesser extent constitutively active MEK (R4F) rescued RA-induced cell death in Gab2-silenced cells (Fig. 5 E).

Previous studies showed that RA activates the p38 and JNK pathways (Alsayed et al., 2001; Wang et al., 2001). We tested the possibility that bFGF may regulate RA-induced cell death through p38 and JNK. Our results demonstrated that although bFGF or RA could transiently activate JNK and p38 in P19 cells, inhibitor studies showed that these kinases were not important components of the bFGF survival pathway (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200505061/DC1).

The PH domain of Gab2 is required for its function

Gab2 undergoes growth factor–dependent phosphorylation at multiple tyrosines including those that dock p85/PI3K. We determined how a Gab2 mutant lacking p85 binding sites (3YF) responded to bFGF stimulation. Parental P19 cells were transiently cotransfected with WT or 3YF along with FLAG-AKT. The 3YF mutant showed decreased bFGF-induced tyrosine phosphorylation compared with WT. Moreover, AKT activation in 3YF-overexpressing cells was reduced compared with

A

	Control shRNA					Gab2 shRNA					Gab3 shRNA					
bFGF (m)	0	2	5	15	30	0	2	5	15	30	0	2	5	15	30	

pGSK3 IVK

Fold 0.06 0.06 0.42 1.0 0.64 0.01 0.12 0.22 0.23 0.23 0.08 0.07 0.5 0.98 0.58

t-AKT IP: FLAG

IP: Gab2
IB: Gab2

B

IP: FLAG

bFGF(m)	0	5	15	0	5	15	0	5	15	IB:

pAKT-T308
pAKT-S473
FLAG

IP: Gab2

Gab2

Control shRNA + + + - - - - - -
Gab2 shRNA - - - + + + + + +
WT-Gab2 - - - - - - + + +
FLAG-AKT + + + + + + + + +

C

	Control shRNA			Gab2 shRNA			Gab2 shRNA +WT-Gab2			
bFGF (m)	0	5	15	0	5	15	0	5	15	

~pERK IB: Myc

IP: Gab2
IB: Gab2

D

	Control shRNA						Gab2 shRNA						Gab2 shRNA+WT-Gab2					
bFGF (m)	0	5	15	30	60	180	0	5	15	30	60	180	0	5	15	30	60	180

~pERK IB: HA

E

Control shRNA + - - - -
Gab2 shRNA - + + + +
MEK1-R4F - - + - +
AKT1-E40K - - - + +

Figure 5. **Gab2 mediates survival through the AKT pathway.** (A) P19 cells were transiently transfected with plasmids as indicated and stimulated with bFGF. FLAG-tagged AKT was precipitated and an in vitro AKT kinase assay was performed as described in Fig. 4 A. Gab2 levels were measured to demonstrate efficient knockdown of Gab2. (B) P19 cells were transiently transfected with the indicated plasmids and stimulated by bFGF. FLAG-AKT precipitates were probed sequentially with α-pAKT (T308), α-pAKT (S473), and α-FLAG antibodies. (C and D) P19 cells were transiently transfected with Myc- or HA-ERK and the indicated shRNA plasmids, and then bFGF stimulated. Total cell lysates were probed with α-Myc (C) or α-HA (D) antibody to detect phosphorylation-induced mobility shift of ERK. (E) P19 cells were transfected with various combinations of plasmids and treated as described in Fig. 2 B. Shown is the percentage of living to total cells (mean ± SD; n = 3; **, P < 0.005).

vector control, indicating that the 3YF mutant exerts a dominant-negative effect over endogenous Gab2 (Fig. 6 A).

The PH domain of Gab2 is important for recruitment to the phagocytic cup (Gu et al., 2003). To examine the role of the PH domain in bFGF signaling, we transiently overexpressed Gab2 mutants, either ΔPH (which lacks the PH domain) or ΔPH/3YF (a combination of 3YF and ΔPH mutations). ΔPH and ΔPH/3YF mutants showed markedly reduced bFGF-induced tyrosine phosphorylation (Fig. 6 B). AKT activation was also greatly reduced in cells expressing either mutant, compared with cells expressing WT Gab2, but was similar to vector-transfected cells. These results indicate that the PH domain of Gab2 is required for optimal bFGF-induced tyrosine phosphorylation and provide an explanation for the dominant-negative effect of 3YF. Consistent with their degree of AKT activation, 3YF, ΔPH, and ΔPH/3YF were unable to reverse the hypersensitivity to RA-induced cell death caused by Gab2 silencing (Fig. 6 C).

RA-induced neuronal differentiation is accompanied by marked Gab2 up-regulation

The P19 cell line is a good in vitro model to study RA-induced neuronal differentiation (McBurney et al., 1982). Given that

bFGF has a critical role in protecting P19 cells against RA-associated cell death, we determined the effect of depleting endogenous bFGF during differentiation. We used neuron-specific β-III tubulin as a marker for neuronal differentiation. By immunofluorescence, only 50% as many cells survived after differentiation in the presence of bFM-1 compared with its absence (Fig. 7 A, left), and, of these, only ~40% were neurons, compared with ~90% in cultures where bFGF was not depleted (Fig. 7 A, right). Next, we investigated if Gab2 expression was altered during neuronal differentiation. Gab2 levels increased dramatically when P19 cells differentiated into neuron-like cells (Fig. 7 B). β-III tubulin expression showed kinetics similar to Gab2 expression. We then determined if bFGF affected the kinetics of Gab2 expression during differentiation. The presence of bFGF hastened the appearance of Gab2 as well as β-III tubulin (Fig. 7 B). Thus, depleting bFGF levels in the conditioned medium decreased the total number of surviving cells as well as the percentage of neurons (Fig. 7 A), whereas increasing the levels of bFGF by exogenous addition had the opposite effect (Fig. 7 B). These findings suggest that bFGF in combination with RA facilitated an earlier commitment of P19 cells to the neural fate. The increase in Gab2 levels could be a specific response to RA treatment or intrinsic to the neuronal differentiation program in P19 cells.

Figure 6. **bFGF-induced optimal AKT activation requires Gab2.** (A) P19 cells were transiently cotransfected with the indicated Gab2 constructs and FLAG-AKT, and then stimulated by bFGF. HA-Gab2 precipitates were sequentially blotted with α-PY and α-HA antibodies. FLAG-AKT precipitates were probed sequentially with α-pAKT and α-FLAG antibodies. (B) P19 cells were transiently transfected and stimulated as described in A. HA-Gab2 precipitates were probed sequentially with α-PY and α-HA antibodies. FLAG-AKT precipitates were used in an in vitro AKT kinase assay as described for Fig. 4 A. (C) Gab2 shRNA was cotransfected with vector or various Gab2 constructs into P19 cells. Cells were treated as described in Fig. 2 B. Shown is the percentage of surviving to total cells (mean ± SD; n = 3; **, P < 0.005). (bottom) A Gab2 blot of total cell lysates. White lines indicate that intervening lanes have been spliced out.

We then asked if silencing of Gab2 affected RA-induced neuronal differentiation in P19 cells. Stable clones expressing either control or Gab2 shRNA were induced by RA to differentiate. Both Gab2 and β-III tubulin expression increased in fully differentiated cells expressing control shRNA (Fig. 7 C). Similar to the effect of bFM-1, cells where Gab2 was stably silenced showed significantly reduced expression of β-III tubulin, indicating that Gab2, like bFGF, plays a role in neuronal differentiation. Because RA induces P19 to differentiate into both neuron- and glia-like cells, we tested the expression pattern of Gab2. After differentiation, cells expressing control or Gab2 shRNA were doubly stained with antibodies to Gab2 and β-III tubulin (Fig. 7 D). Gab2 was present in β-III tubulin–positive cells. Consistent with a role for Gab2 in protecting cells against RA-induced cell death, when Gab2 was stably silenced, fewer cells survived differentiation, compared with cells expressing

control shRNA (Fig. 7 E, left). Moreover, fewer β-III tubulin–positive cells emerged from the Gab2-silenced clone after RA treatment (Fig. 7 E, middle) and these cells were abnormal in appearance, showing greatly shortened neurite extensions (Fig. 7 D; and Fig. 7 E, right). These data demonstrate that in P19 cells, Gab2, like bFGF, is important for neuronal differentiation.

To further confirm a function for Gab2 in embryonic neuronal differentiation, ES cells were induced to differentiate into neurons using the 4−/4+ RA protocol (Bain et al., 1995). First, we verified that the FGF signaling pathway was similarly important in ES cells. We determined the number of ES cells that survived differentiation in the presence or absence of PD173074. For both P19 and ES cells (Fig. 8 A), 50 nM PD173074 was sufficient to reduce the number of surviving cells by 50%. Next, we examined Gab2 expression in ES cells. Gab2 was low in proliferating ES cells, but expression increased significantly upon RA induction (Fig. 8, B and C), similar to that described for P19 cells (Fig. 7 B). Immunofluorescence further showed that most cells stained positive for Gab2 and β-III tubulin. A few were Gab2 positive and β-III tubulin negative, whereas others were negative for both (Fig. 8 D). Together, these results demonstrate that Gab2 and bFGF exert similar effects during RA-induced neuronal differentiation in P19 cells and ES cells and that Gab2 is likely to be functioning as a bFGF effector.

The role of Gab2 in NSCs

So far, we have shown that Gab2 is required for bFGF-mediated cell survival and that Gab2 expression is increased during RA-induced embryonic neuronal differentiation, which is further enhanced by the addition of bFGF. To study the role of Gab2 in stem cells committed to a neural fate, NSCs were isolated from the SVZ of postnatal mice and cultured as floating neurospheres. Neurospheres are dependent on either bFGF or EGF for growth (Reynolds and Weiss, 1992), and upon removal of growth factors they can be induced to differentiate into neurons, astrocytes, and oligodendrocytes. Addition of RA is reported to increase the number of cells differentiating into neurons (Takahashi et al., 1999). Primary neurospheres were dissociated and plated in medium containing 1% FBS alone or with RA or RA and 10 ng/ml bFGF and allowed to differentiate for 7 d. Removal of bFGF greatly increased the death of NSCs (Fig. 9 A, left). Addition of RA to NSCs further reduced cell survival. After 7 d of differentiation, 42% of surviving cells in the presence of RA were neurons, compared with 35% in its absence, although this difference was not statistically significant (Fig. 9 A, right). Previous observations had shown that the maximal enhancing effect of RA is apparent only at >12 d after RA removal (Takahashi et al., 1999). Cell viability was markedly increased when bFGF was added along with RA. However, the percentage of neurons was less, with a concomitant increase in glia identified by glial fibrillary acidic protein (GFAP) staining (Fig. 9 B). This is consistent with a previous report that high concentrations (10 ng/ml) of bFGF promote glial generation (Qian et al., 1997).

We next examined the role of Gab2 in NSCs. Undifferentiated neurospheres stained strongly for Gab2 (Fig. 9 B, top),
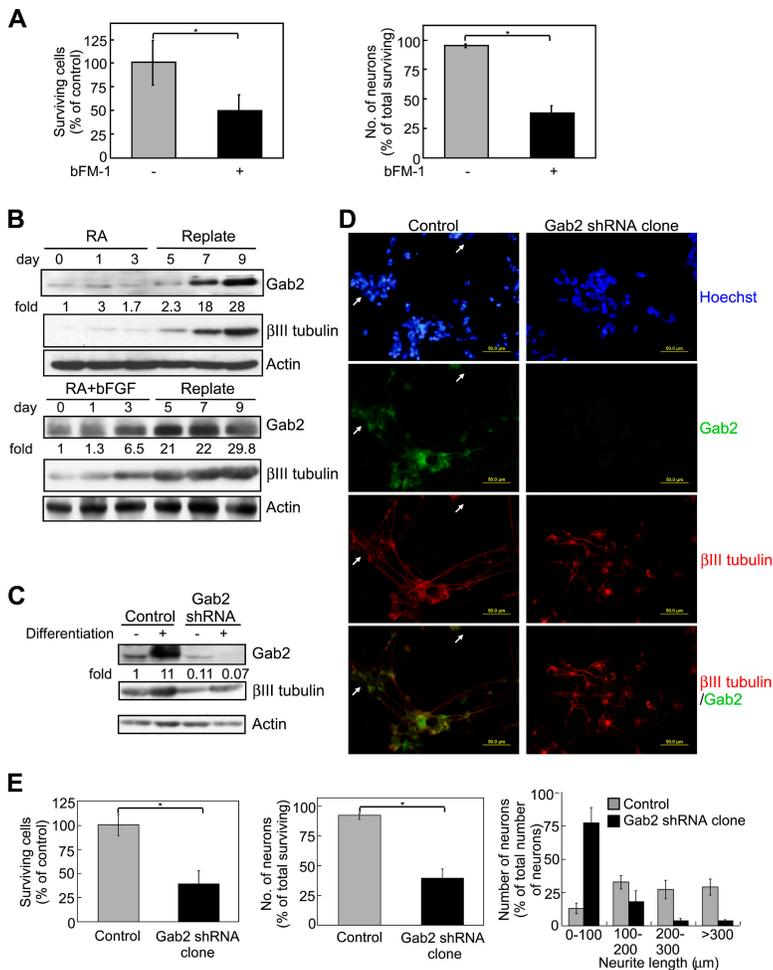
Figure 7. **Gab2 regulates RA-induced neuronal differentiation in P19 cells.** (A) P19 cells were induced to differentiate by RA with or without 20 ng/ml bFM-1. Cells surviving at the end of the differentiation protocol were stained with α-β-III tubulin to visualize neurons and with Hoechst dye to visualize all cells. Total cell number and the number of β-III tubulin–positive cells were obtained from 10 different fields. Greater than 500 cells were counted for each experiment. (left) Surviving cells are shown as a percentage of that in the absence of bFM-1. (right) β-III tubulin–positive cells are expressed as a percentage of total surviving cells. Results are plotted as the mean ± SD ($n = 4$; *, $P < 0.05$). (B) P19 cells were treated with RA in the absence (top) or presence (bottom) of bFGF for the indicated times. Cells were harvested and blotted for Gab2 and β-III tubulin expression. Fold induction was calculated by normalizing Gab2 to actin levels. (C) Stable clones transfected with control or Gab2 shRNA-2 were induced to differentiate by RA. Undifferentiated cells were harvested at the beginning of the experiment and differentiated cells 9 d later. (D) 9 d after initiation of RA treatment, stable clones expressing control or Gab2 shRNA were doubly stained with anti–β-III tubulin (Cy3) and rabbit α-Gab2 (AF 488). Nuclei were visualized by Hoechst staining (blue). Gab2 was primarily in cells that were β-III tubulin positive. A few cells were Gab2 and β-III tubulin negative (arrows). Bars, 50 μm. (E) The number of surviving cells (left) and neurons (middle) were estimated as described in A 9 d after RA induction. The lengths of neurites were measured using a micrometer scale bar (right). 50 β-III tubulin–positive cells were measured for each experiment. Shown is the mean ± SD ($n = 4$; *, $P < 0.05$).

indicating that Gab2 expression is already elevated in NSCs. Because neurospheres in culture are considered to be heterogeneous (Pevny and Rao, 2003), we cannot exclude the possibility that there are already more committed progenitors in our cultures, although Gab2 staining appeared to be relatively homogeneous. When induced to differentiate, Gab2 was again detected in neurons (Fig. 9 B). Several small, phase-dark cells also stained positive for Gab2, but Gab2 was barely detectable in GFAP-positive astrocytes. To test if Gab2 is required for bFGF-mediated proliferation, neurospheres were transduced by a lentivirus expressing control or Gab2 shRNA with GFP as a marker. Gab2 expression was greatly reduced by Gab2 shRNA, compared with cells transduced by a control shRNA lentivirus or to untransduced cells (Fig. 9 C). A clonal assay was conducted to determine how Gab2 silencing affected neurosphere proliferation. Transduced neurospheres were grown in the presence of bFGF alone for 8 d. Silencing of Gab2 in NSCs markedly diminished their ability to generate neurospheres, and the size of neurospheres was significantly smaller compared with those transduced with a control shRNA (Fig. 9 D). Thus, Gab2 is required for bFGF-mediated proliferation of NSCs.

To determine if Gab2 is required for neuronal survival during NSC differentiation, transduced neurospheres were differentiated in the presence of 1% FBS and neuronal apoptosis was measured using an antibody that recognizes cleaved caspase-3. 72 h after differentiation induction, 32% of GFP-positive neurons were apoptotic in Gab2-silenced cells compared with 8% in cells transduced with a control shRNA (Fig. 9 E). Consistently, Gab2 suppression reduced the number of surviving, GFP-positive neurons by 60–70% (Fig. 9 E), which is to be contrasted to a difference of only 27% in the GFP-positive, nonneuronal population. This latter difference is in agreement with minimal Gab2 expression in astrocytes (Fig. 9 B) and may reflect the difference in transduction efficiencies (control, 60%; Gab2 shRNA, 50% at the time of plating). Together, our data implicate Gab2 as being essential for optimal neuronal generation, in part by providing survival signals during neuronal differentiation. Based on the present findings, we cannot exclude an additional role for Gab2 in specifying the neuronal fate during commitment.

Discussion

Gab1, Gab2, and Gab3 null mice have profoundly different phenotypes ranging from embryonic lethality (Gab1) to apparent normalcy (Gab3; Itoh et al., 2000; Gu et al., 2001; Seiffert et al., 2003), consistent with the Gab proteins having distinct functional roles in development. In this work, we used RNA interference to silence Gab2 and Gab3 in the P19 EC cell line and showed that these proteins have nonoverlapping functions in

Figure 8. **Gab2 is highly expressed during ES neuronal differentiation.** (A) P19 and ES cells were induced by RA to undergo differentiation in the presence or absence of 50 nM PD173074. At the end of the differentiation protocol, cell viability was measured as described in Fig. 7 A (*n* = 2). D3 (B) and J1 (C) ES cells at the indicated times during the 4−/4+ RA differentiation protocol were harvested and analyzed for Gab2 and β-III tubulin expression. (D) D3 cells were induced to differentiate as described in B. 12 d after initiating differentiation, cells were multiply stained with α-β-III tubulin (Cy3), purified rabbit α-Gab2 (AF 488), and Hoechst dye (blue). Arrows point to cells that are doubly negative for Gab2 and β-III tubulin. Other cells stained for Gab2 only (arrowheads). Bars, 50 μm.
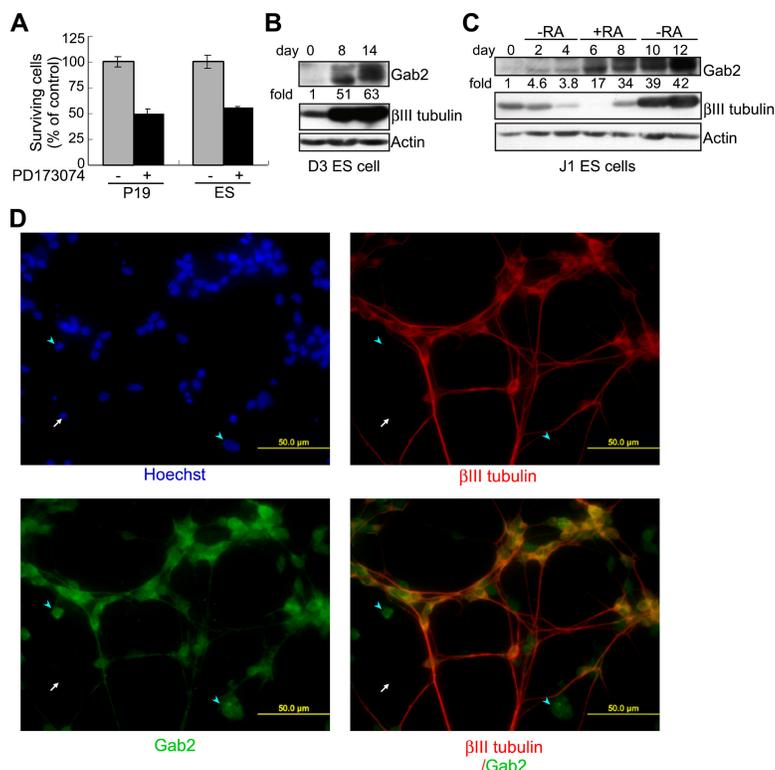
bFGF survival signaling. Gab2, but not Gab3 suppression, reduced bFGF-mediated AKT activity and abrogated bFGF's ability to protect P19 cells from RA-induced cell death. In addition, Gab2 relayed the bFGF signal to AKT and not ERK, and it required both its PH domain and p85/PI3K binding sites to signal downstream. These results support the conclusion that Gab2 is a critical positive regulator of bFGF-stimulated signaling and that it modulates bFGF signaling mainly through the AKT pathway. Using the EC and ES system, we also demonstrated for the first time that Gab2 expression increased dramatically during embryonic neural differentiation, and in EC cells, Gab2 silencing significantly affected this process. Gab2 was already highly expressed in NSCs, suggesting that up-regulation of Gab2 may occur at the neural commitment stage, before neuronal differentiation. Lastly, Gab2 silencing in NSCs markedly reduced the ability of bFGF to support neurosphere proliferation and inhibited neuronal survival when NSCs were induced to differentiate. As Gab2 has been reported to participate in mast cell and macrophage differentiation (Gu and Neel, 2003), it may have a more general role in cellular differentiation.

During development, various extracellular signaling molecules modulate neuronal differentiation. RA is one such molecule. RA is teratogenic when administrated in excess to pregnant animals and affects multiple systems including the central nervous system (Maden and Holder, 1992). In *Xenopus laevis*, RA regulates pattern formation of the vertebrate neural plate, by up-regulating prepattern and neurogenic genes, and by down-regulating genes that inhibit neurogenesis (Franco et al., 1999). FGFs are also important factors of neuronal differentiation. bFGF participates in the development of the cerebral cortex and supports the proliferation of NSCs from the SVZ

(Reynolds and Weiss, 1992; Dono et al., 1998). The FGF and RA pathways have been shown to have opposing effects in vivo (Appel and Eisen, 2003). During spinal cord development in the chick embryo, FGF blocks expression of class I HD/bHLH transcription factors and Sonic hedgehog. This inhibits neurogenesis and maintains the caudal region as a stem zone. On the other hand, RA, arising from somites, antagonizes the FGF signal and promotes neurogenesis by up-regulating class I genes. Thus, FGF and RA coordinate the initiation of neurogenesis.

Gab2 is expressed in the brain but little is known about its function there. Our results firmly establish that Gab2 has a role in neural function and provides a link between bFGF and RA in embryonic neural differentiation. bFGF plays a complex role during neural development. It supports the proliferation and survival of ES cells and is needed for the expansion and maintenance of neuroectodermal and neural precursor cells (Reynolds and Weiss, 1992). When neural precursor cells are induced to differentiate, the ratio of neurons to other cell types in culture appears to depend on the levels of bFGF present, low doses (0.1 ng/ml) favoring neuronal differentiation and high doses (10 ng/ml) glial generation (Qian et al., 1997). In EC cells, Gab2 expression is markedly increased during RA-induced neural differentiation and is further enhanced by the addition of bFGF. Depletion of endogenous bFGF gives rise to a phenotype which is similar to that induced by Gab2 silencing. This leads to the interesting possibility that Gab2 may play a role in bFGF-dependent maintenance/expansion of neural precursors. Consistently, NSCs that are already committed to the neural fate but still retain the potential to differentiate, show high levels of Gab2 expression and Gab2 silencing significantly reduced NSC proliferation. bFGF may further modulate neurogenesis
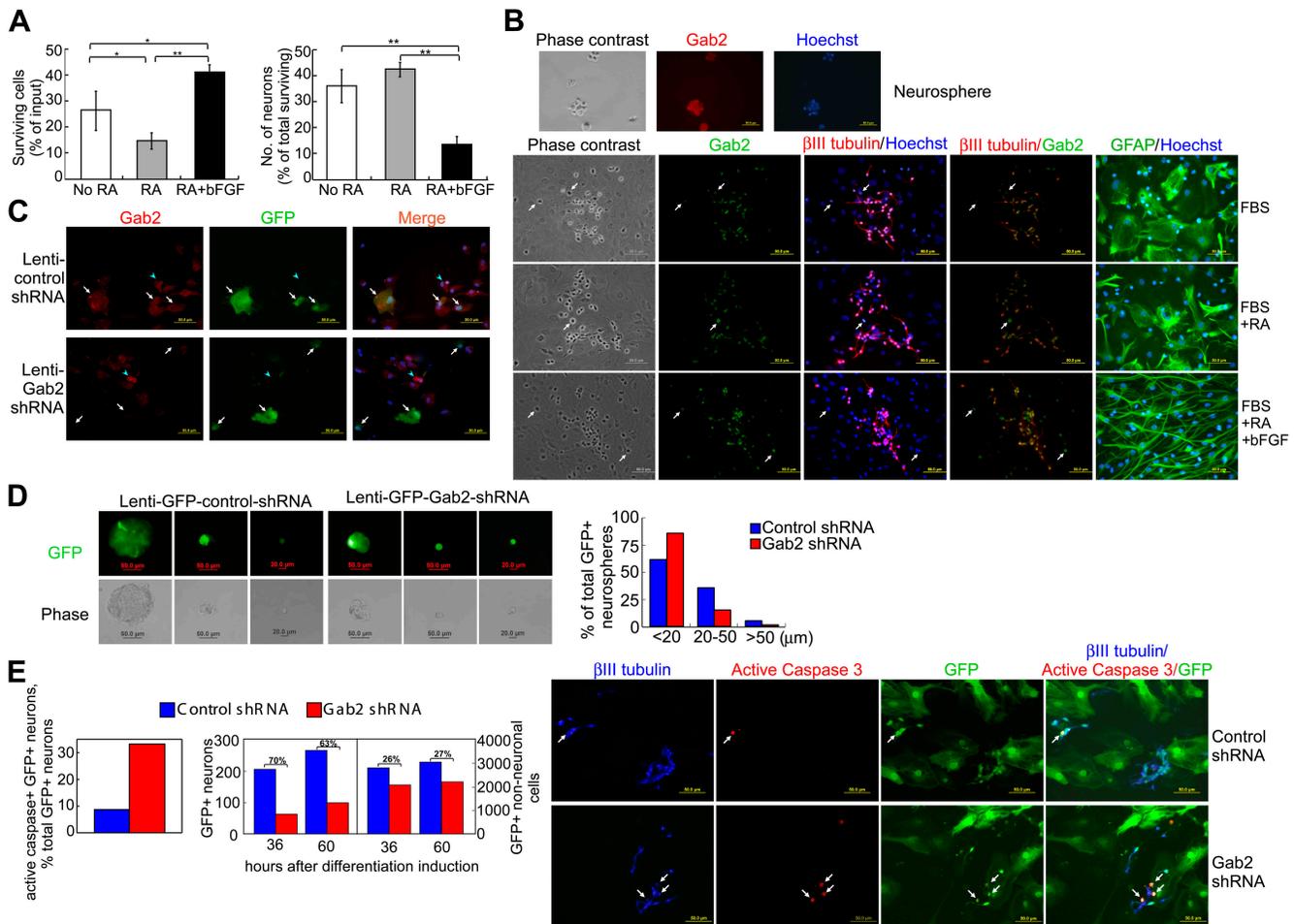
Figure 9. **Gab2 knockdown impairs bFGF-mediated NSC proliferation and neuronal differentiation.** (A) Primary NSCs differentiated for 7 d in the presence of 1% FBS alone or with 1 μM RA ± 10 ng/ml bFGF. Total cell number and the number of β-III tubulin–positive cells were obtained from 10 different fields. (left) Greater than 1,000 cells were counted for each experiment. Cell viability is shown as a percentage of number of surviving to input cells (means ± SD; $n = 3$; *, $P < 0.05$; **, $P < 0.005$). (right) 400–800 β-III tubulin–positive cells were counted and plotted as a percentage of total cell number (mean ± SD; $n = 3$; **, $P < 0.005$). (B) NSCs were differentiated as described in A. Cells were multiply stained with rabbit α-Gab2 (AF 488), α-β-III tubulin (Cy3), and Hoechst dye (blue). Several cells stained for Gab2 only (arrows). Separate samples were also stained with α-GFAP (AF 488). (C) Neurospheres were transduced by control or Gab2 shRNA lentivirus and after 48 h stained with α-Gab2 and Hoechst. Transduced cells are identified by GFP expression (arrows). Arrowheads point to nontransduced cells. (D) Primary NSCs were transduced by control or Gab2 shRNA lentivirus, dissociated, and plated into uncoated 96-well plates under limiting dilution conditions (10 cell/well) in the presence of 20 ng/ml bFGF for 8 d. GFP-positive neurospheres were scored by size ($n = 400$). (E) NSCs were transduced and plated in the presence of 1% FBS. At 72 h after plating, GFP-positive cells that also stained positive for β-III tubulin (AF 350) and active caspase 3 (Cy3) were obtained from 60 fields. Greater than 100 apoptotic neurons were obtained for Gab2-silenced cells. The percentage of apoptotic neurons was determined by counting all GFP, β-III tubulin–positive cells from the same fields. Arrows point to apoptotic, GFP-positive neurons. The number of viable, GFP-positive neurons was estimated in a similar experiment. Cells that were doubly positive for GFP and β-III tubulin were obtained from 50 random fields and a minimum of 200 cells were obtained for NSCs transduced with either control or Gab2 shRNA virus. Non-neuronal, GFP-positive cells were also obtained from the same fields and >2,000 cells were counted. (left) Shown are the results from a representative experiment out of at least three independent experiments. Plotted are the actual cell numbers. Percent refers to the percent reduction in Gab2-silenced cells compared to control cells.

when NSCs are induced to differentiate. In the presence of exogenous bFGF, more cells survive but there is a concomitant reduction in the ratio of neurons to other cells, consistent with previous observations that high dose bFGF promotes glial formation. In the absence of exogenous bFGF, low levels of bFGF may still be present in the conditioned medium because neurons and astrocytes are reported to secrete bFGF (Reuss and von Bohlen und Halbach, 2003). Thus, the effect on differentiation due to Gab2 suppression could still reflect a bFGF effect. However, neurotrophins are also secreted by NSCs and their receptors are up-regulated during differentiation (Barnabe-Heider and Miller, 2003). Therefore we cannot exclude the

possibility that Gab2 also functions downstream of Trk receptors to modulate the fate specification of multipotential progenitors. Irrespective of which upstream signal Gab2 is responding to during NSC differentiation, our data clearly support an essential role for Gab2 in mediating neuronal survival. Interestingly, because Gab2 expression was prominent in neurons but not astrocytes after NSC differentiation, our findings also argue for a possible role of Gab2 in lineage selection.

A key finding of our studies is that Gab2 regulates the bFGF survival pathway mainly through AKT. Both the p85 binding sites and the PH domain of Gab2 are crucial for bFGF-stimulated AKT activation. Suppression of Gab2 does not com-

pletely eliminate AKT activation, indicating that Gab2-independent pathways also exist. Gab3 is not a major contributor because Gab3 silencing does not affect bFGF-induced AKT activation. Deletion of the PH domain significantly reduces bFGF-mediated Gab2 tyrosine phosphorylation and AKT activation, suggesting that Gab2 needs to be recruited to the plasma membrane via PIP_3 before it can become tyrosine phosphorylated. Altogether, these results support a model in which bFGF stimulates a Gab2-independent pathway to induce early PI3K–AKT activation. PIP_3 generated during this early phase then binds to the PH domain of Gab2, recruiting Gab2 to the plasma membrane, whereby it becomes tyrosine phosphorylated at sites including those that dock p85. In this fashion, an amplification loop is created resulting in further AKT activation. This model is consistent with the finding that 3YF-Gab2 when overexpressed, functions in dominant-negative fashion, possibly by sequestering PIP_3 and blocking signaling to AKT.

As we did not observe any defect in bFGF-mediated ERK activation in Gab2-silenced P19 cells, FGFR probably uses other proteins such as FRS2 to activate the ERK pathway. This is supported by the report that FRS2 is required for FGF1-induced ERK activation. Moreover, similar to our observations on Gab2, Gab1 is essential for FGF1-induced PI3K–AKT, but not ERK, activation (Lamothe et al., 2004). Although Gab2 does not appear to play a major role in bFGF-induced ERK activation, it clearly contributes to ERK activation in response to other stimuli. For example, while IgE-provoked ERK activation in Gab2 knockout mice is not affected (Gu et al., 2001), Erk activation induced by BCR/ABL is clearly decreased (Sattler et al., 2002). Thus, depending on the stimulus, Gab2 may have differential effects on ERK activation.

Until now, Gab2 was mainly considered to be a regulator of hematopoiesis. Data shown in this study provide the first demonstration that Gab2 also functions in the nervous system by acting as a critical link between bFGF and the PI3K–AKT pathway. No gross brain defect was reported in Gab2 null mice; however, bFGF−/− mice were also initially reported to have normal brain morphogenesis. It was only recently that these mice were found to have a significant decrease in the number of neurons in the cerebral cortex (Dono et al., 1998). It will be interesting to see if an in-depth investigation of Gab2 knockout mice will reveal defects in neural development and neurological function.

Materials and methods

Reagents and antibodies

bFGF and EGF were obtained from PeproTech. All-trans RA and heparin were obtained from Sigma-Aldrich. Cell culture reagents were purchased from Invitrogen. The inhibitors used were as follows: U0126 (Cell Signaling); LY (LC Laboratories); SP600125 and SB203580 (Calbiochem); PD173074 (a gift of K. Kilgore, Pfizer Global Research and Development, Ann Arbor, MI). The antibodies used were as follows: JNK, AKT, myc (9E10), and actin antibodies (Santa Cruz Biotechnology, Inc.); PY (4G10), and bFM-1 (UBI); pAKT, pGSK3, pERK, active caspase-3 antibodies, and α-p-p38 antibody–conjugated beads (Cell Signaling); FLAG (M2) and GFAP antibodies (Sigma-Aldrich); HA and β-III tubulin antibodies (BABCO/Covance). Rabbit α-Gab2 was purchased from UBI and goat α-Gab2 from Santa Cruz Biotechnology, Inc. Cy3-conjugated secondary antibodies were from obtained from Jackson ImmunoResearch Laboratories and AlexaFluor (AF)-conjugated secondary antibodies were obtained from Molecular Probes.

Cell culture, DNA constructs, and transfection

P19 cells were cultured in αMEM/10% FBS. The D3 and J1 ES cells (gifts of S. O'Shea, University of Michigan, Ann Arbor, MI) were cultured in DME/10% FBS and 10^3 U/ml LIF (Chemicon) on 0.1% gelatin-coated tissue culture dishes. WT and 3YF (Y441F, Y465F, Y574F) Gab2 (gifts of H. Gu, Harvard University, Boston, MA) have been previously described (Gu et al., 2001). A ΔPH mutant was generated by deletion of residues 6–115. A Myc- or HA-tagged ERK and FLAG-tagged WT AKT plasmid has been described previously (Lee and States, 2000). The shRNA pU6 vector and the control vector, pU6-XASH targeting an irrelevant frog protein, were provided by D. Turner (University of Michigan, Ann Arbor, MI; J.Y. Yu et al., 2002). We designed shRNA constructs to target against the 3′-UTR of Gab2 and Gab3. The Gab2 and Gab3 target sequences are as follows: Gab2 shRNA-1 (5′-GTTCAAGAGCCATGAGGCAGCTGCCC-3′), Gab2 shRNA-2 (5′-CCTATGACTTCTGTCAATAC-3′), Gab2 shRNA-3 (5′-CAACATCAGGGACCTGACCCTTTCCCAT-3′), Gab3 shRNA-1 (5′-AAGTGTGGGCTTGGGTGTGATT-3′), and Gab3 shRNA-2 (5′-CTTCCTC-TTCTCTTTGTCCTCAGCAC-3′). Based on results in Fig. S1, Gab2 shRNA-2 and Gab3 shRNA-2 were selected for subsequent transfection. To establish stable P19 Gab2 knockdown cell lines, the Gab2 shRNA-2 was cotransfected with pPUR (CLONTECH Laboratories, Inc.) into P19 cells and cells were selected in medium containing 1 μg/ml puromycin. For transient transfection, 0.8 μg of the shRNA construct was cotransfected with 0.2 μg of a reporter plasmid (Myc-, HA-ERK, or FLAG-AKT) into P19 cells. Transfected cells were allowed to express for 24 h before starvation and stimulation.

In vitro neuronal differentiation

Differentiation of P19 cells was induced as described previously (McBurney et al., 1982). ES cells were differentiated using the 4−/4+ RA protocol (Bain et al., 1995). In experiments to inhibit endogenous bFGF effects, 20 μg/ml bFM-1 or 50 nM PD173074 was added at the beginning of the differentiation process.

Isolation and culture of NSCs

NSCs from the SVZ of CD1 mice (Charles River Laboratories) at postnatal day 14 were isolated and cultured as described previously (Gabay et al., 2003). NSCs were grown as floating neurospheres in DME/F12 medium containing N2 and B27 supplements plus 20 ng/ml bFGF, 5 μg/ml heparin, and 20 ng/ml EGF for 7 d before being used for differentiation experiments. To induce differentiation, neurospheres were dissociated by trituration and $5–7.5 \times 10^4$ cells were plated onto coated glass coverslips or 24-well plates in DME/F12/1% FBS. In some wells, 1 μM RA ± 10 ng/ml bFGF was added.

Lentiviral transduction

Control XASH shRNA or Gab2 shRNA was ligated into pLentiLoX (a gift of L. van Parijs, Massachusetts Institute of Technology, Boston, MA; Rubinson et al., 2003). 293T cells were cotransfected with pMDLg/pRRE, pCMV-VSVG, pRSV-REV, and either pLentiLoX-XASH or pLentiLoX-Gab2 shRNA by calcium phosphate precipitation. VSVG-pseudotyped lentiviruses were collected 48 after transfection and concentrated by ultracentrifugation at 36,000 rpm for 90 min. The viral titer was determined to be $2–10 \times 10^8$ TU/ml following an established protocol (Rubinson et al., 2003). To assess the effect of Gab2 silencing in NSCs, neurospheres were first dissociated into single cells and allowed to recover in medium containing growth factors for 8 h. Lentiviruses were then added for 12 h at a multiplicity of infection of 20. Neurospheres were further allowed to recover for 36 h before plating for clonal expansion (Fig. 9 D) or differentiation (Fig. 9 E) as described in the figure legends.

Stimulation, immunoprecipitation, and immunoblotting

Cells were starved in αMEM for 24 h and in all experiments stimulated with both bFGF (100 ng/ml) and heparin (5 μg/ml) for the times indicated in the figure legends. Lysis, immunoprecipitation, and blotting were performed as described previously (Lee and States, 2000). Protein concentration was determined with the assay kit (Bio-Rad Laboratories).

AKT kinase assay

Starved cells were stimulated with bFGF and lysed. 500 μg of lysates was precipitated with 1 μg of α-AKT–conjugated protein G–Sepharose and used in an in vitro kinase assay according to the manufacturer's protocol (Cell Signaling). After SDS-PAGE and transfer to PVDF, the bottom part of the membrane was probed with α-p-GSK3 antibody, whereas the top part containing AKT was probed sequentially with α-p-AKT and α-total AKT antibodies.

Cell death assays

P19 cells were seeded in 12-well plates and transfected with various constructs. 24 h later, the transfected cells were treated with 1 μM RA ± 10 ng/ml bFGF for 48 h. Dead cells were identified by Trypan blue exclusion and by Hoechst 33258 (Sigma-Aldrich) staining. To block endogenous bFGF, bFM-1 or PD173074 was preincubated with P19 cells for 1 h before RA treatment. FITC-labeled Annexin-V (BD Bioscience) staining of live cells was used to confirm the presence of apoptosis. To measure proliferation/viability, P19 cells were starved for 16 h and treated with inhibitors for 1 h before the addition of RA ± bFGF. The number of surviving cells was estimated 48 h later by the MTS assay (CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay; Promega). To measure apoptosis in neurons, differentiated NSCs were stained with antibodies to active caspase-3 and β-III tubulin.

Immunostaining

To examine Gab2 translocation, P19 cells were plated on glass coverslips and transfected with HA-Gab2. Cells were starved for 16 h in αMEM before stimulation with bFGF for 15 min. Immunostaining was performed essentially as described previously (Gu et al., 2003). For double staining, cells were seeded onto glass coverslips coated with poly-D-lysine and fibronectin or laminin. We immunopurified rabbit α-Gab2 by incubating the IgG fraction with 2 × 1 cm nitrocellulose chips on which overexpressed Gab2 was immobilized. We performed double staining by adding antibodies sequentially. The following dilutions were used: purified rabbit α-Gab2 (1:260), HA (1:800), TuJ1 (1:4,000), GFAP (1:500), active caspase-3 (1:100), AF 488 goat α-rabbit (1:150), AF 350 goat α-mouse (1:100), Cy3-goat α-mouse (1:4,000), and Cy3-goat α-rabbit (1:500). Epifluorescence was visualized with an Olympus IX50 with LC Plan Fl 40×/0.60 Ph2 or LC Ach 20×/0.4 PhC objective. Photomicrographs were acquired digitally using the DP Controller for a cooled charge coupled device camera (DP70; Olympus). Controls with secondary antibodies only or with one primary along with one or both secondary antibodies accompanied each experiment. NIH Image v1.62 was used to measure neurite lengths using a stage micrometer scale provided by the microscopy image laboratory (University of Michigan). ImageJ was used to perform cell counts on digital images.

Statistical analysis

p-values were calculated using the two-sided t test.

Online supplemental material

Supplemental data provide information on primer and PCR conditions used in Figs. 1 and 2 and methods to test the efficacy and specificity of shRNA constructs and measurements of JNK and p38 activity. Fig. S1 shows the efficacy and specificity of Gab2 and Gab3 shRNA constructs. Fig. S2 shows JNK and p38 kinase activities during RA-induced cell death. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200505061/DC1.

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