A Novel Role for Gab1 and SHP2 in Epidermal Growth Factor-Induced Ras Activation

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Running title: SHP2 regulates Gab1/RasGAP interaction

The abbreviations used are: EGF, epidermal growth factor; EGFR, EGF receptor; Gab1-ΔPQ, Gab1 deleted between Pro161 and Gln317; GAP, GTPase-activating protein; GAP3S, SH2-SH3-SH2 domains of RasGAP; GST, glutathione S-transferase; HA, hemagglutinin epitope tag; HGF, hepatocyte growth factor; IB, immunoblot; IP, immunoprecipitation; PDGF, platelet-derived growth factor; PH, pleckstrin homology; PI3K, phosphoinositide 3-kinase; RBD, Ras-GTP binding domain; SH, Src homology; SHP2-C/S, SHP2 catalytically inactive mutant; WT, wild-type.
Summary

SHP2 was recently found to downregulate PI3K activation by dephosphorylating Gab1 but the mechanisms explaining the positive role of the Gab1/SHP2 pathway in EGF-induced Ras activation remain ill defined. Substrate trapping experiments now suggest that SHP2 dephosphorylates other Gab1 phosphotyrosines located within a central region displaying four YXXP motifs. Because these sites are potential docking motifs for RasGAP, we tested whether SHP2 dephosphorylates them to facilitate Ras activation. We observed that a Gab1 construct preventing SHP2 recruitment promoted membrane relocation of RasGAP. Moreover, a RasGAP inactive mutant restored the activation of Ras in cells transfected with SHP2-inactivating Gab1 mutant or in SHP2-deficient fibroblasts, supporting the hypothesis that RasGAP is a downstream target of SHP2. To define whether Gab1 was a RasGAP-binding partner, a Gab1 mutant deleted of four YXXP motifs was produced. The deletion suppressed RasGAP redistribution and restored the defective Ras activation caused by SHP2-inactivating mutations. Moreover, Gab1 was found to interact with RasGAP SH2 domains, only under conditions where SHP2 is not activated. To identify RasGAP-binding sites, Tyr to Phe mutants of Gab1 YXXP motifs were produced. Gab1 constructs mutated on Tyr 317 were severely affected in RasGAP binding and the most active in compensating for Ras defective activation and in blocking RasGAP redistribution induced by SHP2 inactivation. We have thus localized on Gab1 a Ras negative regulatory tyrosine phosphorylation site involved in RasGAP binding and showed that an important SHP2 function is to downregulate its phosphorylation to disengage RasGAP and sustain Ras activation.
Introduction

The GTPase Ras is a crucial signaling relay of receptor tyrosine kinases, and the mechanisms controlling its activation by growth factors appear well understood: for example the activated epidermal growth factor (EGF) receptor (EGFR) autophosphorylates on tyrosine residues, which creates docking sites for phosphotyrosine-binding domains (e.g., SH2) of adaptor proteins, including Grb2 and Shc. Because Grb2 is constitutively associated with Sos, a guanine nucleotide exchange factor of Ras, the binding of Grb2 to phosphorylated EGFR results in the recruitment of Sos to the plasma membrane and has been proposed as a model for activation of membrane-bound Ras. Once activated, Ras can stimulate several effectors, notably the protein Ser/Thr kinase Raf1 and the downstream activated mitogen-activated protein kinases Erk1/2, major regulators of cell proliferation, differentiation and survival (1).

Although this pathway has reached a canonical status, the docking protein Gab1 and one of its binding partners, SHP2, were recently found to participate in Ras/Erk activation in response to EGF and other growth factors (2-5). SHP2 is a protein tyrosine phosphatase with two SH2 domains and phosphorylation sites with affinity for Grb2 SH2 domains. Its catalytic activity is stimulated by engagement of its SH2 domains with specific phosphorylated motifs that, in the case of EGF signaling, are provided by Gab1. Gab1 contains an N-terminal pleckstrin homology (PH) domain, multiple Ser/Thr and Tyr phosphorylation sites, and two Grb2 SH3-binding motifs. In response to EGF, Gab1 is recruited through Grb2 in the vicinity of the activated EGFR, and becomes phosphorylated. As a result, Gab1 attracts and activates two partners that are essential for EGFR-mediated biological responses: SHP2 (6) and the p85/p110 subtype of phosphoinositide 3-kinase (PI3K), which lipid products are also involved in Gab1 recruitment (5,7).
It is now well accepted that SHP2 is functionally an atypical phosphatase that plays a positive role in the biological responses to growth factors, and the Ras/Erk pathway represents the major signaling module positively regulated by this phosphatase (2-6). Yet, the mechanisms underlying this regulation have remained elusive. SHP2 has been first proposed to act as an adaptor in the case of platelet-derived growth factor (PDGF) signaling since it can bind to the PDGF receptor then become phosphorylated on its Grb2-binding sites (8). SHP2 could hence participate in Ras activation by contributing to the recruitment of Grb2/Sos in the vicinity of the PDGF receptor. However, this model has been recently challenged by several reports. First, the phosphorylation of SHP2, required to bind Grb2, seems marginal, if not undetectable, under EGF stimulation (2,5,9). In addition, the catalytic activity of SHP2 is important for Ras/Erk activation induced by EGF, insulin or hepatocyte growth factor (HGF), which implies that SHP2 does not simply function as an adaptor protein in response to these growth factors (5,10,11). This may be also true for PDGF signaling since a recent study has identified SHP2 mutants with normal Grb2-binding ability but defective in mediating PDGF-induced Erk activation (9).

Thus, the elucidation of SHP2 function in Ras activation seems to require the identification of novel substrate(s) which phosphorylation is detrimental to Ras activation. Recently, SHP2 was found to dephosphorylate Cbp/PAG and paxillin, two docking proteins that, when phosphorylated, can restrain Src activation by promoting its interaction with its regulatory kinase Csk (12,13). Nevertheless, downstream of Src, how Ras can benefit from this activation remains poorly documented. In addition, SHP2 was reported to promote Erk activation in response to fibroblast growth factor by dephosphorylating Sprouty2, a protein that, when phosphorylated, can act as a trap for Grb2 SH2 domain (14). Nevertheless, it seems unlikely that this mechanism could also occur under EGF stimulation since SHP2 is not known to maintain the interaction between the activated EGFR and Grb2 (2,5).
Evidences that Gab1 itself is a SHP2 substrate have been reported, suggesting that this interaction might participate in Ras activation. First, in *Drosophila*, genetic analysis have led to the identification of Dos, a Gab1 homolog, as a substrate of Csw, the SHP2 homolog (15,16). More recently, a "substrate-trapping" approach in mammalian cells has shown that Gab1 can form stable complexes with SHP2 catalytically inactive mutants, which is a strong indication that Gab1 can be also a SHP2 substrate (17). In agreement, SHP2 was found to control PI3K activation in response to EGF by dephosphorylating the PI3K-binding sites of Gab1 (18). However, the multiplicity of Gab1 phosphorylation sites (19) suggests that the phosphatase could dephosphorylate Gab1 phosphotyrosine residues which could be unfavorable for Ras activation. For example, these sites could provide docking signals for Ras negative regulatory proteins, and then SHP2 function would be to control their recruitment in EGFR signaling complexes by dephosphorylating Gab1.

A good candidate for such a Ras negative regulator controlled through dephosphorylation of its docking site(s) is p120-RasGAP, the GTPase-activating protein that inactivates Ras by turning on its intrinsic enzymatic activity. RasGAP contains two SH2 domains that have binding affinity for the phosphorylated YXXP motif. The major binding partners of RasGAP are DOK proteins, a family of adaptors preferentially expressed in lymphoid tissues. Their prototype, DOK1, contains six YXXP motifs (20). Strikingly, Gab1 also contains six of these motifs. Yet, it is not known whether Gab1 can associate with RasGAP. Various candidates can potentially recognize Gab1 YXXP motifs, including the adaptors Nck and Crk, and the phospholipase Cγ. However, thus far, these proteins have been identified as Gab1 binding partners only in response to HGF stimulation (21,22). Under EGF stimulation, these sites have not been allocated to any particular effector, even though some of them appear to be phosphorylated *in vitro* by the EGFR (22,23).
In this report, we have addressed the question of whether Gab1 YXXP motifs provide docking sites for RasGAP and constitute target sites for dephosphorylation by SHP2. Using substrate trapping and site-directed mutagenesis, we have obtained evidence that Gab1 YXXP motifs can recruit RasGAP and that an essential function of SHP2 is to downregulate this interaction, thus allowing an efficient activation of Ras in response to EGF.
EXPERIMENTAL PROCEDURES

Materials—Human recombinant EGF was from Calbiochem. Monoclonal antibody against Myc epitope tag (clone 9E10) was from Santa Cruz Biotechnology, Inc. Monoclonal anti-EGFR (clone LA1) and polyclonal anti-Gab1 (Catalog # 06-579) and anti-RasGAP (Catalog # 06-157) were from Upstate Biotechnologies, Inc. The monoclonal anti-HA epitope tag (clone 12CA5) was from Roche Molecular Biochemicals. The monoclonal anti-SHP2 antibody was from BD Transduction Laboratories (clone 79). The polyclonal antibody against phospho-Gab1-Tyr 307 was from Cell Signaling Technology (Catalog # 3234). The monoclonal anti-phosphotyrosine antibody was produced from the culture medium of the 4G10 hybridoma (kindly provided by Dr. P. Mayeux, Paris, France). Cell culture reagents were from Biowhittaker, Inc.

Expression Plasmids and Site-directed Mutagenesis—The plasmids encoding Gab1-Y627F, Gab1-YF3, SHP2-WT, SHP2-C/S, HA-tagged WT Ras (HA-Ras) and GST-p85 have been already described (5,24,25). A pcDNA3 vector encoding RasGAP-WT was kindly provided by Dr. A. Yoshimura (Fukuoka, Japan). To produce the RasGAP inactive mutant, we changed Arg 789 to Gln, alone or in combination with Arg 903, using the QuickChange multisite-directed mutagenesis kit (Stratagene). Novel Gab1 constructs used in this study (YF3/Y627F, ΔPQ±Y627F, Y242/259F±Y627F, Y307/317F±Y627F, Y242F±Y627F, Y317F±Y627F) were created using the regular QuickChange kit that was more reliable (sequences of mutagenic primers available on request). All mutants were verified by sequencing, expression, and EGF-induced phosphorylation and membrane relocation.

Cell culture, Transfection and Stimulations—This study was performed both in Vero cells (a non transformed monkey kidney cell line, ATCC CCL 81) and in two immortalized mouse embryo fibroblast cell lines (MEF) expressing either the endogenous SHP2 (WT) or a truncated, non-recruitable form of the phosphatase (ΔSHP2) (26). Cells were maintained in
Dulbecco's modified Eagle's medium supplemented with 7.5% fetal bovine serum and antibiotics. For transient transfection of Vero cells, subconfluent 60-mm plates were incubated 3 hours with 2 ml of Dulbecco's modified Eagle's medium containing 2 µg of total DNA and 6 µl of each LipofectAMINE and Plus reagent (Invitrogen, Inc.). In MEF, two 100-mm plates per assay were used, each containing 4 ml medium, 4 µg of total DNA and 20 µl of each LipofectAMINE and Plus reagent. Before stimulation, cells were blocked overnight by serum starvation. Cells were stimulated for 5 min with 10 (Vero) or 30 (MEF) ng/ml EGF.

**Cell lysis, Immunoprecipitations and Immunoblotting**—Cells were scraped off in lysis buffer containing 20 mM Tris, pH 7.4, 150 mM NaCl, 10 mM EDTA, 10% glycerol, 1% Nonidet P-40, 10 µg of each aprotinin and leupeptin and 1 mM orthovanadate. After shaking for 15 min at 4°C followed by a 13,000 xg centrifugation for 15 min, soluble material was incubated with the appropriate antibody for 2 hours at 4°C. The antigen-antibody complexes were incubated with protein A- or protein G-Sepharose (Sigma) for 1 h, then collected by brief centrifugation and washed three times with lysis buffer containing 0.1% Nonidet P-40, 1 µg/ml of each aprotinin and leupeptin, 0.1 mM orthovanadate. Proteins were then resolved by SDS-PAGE and immunoblotting using a standard procedure. Blots were developed using chemiluminescence (Amersham Biosciences, Inc.). For immunoblotting analysis of cell lysates, cells were directly scraped off in electrophoresis sample buffer, then boiled and processed for immunoblotting.

**GTP-Ras Affinity Precipitation Assay in Transfected Vero and MEF Cells**—The assay was performed essentially as described using the recombinant Ras-binding domain (RBD) of Raf1 as an affinity probe for GTP-Ras (5,27). The RBD was expressed as GST fusion protein in *E. coli* and extracted using glutathione-Sepharose beads (Sigma). To measure Ras activation in Vero cells, they were cotransfected with 0.2 µg and 1.8 µg of plasmid encoding
HA-tagged RasWT and the indicated effector, respectively. In MEF cells, we have used 0.4 µg of HA-Ras vector and 3.6 µg of plasmid encoding Gab1 or RasGAP constructs.

Following stimulation, cells were scrapped off in 1 ml lysis buffer containing 50 mM Tris pH 8.0, 150 mM NaCl, 10 mM MgCl₂, 0.5% DOC, 1% Nonidet P-40, 0.1% SDS, 1 mM PMSF, 1 µg/ml of each aprotinin and leupeptin. Cleared lysates were incubated at 4°C for 30 min with 10 µg of GST-RBD bound to glutathione-Sepharose beads. Beads were washed three times in lysis buffer, and then boiled in standard electrophoresis sample buffer and proteins were resolved by SDS-PAGE, followed by immunoblotting with anti-HA antibody.

**GST-RasGAP Binding Assay**—The GST fusion proteins containing the SH2-SH3-SH2 domains of RasGAP or only the C-terminal SH2 domain have been already described (28) (kindly provided by Dr. J. Nunez, Marseille, France). The fusion proteins were expressed in *E. coli* and extracted using glutathione-Sepharose beads (Sigma). The pulldown was conducted like the GTP-Ras assay, except that 3 µg of fusion protein were used and incubated during one hour with cleared lysates.

**Membrane Fractions**—Fractions containing membrane-associated proteins were prepared essentially as described (29). Cells were scrapped off in lysis buffer containing 20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 10 mM EGTA, 2 mM MgCl₂, 10 µg/ml of each aprotinin and leupeptin, 1 mM orthovanadate, and were Dounce homogenized. The lysate was spun at 13,000 xg for 30 min and the pellet was resuspended in lysis buffer. The sample was then centrifuged again and the pellet was washed once then dissolved in lysis buffer supplemented with 1.2% Triton X-100. The insoluble material was spun out and the supernatant was taken as the solubilized membrane fraction.

**Statistics**—The results shown are representative of at least two independent experiments performed in duplicates, unless otherwise indicated.
RESULTS

Evidence that Gab1 PI3K-binding sites are not SHP2 targets in Ras activation and that SHP2 dephosphorylates other Gab1 sites—SHP2 was recently shown to downregulate the EGF-induced PI3K/Akt pathway by dephosphorylating Gab1 YXXM p85-binding motifs (18). In agreement with this, we observed an increase of both PI3K association with Gab1 and Akt activation in Vero cells transfected with a Gab1 mutant (Y627F) unable to recruit SHP2 (5) and in SHP2-deficient fibroblasts (data not shown). Although unlikely, it was necessary to exclude that this dephosphorylation could be somehow positive for Ras activation. We thus changed the Tyr of the three YXXM motifs to Phe in the Gab1-Y627F construct to produce the so-called YF3/Y627F mutant. The Y627F mutation has a strong dominant negative effect on EGF-induced Ras/Erk activation (4,5). We therefore assumed that if Gab1 YXXM motifs were SHP2 targets in Ras activation, their mutation should suppress or reduce the dominant negative effect of the Y627F mutation. However, Gab1-YF3/Y627F inhibits Ras activation even stronger than Gab1-Y627F (Fig. 1A), which excludes that dephosphorylation of Gab1 YXXM motifs by SHP2 could produce a positive effect on Ras activation.

We next hypothesized that SHP2 could dephosphorylate Gab1 not only on PI3K-binding sites. To test this hypothesis, we have performed substrate trapping experiments using the SHP2 catalytically inactive mutant C459S (C/S) that can form stable complexes with potential SHP2 substrates (17). The experiment was performed by cotransfecting SHP2-C/S with Gab1 mutants lacking different Tyr residues, and the existence of an interaction between these proteins was determined by coimmunoprecipitation followed by immunoblotting analysis. Of course, all Gab1 mutants tested in substrate trapping experiments were prepared from the Gab1-Y627F template to eliminate the binding of SHP2-C/S through its SH2 domains. Fig. 1B shows that SHP2-C/S, but not SHP2-WT,
coimmunoprecipitates with Gab1-Y627F when cells were stimulated with EGF, as expected since it was already demonstrated that SHP2 dephosphorylates Gab1 PI3K-binding motifs (18). More interestingly, disruption of all PI3K-binding sites in the Gab1-YF3/Y627F mutant reduces the interaction between Gab1-Y627F and SHP2-C/S, but only to a limited extent. This suggests that SHP2-C/S traps Gab1 residues different from PI3K-binding sites, implying that SHP2-Gab1 interaction is not limited to PI3K regulation.

SHP2 dephosphorylates Gab1 on residue(s) located between Pro 161 and Gln 318 We attempted to identify these novel sites recognized by SHP2-C/S, assuming that one of them could be involved in recruiting a negative regulator of Ras activation. In addition to PI3K- or SHP2-binding sites, Gab1 sequence contains 15 Tyr residues, which virtually excludes a systematic mutagenesis approach. However, a central region located between Pro 161 and Arg 318 displays four out of the six potential RasGAP-binding YXXP motifs (Fig. 2A). Because this region does not include any known domain required for Gab1 recruitment, we deleted it as a first approach to determine whether it contained Tyr residues trapped by SHP2-C/S. This deletion was introduced in Gab1-WT and in the Gab1-Y627F background to perform substrate trapping experiments. To verify first the ability of these so-called ΔPQ mutants to be recruited, we have examined both their membrane translocation and phosphorylation in response to EGF. Fig. 2B shows that these mutants behave similarly to other Gab1 constructs in terms of EGF-induced relocation to membrane fractions, which confirmed that the deleted region is not required for Gab1 recruitment. In contrast, the phosphorylation of ΔPQ mutants is strongly reduced in comparison with that of Gab1-WT or Y627F, suggesting that the deleted region contains significant phosphorylation sites (Fig. 2C). Following this characterization, the Gab1-Y627F/ΔPQ construct was cotransfected with SHP2-C/S to perform a substrate trapping experiment, in comparison with Gab1-Y627F. As shown in Fig. 2D, the deletion severely reduces the interaction between SHP2-C/S and...
Gab1, strongly suggesting that SHP2 dephosphorylates Tyr residue(s) located between Pro 161 and Arg 318.

A RasGAP dominant negative mutant bypasses the Gab1/SHP2 pathway—Because the Gab1 Pro161-Arg318 region displays potential RasGAP-binding motifs, the previous data suggest that SHP2 facilitate Ras activation by downregulating a putative association between Gab1 and RasGAP, implying that RasGAP should be a downstream target of Gab1 and SHP2. To test this hypothesis, we have first studied the association of RasGAP with membrane fractions as readout of RasGAP mobilization. As shown in Fig. 3A, when cells are transfected with Gab1-WT, EGF induces at best a minor redistribution of RasGAP. The membrane redistribution of Gab1 itself was used to control the quality of cell stimulation and membrane extraction. In contrast to Gab1-WT, transfection of Gab1-Y627F resulted in an important membrane relocation of RasGAP in response to EGF, which strongly suggests that the Gab1/SHP2 pathway downregulates RasGAP recruitment under normal conditions.

To test this hypothesis further, we have produced a RasGAP inactive mutant to determine whether its overexpression could attenuate the requirement for the Gab1/SHP2 pathway in Ras activation. The RasGAP mutant was designed to produce a protein unable to interact with Ras but normally recruitable through its other interaction domains, according to structure-function studies (30,31). We have thus performed site-directed mutagenesis to change RasGAP Arg 903 to Gln, alone or in combination with Arg 789. To analyze the effect of these mutations on GAP activity, the constructs were simply overexpressed in Vero cells and their ability to interfere with Ras activation was compared with that of RasGAP-WT. As shown in Fig. 3B, overexpression of the single mutant R903Q nearly abolishes the activation of Ras induced by EGF, similarly to the effect of RasGAP-WT overexpression. This indicates that the single mutant retained a significant GAP activity. In contrast,
overexpression of the double mutant R789/903Q (2RQ) produces a minor effect on Ras activation, which shows that this mutant has lost most of its GAP activity.

Therefore the 2RQ construct could be used as a RasGAP dominant negative mutant to define whether RasGAP was a downstream target of SHP2. This was achieved by testing the capacity of 2RQ to compensate for the defective Ras activation caused by SHP2 inactivation. This mutant was first transfected in Vero cells to determine whether it could revert the Ras inhibitory effect of the Gab1-Y627F mutation. As shown in Fig. 3C, the 2RQ mutant clearly restores the EGF-induced activation of Ras impaired by Gab1-Y627F. Importantly, this mutant does not increase the level of GTP-Ras in unstimulated cells (Fig. 2B), which indicates that the restoration of the EGF-induced response is not due to an unspecific effect on basal Ras activation. Moreover, to control that the 2RQ mutant has no action upstream of Gab1 or SHP2, we have verified that its overexpression does not influence the EGF-induced Gab1 phosphorylation (Fig. 3D), neither of the binding of Gab1-WT to SHP2 (data not shown).

To determine whether the 2RQ mutant could alleviate the requirement for SHP2 in another cellular model, a similar experiment was conducted in mouse embryo fibroblast cells (MEF) expressing a truncated non-recruitable form of SHP2 (MEF-ΔSHP2). These cells display a reduced activation of Ras in response to EGF, compared to MEF-WT (2). As shown in Fig. 3E, overexpression of the 2RQ mutant in MEF-ΔSHP2 strongly stimulates the activation of Ras induced by EGF, without influencing the signal in unstimulated cells. Taken together, these data support the hypothesis that RasGAP is a downstream target of the Gab1/SHP2 pathway.

_Gab1 can bind to RasGAP SH2 domains through the Pro161-Arg318 region—_ Since Gab1 displays potential RasGAP-binding motifs, we have explored the possibility that they represent target sites for dephosphorylation by SHP2. The first step was to study the...
ability of Gab1-ΔPQ to interfere with RasGAP membrane relocation. We assumed that if the Gab1 Pro161-Arg318 region contained the sites promoting RasGAP relocation when SHP2 is not recruited, its deletion should suppress RasGAP redistribution produced by both expression of the Y627F mutation and EGF treatment (Fig. 3A). As shown in Fig. 4A, transfection of Gab1-ΔPQ had only a minor effect on RasGAP relocation in comparison with Gab1-WT, as expected since this relocation is poorly induced under conditions that allow SHP2 activation (Fig. 3A). More interestingly, introduction of the deletion in the Y627F background (Gab1-ΔPQ/Y627F) completely suppresses the RasGAP relocating effect of the Y627F mutation. This strongly supports the view that the deleted region contains sites allowing the downregulation by SHP2 of RasGAP recruitment.

To investigate further the association between Gab1 and RasGAP, we performed an interaction assay with GST fusion proteins containing all or part of the SH2-SH3-SH2 region of Ras-GAP. Fusion proteins were immobilized on beads and used as affinity reagents. Proteins precipitated with the beads were then analyzed by immunoblotting. We first determined whether the beads could capture endogenous Gab1 in lysates of MEF-ΔSHP2 cells, a cell line that supposedly provided optimal conditions to observe an interaction negatively regulated by SHP2. Fig. 4B shows that, when the whole RasGAP SH2-SH3-SH2 region (3S) is used as bait, Gab1 is significantly precipitated from EGF-treated ΔSHP2 cells. As a positive control, beads loaded with PI3K (p85α) SH2 domains were used to capture Gab1 from MEF cells (Fig. 4C), which validates the assay conditions and confirms that SHP2 downregulates PI3K interaction with Gab1 (18). As a negative control, Gab1 is much less precipitated when the experiment is performed with MEF-WT cells. Fig. 4B also shows that beads carrying only the C-terminal SH2 domain of RasGAP (CT) do not precipitate Gab1. These data imply that phosphorylated Gab1 interact with RasGAP SH2 domains, and that this association is regulated by SHP2.
To localize Gab1 RasGAP-binding sites, we have first used the ΔPQ mutant. After transfection in Vero cells, its ability to associate with RasGAP was assessed using the GST-RasGAP-3S interaction assay. To control the pulldown specificity, the experiment was also performed with transfected Gab1-Y627F and Gab1-YF3 constructs and, as bait, the fusion protein GST-p85α. As shown in Fig. 4D, the RasGAP SH2-SH3 region efficiently precipitates all Gab1 mutants, except the one carrying the deletion of the Pro161-Arg318 region. As a control (Fig. 4E), deletion of this region does not prevent, and even improves the interaction between Gab1 and p85 SH2 domains, an increase that may be due to a reduced steric hindrance between p85 and proteins binding to the deleted region. Consequently, these results indicate that the deletion specifically alters Gab1 RasGAP-binding ability. In addition, Fig. 4D shows that Gab1-Y627F interacts more strongly than Gab1-WT with RasGAP SH2 domains, which is also in favor of Gab1 dephosphorylation by SHP2 on RasGAP-binding sites. We conclude from these experiments that RasGAP SH2 domains associate with Gab1 through motifs located within the Pro161-Arg318 region.

Gab1 deletion mutant deficient for RasGAP-binding attenuates the requirement for SHP2 in Ras activation—The previous data support the notion that SHP2 participates in Ras activation by controlling the Gab1-mediated recruitment of RasGAP. Therefore, if this actually works, Ras activation should become independent of SHP2 in conditions where the EGFR recruits only Gab1 molecules lacking RasGAP-binding sites. To test this assumption, we have determined whether overexpression of Gab1-ΔPQ in Vero cells could bypass SHP2 function as a positive regulator of Ras activation. As shown in Fig. 5A, in the Gab1-WT background, the deletion has a stimulatory effect on Ras activation, which is not unexpected if the deleted region contains RasGAP-binding motifs. More interestingly, in the Gab1-Y627F background, the ΔPQ deletion completely suppresses the Ras inhibitory effect of the Y627F mutation, strongly suggesting that the deleted region displays Ras negative-
regulatory sites dephosphorylated by SHP2. To confirm this finding, a similar experiment was performed in MEF-ΔSHP2 cells. As shown in Fig. 5B, transient transfection of Gab1-ΔPQ/Y627F strongly stimulates the defective Ras activation in EGF-treated MEF-ΔSHP2. Altogether, these results designate the existence in the ΔPQ region of Ras negative-regulatory sites that must be dephosphorylated by SHP2 to achieve a full activation of Ras.

Further identification of Gab1 RasGAP-binding sites—To localize more precisely the Gab1 Tyr residue(s) involved in RasGAP binding, each of the two Tyr tandems of the ΔPQ region (Fig. 2A) was replaced with Phe to produce the so-called Y242/259F and Y307/317F mutants. These mutants were constructed both in the Gab1-WT and Y627F background. They were first tested for their ability to bind RasGAP using the GST-GAP3S pulldown assay. As shown in Fig. 6A, the double mutation Y242/259F produces at best a minor effect on the Gab1 binding to RasGAP, whereas the construct mutated on the Tyr 307/317 tandem has an affinity for RasGAP nearly abolished, suggesting that these Tyr residues constitute major RasGAP binding sites. We have next compared the double mutants ability to interfere with RasGAP membrane relocation induced by the Y627F mutation and EGF stimulation. Fig. 6B shows that the Y307/317F mutant completely suppresses this translocation, while the Y242/259F mutant has a minor effect, which confirms that Tyr 307 and 317 are essential for RasGAP recruitment. The Gab1 double mutants were also compared by their capacity to restore the defective Ras activation in MEF-ΔSHP2 cells. Transient transfection of the Y307/317F construct, but not of the Y242/259F mutant, strongly enhances Ras activation induced by EGF, which further strengthens the identification of the RasGAP-binding site on Tyr 307 and 317. Finally, we compared the potency of the effects produced by the double Tyr mutants with that of the Gab1-ΔPQ deletion mutant, using a semi-quantitative densitometric analysis performed on immunoblots from GST-RasGAP pulldown and Ras activation experiments. The Y307/317F mutant shows characteristics similar to the ΔPQ
mutant in terms of loss of RasGAP binding capacity (Fig. 6D) and of ability to restore Ras
activation in MEF-ΔSHP2 (Fig. 6E). Taken together, these results demonstrate that Tyr 307
and 317 constitute major RasGAP-binding sites on Gab1, and support the concept that these
sites must be dephosphorylated by SHP2 to achieve an efficient activation of Ras.

Next we attempted to narrow the localization of the RasGAP-binding site to a single
Gab1 tyrosine. To discriminate Tyr 307 and 317, we have first taken advantage of an
antibody that recognizes the phosphorylated form of Gab1 Tyr 307. We have thus compared
the EGF-induced phosphorylation of this site in cells expressing Gab1-WT or Gab1-Y627F,
assuming that if Tyr 307 represents a RasGAP docking site, target of SHP2, its
phosphorylation should be enhanced in cells unable to recruit SHP2. However, Fig. 7A
shows that this is not the case since Tyr 307 phosphorylation was decreased in cells
expressing Gab1-Y627F. Similar results were obtained by comparing Tyr 307
phosphorylation on endogenous Gab1 in MEF-ΔSHP2 and MEF-WT (data not shown).
Since these results allowed to exclude Tyr 307 as a SHP2 dephosphorylation target, we have
produced a Phe mutant of Tyr 317 in the Y627F background to study first its ability to bind
RasGAP. As shown in Fig. 7B, the Y317F/Y627F mutant has an affinity for RasGAP
severely compromised compared to Gab1-Y627F alone or to Gab1-Y242F/Y627F. In
agreement, the Y317F mutation suppressed the membrane relocation of RasGAP induced by
the SHP2-inactivating mutation Y627F (Fig. 7C), which confirmed that Tyr 317 mediates
RasGAP recruitment. Finally, we have studied the ability of Gab1-Y317F to restore Ras
activation in MEF-ΔSHP2 cells Transient transfection of this mutant reinforced the GTP
loading of Ras induced by EGF (Fig. 7D). Altogether, these results indicate that Tyr 317 is
an important RasGAP binding motif that must be dephosphorylated by SHP2 to achieve a
full activation of Ras.
DISCUSSION

A number of recent data indicated that Gab1 is not only an activator but also a substrate of SHP2, suggesting that a further examination of the interaction between the phosphatase and the major proximal signaling relay of the EGFR could provide insights on pending issues of Ras activation. Thus far, it was only known that SHP2 can dephosphorylate Gab1 PI3K-binding sites (18), and, although unlikely, it was necessary to exclude that dephosphorylation of Gab1 YXXM motifs could be somehow positive for Ras activation. Indeed, identical motifs present on the PDGF receptor were recently found to exert, besides PI3K activation, a negative regulatory function by targeting the receptor to lysosomal degradation (32). In addition, although PI3K activation may participate in Ras activation (5), one could not exclude an unsuspected antagonism between PI3K hyperactivation and Ras, due for example to an illegitimate recruitment of PH domain-containing proteins. However, in the case of Gab1, we have found that mutations of its YXXM motifs did not alleviate, but aggravated, the deficient activation of Ras due to an SHP2-inactivating mutation, which experimentally confirmed that PI3K-binding sites dephosphorylation cannot produce a positive effect on Ras activation.

We have next considered the possibility that SHP2 could dephosphorylate Gab1 on other Tyr residues. Using substrate trapping as an efficient method to identify SHP2 substrates (17), we have observed that catalytically inactive SHP2-C/S was still able to associate with a Gab1 mutant lacking both SHP2- and PI3K-binding sites. Among the remaining Tyr residues, six were contained within YXXP motifs that, once phosphorylated, have a strong potential for recruiting signaling proteins. They were initially considered as possible docking sites for the SH2 domains of Nck, Crk and phospholipase Cγ (19). However, these proteins have not been reported to associate with Gab1 under EGF stimulation. Therefore, other binding candidates must be considered and RasGAP appeared
to be an attractive partner. The reason why RasGAP was ignored so far might be due to the initial findings that its SH2 domains recognize the YXXPXD motif, found on the PDGF receptor for example (33), but absent on Gab1. Nevertheless, recent data obtained with DOK or the EGFR itself showed that the shorter YXXP sequence is sufficient to bind RasGAP (34,35).

It was thus important to investigate the role of Gab1 YXXP motifs, both as potential RasGAP-binding motifs and target substrates for SHP2. As a first approach, we have deleted Gab1 of a supposedly "inert" region located between Pro161 and Gln318. This allowed us to eliminate four YXXP motifs in a single round of PCR, a strategy that apparently did not alter Gab1 recruitability, while avoiding the multiplication of secondary mutations due to successive PCR, if these sites had to be mutated one after the other. The experiments with this deletion mutant Gab1-ΔPQ provided several data in favor of the hypothesis that Gab1 displays RasGAP-binding motifs that must be dephosphorylated by SHP2 to achieve a full activation of Ras: 1) the ΔPQ mutant lose the capacity to be trapped by SHP2-C/S in comparison with full-length Gab1; 2) it suppressed the membrane translocation of RasGAP induced by a Gab1 mutant preventing SHP2 recruitment; 3) the interaction of Gab1 with RasGAP SH2 domains was lost in the ΔPQ mutant, whereas its affinity for p85 was maintained; 4) the ΔPQ deletion abolished the requirement for SHP2 in two different cellular models: it suppressed the dominant negative effect of the Gab1-Y627F mutation in Vero cells and restored the defective Ras activation in EGF-treated MEF-ΔSHP2 cells.

The next step was to localize the particular(s) YXXP motif involved in RasGAP binding within the Pro161-Arg318 region. We generated a series of Gab1 constructs carrying Tyr to Phe mutations on each of the two YXXP tandem motifs, which allowed us to propose that Tyr 317 is an important residue for binding RasGAP. Indeed, mutation of this residue, both in the single Y317F and the double Y307/317F mutants, strongly disrupted Gab1 association
with RasGAP SH2 domains. Moreover, the constructs mutated on Tyr 317 prevented the membrane relocation of RasGAP induced by overexpression of Gab1 SHP2-inactivating mutant, and restored Ras activation in MEF-ΔSHP2. The participation of Tyr 307 in this process could be excluded since it appeared hypophosphorylated when SHP2 was not activated (Fig. 7A). We could thus infer from these data that Tyr 317 represents an important target site for dephosphorylation by SHP2 that can thereby downregulate RasGAP recruitment. Nevertheless, a different approach based on "phosphoproteomics" would be most informative by comparing the actual phosphorylation sites of Gab1 between normal and SHP2-deficient cells, and further defining the spatio-temporal characteristics of this signaling pathway.

The notion that RasGAP can be a downstream target of SHP2 in EGFR signaling has been recently suggested, on the basis that RasGAP has affinity for a site on the EGFR that can be "substrate-trapped" by catalytically inactive SHP2 (35). Although we propose here an alternative mechanism to control RasGAP recruitment, our study adds further support with novel elements to the existence of a link between SHP2 and RasGAP. First, we have been able to observe that overexpression of a Gab1 mutant unable to launch SHP2 activation (Y627F) resulted in a redistribution of RasGAP in membrane fractions under EGF stimulation (Fig. 3A), which was a good indication that RasGAP was a downstream target of the Gab1/SHP2 pathway. Moreover, on the basis of structure-function studies that identified Ras-RasGAP interaction sites (30,31), we have produced for the first time a RasGAP inactive mutant that behaved as a RasGAP dominant negative mutant. Indeed, when overexpressed to "inactivate" endogenous RasGAP, it restored the defective activation of Ras in two different cellular models of SHP2 deficiency. This occurred without modification of the basal level neither of activated Ras nor of the signaling upstream of SHP2, which attested to the specificity of this approach. Consequently, RasGAP appears to be an
important downstream target of the phosphatase on the pathway linking EGFR to Ras activation, similarly to SHP2 function downstream of other receptors like the PDGFR or Torso in fly (33,36). However, to our knowledge, this is the first demonstration that SHP2 controls the recruitment of RasGAP not directly bound to a receptor but to a downstream effector.

Based on the results presented in this study and from previous reports on the subject, we propose the model displayed in Fig. 8 to illustrate the role of SHP2 upstream from Ras. We confirm that the phosphatase can modulate PI3K activation by dephosphorylating Gab1 PI3K-binding sites (18) but the physiological significance of this dephosphorylation seems secondary, considering the overall positive role of SHP2 in EGFR-mediated biological responses. In contrast, we have located on Gab1 Ras negative-regulatory tyrosine phosphorylation sites involved in the binding to RasGAP. We propose that an important function of SHP2 is to dephosphorylate these sites to disengage RasGAP from Ras activation complexes. Quite interestingly, our model is fully compatible with time-course studies showing that SHP2 is much less involved in the earliest phase of Ras activation than in maintaining the level of this activation (2,35). Indeed, according to our model, SHP2 is not necessary for the initiation of events leading to Ras activation since it does not influence the binding of Grb2/Sos to the EGFR. Next, when Gab1 phosphorylation occurs, RasGAP can be recruited, then SHP2 becomes essential to downregulate this recruitment and to prevent at this moment the abortion of Ras activation.

A question that remains unanswered relates to the evolutionary benefits of a signaling module consisting of a scaffolding protein (Gab1) recruiting both a negative effector (RasGAP) and a protein (SHP2) that downregulates the binding of this effector. Because Gab1, similarly to DOK, contains multiple potential RasGAP-binding sites, we speculate that these sites may provide a safety system against an illegitimate ignition of the Ras
pathway possibly induced by unspecific phosphorylations. Indeed, the mechanism described herein suggests that only a specific pattern of EGFR and Gab1 phosphorylation allowing both to recruit Grb2/Sos and to disengage RasGAP can lead to a sustained activation of Ras. In addition, similarly to what was envisaged in *Torso* signaling (36), this mechanism might allow the modulation by SHP2 of the intensity and duration of Ras activating signals emanating from the EGFR. On the other hand, recent studies have suggested that RasGAP may have Ras-independent functions, for example in cell migration or even Akt activation (37,38). Therefore it could be important for EGF bioactivity to launch RasGAP-dependent pathway(s) in a very early stage of EGFR activation, then to disengage RasGAP to reach an adequate level of Ras activation. Further molecular studies of the spatio-temporal regulation of RasGAP recruitment and associated downstream responses are required to address this question.

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FIGURE LEGEND

Fig. 1. Evidence that Gab1 PI3K-binding sites are not targets of SHP2 in Ras activation and that SHP2 dephosphorylates other Gab1 sites. A, Vero cells were cotransfected as described in Experimental Procedures with HA-tagged Ras and the indicated Gab1-Myc construct: wild-type (WT); mutated on its SHP2-binding site (Y627F); mutated on its three p85-binding sites (YF3); mutated on both its p85- and SHP2-binding sites (YF3/Y627F). After stimulation, cells were lysed and incubated with beads bound to a GST fusion protein containing the Ras binding domain of Raf (GST-RBD) to precipitate GTP-bound Ras. The amount of activated HA-Ras associated with the beads was determined by anti-HA immunoblotting (IB HA, top). Lysates were also subjected to anti-HA (middle) and anti-Myc immunoblotting (bottom) to control the expression level of HA-Ras and Gab1-Myc constructs, respectively. Lane WT(-RBD), a control was performed using a lysate of stimulated cells transfected with Gab1-WT and incubated with GST alone. B, Vero cells were cotransfected with wild-type (WT) or catalytically inactive SHP2 (C/S) and one of the indicated Gab1-Myc construct. Following stimulation with EGF when indicated, cells were subjected to anti-SHP2 immunoprecipitation, followed by anti-Myc (top) and anti-SHP2 (middle) immunoblotting. Gab1-Myc expression was analysed by anti-Myc immunoblotting of total cell lysates (bottom). Far-right lane, immunoprecipitation performed without anti-SHP2 antibody.

Fig. 2. Gab lacking the Pro161-Arg318 region remains mobilizable by EGF but much less trappable by SHP2-C/S. A, schematic representation of Gab1 WT and ΔPQ mutant displaying the potential RasGAP-binding motifs (YXXP) and the domains functionally identified as important for EGF signaling (PH domain; binding sites for: Grb2-SH3 domains, Met, PI3K and SHP2 SH2 domains). B, C, functionality of Gab1-ΔPQ mutants: Vero cells were transfected with the indicated Gab1-Myc construct (ΔPQ/Y627F is both...
deleted between Pro 161 and Arg 318 and unable to bind SHP2). Following stimulation, membrane fractions were prepared as described in Experimental Procedures and analysed by anti-Myc immunoblotting (top). Cell lysates were also directly subjected to anti-Myc immunoblotting to verify that equal amounts of transfected Gab1 were present in each sample (bottom). C, anti-Myc immunoprecipitates were performed from cells transfected with the indicated Gab1-Myc construct, followed by anti-phosphotyrosine immunoblotting (top). Bottom, control of the expression level of constructs in lysates. D, Vero cells were cotransfected with 1 µg of each plasmid DNA encoding SHP2-C/S and one of the indicated Gab1-Myc constructs. Following stimulation or not with EGF as shown, cells were processed for SHP2 immunoprecipitation, followed by anti-Myc (top) and anti-SHP2 (middle) immunoblotting. Bottom, control of the expression level of Gab1-Myc constructs in lysates. Lane Y627F(–Ab), a control immunoprecipitation performed without adding anti-SHP2 antibody.

Fig. 3. Generation of a RasGAP inactive mutant bypassing the requirement for SHP2 in Ras activation. A, membrane fractions were prepared from Vero cells transfected with different Gab1-Myc constructs and stimulated or not with EGF as indicated. Fractions were analysed by immunoblotting anti-RasGAP (top), anti-Myc (middle), and anti-EGFR to control gel loading (bottom). B, Vero cells were cotransfected with HA-tagged Ras and plasmids encoding the indicated RasGAP constructs (v, empty vector). Following stimulation, cells were lysed and processed for the GST-RBD pulldown assay and analysed by anti-HA immunoblotting (top). Lysates were also directly subjected to anti-HA (middle) and anti-RasGAP (bottom) immunoblotting to control construct expression. C, Vero cells were cotransfected in a 1/4/4 ratio with plasmids encoding, respectively, HA-Ras, Gab1-Myc-WT or -Y627F, and either empty vector (-) or the RasGAP-R789/903Q (2RQ) construct (+), as indicated. Following stimulation or not with EGF, cells were subjected to the GST-RBD
pulldown assay. The amount of activated HA-Ras precipitated was determined by anti-HA immunoblotting (top). Corresponding lysates were subjected to anti-HA, anti-RasGAP and anti-Myc immunoblotting (IB), as indicated, to control the expression level of each transfected protein. Far-right lane: a control pulldown assay was performed with GST alone on the indicated cell lysate. D, Vero cells were cotransfected as described in C and then subjected to anti-Myc immunoprecipitation to control the phosphorylation of Gab1-Myc constructs using anti-phosphotyrosine (IB P-Tyr) and anti-Myc immunoblotting as indicated. Two bottom panels, control of expression of the transfected constructs in lysates. E, MEF-ΔSHP2 cells were cotransfected with HA-tagged Ras and either empty vector (v) or the RasGAP-2RQ mutant. After EGF treatment, cleared lysates were processed for the GST-RBD pulldown assay followed by anti-HA immunoblotting analysis (top). The expression of transfected constructs was controlled in lysates by anti-HA (middle) and anti-RasGAP (bottom) immunoblotting.

Fig. 4. A specific interaction between Gab1 and RasGAP involving the Gab1 Pro161-Arg318 region. A, membrane fractions were prepared from Vero cells transfected with the indicated Gab1-Myc construct and stimulated or not with EGF, as shown. Fractions were analysed by anti-RasGAP (top), anti-Myc (middle), and anti-EGFR (bottom) immunoblotting to control gel loading. B, MEF-WT or -ΔSHP2 cells were stimulated with EGF as indicated. Cells were then lysed and cleared lysates were incubated with beads bound to a GST fusion protein containing either the whole RasGAP SH2-SH3-SH2 region (GST-GAP-3S) or only the C-terminal SH2 domain (GST-GAP-CT), as indicated. The amount of endogenous Gab1 associated to the beads was analysed by anti-Gab1 immunoblotting (top). Crude lysates were also subjected to anti-Gab1 immunoblotting to verify that equal amounts of Gab1 were present in each sample (bottom). C, as a positive control, the pulldown assay was performed as in B, using a GST fusion protein containing the SH2 domains of PI3Kα.
D, Vero cells expressing the indicated Gab1-Myc construct were treated or not with EGF and subjected to the GST-RasGAP-3S pulldown assay, followed by anti-Myc immunoblotting \((\text{top})\). Gab1-Myc expression was controlled in total cell lysates \((\text{bottom})\). E, same experiment as in \(D\), except that the pulldown was performed with the GST-p85 fusion protein.

Fig. 5. The Gab1 deletion mutant deficient for RasGAP binding attenuates the requirement for SHP2 in Ras activation. \(A\), Vero cells were cotransfected with HA-tagged Ras and the indicated Gab1-Myc construct. Following stimulation, cells were lysed and incubated with GST-RBD beads. The amount of HA-Ras precipitated with the beads was analysed by anti-HA immunoblotting \((\text{top})\). Lysates were also subjected to anti-HA \((\text{middle})\) and anti-Myc \((\text{bottom})\) immunoblotting to control HA-Ras and Gab1-Myc expression, respectively. \(B\), same experiment as in \(A\) in MEF-ΔSHP2 cells cotransfected as described in Experimental Procedures with HA-tagged Ras and either empty vector \((\text{v})\) or Gab1-Myc-ΔPQ/Y627F.

Fig. 6. Localization of Gab1 RasGAP-binding site on the second YXXP tandem. \(A\), Vero cells were transfected with one of the indicated Gab1 constructs, including Gab1 deficient for SHP2 binding and carrying Tyr to Phe mutations on the first or second tandem Tyr-X-X-Pro motifs \((Y627F + Y242/259F\) and \(Y627F + Y307/317F\), respectively). Following stimulation, cleared lysates were subjected to the GST-RasGAP-3S pulldown assay and the amount of Gab1 precipitated was analysed by anti-Myc immunoblotting \((\text{top})\). Crude lysates were subjected to anti-Myc immunoblotting to verify the expression of transfected constructs \((\text{bottom})\). Lane \(WT(-GAP3S)\): a control performed using beads loaded with GST alone. \(B\), membrane fractions were prepared from Vero cells transfected with the indicated Gab1-Myc construct and stimulated or not with EGF, as shown. Fractions were analysed by anti-RasGAP \((\text{top})\) and anti-EGFR \((\text{bottom})\) immunoblotting to control gel loading. \(C\), MEF-ΔSHP2 cells
were cotransfected with HA-Ras and the indicated Gab1-Myc constructs. Following stimulation, cells were subjected to the GST-RBD pulldown assay to measure the amount of activated HA-Ras, as described in Fig. 5. D, E, Graphs representing the data from GST-RasGAP3S pulldown (D) and Ras activation (E) assays performed in Vero or MEF-ΔSHP2 cells, respectively, expressing the indicated Gab1 constructs and stimulated with EGF. Data were quantified using a Bio-Rad gel analysis device and the software NIH-IMAGE (mean ± S.E. from three independent experiments).

Fig. 7. Gab1 Tyr 317 constitutes the major docking site for RasGAP. A, Vero cells were transfected with Gab1-Myc-WT or -Y627F and stimulated or not with EGF during the indicated time. Cells were then subjected to anti-Myc immunoprecipitation, followed by anti-phospho-Gab1-Y307 (top) and anti-Myc (middle) immunoblotting. Bottom, control of the expression level of Gab1-Myc constructs in lysates. Lane WT-Ac, a control immunoprecipitation performed without adding anti-Myc antibody. B, Vero cells expressing the indicated Gab1-Myc constructs were subjected to the GST-GAP-3S pulldown assay. The experiment was analysed as in Fig. 6A. C, membrane fractions were prepared from Vero cells transfected with the indicated Gab1-Myc constructs and stimulated or not with EGF. Fractions were analysed by anti-RasGAP (top) and anti-EGFR (bottom) immunoblotting to control gel loading. D, MEF-ΔSHP2 cells were cotransfected with HA-tagged Ras and either empty vector (v) or the indicated Gab1-Myc construct. Following stimulation, cells were subjected to the GST-RBD pulldown assay to measure the amount of activated HA-Ras, as described in Fig. 6C.

Fig. 8. Model outlining the role of Gab1 and SHP2 in EGF-induced Ras activation. Once Gab1 is associated to the activated EGFR, through Grb2 or PI3K lipid products, it recruits and activates PI3K and SHP2 (5,7). The phosphatase can then modulate PI3K activation by dephosphorylating Gab1 PI3K-binding sites (18), a regulation that appears
secondary considering the positive role of SHP2 in this pathway. In contrast, we have located on Gab1 Ras negative-regulatory tyrosine phosphorylation sites involved in the binding of RasGAP. To achieve a full activation of Ras, SHP2 must dephosphorylate these sites to disengage RasGAP from Ras activation complexes.
Figure 1
Figure 2
Figure 3
Figure 4

A

GST-GAP:

WT  Y627F  ΔPQ  ΔPQ/Y627F

EGF:

-       +         -       +        -     +       -        +

IB RasGAP

IB Myc

IB EGFR

Membrane fractions

B

MEF:  WT  ΔSHP2

GST-GAP:  CT  3S  CT  3S

EGF:  -  +  -  +  -  +  -  +

IB Gab1

GST-GAP pulldown

Lysates

C

MEF:  WT  ΔSHP2

EGF:  -  +  -  +

IB Gab1

GST-p85 pulldown

Lysates

D

Gab1-Myc:  WT  Y627F  YF3  ΔPQ

EGF

IB Myc

GST-GAP3S pulldown

Lysates

E

Gab1-Myc:  WT  Y627F  YF3  ΔPQ

EGF

IB Myc

GST-p85 pulldown

Lysates
Figure 5

A

| Gab1-Myc: | WT | Y627F | ΔPQ | ΔPQ/Y627F |
|-----------|----|-------|-----|-----------|
| IB HA     |    |       |     |           |
| GST-RBD pulldown | | | | |
| HA-RasGTP | | | | |
| IB Gab1-Myc | | | | |
| Lysates   | | | | |

B

| EGF: | - | + |
|------|---|---|
| IB HA | | |
| GST-RBD pulldown | | |
| HA-RasGTP | | |
| IB Myc | | |
| Gab1 | | |
| Lysates | | |
Figure 6

A

Gab1-Myc: WT Y627F Y242/259F Y307/317F WT (-GAP3S)

IB Myc

GST-GAP3S pulldown

Lysates

B

Gab1-Myc: WT Y627F Y627F+ Y242/259F Y307/317F

EGF: - + - + +

IB RasGAP

IB EGFR

Membrane fractions

C

Gab1-Myc: WT Y242/259F Y307/317F WT (-RBD)

IB HA

GST-RBD pulldown

Lysates

D

E

Gab1/GST-GAP3S assoc. (% of max)

Gab1: WT Y627F ΔPQ Y242/259F Y307/317F

Ras activation (fold increase)

Gab1: WT ΔPQ Y242/259F Y307/317F
Figure 7
Figure 8
A novel role for Gab1 and SHP2 in epidermal growth factor-induced Ras activation
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