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Owing to the wrong counting of the amino acids in the murine Gab1 sequence, some amino acids were not correctly labelled: serine 551 (Ser551) should be counted as Ser552, Ser567 should be counted as Ser568 and tyrosine 580 (Tyr580) should be counted as Tyr590.

The authors apologize for this mistake.

A new mechanism for the regulation of Gab1 recruitment to the plasma membrane

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Summary

Adaptor proteins involved in signal transduction fulfil their cellular functions by bringing signalling molecules together and by targeting these signalling components to defined compartments within the cell. Furthermore, adaptor proteins represent a molecular platform from which different signalling pathways are initiated. Gab1 is an adaptor protein that recruits the p85 subunit of the phosphatidylinositol 3-kinase, the adaptor Grb2, the adaptor and phosphatase SHP2 and the GTPase-activating protein Ras-GAP. Gab1 thus contributes to the activation of the PI3K cascade and the MAPK cascade through many growth factors and cytokines. The recruitment of Gab1 to phosphatidylinositol (3,4,5)-trisphosphate within the plasma membrane by its pleckstrin-homology domain is regarded as a major regulatory step for the activation of Gab1. Here, we present a new more complex mechanism for Gab1 translocation that involves and depends on the activation of ERK. We demonstrate that the presence of PI3K activity in the cell is not sufficient for binding Gab1 to the plasma membrane. Instead, additional MAPK-dependent phosphorylation of Ser551 in Gab1 is crucial for the recruitment of Gab1 to the plasma membrane. This mechanism represents a new mode of regulation for the function of PH domains.

Supplementary material available online at
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Key words: Gab1, Interleukin 6, Adaptor protein, Cytokine, Signal transduction

Introduction

Signal transduction depends on the sequential activation of signalling components. On one hand this allows rapid signal amplification and on the other hand very complex regulation is possible to integrate information from different sources. Recently, research has focused on the cross-talk of hormones or cytokine signalling. Synergistic activation, as well as mutual inhibition of signalling pathways by different stimuli, is possible. Key events in signal transduction are the activation of proteins and lipids by altering their phosphorylation status by kinases or phosphatases. Alternatively, the activities can be regulated by limited proteolysis or lipase activities, respectively. Targeting to specific cellular compartments, binding of second messengers, conformational changes and regulated protein-protein or protein-lipid interactions are additional mechanisms in signal transduction. Adaptor proteins contribute to all these events.

Once adaptor proteins are activated, they present a signalling platform and can be bound by signalling proteins, which activate each other. Different signalling pathways can be initiated at these adaptors in parallel, sequentially or alternatively. In addition, the cellular distribution of adaptor proteins can be regulated. Hence, the translocation of an adaptor is crucial to bring an adaptor-bound enzyme to its substrate within the cell. Adaptor proteins therefore orchestrate signal transduction by recruiting signalling components to a defined compartment within the cell.

Interleukin 6 (IL-6) is a cytokine with a broad function in the immune response. It acts as a differentiation factor on leukocytes, regulates cell proliferation of plasma cells and survival of neuronal cells, acts as migration factor on T-cells and monocytic cells and is the major mediator of acute phase gene expression (Kamimura et al., 2003). IL-6 binds to a receptor complex composed of IL-6Rα and the signal transducing subunit gp130. Cells that do not express IL-6Rα can be stimulated with IL-6 in complex with the soluble agonistic IL-6Rα (*trans*-signalling). The soluble form of IL-6Rα is derived from apoptotic neutrophils by shedding (Scheller et al., 2006).

Assembly of the receptor complex leads to activation of the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway as well as to activation of the mitogen-activated protein kinase (MAPK)- and phosphatidylinositol 3-kinase (PI3K)-cascade (Heinrich et al., 1998). Whereas the mechanism for JAK/STAT activation is quite well elucidated, the mechanisms leading to the activation of the STAT-independent cascades are poorly understood. The current view for extracellular-regulated kinase (ERK) activation favours the recruitment of the phosphatase and adaptor protein SHP2 (SH2 domain-containing phosphatase 2) to tyrosine-phosphorylated gp130 (Fukada et al., 1996; Schiemann et al., 1997). Similarly to other pathways, the binding of the adaptor Grb2 (growth factor receptor-bound protein 2, GRB2) to SHP2 as well as a role of the adaptor protein Gab1 (Grb2-associated binder 1; GAB1) is speculated. In cells from Gab1-deficient embryos, ERK activation is reduced in response to IL-6 signalling. Gab1 knockout in mice leads to defects in the development of the heart, placenta and skin, and the death of the animal in utero (Itoh et al., 2000). Although a crucial role of PI3K, SHP2 and Ras in Gab1-mediated ERK activation has been proposed (Takahashi-Tezuka et al., 1998), the mechanism of Gab1 activation remains obscure.

The Gab1 adaptor protein is involved in the signal transduction of many growth factors and interleukins and mediates activation of the MAPK cascade as well as the PI3K cascade (Gu and Neel, 2003; Nishida and Hirano, 2003). Gab1 contains tyrosine motifs, which

in their phosphorylated forms represent binding sites for the tyrosine phosphatase and adaptor protein SHP2, for the p85 regulatory subunit of the PI3K, for phospholipase C γ (PLCγ), as well as for the Ras-GTPase activating protein Ras-GAP (Gu and Neel, 2003; Montagner et al., 2005). All these proteins interact with Gab1 through their SH2 domains, which specifically bind to tyrosine-phosphorylated motifs within Gab1. SHP2 has been attributed an additional regulatory function by dephosphorylating the PI3K-binding site (Zhang et al., 2002) and the Ras-GAP binding site (Montagner et al., 2005) in Gab1.

The recruitment of Gab1 to the plasma membrane is regarded as a crucial step for Gab1 activation. Gab1 is targeted by its pleckstrin homology (PH) domain to phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P_3] at the plasma membrane (Itoh et al., 2000; Maroun et al., 1999; Rodrigues et al., 2000; Sachs et al., 2000). PtdIns(3,4,5)P_3 derives from PtdIns(4,5)P_3 by PI3K-mediated phosphorylation of the 3-hydroxy group of the inositol moiety. The presence of PtdIns(3,4,5)P_3 in the plasma membrane is regulated by a balance of PI3K and lipid phosphatases, such as PTEN (phosphatase and tensin homologue deleted from chromosome 10) and SHIP (SH2 domain containing inositol phosphatase). PTEN dephosphorylates PtdIns(3,4,5)P_3 at the phosphate at position 3 whereas SHIP targets the phosphate at position 5 of PtdIns(3,4,5)P_3 (Koyasu, 2003). These phosphatases are attributed tumour suppressor functions, whereas PI3K induces anti-apoptotic pathways.

Gab1 also interacts with growth factor and cytokine receptors. A so-called Met-binding domain in Gab1 enables a direct interaction with the transmembrane HGF receptor cMet (Schaeper et al., 2000; Weidner et al., 1996). The interaction with most cytokine receptors is mediated via Grb2 (Lock et al., 2000). In fact, Gab1 (Grb2-associated binder 1) is named after its constitutive interaction with Grb2 (Holgado-Madruga et al., 1996). Grb2 exerts a bridging function through its SH2 domain, which binds to the phosphorylated tyrosine motifs within the activated receptor, whereas the Grb2-SH3 domain interacts with the proline-rich domain of Gab1. The recruitment to the plasma membrane via receptor interaction or via binding PtdIns(3,4,5)P_3 at the membrane is mandatory for tyrosine phosphorylation of Gab1 and the subsequent binding of the effector enzymes and downstream signalling.

Here, we present data on a complex mechanism of Gab1 activation and its translocation to the plasma membrane, which involves and depends on the activation of ERK.

Results

The SHP2-binding site in Gab1 is crucial for ERK activation

We evaluated the requirement of the SHP2 interaction site in Gab1 for Gab1-mediated activation of the MAPK cascade. We set up experiments to analyse the potential of the Gab1 wild-type protein and a Gab1 protein harbouring mutations in the SHP2 binding sites (ΔSHP2) to increase IL-6-dependent ERK activation when expressed in HEK393T cells. HEK293T cells were transiently transfected with expression vectors for the respective Gab1 proteins and for EpoR-gp130 chimeric receptors composed of the extracellular part of the EpoR and the transmembrane and cytoplasmic part of gp130. These well-established chimeric receptors allow us to selectively stimulate the population of transfected cells without activating endogenous wild-type gp130.

In the absence of exogenously expressed Gab1, stimulation-dependent ERK activation was obvious (Fig. 1A, 2nd panel, lane 2). Expression of wild-type Gab1 further enhances the amount of phosphorylated ERK (lane 4). By contrast, the Gab1 mutant ΔSHP2 failed to further enhance ERK activation (lane 6).

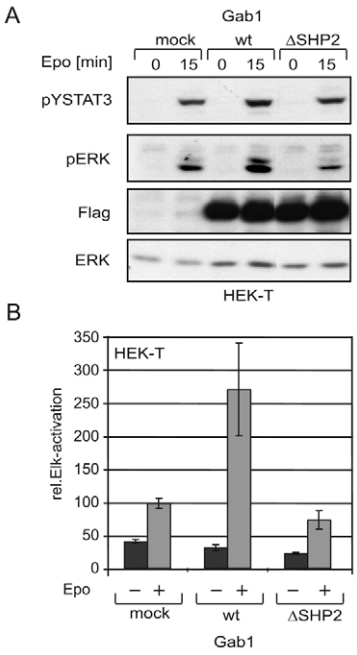


Fig. 1. The Grb2 and SHP2 binding sites in Gab1 are crucial for ERK activation. (A) HEK293T cells were cotransfected with expression vectors for Gab1, Gab1ΔSHP2 or empty vector together with vectors encoding EpoR-gp130 chimeric receptors. Two days after transfection, cells were starved in DMEM without FCS and subsequently stimulated with Epo (7 U/ml) for 15 minutes to induce IL-6 signalling. Cell lysates were prepared and proteins separated by SDS-PAGE. After western blotting, the membranes were stained for activated STAT3 (upper panel) to control stimulation of the cells, and for the activated forms of ERK1/2 (second panel). After stripping, the membrane was stained for exogenous Gab1 (Flag) and ERK to monitor Gab1-transfection efficiency and equal loading of the gel (ERK). (B) HEK293T cells were transfected with expression vectors encoding EpoR-gp130 together with vectors for wt-Gab1, Gab1ΔSHP2 or empty vector. MAPK activity was monitored by using an Elk1 transactivation approach (PathDetect Elk1 trans Reporting System, Stratagene). For the Elk1 reporting system, cells were transfected with a Gal4-driven luciferase reporter and an expression vector encoding a fusion protein composed of the DNA-binding domain of Gal4 and the transactivation domain of the Elk1 transcription factor (pFA2-Elk1). Activation of MAPK leads to phosphorylation and activation of the Elk-transactivation domain and subsequent increase of Gal4/Elk1-dependent luciferase reporter activity. Modulation of MAPK activity by Gab1 mutants was monitored by luciferase activity. 6 hours after transfection, cells were stimulated with Epo (7 U/ml) for 16 hours to induce IL-6 signalling. Transfection efficiency was controlled by cotransfection of vector for constitutive expression of β-galactosidase. Luciferase activity was normalised to β-galactosidase activity of each sample in triplicate. Results are given as the mean ± s.e.m.

To confirm these data, we analysed Elk-dependent reporter gene expression. Elk is a transcription factor activated by ERK-dependent phosphorylation within the transactivation domain. We also tested the competence of coexpressed wild-type Gab1 or of the Gab1 mutant ΔSHP2 to increase transcriptional activity of Elk, which was initiated through the activation of EpoR-gp130 chimera (Fig. 1B). Similarly to the previous experiment, the wild-type Gab1 protein further increased Elk-dependent reporter gene induction. By contrast, the Gab1 mutant ΔSHP2 did not enhance Elk-mediated gene induction when compared with cells that were not transfected with Gab1 cDNAs (mock). From all these data we draw

the conclusion that SHP2 binding to Gab1 is crucial for the function of Gab1 to regulate MAPK activation. We controlled the cell-surface expression of EpoR/gp130 in the absence and presence of the Gab1 mutants by FACS and did not find any differences that could explain the observed differences in ERK activation (supplementary material Fig. S1).

The PH domain and PI3K activity are essential for Gab1 activation

The data above demonstrate the relevance of the SHP2-binding site within Gab1 for ERK and Elk activation. Thus, we analysed the phosphorylation of Tyr627 within this binding site in the following experiments. Since PH domain-independent Gab1 activation has been discussed for other signalling pathways, we compared ERK activation and Gab1 tyrosine phosphorylation in the presence of the wild-type Gab1 with ERK activation in the presence of a Gab1 mutant, which lacks the PH domain (ΔPH) and in the presence of the Gab1-PH domain alone (PH). Cells expressing these proteins with the EpoR-gp130 chimeric receptors were stimulated, and the cell lysates were analysed by western blotting. The blot was stained for the phosphorylated form of STAT3 to demonstrate stimulation. Fig. 2A clearly shows enhanced ERK activation and Gab1 phosphorylation in cells expressing the wild-type Gab1 protein. By contrast, expression of the Gab1 mutant ΔPH as well as the isolated PH domain did not lead to phosphorylation of ERK and Gab1. These data indicate that the PH domain of Gab1 is crucial but not sufficient for Gab1 activation.

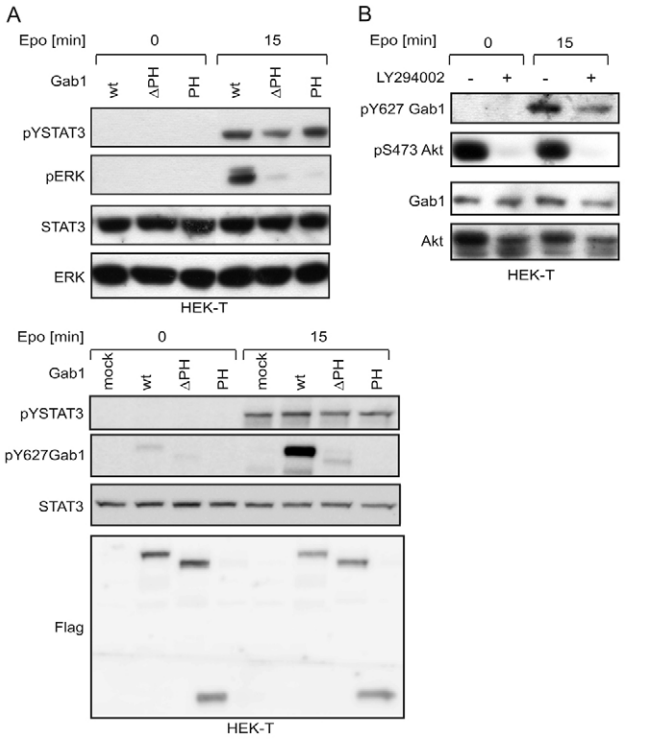


Fig. 2. The PH domain in Gab1 and PI3K activity are required for Gab1 activation. (A) HEK293T cells were cotransfected with expression vectors for wt-Gab1, Gab1ΔPH or the isolated PH domain, together with vectors encoding EpoR-gp130 chimeric receptors. Two days after transfection, cells were starved in DMEM without FCS for 3 hours and subsequently stimulated with Epo (7 U/ml) for 15 minutes. Cell lysates were prepared and proteins separated by SDS-PAGE. After western blotting, the membranes were stained for activated STAT3 to control stimulation of the cells and for the activated forms of ERK1/2 and Gab1. After stripping, the membrane was stained for Gab1 (Flag), STAT3 and ERK to monitor Gab1-transfection efficiency and equal loading of the gels (STAT3, ERK). (B) HEK293T cells were cotransfected with expression vectors for Gab1 and EpoR-gp130 chimeric receptors. Cells were treated as above and then with the PI3K inhibitor LY294002 (40 μM) for 45 minutes prior to stimulation with Epo (7 U/ml) for 15 minutes. After western blotting, the membranes were stained for Gab1 phosphorylated at Y627 and for Akt phosphorylated at Ser473 (pS473Akt) to monitor PI3K activity. After stripping, the membrane was stained for Gab1 and Akt to monitor Gab1-transfection efficiency and equal loading of the gel. The shift in Akt mobility is due to phosphorylation of the protein. Owing to the use of Gab1-GFP fusion proteins in the following experiments and to better distinguish endogenous from exogenous Gab1 proteins, the latter were expressed as GFP-fusion proteins to reduce mobility in SDS-PAGE.

The Gab1 PH domain is known to interact with PtdIns(3,4,5)P_3 that is generated by PI3K at the plasma membrane. The importance of the PH domain for Gab1 activation suggests PI3K activity to be crucial for IL-6-dependent Gab1 activation.

To validate PI3K-dependent Gab1 activation, we tested the effect of the PI3K inhibitor LY294002 on Gab1 Y627 phosphorylation. HEK293T cells were incubated with the inhibitor and IL-6 signalling was initiated through the EpoR-gp130 chimeric receptors. Cell lysates were then analysed by western blotting (Fig. 2B). Inhibition of PI3K by LY294002 was confirmed by reduced phosphorylation of the downstream target AKT, used as a monitor for PI3K activity. Corroborating the results shown in Fig. 2A, inhibition of PI3K reduced Gab1 phosphorylation induced by IL-6 signalling. All these data provide evidence that the PH domain, as well as PI3K activity, is involved in Gab1 activation.

IL-6-induced translocation of Gab1 to the plasma membrane depends on basal PI3K activity

Since the Gab1 PH domain is crucial for tyrosine phosphorylation of Gab1, we focused on the mechanism of IL-6-induced Gab1 activation and its translocation to the plasma membrane using live-cell microscopy. Translocation of Gab1-GFP fusion proteins could be demonstrated in HEK293T cells after induction of IL-6 signalling through EpoR-gp130 chimeric receptors. The confocal microscopy data shown in Fig. 3A demonstrate predominant Gab1 localisation at the plasma membrane after 20 minutes of stimulation.

We made use of the two cell lines HEK293 and HEK293T, which differ in basal PI3K activity. HEK293T cells are characterised by high basal PI3K activity as demonstrated by AKT phosphorylation. By contrast, no AKT phosphorylation was detectable in HEK293 cells (Fig. 3B). Gab1 was found in the cytoplasm in non-stimulated cells (Fig. 3C). Interestingly, Gab1 translocation to the plasma membrane in response to IL-6 signalling was only apparent in HEK293T cells but not in HEK293 cells. Since this discrepancy correlates with enhanced basal PI3K activity in HEK293T cells, we speculated that IL-6-induced Gab1 translocation requires basal PI3K activity in the cell. Therefore, we pre-incubated HEK293T cells with the PI3K inhibitor LY294002 and analysed the cellular distribution of Gab1 prior to and after stimulation. As shown in Fig. 3D, no translocation of Gab1 was detectable in the presence of the PI3K inhibitor LY294002. These observations were also confirmed with the structurally distinct PI3K inhibitor wortmannin (supplementary material Fig. S2). These results suggest that IL-6-induced recruitment to the plasma membrane strongly depends on PI3K activity, indicative of PH-domain-dependent rather than simple receptor-interaction-mediated membrane recruitment.

To test our hypothesis that basal PI3K activity is crucial for IL-6-induced translocation of Gab1, we enhanced PI3K activity in HEK293 cells by supplementing the medium with 10% FCS or by

expression of a constitutive active mutant of PI3K (p110γ-CAAX) (Fig. 3E,F). Fig. 3F demonstrates that both approaches were able to increase constitutive PI3K activity and thus, IL-6 signalling induces translocation of Gab1 to the plasma membrane, even in HEK293 cells. Gab1 accumulation at the membrane could not be detected in non-stimulated cells (Fig. 3E).

Isolated Gab1-PH domain is located at the plasma membrane in unstimulated cells and translocation of full-length Gab1 is blocked

Intriguingly, the basal PI3K activity in unstimulated cells, as observed in HEK293T cells or in HEK293 cells cultivated in the presence of FCS or expressing constitutive active PI3K, does not lead to constitutive Gab1 recruitment to the plasma membrane. We therefore speculated that the full-length Gab1 protein has properties that prevent Gab1 translocation to the plasma membrane, despite PI3K activity in unstimulated cells. Consequently, we tested whether the isolated PH domain of Gab1 could be found at the plasma membrane in unstimulated HEK293T cells. Fusion proteins of GFP linked to the Gab1 PH domain or the full-length protein were expressed in HEK293T cells and the cellular distribution of these proteins in living cells was analysed by laser-scanning microscopy (Fig. 4A). The PH domain was clearly located at the plasma membrane, whereas the full-length protein was found in the cytoplasm. These observations indicate that membrane targeting of the full-length Gab1 protein is blocked by protein regions within Gab1 that are not present in the isolated PH domain.

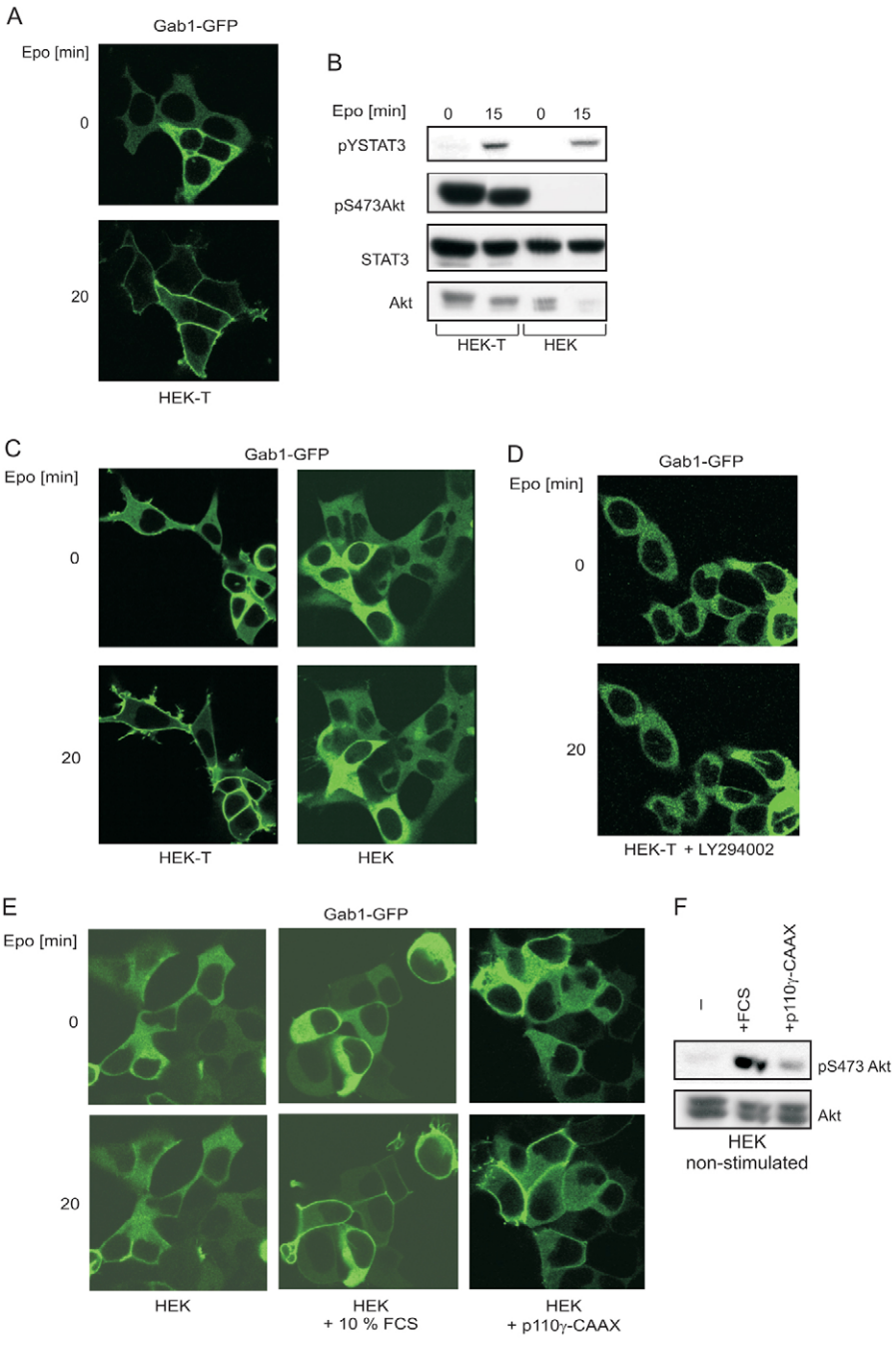


Fig. 3. IL-6-induced translocation of Gab1 to the plasma membrane depends on basal PI3K activity. (A) HEK293T cells were transfected with expression vectors for Gab1-GFP together with vectors encoding EpoR-gp130 chimeric receptors. 24 hours after transfection, cells were transferred to poly-L-lysine-coated coverslips. The next day cells were starved for 3 hours in KRH and transferred into the perfusion chamber of a laser-scanning microscope. After 20 minutes, the cells were stimulated with Epo (7 U/ml). Gab1 localisation is shown before stimulation and after 20 minutes of Epo stimulation. (B) HEK293 and HEK293T cells were cotransfected with expression vectors for Gab1 and vectors encoding EpoR-gp130 chimeric receptors. Two days after transfection, cells were starved in DMEM without FCS for 3 hours and subsequently stimulated with Epo (7 U/ml) for 15 minutes. Cell lysates were prepared and equal amounts of protein separated by SDS-PAGE. After western blotting, the membranes were stained for activated STAT3 to control stimulation of the cells and for Akt phosphorylated at Ser473 (pS473Akt) to monitor PI3K activity. After stripping, the membrane was stained for STAT3 and AKT to monitor equal loading of the gel. (C,D) HEK293 and HEK293T cells were treated and Gab1 localisation analysed as described above in A (C) or pretreated with the PI3K-inhibitor LY 294002 (40 μM) for 45 minutes (D). (E) HEK293 cells were treated and Gab1 localisation analysed as described above in A but cultivated in 10% FCS or transfected with an expression vector for constitutive active PI3K (p110γ-CAAX). (F) As for E, HEK293 cells were cultivated in the absence of FCS (–, 1st lane), in the presence of 10% FCS (2nd lane), or transfected with an expression vector for constitutive active PI3K (p110g-CAAX) (3rd lane). To monitor PI3K activity, cell lysates were prepared and equal amounts of protein separated by SDS-PAGE. After western blotting, the membranes were stained for Akt phosphorylated at Ser473 to monitor PI3K activity. After stripping, the membrane was stained for Akt to ensure equal loading of the gel.

The region between Leu545 and Glu587 in Gab1 blocks membrane recruitment of full-length Gab1

Membrane targeting of full-length Gab1 could be blocked by binding of any of the Gab1-interaction partners. Therefore, we determined whether the binding sites in Gab1 for PI3K, SHP2 or Grb2 are involved in counteracting Gab1 membrane binding in unstimulated cells. Gab1-GFP fusion proteins harbouring mutations within the binding sites for PI3K (ΔPI3K), SHP2 (ΔSHP2) or Grb2 (ΔGrb2) (Schaeper et al., 2000) were expressed in HEK293T cells. The cellular distribution of these proteins was compared with those of wild-type Gab1 and of the Gab1 mutant lacking the PH domain (ΔPH) by laser-scanning microscopy. Fig. 4B shows that none of the mutants was found at the plasma membrane in unstimulated cells. In response to IL-6 signalling, all proteins except Gab1ΔPH translocate to the plasma membrane.

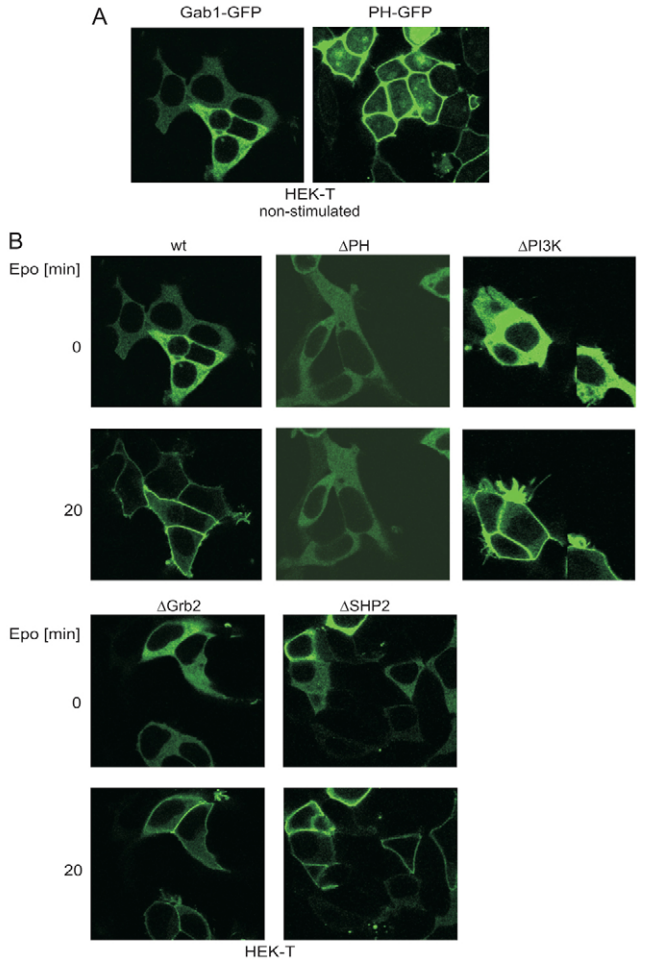


Fig. 4. Constitutive membrane localisation of the isolated PH domain. (A) HEK293T cells were transfected with expression vectors for Gab1-GFP or the isolated PH domain fused to GFP together with vectors encoding EpoR-gp130 chimeric receptors. 24 hours after transfection, cells were transferred to poly-L-lysine-coated coverslips. The next day, cells were starved for 3 hours in KRH and transferred into the perfusion chamber of a laser-scanning microscope. After 20 minutes, the cells were stimulated with Epo (7 U/ml). Gab1 localisation is shown before stimulation and after 20 minutes of Epo stimulation. (B) HEK293T cells were transfected with expression vectors for Gab1-GFP wild type or the mutants Gab1-ΔPH-GFP, Gab1-ΔPI3K-GFP, Gab1-ΔGrb2-GFP or Gab1-ΔSHP2-GFP, together with vectors encoding EpoR-gp130 chimeric receptors. Cells were stimulated and Gab1 localisation analysed as described in A.

Next, we tried to identify the region that blocks association of the PH domain with the plasma membrane in the full-length protein. We started to identify the responsible region within Gab1 by analysing the cellular distribution of Gab1-GFP deletion mutants in unstimulated HEK293T cells. For comparison the isolated PH domain fused to GFP was added to this experiment (Fig. 5A). Fig. 5B demonstrates localisation of the PH domain at the plasma membrane. Sequential extension of the construct did not change the cellular distribution until the Gab1 mutant exceeded the N-terminal 545 amino acids (Δ545). Addition of the next C-terminal 42 amino acids totally changed the cellular distribution of the fusion protein: this Gab1-GFP construct harbouring the N-terminal 587 residues was found in the cytoplasm. These observations suggest that the region between Leu545 and Glu587 in Gab1 blocks membrane localisation of Gab1 in unstimulated cells by affecting the accessibility of the PH domain in Gab1 either directly, or by binding an inhibitory protein.

Translocation of the full length Gab1 protein to the plasma membrane in response to IL-6-signalling is triggered by MAPK activity

How then does IL-6 signalling release the block to facilitate Gab1 membrane translocation? The region between Leu545 and Glu587 bears an ERK target site (Lehr et al., 2004). Thus, we hypothesised that activation of the MAPK cascade might trigger the translocation of Gab1 in response to IL-6. We studied the effect of the MEK-inhibitor U0126 on the cellular distribution of Gab1-GFP in the presence and absence of IL-6 signalling. Interestingly, in the presence of the MEK inhibitor, Gab1-GFP did not translocate to the plasma membrane in stimulated cells (Fig. 6A), whereas the inhibitor did not affect the cellular distribution in non-stimulated cells. As shown in Fig. 6B, U0126 strongly affects stimulation-dependent activation of ERK but not STAT3. These observations were also confirmed with the structurally distinct inhibitor PD98059 (supplementary material Fig. S3).

To further corroborate a crucial contribution of MAPK-cascade activation we tried to replace cytokine stimulation by expressing constitutively active mutants of MEK or the upstream kinase MEKK (Fig. 6D). As demonstrated in Fig. 6C, expression of these constitutively active mutants of both proteins directs Gab1 to the plasma membrane. This observation indicates that in the presence of basal PI3K activity, the activation of the MAPK-cascade is sufficient to relieve the block in Gab1 and consequently changes the intracellular Gab1 localisation from the cytoplasm to the plasma membrane.

MAPK activation in response to IL-6 signalling strongly depends on the Tyr759, whereas STAT activation is mediated through the four membrane distal tyrosine motifs of the signal-transducing receptor subunit gp130 (Fukada et al., 1996; Schmitz et al., 2000). We next tested whether Tyr759 is crucial for the translocation of Gab1 to the plasma membrane. The EpoR-gp130 chimeric receptors used to induce IL-6 signalling in the previous experiments were mutated: Tyr759 was replaced by Phe in the receptor EpoR-gp130(YFYYYY). All cytoplasmic tyrosine motifs except Tyr759 were mutated in the EpoR-gp130(FYFFFF) chimeric receptor. As in the previous experiments, the receptors and Gab1-GFP were expressed in HEK293T cells, and the cellular distribution of Gab1 was analysed in the presence or absence of IL-6 signalling. The results shown in Fig. 6E illustrate that the Tyr759 in gp130 is crucial and sufficient for translocation of Gab1 to the plasma membrane in response to IL-6 signalling. None of the other cytoplasmic

tyrosine motifs in gp130 is involved in the regulation of the cellular distribution of Gab1.

All these data from three independent experimental approaches confirm our hypothesis that activation of the MAPK cascade by IL-6 regulates the cellular distribution of Gab1.

Charged amino acids at position 551 or 567 regulate the cellular distribution of Gab1

Finally, we analysed whether the ERK target sites in the Gab1 region between Leu545 and Glu587 are indeed involved in the regulation of the cellular localisation of Gab1 in response to IL-6 signalling. Therefore Ser551 and Ser567 were replaced with alanine and the corresponding Gab1 mutant expressed in HEK293T cells. Stimulation was performed via the EpoR-gp130 chimeric receptors as in the previous experiments. Fig. 7A clearly demonstrates that this mutant does not change its cellular distribution in response to IL-6 signalling, whereas the wild-type Gab1 could be found at the plasma membrane.

Ser551 and Ser567 within the MAPK target sites appeared to be crucial for the regulation of Gab1, furthermore, activation of the MAPK cascade was found to be crucial for Gab1 regulation. Consequently, we tested whether Glu551 and Glu567 in Gab1 could mimic Ser551 and Ser567 phosphorylation and target Gab1 to the plasma membrane, even in the absence of cytokines. As shown in

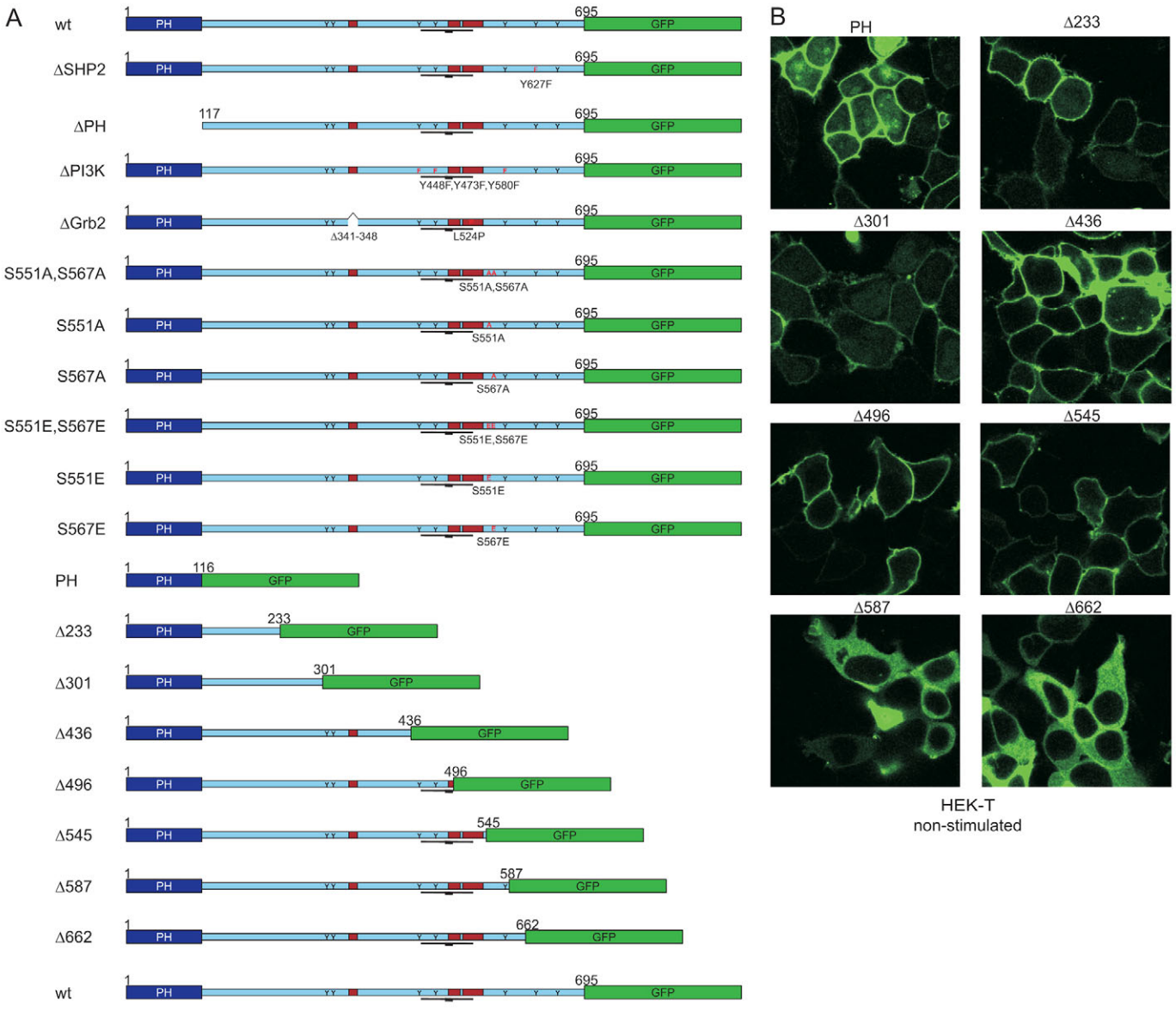


Fig. 5. The region between L545 and E587 in Gab1 blocks membrane recruitment of full-length Gab1. (A) Schematic representation of the analysed Gab1-GFP fusion proteins used in this study. Tyrosine motifs involved in protein binding are indicated by Y. Proline-rich regions are highlighted in red. The Met-binding domain is underlined. The numbers represent the first and last amino acids in the mutant proteins corresponding to the numbering in Gab1 wild-type protein. GFP is fused to the C-terminus of Gab1. (B) HEK293T cells were transfected with expression vectors for Gab1-GFP fusion proteins depicted in A, together with vectors encoding EpoR-gp130 chimeric receptors. 24 hours after transfection, cells were transferred to poly-L-lysine-coated coverslips. The next day cells were starved for 3 hours in KRH and transferred into the perfusion chamber of a laser-scanning microscope. After 20 minutes, the cells were stimulated with Epo (7 U/ml). Gab1 localisation is shown before stimulation and after 20 minutes of Epo stimulation.

the right panel of Fig. 7A, Gab1 was constitutively found at the plasma membrane of unstimulated cells when glutamate substitutes Ser551 and Ser567. These observations suggest that phosphorylation of Ser551 and/or Ser567 in Gab1 regulates the cellular distribution of Gab1. To discriminate the individual contribution of Ser551 and Ser567 in Gab1 for the regulation of Gab1 localisation, single point mutations were prepared. Replacement of Ser551 with alanine was sufficient to abrogate stimulation-dependent membrane recruitment of Gab1. In line with this observation, replacing Ser551 with glutamate was sufficient to mimic cytokine stimulation, leading to constitutive membrane binding of Gab1 (Fig. 7B). By contrast, the replacement of Ser567 with either alanine or glutamate did not change the properties of Gab1 in respect to stimulation-dependent translocation to the plasma membrane (Fig. 7C).

All these observations suggest that phosphorylation of Ser551 in Gab1 through the MAPK cascade, which was initiated by IL-6-signalling through Tyr759 in gp130, regulates the cellular distribution of Gab1.

Discussion

The Gab1 adaptor protein was first identified to contribute to EGF, insulin and HGF signalling (Fixman et al., 1997; Holgado-Madruga et al., 1996; Weidner et al., 1996) and was later also found to act upstream of ERK in IL-6 signalling (Takahashi-Tezuka et al., 1998). The authors provided evidence that PI3K and SHP2 bind to phosphorylated Gab1 in response to IL-6. Using Gab1 mutants that do not interact with SHP2 (Schaeper et al., 2000), we provide evidence of a crucial contribution of SHP2 binding to Gab1 for IL-

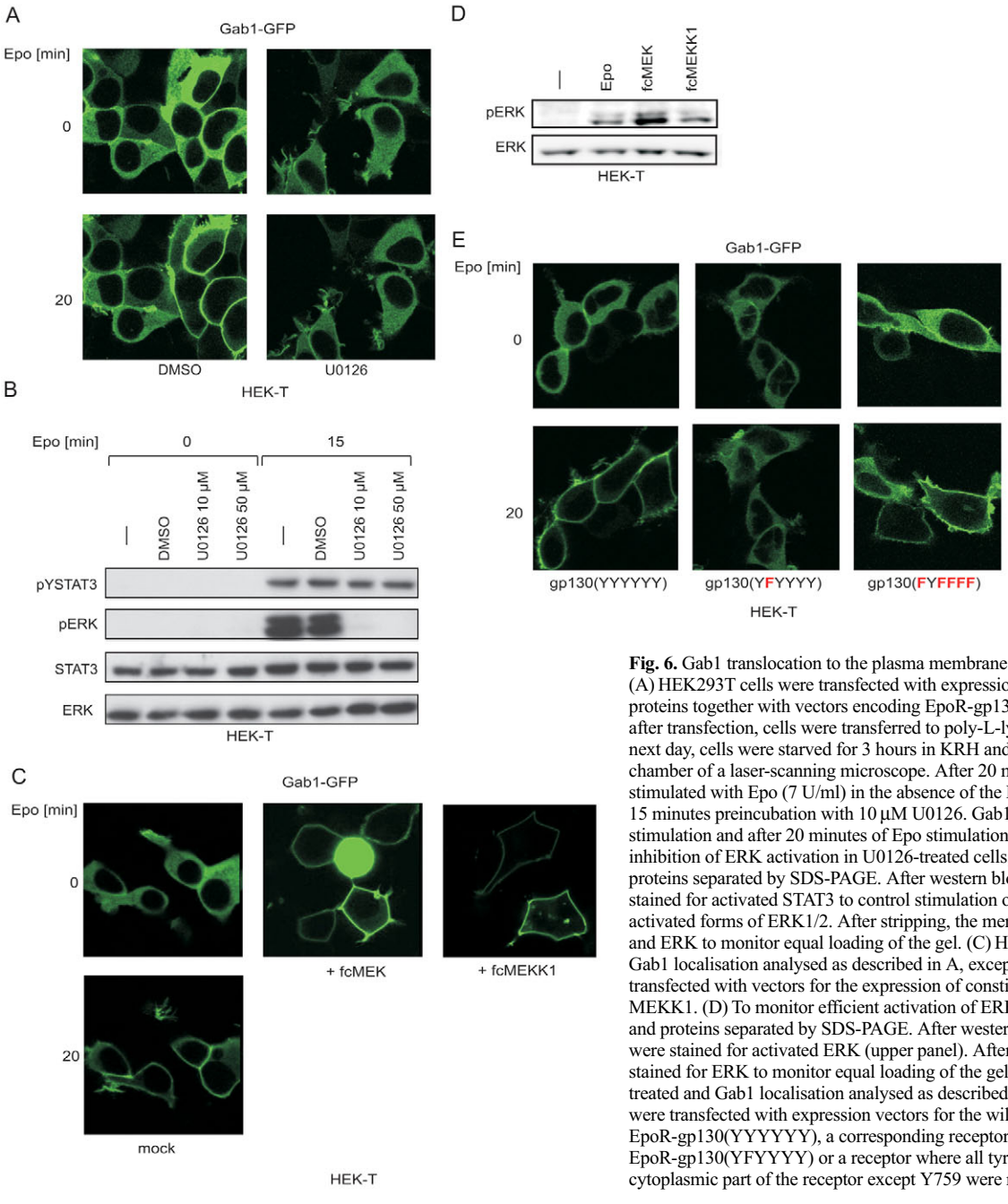
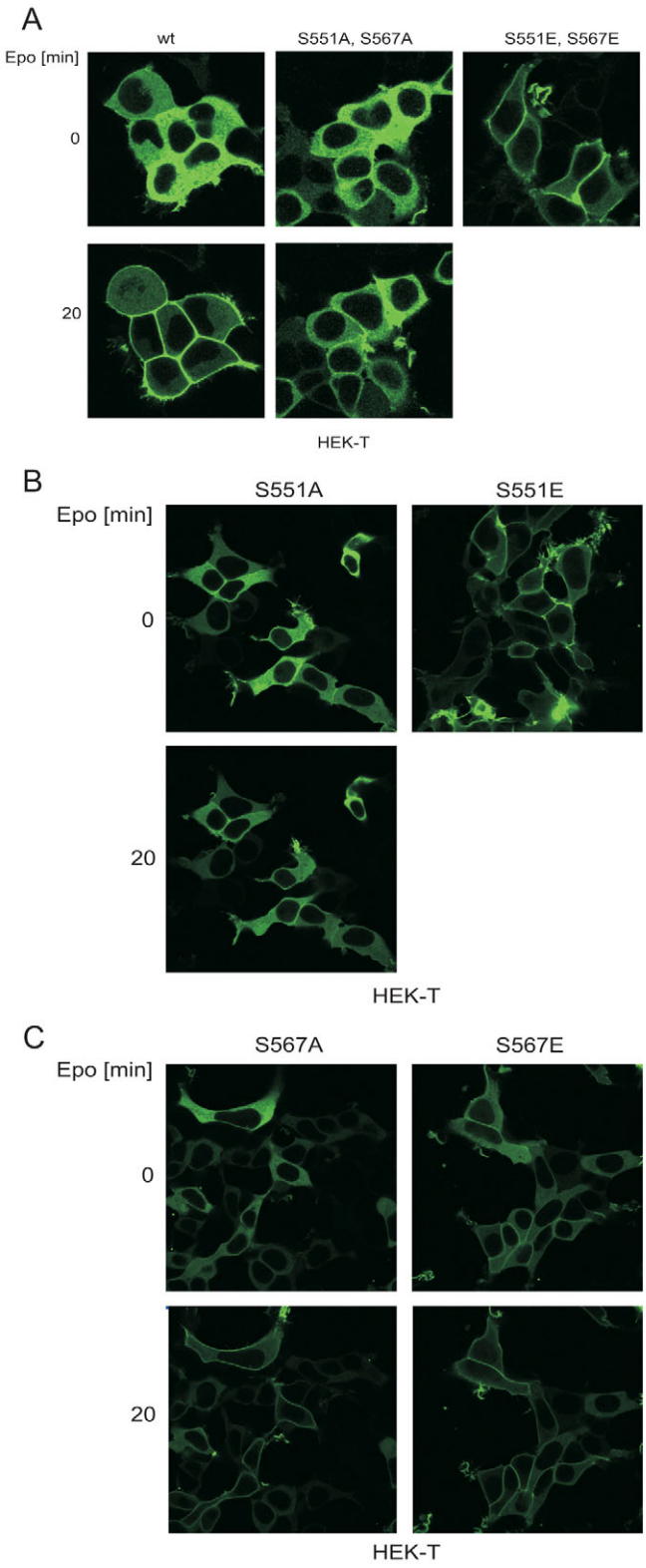


Fig. 6. Gab1 translocation to the plasma membrane is triggered by MAPK activity. (A) HEK293T cells were transfected with expression vectors for Gab1-GFP fusion proteins together with vectors encoding EpoR-gp130 chimeric receptors. 24 hours after transfection, cells were transferred to poly-L-lysine-coated coverslips. The next day, cells were starved for 3 hours in KRH and transferred into the perfusion chamber of a laser-scanning microscope. After 20 minutes, the cells were stimulated with Epo (7 U/ml) in the absence of the MEK inhibitor U0126 or after 15 minutes preincubation with 10 µM U0126. Gab1 localisation is shown before stimulation and after 20 minutes of Epo stimulation. (B) To monitor efficient inhibition of ERK activation in U0126-treated cells, cell lysates were prepared and proteins separated by SDS-PAGE. After western blotting, the membranes were stained for activated STAT3 to control stimulation of the cells, and for the activated forms of ERK1/2. After stripping, the membrane was stained for STAT3 and ERK to monitor equal loading of the gel. (C) HEK293T cells were treated and Gab1 localisation analysed as described in A, except that cells were additionally transfected with vectors for the expression of constitutively active MEK or MEKK1. (D) To monitor efficient activation of ERK, cell lysates were prepared and proteins separated by SDS-PAGE. After western blotting, the membranes were stained for activated ERK (upper panel). After stripping, the membrane was stained for ERK to monitor equal loading of the gel. (E) HEK293T cells were treated and Gab1 localisation analysed as described above in A, except that cells were transfected with expression vectors for the wild-type chimeric receptor EpoR-gp130(YYYYYY), a corresponding receptor with a Y759F exchange EpoR-gp130(YFYYYY) or a receptor where all tyrosine residues within the cytoplasmic part of the receptor except Y759 were replaced by F.

6-induced ERK activation (Fig. 1). We observed that ERK activation, as well as phosphorylation of the SHP2 binding site within Gab1, strongly depends on the Gab1 PH domain (Fig. 2), suggesting that Gab1 phosphorylation occurs at the membrane. In our study, we focused on the regulation of Gab1 plasma membrane recruitment in response to IL-6 because this issue although not well studied, is essential for understanding Gab1 function in general and IL-6 signalling in particular.



Recruitment of Gab1 to the plasma membrane could be facilitated by direct receptor interaction or by binding PtdIns(3,4,5)P_3 at the plasma membrane through the N-terminal PH domain in Gab1 (Gu and Neel, 2003). Detailed studies on HGF signalling revealed that Gab1 interacts through a so-called Met-binding domain with the HGF-receptor cMet (Weidner et al., 1996). Very recently Sampaio and colleagues discovered that the signal strength of EGF dictates whether Gab1 is recruited to the EGF receptor or to PtdIns(3,4,5)P_3 in the plasma membrane. Strong EGF stimulation induces PtdIns(3,4,5)P_3-independent receptor binding whereas weak EGF stimulation induces PtdIns(3,4,5)P_3-dependent membrane recruitment of Gab1 (Sampaio et al., 2007). Here, we present data on IL-6 signalling demonstrating that Gab1-mediated ERK activation and Gab1 phosphorylation, as well as Gab1 recruitment to the membrane, strongly depend on basal PI3K activity and the PH domain in Gab1 (Fig. 3). Thus, IL-6 signalling resembles low rather than strong EGF signalling, suggesting that EGF is a much more potent activator of the MAPK and PI3K cascades than IL-6.

The current view for the regulation of PH domain-dependent recruitment of proteins to PtdIns(3,4,5)P_3 in the plasma membrane simply depends on the regulation of PtdIns(3,4,5)P_3 availability through PI3K, SHIP and PTEN. Here, we present data on a new regulatory mechanism for a PH-domain-mediated recruitment of Gab1 to the plasma membrane, which crucially depends on the activation of the MAPK cascade. PI3K activity was required, but surprisingly was not sufficient for IL-6-induced translocation of full-length Gab1 from the cytoplasm to the plasma membrane, whereas it was sufficient to target the isolated Gab1 PH domain to the membrane. Mutations in the binding sites for Grb2, SHP2 or PI3K did not affect Gab1 membrane targeting, suggesting that membrane targeting of Gab1 is an event upstream of binding these proteins (Fig. 4).

We were able to define the regulatory region within Gab1 that blocks the membrane recruitment of the full-length protein, and most importantly, identified the trigger that relieves this block (Fig. 5). The regulatory region between residues 545 and 587 in Gab1 is highly conserved and bears previously characterised MAPK target sites (Lehr et al., 2004). Indeed, activation of the MAPK cascade by constitutive active MEK or MEKK or by IL-6 enables Gab1 to be recruited to the plasma membrane. Furthermore, the IL-6-induced release depends on Tyr759 in the cytoplasmic part of the IL-6 receptor component gp130, which is known to be crucial for IL-6-mediated ERK activation (Fukada et al., 1996; Schiemann et al., 1997) (Fig. 6). The relevance of these MAPK-target sites in Gab1 for plasma membrane targeting was further confirmed by loss-of-function and also by gain-of-function mutations of the respective serine residues in Gab1 (Fig. 7). Previous studies from the Cantley

Fig. 7. Charged amino acids at residue 551 mimic the ERK stimulus to induce translocation of Gab1 to the plasma membrane. HEK293T cells were transfected with expression vectors for Gab1-GFP wild-type proteins or mutants where Ser551 and Ser567 are replaced by alanine or glutamate (A), or mutants where only Ser551 was replaced by alanine or glutamate (B), or mutants where only Ser567 was replaced by alanine or gluatmate (C) together with vectors encoding EpoR-gp130 chimeric receptors. 24 hours after transfection, cells were transferred to poly-L-lysine-coated coverslips. The next day, cells were starved for 3 hours in KRH and transferred into the perfusion chamber of a laser-scanning microscope. After 20 minutes the cells were stimulated with Epo (7 U/ml). Gab1 localisation is shown before stimulation and after 20 minutes of Epo stimulation.

laboratory demonstrated that Gab1 phosphorylation by MAPK positively regulates HGF signalling but negatively influences EGF signalling (Yu et al., 2002; Yu et al., 2001). A detailed study on the individual MAPK target sites in Gab1 demonstrates that insulin-mediated PI3K activity and Gab1 binding is reduced in response to MAPK activation (Lehr et al., 2004). All these effects appear to be downstream of the membrane recruitment analysed in our study, because we could not observe a relevant function of the PI3K-binding site in Gab1 for IL-6-induced membrane targeting.

The data presented here are fully in line with our hypothesis that membrane recruitment of Gab1 is the initial step for a positive-feedback loop. Receptor-mediated ERK activation relieves the block of the PH domain in Gab1 and consequently induces the translocation of Gab1 from the cytoplasm to the plasma membrane. Here, Gab1 is phosphorylated by kinases found at the membrane and then recruits SHP2 and PI3K, leading to enhanced MAPK activity and to an increase in PtdIns(3,4,5)P_3 in the plasma membrane. Both activities would further boost cytokine-induced signalling.

This scenario would explain the requirement for Gab1 membrane recruitment in the case of weak EGF stimulation as discussed above (Sampaio et al., 2007), whereas under strong stimulation, no positive-feedback loop is required to transduce the signal.

In summary, we present data demonstrating that PH domains can be regulated by MAPK activity. The view that only the PtdIns(3,4,5)P_3 content in the membrane determines recruitment of PtdIns(3,4,5)P_3-binding PH domain-containing proteins appears to be too simple. The new regulatory mechanisms for the function of PH domains presented in our study should now also be considered. It remains an intriguing question as to whether PH domains in non adaptor proteins are regulated similarly.

Materials and Methods

Materials

Restriction enzymes were purchased from New England Biolabs (Ipswich, MA). Oligonucleotides were synthesised by MWG-Biotech (Ebersberg, Germany). Recombinant erythropoietin (Epo) was a generous gift from J. Burg and K. H. Sellinger, Roche (Mannheim, Germany). Antibodies specific for activated/phosphorylated STAT3, ERK, AKT and Gab1 were obtained from Cell Signaling Technology (Frankfurt/Main, Germany). Antibodies to STAT3, ERK1, ERK2 and AKT were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Gab1 antibodies were from Upstate Biotechnology (New York, NY) and antibodies against the Flag-Tag (M2) were from Sigma. The PI3K inhibitor LY294002 was from Calbiochem (Cambridge, MA) whereas the MEK-inhibitor U0126 was from Promega (Madison, WI). DMEM, DMEM/NUT-MIX-F12, OPTIMEM and antibiotics were from Gibco (Eggenstein, Germany). FCS was provided by Cytogen (Berlin, Germany).

Western blot

For the isolation of cellular proteins, confluent cell cultures were lysed in 300 µl RIPA-lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% NP-40, 15% glycerol, 1 mM NaF and 1 mM Na_3VO_4) supplemented with 10 µg/ml of each aprotinin, pepstatin and leupeptin as well as 0.8 µM Pefabloc. Proteins were separated by SDS-PAGE and transferred to a poly(vinylidene difluoride) (PVDF) membrane (PALL, Dreieich, Germany). Antigens were detected by incubation with specific primary antibodies (1:1000) and horseradish-peroxidase (HRP)-coupled secondary antibodies (1:10,000) (DAKO, Hamburg, Germany). The membranes were developed with an Immobilon Western Chemiluminescent HRP Substrate (Millipore Corporation, Billerica, MA).

Cell culture

HEK293 and HEK293T cells were from DSMZ (Braunschweig, Germany). These cells as were cultivated in DMEM supplemented with 10% FCS, 60 mg/ml penicillin, 100 mg/ml streptomycin at 37°C in a water-saturated atmosphere containing 5% CO_2.

Transfection

HEK293 cells and HEK293T cells were transiently transfected with 0.5-2 µg DNA using Lipofectamine 2000 transfection reagent (Life Technologies (Karlsruhe, Germany) as outlined in the manufacturer's instructions. Transfections were adjusted with control vectors to equal amounts of DNA.

Elk transactivation assay

MAPK activity was monitored by screening for phosphorylated ERK1/2 by western blotting or by Gal4/Elk-reporter analyses. Experiments were performed as described using Stratagene's instructions for the PathDetect Elk1 *trans* Reporting System (Stratagene Europe, Amsterdam). Briefly, a luciferase reporter pFR-Luc containing a Gal4-promoter element was cotransfected with an expression vector for a fusion protein composed of the DNA-binding domain of Gal4 and the transactivation domain of the Elk1 transcription factor (pFA2-Elk1). Transfection efficiency was monitored by cotransfected β-galactosidase expression vector as described below. Activated MAPKs lead to phosphorylation of the Elk transactivation domain and thus to the expression of luciferase.

Cell lysis and luciferase assays were carried out using the luciferase kit (Promega, Madison, WI) as described by the manufacturer. All transient expression experiments were done at least in triplicate. Luciferase activity values were normalised to transfection efficiency monitored by the cotransfected β-galactosidase expression vector (pCR3lacZ; Pharmacia, Uppsala, Sweden) (1 µg) as described in the manufacturer's instructions.

Expression vector

Expression vectors for EpoR-gp130 chimeric receptors [pRcCMV-EG(YYYYYY), pRcCMV-EG(Y**F**YYYY), pRcCMV-EG(F**Y**FFFF)] were previously described (Anhuf et al., 2000) and encode EpoR-gp130 chimeric receptors where none (YYYYYY), the SHP2/SOCS3 binding Tyr759 (YFYYYY), or all except Tyr759 (FYFFFF) were exchanged with phenylalanine. pBAT-Gab1 wt encodes Gab1 wild-type protein and was described by Weidner et al. (Weidner et al., 1996). pBAT-Gab1ΔSHP2 encodes a Gab1 mutant where Y627 has been replaced by F and therefore does not bind SHP2 (Schaeper et al., 2000). The expression vectors for all GFP-fusion Gab1-fusion proteins were derived from the pd2eGFP-N1 expression vector (Clontech, Mountain View, CA) and correspond to the mutants provided, described and characterised in detail (Schaeper et al., 2000). Briefly, pd2eGFP-N1-Gab1 encodes wild-type Gab1 fused to eGFP. pd2eGFP-N1-Gab1-ΔSHP2 encodes a Gab1 mutant where Tyr627 has been replaced by Phe and therefore does not bind SHP2. The Gab1 mutant which does not bind Grb2 contains a deletion of residues 341-348 and an exchange of Leu524 to Pro and is encoded by pd2eGFP-N1-Gab1-ΔGrb2. pd2eGFP-N1-Gab1-ΔPI3K encodes a Gab1 mutant which does not interact with PI3K because of an exchange of Tyr448, Tyr473 and Tyr580 to Phe. The Gab mutant lacking the PH domain lacks the N-terminal 116 residues of Gab1 and is encoded by pd2eGFP-N1-Gab1-ΔPH. peGFP-N1-Gab1-PH encodes the 116 N-terminal amino acids of Gab1. All deletion mutants depicted in Fig. 5A as well as the Ser to Ala or Ser to Glu Gab1 mutants were cloned by PCR mutagenesis based on pd2eGFP-N1-Gab1. All Gab1 constructs used in this study contain an N-terminal Flag tag. The sequences of all constructs were verified by DNA sequencing. The individual Gab1-constructs are depicted in Fig. 5A.

pcDNA3-p110γCAAX was kindly provided by I. Rubio and described elsewhere (Hehl et al., 2001). pfcMEK1 and pfcMEKK1 for the expression of constitutive MEK1 and MEKK1 were purchased from Stratagene (Waldbronn, Germany).

Live cell imaging

24 hours after transfection, cells were seeded in 6 cm plates on 42 mm poly-L-lysine-coated coverslips and cultured for additional 24 hours. Subsequently, coverslips were transferred into a closed perfusion chamber (Pecon, Erbach, Germany). Live cell imaging was performed with a confocal laser-scanning microscope (Zeiss LSM510, Zeiss, Jena, Germany). The temperature of the chamber and of the objective of the microscope was adjusted to 37°C. The medium in the chamber was Krebs-Ringer-HEPES (KRH) containing 130 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5.5 mM glucose, 10 mM HEPES pH 7.4. Before stimulation, the cells in the chamber were left untreated for 10 minutes. After stimulation, one picture per minute was taken for the following 20 minutes. The GFP-fusion proteins were excited using laser light of 488 nm. Emission was detected using a 503-530 nm filter.

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