Scaffolding Protein Grb2-associated Binder 1 Sustains Epidermal Growth Factor-induced Mitogenic and Survival Signaling by Multiple Positive Feedback Loops

Received for publication, January 17, 2006, and in revised form, April 13, 2006. Published, JBC Papers in Press, May 9, 2006, DOI 10.1074/jbc.M600482200

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Grb2-associated binder 1 (GAB1) is a scaffold protein involved in numerous interactions that propagate signaling by growth factor and cytokine receptors. Here we explore in silico and validate in vivo the role of GAB1 in the control of mitogenic (Ras/MAPK) and survival (phosphatidylinositol 3-kinase (PI3K)/Akt) signaling stimulated by epidermal growth factor (EGF). We built a comprehensive mechanistic model that allows for reliable predictions of temporal patterns of cellular responses to EGF under diverse perturbations, including different EGF doses, GAB1 suppression, expression of mutant proteins, and pharmacological inhibitors. We show that the temporal dynamics of GAB1 tyrosine phosphorylation is significantly controlled by positive GAB1-PI3K feedback and negative MAPK-GAB1 feedback. Our experimental and computational results demonstrate that the essential function of GAB1 is to enhance PI3K/Akt activation and extend the duration of Ras/MAPK signaling. By amplifying positive interactions between survival and mitogenic pathways, GAB1 plays the critical role in cell proliferation and tumorigenesis.

Signaling through the epidermal growth factor (EGF)3 receptor (EGFR) is crucial for many cellular processes, including growth, cell-cycle progression, differentiation, and apoptosis (1, 2). Stimulation by growth factors of the EGFR family causes dimerization of EGFR monomers and activates their intrinsic tyrosine kinase activity. Subsequent transphosphorylation of multiple tyrosine residues on the cytoplasmic tail of EGFR conveys a biochemical message to various adaptor proteins and enzymes with specific phosphotyrosine binding domains. EGFR-mediated phosphorylation and activation of multiple binding partners initiates signal propagation through a number of interacting branches, including the mitogen-activated protein kinase (MAPK) cascade and the phosphatidylinositol 3-kinase (PI3K)/Akt survival pathway (see Fig. 1).

The adaptor proteins Grb2 (growth factor receptor-binding protein 2) and Shc (Src homology 2 (SH2) and collagen domain protein) play key roles in signaling downstream of EGFR. Grb2 associates with activated EGFR either directly or through tyrosyl-phosphorylated Shc (3). This association is mediated by the SH2 domain of Grb2 that binds to specific phosphotyrosine residues on EGFR or Shc. Simultaneously, through its N-terminal SH3 domain, Grb2 associates with the cytoplasmic guanine nucleotide exchange factor (GEF) SOS (homolog of the Drosophila melanogaster Son of sevenless) (4, 5). EGF-induced recruitment of the SOS-Grb2 complex to the plasma membrane is critical for the initiation of the MAPK/ERK pathway (Raf/MEK/ERK cascade) (6–8). SOS catalyzes the transformation of an inactive GDP-bound form (Ras-GDP) of the small membrane-anchored GTPase Ras into its active GTP-bound form (Ras-GTP). Subjected to multiple controls, Ras acts as the gatekeeper of the MAPK/ERK cascade and a critical switch that responds to a number of signals that determine the cell’s fate (9–11). Signaling of activated Ras is turned off by the activation of GTPase-activating protein (RasGAP/p120-GAP), which stimulates GTP hydrolysis by Ras (12). Inhibitory feedback phosphorylation of SOS by ERK provides an additional mechanism for the inhibition of Ras signaling (13–15).

EGF-induced membrane recruitment of the SOS-Grb2 complex can be mediated not only by EGFR but also by the Grb2-associated binder (GAB) adaptor proteins (3, 16). The GAB proteins are also critical components of a major route of PI3K activation by EGFR and are involved in the recruitment of the p85 regulatory subunit of PI3K to the plasma membrane (17–19). All members of GAB family contain the N-terminal pleckstrin homology (PH) domain that mediates membrane targeting, several proline-rich motifs serving as binding sites for SH3 domain-containing proteins, such as Grb2 and the soluble tyrosine kinase Src, and multiple tyrosine phosphorylation sites that recruit a variety of effectors, including PI3K, RasGAP, and...
protein-tyrosine phosphatase SHP2 (20–22). The relative abundance of GAB1/2/3 isoforms varies in different cell types (23). Here we use HEK293 cells and focus on the functional role of GAB1 in EGF-induced signaling.

The association of GAB1 with EGFR is thought to occur predominantly via Grb2 (19), resulting in tyrosine phosphorylation of GAB1 on several sites that bind SH2 domains of p85, RasGAP, and SHP2. GAB1-mediated recruitment of p85 leads to PI3K activation and the production of phosphatidylinositol (3,4,5)-triphosphate (PIP3) in the plasma membrane. This GAB1-PI3K interaction generates positive feedback in PI3K stimulation (see Fig. 1): the PH domain of GAB1 binds PIP3, and this leads to a further recruitment of GAB1 to the membrane, which further activates PI3K (18). In addition, the plasma membrane recruitment of GAB1 influences the Ras/PI3K pathway in multiple ways (see Fig. 1). GAB1 can bind the Grb2-SOS complex, which activates Ras, and tyrosyl-phosphorylated GAB1 can bind RasGAP, which negatively regulates Ras. Intriguingly, protein phosphatase SHP2, which binds to GAB1, was reported to be a positive regulator of the MAPK pathway (20, 22, 24–27). This positive effect is related to the formation of the GAB1-SHP2 complexes and subsequent dephosphorylation of the docking sites on GAB1 involved in RasGAP binding (27), which results in an elevation of active Ras-GTP.

GAB1−/− mice die early in the embryonic development due to a combined effect of loss-of-function mutations in multiple receptor-tyrosine kinase pathways (22, 28). Some of these effects can be explained by the essential function of GAB1 in activation of the PI3K/Akt survival pathway and by the findings that cells from GAB1−/− mice or with GAB1 mutants lacking SHP2 binding sites showed impaired MAPK/ERK activation (19, 20, 25, 29, 30). However, reports on the significance of GAB1 and PI3K for MAPK activation have been controversial. The expression of the constitutively active catalytic subunit p110α of PI3K stimulated the MAPK pathway in some cells but not in the others (31). Likewise, treatment with the PI3K inhibitors wortmannin and LY294002 led to inhibition of Ras and ERK activation in the non-transformed Vero cells (24) but had no effect on ERK-PK in opossum kidney cells (32), bovine tracheal myocytes (33), and rat hepatocytes (data from this laboratory, not shown), and even increased ERK activation in the ErbB4-expressing Chinese hamster ovary cells; the latter was attributed to inhibitory phosphorylation of Raf1 by Akt, the downstream target of PI3K (34). Thus, although many mechanisms of the GAB involvement in PI3K-MAPK cross-talk are understood at the molecular level, their physiological control within the overwhelmingly complex network of protein and protein-lipid interactions remains elusive. Systems Biology approaches may help link both well known and conjectured molecular mechanisms to physiological responses of complex cellular networks through quantitative, predictive, and testable mathematical models.

In the present report, we combined experimental analysis of EGF-induced signaling with the development of a comprehensive computational model of the EGFR network. Our goal is to unlock the functional role of the scaffold adaptor protein GAB1 in the activation of the Ras/MAPK and PI3K/Akt pathways and in the interactions between these pathways. This combined experimental and computational modeling approach enabled us to assess how GAB1 modulates multiple feedforward and feedback regulatory mechanisms that control the MAPK temporal dynamics and couple EGF-induced Ras/MAPK and PI3K/Akt signaling. The model described similarities and differences in the changes of the Ras/MAPK dynamics after GAB1 suppression or PI3K inhibition, and these predictions were verified experimentally. The model predicts that the relative abundance of EGF, GAB, and SHP2 is a critical controlling factor in the PI3K and MAPK pathway interactions. Our findings support the view that GAB1 serves as an additional signaling platform initiating the Ras/MAPK pathway. This GAB1 role is especially important at low EGF and/or EGFR levels. Our results have important ramifications for the identification of therapeutic targets in the PI3K/Akt survival and MAPK proliferation pathways.

MATERIALS AND METHODS

Reagents and Commercial Antibodies—All routine reagents were obtained from Sigma (St. Louis, MO) unless otherwise noted. The following primary monoclonal and polyclonal antibodies were used: anti-EGF receptor, anti-Akt1/PKBα PH domain, anti-α-tubulin (clone DM1A) (all three from Upstate Biotechnology, Lake Placid, NY); anti-phospho-Akt (Ser-473), anti-ERK, anti-phospho-ERK (Thr-202/Tyr-204), anti-MEK1/2, anti-phospho-MEK1/2 (Ser-217/221), anti-SHP2 (all from Cell Signaling, Beverly, MA); anti-phospho-EGF receptor (Tyr-1173) (BioSource, Camarillo, CA); anti-GAB1 (H-198), anti-phospho-GAB1 (Tyr-627), anti-Grb2 (C-23) (all from Santa Cruz Biotechnology, Santa Cruz, CA); anti-Ras (BD Transduction Laboratories, San Diego, CA); and anti-glycerolaldehyde-3-phosphate dehydrogenase (clone 6C5) (Chemicon, Temecula, CA). Secondary horse anti-mouse horseradish peroxidase-linked IgG antibodies were purchased from Cell Signaling (Beverley, MA); peroxidase-conjugated ImmunoPure goat anti-rabbit IgG (H+L) and rabbit anti-sheep IgG (H+L) were from Pierce.

Cell Culture and Transfection—Human embryonic kidney HEK293 and epidermoid carcinoma A431 cell lines obtained from ATCC (Manassas, VA) were cultured in Dulbecco’s modified Eagle’s medium/F-12 medium supplemented with 10% fetal bovine serum (both from Invitrogen) and penicillin-streptomycin solution (100 μg/ml each, Fisher) in a humidified 5% CO2 incubator at 37 °C. Cells were split 1:3 every 3 days to maintain subconfluency.

One hour or less before transfection adherent cells were trypsinized and resuspended in antibiotics-free serum-reduced Opti-MEM I medium (2 × 105 cells/ml, Invitrogen). The cells were set aside in the incubator at 37 °C while a master mix of transfection complexes was prepared according to the manufacturer’s instructions, with 6.25 μl of 20 μM stock siRNA and 7 μl of siPORT NeoFX transfection reagent (Ambion, Austin, TX) in Opti-MEM I medium per well. 200 μl of newly formed transfection complexes was dispensed into empty wells of 6-well culture plates (Nunc, Denmark) and subsequently gently mixed with a 2.3-ml cell overlay volume giving a final concentration of 50 nm GAB1 siGENOME SMARTpool siRNA (Dharmacon, Lafayette, CO). Control cells were transected
with siCONTROL Non-Targeting siRNA Pool (Dharmacon) or left untreated. Control and transfected cells were incubated in normal cell culture conditions for 12–24 h before 1 ml of complete medium with antibiotics was added into each well. After 48 h, the medium was removed by suction, adhered cells were washed with phosphate-buffered saline, and 2.5 ml of fresh complete medium was poured into each well. 60 h after transfection the complete medium was replaced with 2.5 ml of fetal bovine serum-free medium, and cells were starved for 12 h. The phenotypic and functional efficiency of RNA interference-based knockdown of GAB1 protein expression was measured at protein level by Western blotting analysis. Routinely, 70–85% of GAB1 protein depletion was achieved.

**Cell Stimulation, Treatment with Inhibitors, and Protein Extraction**—72 h after from transfection with siRNA and 12-h serum starvation, cells were stimulated with 20 nM EGF for the indicated time periods. 30 min before stimulation cells were incubated with 200 nM PI3K inhibitor wortmannin (Cell Signaling), 5 μM MEK inhibitor U0126, or 5 μM ERK inhibitor 5-iodotubericidin (the latter two from Calbiochem) as indicated. Cells were scraped in 700 μl per well of ice-cold lysis buffer (150 mM NaCl, 2.5 mM EGTA (pH 7.4), 25 mM Hepes (pH 7.4), 5% glycerol, 1% Igepal CA-630, 10 μl/ml phosphatase inhibitor mixture Set II (Calbiochem), and one Complete protease inhibitor mixture tablet per 10 ml (Roche Applied Science, Indianapolis, IN)). Alternatively, in experiments without siRNA transfection, HEK293 or A431 cells were seeded in 6-well plates at a concentration of 3 × 10⁵ cells per well incubated for 24 h and starved 12 h before treatment with inhibitor(s) and stimulation with a ligand.

Cells were collected in tubes, vigorously vortexed for 10 s, and solubilized for 30 min on ice. Detergent-insoluble material was removed by centrifugation at 10,000 × g for 10 min at 4 °C. Protein concentrations were determined using the bicinchoninic acid solution (Pierce). Proteins from each total cell lysate were immediately dissolved in 5 volumes of 1× Laemmli buffer (Bio-Rad Laboratories) with 5% β-mercaptoethanol and boiled for 5 min at 95 °C.

**Preparation of Cytoplasmic and Membrane Fractions with Digitonin**—Following EGFR stimulation, the starvation medium was replaced with 1 ml of ice-cold permeabilization buffer containing 150 mM NaCl, 2.5 mM EGTA (pH 7.4), 25 mM Hepes (pH 7.4), 5% glycerol, 150 μg/ml digitonin (Sigma), 10 μl/ml phosphatase inhibitor mixture Set II and one Complete protease inhibitor mixture tablet per 10 ml. The cells were left on ice for 10 min, collected into Eppendorf tubes, and fractionated by centrifugation at 10,000 × g for 2 min. Cytoplasmic fractions (supernatant) were collected in separate tubes and used for preparation of SDS-PAGE samples as described above. Membrane fractions (pellet) were immediately mixed with 200 μl/tube of ice-cold lysis buffer (see above), vigorously vortexed for 10 s, and solubilized for 30 min on ice. Detergent-insoluble material was removed by centrifugation at 10,000 × g for 10 min at 4 °C, and supernatant was used for preparation of SDS-PAGE samples as described above. The efficiency of digitonin cell permeabilization was determined by trypan blue dye exclusion. 100% of the cells were permeabilized by 150 μg/ml digitonin in 10 min.

**Immunoprecipitation and Immunoblotting**—300 μg of each total cell lysate was incubated with the indicated antibody (2–5 μg) while rocking for 2 h at room temperature and then incubated with 30 μl of protein-A-Sepharose 4B beads (Sigma) overnight at 4 °C. Tyrosine-phosphorylated proteins from total cell lysates were collected with 20 μl of monosclonal anti-phosphotyrosine-agarose beads (clone PT-66, Sigma) overnight at 4 °C. The beads were washed with phosphate-buffered saline three times, and proteins were eluted in 2× Laemmli buffer (Bio-Rad), boiled for 5 min at 95 °C, and spun down.

The resulting immunoprecipitates or proteins from total cell lysates were then subjected to SDS-PAGE using Novex 8% Tris-glycine (Invitrogen) or NuPAGE 4–12% Bis-Tris (Invitrogen) gels. Gel areas with separated phosphorylated proteins of interest and housekeeping proteins were manually cut based on their molecular weight, combined, and transferred onto the same nitrocellulose membrane (Bio-Rad) to avoid transfer-based variability. The membrane was rinsed with distilled water, blocked with 3% bovine serum albumin in TBST (5 mM Tris HCl (pH 8.0), 138 mM NaCl, 26.8 mM KCl, 0.05% Tween 20) for 2 h at room temperature, and then probed with indicated primary antibodies overnight at 4 °C. Antibodies against housekeeping proteins (glyceraldehyde-3-phosphate dehydrogenase or Grb2) were used to normalize the sample loading. Additionally, each membrane was visualized using phosphorylated proteins was stripped with Restore Western blot stripping buffer (Pierce) for 12 min at room temperature, washed in TBST for 8 min, blocked, and probed with an antibody against the relevant total protein as described above.

The membranes were washed extensively with TBST, followed by incubation with respective secondary antibodies for 1 h at room temperature. After final washing with TBST for 45 min the bands were visualized by using an Image Station 440CF (Eastman Kodak Scientific Imaging Systems, New Haven, CT) using enhanced chemiluminescence detection with SuperSignal West Dura Extended Duration substrate (Pierce). The Western blot analyses shown in the figures are representative of three or more individual experiments. Densitometric analysis was performed using Kodak Digital Science software. The results are represented in plots as means ± S.E.

**Ras Assay**—For Ras-GTP measurement, 300 μg of each supernatant was incubated with 20 μl of Raf-1 Ras binding domains bound to glutathione-agarose beads (Ras Assay Reagent was from Upstate Biotechnology) overnight on a rocking platform at 4 °C. Protein complexes were collected by brief centrifugation and washed three times with ice-cold lysis buffer. Ras-GTP was released from agarose beads by adding 2× Laemmli buffer and boiling for 5 min at 95 °C. 20 μg of the sample was separated by SDS-PAGE using Novex 14% Tris-glycine gels (Invitrogen) and subjected to immunoblotting assay as described above.

**Computational Modeling Analysis of the EGFR Signaling Network**—Fig. 1 presents a flow chart of signaling branches analyzed in a computational model of the EGFR network. We previously developed and validated in silico models of some parts of this network (11, 35–38). The initial part of the EGFR network includes activation and subsequent tyrosine phosphorylation and dephosphorylation of EGFR. It also incorporates...
interactions of phosphorylated EGFR with its immediate adapter and target proteins (including Shc, Grb2, RasGAP, Src, phospholipase Cγ, and p85) and the generation of the Ras-activating and -deactivating signals via two canonical branches, which involve the formation of the EGFR-Grb2-SOS/EGFR-Shc-Grb2-SOS and EGFR-RasGAP complexes, respectively (a detailed kinetic description of this signaling module is given in Supplementary Table S1). Ranges of kinetic parameters were constrained based on the literature data and in vivo measurements of the signaling kinetics. A number of model predictions were validated (11, 35, 37, 39, and, in addition, our kinetic description of this pathway was tested and used by other groups (34, 40, 41).

The processes of EGFR internalization, degradation, and de novo synthesis are listed in Supplementary Table S1. The incorporation of multiple signaling branches initiated by the scaffold GAB1 (supplemental Table S2) extends our previous models (11, 35, 37) and brings about additional regulatory interactions critical for the function of the EGFR network. A distinctive feature of this refined and extended model is the employment of a novel, domain-oriented approach that greatly facilitates the analysis of signaling networks involving scaffold proteins (42, 43). In the model, scaffolds are GAB1, which has six binding sites, and Grb2, which possesses three binding domains. Owing to their multiple domains, scaffolds generate a great variety of heterogeneous multiprotein complexes, each involved in multiple parallel reactions. For instance, some GAB1 forms reside in the cytoplasm, whereas others are recruited to the plasma membrane through binding of the PH domain of GAB1 to PIP3 or the GAB1 proline-rich region to the C-terminal SH3 domain of Grb2 associated with phosphorylated EGFR (16, 19). Subsequent phosphorylation of GAB1 at numerous tyrosine residues creates docking sites for PI3K (44), SHP2 (44), RasGAP (27), Grb2, all of which would have to be accounted for in a mechanistic model that describes all “micro-states” of the network. Because of the exceedingly high numbers of micro-states in the EGFR network, previous models merely ignored this combinatorial variety of micro-states and simulated only a small part of feasible states and reactions. Several methods of handling this problem have been proposed, all based on specifying rules to generate species and reactions automatically. Programs implementing these methods include StochSim (47), BioNetGen (50, 51), and Moleculizer (52). However, at the present time only a part of the entire EGFR network can be analyzed using these programs (53).

An alternative “domain-oriented” approach approximates a mechanistic micro-state picture of the EGFR network in terms of “macro-states,” such as the phosphorylation levels and the fractions occupied by binding partners (42, 43). This domain-oriented framework allows for an approximate description of interactions, in which GAB1 and Grb2 are involved, using “macro-variables” (supplemental Table S2). The macro-variables do not follow the fate of all species and reactions that are generated by scaffold signaling, thereby greatly reducing the number of states and equations required for a quantitative analysis of the system behavior. For instance, each GAB1-related macro-variable accounts for the states of only a single docking site on GAB1 and for the association of GAB1 with EGFR (via Grb2) and PIP3. The importance of a domain-oriented, macro-description goes beyond the reduction of network models by providing a direct connection to experimentally observable variables. In fact, macro-variables are quantified in experimental studies by Western blot analysis using site-specific antibodies, whereas micro-states of GAB1 cannot be assessed readily at the present state of the art. The use of this domain-oriented approach facilitates direct testing of our model against the...
GAB1 Couples EGF-induced Signaling Pathways

To earlier suggestions that the role of this recruitment is to increase diffusion-limited (first-encounter) rates, the function of targeting cytoplasmic proteins to the membrane was recently shown to amplify the number of complexes formed between signaling partners (7, 8). This increase is brought about by the concentration of interacting partners in a thin layer adjacent to the plasma membrane with a much smaller volume than the cytoplasmic volume. For a spherical cell of radius of 10 μm and the layer of 10–15 nm adjacent to the plasma membrane, the ratio of the cytosolic volume (Vcyt) to the near-membrane layer volume (Vmemb) equals ~250–300. In the model, the spatial localization effects are accounted for as follows; for all interactions that occur within the membrane layer (Vmemb), the values of the equilibrium association constants assumed for the cytoplasmic interactions (for instance, reported for in vitro experiments with solubilized proteins) are scaled by a factor Vcyt/Vmemb (11).

Complex Feedback Circuitry of the EGF Network—It is instructive to summarize the numerous feedback/cross-talk regulations incorporated in our model of the EGF network. Positive feedback arises from the PIP3-mediated membrane recruitment of GAB1, resulting in further activation of PI3K that produces PIP3 (18). Through PIP2-induced GAB1 recruitment, PI3K also influences GAB1 signaling to the Ras/MAPK pathway. This involves temporal and spatial modulation of GEF and GAP signals by Grb2-SOS and RasGAP interactions with PIP2-GAB1. In addition, SHP2 engaged by phosphorylated GAB1 negatively regulates RasGAP signaling and, thus, facilitates Ras activation. Inhibitory phosphorylation of Raf by Akt (68, 69) is taken into consideration as another reaction responsible for cross-talk between the PI3K/Akt and Ras/MAPK pathways. The MAPK cascade module is embedded in two negative feedback loops. First, activated ERK phosphorylates SOS leading to the disassembly of the Grb2-SOS complexes and switching off the catalytic process of Ras activation (13–15). The second negative feedback arises from inhibitory phosphorylation of GAB1 by ERK (70–73). Although the molecular mechanisms involved in ERK-mediated inhibition of GAB1 tyrosine phosphorylation are not completely understood, available data support the negative feedback assumption. ERK1/2 binds to and phosphorylates GAB1 at six Ser/Thr residues (Thr-312, Ser-381, Ser-454, Thr-476, Ser-581, and Ser-597), four of which are located adjacent to the YVPM motifs that are specific for the binding of PI3K (70, 73). EGF-induced ERK activation decreases the tyrosine phosphorylation of GAB1 and GAB1 association with PI3K (71). Intriguingly, it was recently reported that the tyrosine kinase receptor c-Met binding domain of GAB1 can associate with phosphorylated ERK1/2. Owing to the presence of a putative nuclear localization signal in the GAB1 N-terminal region, the complex of GAB1 with phosphorylated ERK1/2 translocates to the nucleus, which can also be considered as negative feedback that prevents tyrosine phosphorylation of cytoplasmic GAB1 (74). The complex temporal dynamics resulting from these numerous interactions is difficult to assess by merely qualitative arguments. Using an in silico model and testing its predictions against the experiments that exploit specific perturbations to the EGFR network, we
provide insights into the intricate relationships between EGF stimulation and downstream responses.

RESULTS

PI3K Inhibition Reduces ERK Activation Owing to a Disruption of GAB1-PI3K-GAB1-positive Feedback

PI3K Inhibition Suppresses Ras/ERK Signaling—To explore potential cross-talk between the PI3K/Akt and Ras/ERK pathways in HEK293 cells, we first analyzed the temporal dynamics of EGFR and ERK activation by EGF in the presence or absence of the PI3K inhibitor wortmannin (Fig. 2). At saturating EGF concentrations (20 nM), wortmannin (200 nM) completely inhibited phosphorylation of the downstream PI3K target Akt (Fig. 2A) and significantly inhibited activation of ERK, as shown in Fig. 2B for dually, threonine/tyrosine-phosphorylated ERK. In contrast, wortmannin had almost no effect on EGFR activation, as shown in Fig. 2C for tyrosine phosphorylation of Tyr-1173. Likewise, the phosphorylation levels of the other EGFR sites, including Tyr-992, required for RasGAP binding, Tyr-1148 for Shc binding, and Tyr-1068, known as the major binding site for Grb2, were not affected by wortmannin (data not shown). The EGF dose dependence showed that substantial ERK activation occurred at EGF concentrations as low as 0.05 nM. At such low EGF levels, wortmannin also strongly suppressed ERK phosphorylation, which occurred after a lag phase (Fig. 2D). The data obtained at both saturating and low EGF levels show that cross-talk between the PI3K and MAPK pathways occurs downstream of the EGFR receptor. In concert with ERK inhibition, the presence of wortmannin substantially decreases active Ras-GTP fraction throughout the entire measured reaction period, up to 30 min following EGF activation (Fig. 2E). The wortmannin-induced impairment of Ras activation suggests that significant interactions between the PI3K and MAPK pathways occur upstream or at the level of the Ras cycle, but downstream of EGFR (as shown above). Interestingly, our data imply that reported inhibitory phosphorylation of Raf by Akt (68, 69) cannot be the only or major mechanism of cross-talk between the PI3K/Akt and Ras/MEK/ERK pathways in HEK293 cells. In fact, strong inhibition of Raf by Akt would have resulted in activation, rather than inhibition of ERK following PI3K/Akt inhibition by wortmannin.

Inhibition of the MAPK Pathway Increases EGF-induced Akt Activity and Sustains GAB1 Phosphorylation—The specific inhibitor of MEK, U0126, and the specific inhibitor of ERK 2, 5-iodotubericidine, each increased the Akt phosphorylation (Fig. 3A). These data show that, in contrast with the negative effect of PI3K inhibition on the MAPK pathway, inhibition of the MAPK cascade at the level of MEK or ERK facilitates activation of Akt, the downstream target of PI3K. We also found that GAB1 tyrosine phosphorylation is increased when ERK or MEK is inhibited. This confirms that negative feedback from ERK to GAB1 operates in HEK293 cells, and this feedback is not generated by kinases upstream of ERK (Fig. 3B) (71). To understand the mechanisms underlying cross-talk between the PI3K/Akt and MAPK pathways, we further explored effects of wort-
GAB1 Couples EGF-induced Signaling Pathways

mamm-induced PI3K inhibition on GAB1 and SHP2 and employed a computer model of these pathways to analyze and verify the proposed mechanisms.

PI3K Inhibition Reduces GAB1 Tyrosine Phosphorylation and SHP2 Recruitment—Although EGFR phosphorylation is not affected by wortmannin, the level of GAB1 tyrosine phosphorylation decreases dramatically (Fig. 4A). At first glance, this decrease may seem counterintuitive. Because ERK is inhibited by wortmannin, ERK inhibition would reduce the resulting effect of negative feedback from ERK on GAB1 (70, 71, 73) and increase (rather than decrease) GAB1 phosphorylation by EGFR and Src (Fig. 3B). However, in silico examination (Fig. 4B) demonstrates that the major role in the amplification of GAB1 tyrosine phosphorylation is played by the GAB1-PI3K-GAB1-positive feedback loop. In fact, owing to the membrane localization effect (7, 8), the PIP3-GAB1 complex interacts with Grb2 bound to EGFR with much higher affinity than cytoplasmic GAB1 does. Likewise, GAB1 molecules that are tethered to EGFR through Grb2 rapidly become associated with PIP3. The resulting additional production of PIP3 and the recruitment of new GAB1 molecules to the membrane complete the GAB1-PI3K-GAB1-positive feedback loop and further increase GAB1 phosphorylation.

This analysis implies that wortmannin disruption of the GAB1-PI3K-GAB1-positive loop significantly reduces the amount of the membrane-associated GAB1. To demonstrate this directly, we attempted to estimate the relative abundance of the cytosolic and membrane pools of GAB1 using digitonin as a permeabilizing agent (see “Materials and Methods”). In permeabilized, unstimulated cells, >90% of the total GAB1 is present in the cytosol (Fig. 4C). This is not affected by wortmannin treatment. Following EGF stimulation, GAB1 massively translocates to the membrane and its level decreases in the cytosol, in agreement with earlier reports (the small GTPase Ras is observed only in the membrane fraction of digitonin permeabilized cells and used as a loading control, supplemental Fig. S1) (18, 75–77). Translocation of GAB1 to the plasma membrane correlates with ERK activation (Fig. 4C). Importantly, inhibition of PI3K by wortmannin leads to a dramatic decline of the GAB1 level in the membrane fraction (and nearly complete elimination of phospho-Akt).

A substantial reduction of the membrane-associated GAB1 pool causes a significant decline in GAB1 tyrosine phosphorylation (Fig. 4A). The dephosphorylation attenuates binding of GAB1 partners, such as the phosphatase SHP2 (Fig. 4, D and E) (27, 44, 78), and their subsequent phosphorylation by the EGF receptor. The kinetics of SHP2 phosphorylation in the presence and absence of wortmannin is shown in Fig. 4A. Because SHP2 phosphorylation depends on the SHP2 association with GAB1 phosphorylated on Tyr-627 (27, 44, 78), these results are also corroborated by the data obtained with anti-pY627 GAB1 antibody in the cell lysate (Fig. 4A) and with GAB1 antibody in the SHP2 immunoprecipitate (Fig. 4D). Thus, the model establishes the key function of GAB1-PI3K-GAB1-positive feedback, which enhances GAB1 tyrosine phosphorylation, association of SHP2 with GAB1 and subsequent SHP2 phosphorylation, and PIP3 production. Also, the model explains the experimental data shown in Fig. 3 (A and B) that the disruption of negative feedback from ERK to GAB1 by MEK or ERK inhibitors increases and prolongs phosphorylation of the GAB1 and Akt (supplemental Fig. S2, A and B).

Wortmannin Affects the GEF and GAP Signals by Reducing GAB1 Membrane Targeting: Insight from Computational Modeling—The time course of GAB phosphorylation has important ramifications for the temporal dynamics of the GEF and GAP signals that control Ras activation. These two opposing signals are generated by the plasma membrane recruitment of the Grb2:SOS complex and RasGAP, respectively, and are mediated by both EGFR and GAB1. The Grb2:SOS complex can associate with the membrane-bound GAB1-PIP3 via the Grb2 C-terminal SH3 domain and the GAB1 proline-rich domain, whereas binding of RasGAP is mediated by specific phosphotyrosines pTyr-307/pTyr-317 located within the GAB1 central region (27). Phosphorylation of Tyr-627 and Tyr-
659 on GAB1 is required for SHP2 binding to the GAB1/PIP3 complex (44). The phosphatase SHP2 can dephosphorylate the RasGAP docking sites on GAB1, decreasing the GAP signal associated with GAB1.

Computational analysis shows the salient differences in the temporal dynamics of the SOS and RasGAP signals. Upon the onset of EGF stimulation, the SOS signal that activates Ras rises more expeditiously than the deactivating RasGAP signal (Figs. 5, A and B). This modest delay in the GAP signal compared with the GEF signal is brought about by the difference in the on rates of binding to GAB1 assumed in the model and by the fact that RasGAP association with GAB1 occurs only after tyrosine phosphorylation of the GAB1 docking sites, whereas the SOS signal is transmitted by unphosphorylated GAB1 bound to PIP3. This temporal dynamics of the two opposing signals results in the transient Ras-GTP pattern observed experimentally (11, 79).

Wortmannin perturbs both the SOS signal and RasGAP signal, because the GAB1-PIP3 complex is no longer formed (provided PI3K is completely inhibited). However, the computational analysis shows that wortmannin-induced changes to the SOS and RasGAP signaling patterns are markedly different, largely because of the involvement of the phosphatase SHP2, bound to phosphorylated GAB1 (Fig. 4, D and E), in the control of RasGAP signaling. When PI3K is inhibited, the amplitude and duration of the SOS signal greatly decreases (Fig. 5A). At times longer than ~5 min following the onset of EGF stimulation, the SOS signal becomes almost negligible in wortmannin-treated cells. In contrast to the SOS signal, the RasGAP signal is only modestly reduced by wortmannin. In fact, in control cells the recruitment of SHP2 by tyrosyl-phosphorylated GAB1 bound to PIP3 significantly attenuates GAB1-mediated RasGAP signaling (27). As a result, inhibition of PI3K by wortmannin is not as critical for the GAP signal as for the GEF signal (Fig. 5B). These distinct temporal patterns of the SOS and RasGAP signals and their different responses to inhibition of PI3K identified by computational modeling are essential for understanding the Ras/MAPK temporal dynamics and PI3K-MAPK cross-talk.

Computational Analysis of the Temporal Dynamics of ERK Activation in the Presence and Absence of PI3K Inhibition—The ERK activation dynamics depends on a complex interplay of a multitude of regulatory signals and interactions. Using only qualitative arguments, it is almost impossible to predict the ERK dynamics that results from multiple non-linear interactions and feedback loops, and a testable computational model can help us provide insights into key causative relationships. Our computational model incorporated known positive and negative feedback loops of the EGFR network. In silico examination suggests that the major effect
of GAB1-PI3K- and GAB1-SHP2-mediated feedback interactions is a substantial prolongation of the duration of ERK signaling. The time courses of Ras and ERK activation for control and wortmannin-treated cells predicted by the model are shown in Fig. 6. At saturating EGF concentrations, the ERK activation level reaches a peak ~5 min after the onset of stimulation and later declines, first more rapidly and, then, more slowly for up to 30 min (Fig. 6B) in general agreement with our experimental data (Fig. 2B). At low EGF levels, simulations show the lag phase in ERK activation (Fig. 6C), also compatible with the data (Fig. 2D). The model suggests that the wortmannin-induced decrease in ERK phosphorylation is primarily caused by a dramatic decline in the SOS signal (Fig. 5A). Computer simulations match the experimental data at both saturating and low EGF concentrations, yet the predominant role of GAB1 in the prolongation of ERK signaling suggested by the model requires direct experimental validation. To address this need, we first used the model to predict the temporal behavior of responses to EGF stimulation in a situation when the total abundance of GAB1 was decreased. Then, these signaling responses were assessed experimentally in HEK293 cells where the GAB1 protein expression was selectively suppressed using siRNA.

**FIGURE 5. Computer simulations of the GEF and GAP kinetics.** Simulated time course of membrane-bound SOS (A) and membrane-bound RasGAP (B) in the presence (dashed line) and absence of wortmannin (WM). Membrane-bound forms SOS and RasGAP involve a number of complexes that contain EGFR or membrane-associated GAB1 and are listed in the Supplementary material. Stimulation was with 20 nM EGF.

**FIGURE 6. Computer simulations of the wortmannin effects on the Ras-GTP and ERK kinetics.** The calculated time course of the Ras-GTP fraction (A) and the threonine/tyrosine phosphorylated ERK fraction following stimulation with 20 nM (B) or 1 nM (C) EGF in the absence and presence (dashed line) of wortmannin (WM).

**GAB1 Coupled EGF-induced Signaling Pathways**

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**Computational Modeling Predictions and in Vivo Validation of the GAB1 Function in the Control of the EGFR Network Temporal Dynamics**

GAB1 Suppression Drastically Reduces the Duration of ERK Signaling—The effects of GAB1 suppression and wortmannin on EGF-induced responses might be expected to be
GAB1 Couples EGF-induced Signaling Pathways

FIGURE 7. Effects of GAB1 depletion on EGF-induced ERK activation predicted by the computational model. GAB1 protein abundance was 300 nM in control and 100 nM in GAB1-depleted cells (dashed line). Stimulation with 20 nM (A) and 0.5 nM (B) EGF.

similar, because either perturbation suppresses the positive feedback loops mediated by GAB1. However, in silico analysis also emphasizes important distinctions between these two effects.

Simulations predict that, at saturating EGF concentrations, a 40–70% decrease in the GAB1 protein level would not significantly change the maximal amplitude of ERK phosphorylation (Fig. 7A) in sharp contrast to the wortmannin effect. At low EGF, the calculated ERK activity substantially decreases when the GAB1 protein level is suppressed (Fig. 7B), yet the effect of wortmannin on ERK activation is even more pronounced (Fig. 6C). Similar to wortmannin, GAB1 suppression remarkably shortens the duration of modeled ERK activation (Fig. 7A), largely due to a dramatic decrease in the SOS signal (supplemental Fig. S3). The model explains that the similarity between GAB1 suppression and wortmannin inhibition arises from a decline in PIP3 production caused by either perturbation, whereas the difference is brought about by a pleiotropic role of GAB1 in EGFR signaling. One of the reasons for this difference is the sequestration of the Grb2-SOS complex by cytoplasmic GAB1. Wortmannin does not change the GAB1 abundance but significantly decreases the membrane recruitment of GAB1 (largely mediated by PIP3) and makes the excess of inactive GAB1 a scavenger of Grb2, whose association with GAB1 does not require GAB1 phosphorylation.

Although the signaling dynamics of ERK and MEK change in parallel, at high EGF levels saturation of ERK activation by MEK may occur. In this case, the influence of a partial GAB1 suppression on the maximal phosphorylation level can be greater for MEK than for ERK. In fact, simulations show a moderate, but noticeable decline in the peak of phosphorylated MEK and a dramatic decrease in the duration of the MEK signal in cells where the total GAB1 level is reduced to one-third of the control level (Fig. 8A). Similar to ERK activation, the computed MEK activity is inhibited more strongly by wortmannin than by the GAB1 depletion. These theoretical predictions were tested against the experiments, in which HEK293 cells were transfected with siRNA specific for the GAB1 mRNA. 72 h after siRNA transfection, the GAB1 protein level was 15–30% of the level in control cells. The transfection procedure itself did not cause nonspecific changes in GAB1 protein level, because no depletion of GAB1 was observed in cells transfected with nontargeting siRNA in the presence or absence of EGF stimulation (Fig. 8B). The kinetics of MEK activation was determined in cells where GAB1 was down-regulated by siRNA. The experimental data corroborated in silico predictions of the effects of GAB1 suppression and PI3K inhibition by wortmannin on the temporal patterns of MEK activation (Fig. 8C). These findings are also supported by recent studies on T47D breast cancer cells reported by Meng et al. (80).

In vivo data, obtained in GAB1−/− mouse embryonic fibroblasts, showed a striking similarity to our in silico patterns of ERK and MEK activation (81). In GAB1−/− cells, the ERK phosphorylation level decreased <10% at the maximal value, whereas after reaching the peak this level decayed much faster, and the duration of ERK signaling was greatly diminished compared with GAB1+/− cells. Although the temporal dynamics of MEK and ERK responses was similar, the decline in the peak phosphorylation level was more pronounced for MEK than for ERK (81).

GAB1 Suppression Decreases Akt Activation—The function of GAB1 as an amplifier of growth factor-induced PI3K activation implies that Akt activity will decrease when GAB1 expression is down-regulated. Indeed, in silico and in vivo data demonstrate that Akt phosphorylation declines and is more transient in HEK293 cells, in which the GAB1 protein level was ~15–20% of the level in control cells (Fig. 8, D and E). Interestingly, GAB1 suppression also reduced basal Akt phosphorylation, which is not accounted for by the current model.

In Silico Predictions of EGF-induced Responses in GAB1 Mutants—It is instructive to compare the effects of partial GAB1 suppression with the effects of deletion of different GAB1 docking sites on the EGF-induced response dynamics. Using the model, we first predicted the time course of ERK activation in a GAB1 mutant deficient in the p85 PI3K docking site (Tyr-447, Tyr-472, and Tyr-589). Both the peak phosphorylation level and the duration of ERK activation appeared to be greatly decreased in this GAB1 mutant, owing to the disruption of positive GAB1-PI3K-GAB1 feedback (Fig. 9A). These simulations confirm the data by Rodrigues et al. (18) obtained for the
GAB1 Couples EGF-induced Signaling Pathways

DISCUSSION

GAB1 mutant, in which the three tyrosines involved in the PI3K binding sites were mutated to phenylalanines.

A GAB1 mutant deficient in the PH domain cannot be recruited to the plasma membrane by PIP3 (82). The lack of the GAB1 membrane recruitment by PIP3, suggests that the expression of such a PH domain-deficient mutant would negatively influence GAB1 phosphorylation and ERK activation. Computer simulations corroborate this conjecture and show that the effects of mutations that delete the GAB1 PH domain and wortmannin treatment on responses to EGF are almost identical (supplemental Fig. S4, A and B).

The deletion or substitution of tyrosine residues that form the SHP2 binding site on GAB1 is predicted to have a similar influence on the temporal pattern of ERK activation as GAB1 suppression, rather than wortmannin inhibition. Simulations show that the formation of GAB1-SHP2 complex prolongs the duration of ERK activation but does not significantly increase the ERK-PP maximal level following stimulation with saturating EGF concentrations (Fig. 9B). These in silico predictions are in remarkable agreement with recently reported data in GAB1 Y627F mutants deficient in SHP2 binding (25).

FIGURE 8. Functional consequences of endogenous GAB1 knockdown on EGF-induced MEK and AKT activation in vivo and in silico. A, simulated kinetics of MEK phosphorylation in control cells (solid line), GAB1 suppression (dashed line, [GAB1] = 100 nm), or the presence of wortmannin (WM, dashed-dotted line), 20 nm EGF. B, validation of specific endogenous GAB1 knockdown by siRNA in HEK293 cells. HEK293 cells were transfected with 50 nm GAB1 siRNA, 50 nm non-targeting (NT) siRNA, or left untreated for 72 h as described under “Materials and Methods.” Lysates of quiescent or EGf stimulated (20 nm) cells (5 min) were probed with anti-GAB1 antibodies to estimate the level of GAB1 suppression. Probing for SHP2 and α-tubulin protein levels was used as specificity and loading control. C, time course of MEK phosphorylation in HEK293 cells treated with 20 nm EGF. Cells were either transfected with 50 nm GAB1 siRNA for 72 h (middle three lines) or 200 nm wortmannin for 30 min (bottom three lines) or left untreated (top three lines) before stimulation with EGF. Whole cell lysates were immunoblotted with anti-phospho-Mek 1/2. Equal GAB1 suppression (70%) and sample loading were determined with anti-GAB1 and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies, respectively. Experimental curves of MEK activation were determined in four independent experiments and plotted as the mean ± S.E. Wortmannin (1), GAB1 siRNA (3), and control (C). D, computer simulated and (E) experimental time course of AKT phosphorylation in HEK293 cells treated with 0.5 nm EGF. Cells were depleted of GAB1 as described above or left untreated before stimulation with ligand. Whole cell lysates were immunoblotted with anti-phospho-Akt (Ser-473). Membranes were stripped and reprobed with anti-Akt antibodies to show that GAB1 suppression did not affect Akt protein level. Additionally, equal GAB1 suppression (~85%) and sample loading were determined with anti-GAB1 and anti-Grb2 antibodies, respectively.

GAB1 proteins belong to a family of scaffolds, which is conserved from worms to mammals and plays a vital role in signal transduction downstream of growth factor and cytokine receptors. Versatile GAB functioning was attributed to different molecular mechanisms and GAB-binding partners and effectors, including Grb2, tyrosine kinase receptor c-Met, and other receptor tyrosine kinases, Csk, SHC, PI3K, RasGAP, phospholipase Cγ, PIP3, Src, and ERK (17, 19, 22, 46, 83). However, understanding of a complex network goes beyond a list of binding partners and interactions, and this report presents an integrated in silico analysis and in vivo validation of the GAB1 function in the EGFR network. The combinatorial complexity of feasible protein-protein and protein-lipid complexes impedes conventional mechanistic modeling of the EGFR/Grb2/GAB/PI3K/Ras network (53). Here, we employ a kinetic description that approximates micro-states of a network of scaffold proteins in terms of macro-states of protein domains (42, 43). This description reduced the number of equations and variables from hundreds of thousands to ~350.

Although the challenge of network complexity is partially addressed by a domain-oriented approach, experimental uncertainty of parameter values hampers the use of molecular
level models at a systems scale. The goal of our computational analysis is not to fit an isolated response curve, but to predict and explain an exceedingly large number of diverse patterns of signaling dynamics obtained from our own experiments and literature data. We applied in silico perturbations to our model and wherever possible verified the predicted temporal dynamics in vivo. Our strategy is similar to so-called “pattern-oriented modeling,” when a large number of quantitative and also qualitative patterns help us exclude models that are too simple in structure or too uncertain experimentally (84). Although many parameter values remain uncertain, the structure and regulatory mechanisms incorporated in our model are realistic and robust. In fact, we tested the model by using perturbations resulting from different EGF concentrations, specific inhibitors, GAB1 suppression, and mutant GAB1 proteins. Most importantly, these perturbations corresponded to single parameter changes, which represent the greatest challenge for any model, because a multitude of temporal patterns have to be described without changes in the rate constants. Our model generated reliable predictions of the temporal dynamics of diverse cellular responses with the same set of kinetic parameters.

In silico and in vivo findings of the present report suggest that an essential function of GAB1 is to enhance and sustain the activation of the PI3K/Akt and Ras/MAPK pathways. Simulations and data obtained with the PI3K and MAPK pathway inhibitors (Figs. 2–6) show that GAB1 tyrosine phosphorylation and PI3K/Akt activation are amplified by the tyrosyl-phosphorylated GAB1 → PI3K → PIP3 → tyrosyl-phosphorylated GAB1-positive feedback circuit, whereas negative feedback from ERK shortens the duration of GAB1 tyrosine phosphorylation and decreases Akt activity. The model predicts that the duration of the transient Ras-activating signal is significantly controlled by the PIP3 temporal profile. GAB1-PI3K-positive feedback increases the number of the Grb2-SOS complexes recruited to the plasma membrane via GAB1-PIP3 in phosphotyrosine-independent manner, whereas tyrosyl-phosphorylated GAB1 associated with PIP3 mediates the recruitment of SHP2 and subsequent dephosphorylation of RasGAP binding sites. Both mechanisms extend the time period of Ras activation and are sensitive to perturbations of PI3K activity (e.g. using wortmannin) and also by changes in the GAB1 protein abundance.

This study supports the view that the GAB scaffolds function as signaling platforms that can propagate signaling from growth factors and cytokines similar to their cognate receptors. There-
fore, the sensitivity to wortmannin should critically depend on the abundance of the receptors and GAB1 and the strength of the signal (31, 85). In fact, in HEK293 cells stimulated with low EGF doses, wortmannin-induced suppression of ERK phosphorylation was found more pronounced in silico and in vivo (Figs. 2B, 2D, 6B, and 6C). Our computational analysis shows that an increase in the EGF concentration decreases the wortmannin sensitivity (Fig. 10A). Experiments conducted in A431 cells in vivo, where the EGF level is much higher than in HEK293 cells by one or two orders of magnitude, show that ERK phosphorylation is less sensitive to wortmannin (Fig. 10B).

In conclusion, we presented a synergistic experimental and computational analysis of interactions between mitogenic and survival signaling branches of the EGFR network. This analysis has provided insight into intricate signaling responses of the MAPK and PI3K/Akt pathways to EGF and demonstrated that the essential function of the GAB scaffolds is to enhance PI3K/Akt activation and extend the duration of Ras/MAPK signaling.

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