Signaling behind bars: a role for bar domains

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THE F-BAR PROTEIN PACSIN2
REGULATES GROWTH FACTOR
RECEPTOR ACTIVATION
AND SIGNALING

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Submitted
ABSTRACT

Signaling via growth factor receptors, including the EGF receptor, is key to various cellular processes, such as proliferation, cell survival, and cell migration. In a variety of human diseases, such as cancer, aberrant expression and activation of growth factor receptors can lead to disturbed signaling. Intracellular trafficking is crucial for proper signaling of growth factor receptors. As a result, cell surface levels of growth factor receptors are an important determinant for the outcome of downstream signaling.

BAR-domain-containing proteins represent an important family of proteins that regulate membrane dynamics. In this study we identify a novel role for the F-BAR protein PACSIN2 in the regulation of growth factor receptor signaling. We show that internalized EGF as well as the (activated) EGF receptor are translocated to PACSIN2-positive endosomes. Furthermore, loss of PACSIN2 increased plasma membrane expression of the EGF receptor in resting cells and increased phosphorylation of the EGF receptor, as well as of Erk and Akt, in response to EGF compared to control cells. As a consequence, EGF-mediated cell proliferation was enhanced in PACSIN2-depleted cells.

In conclusion, this study identifies a novel role for the F-BAR-domain protein PACSIN2 in regulating growth factor receptor surface levels and downstream signaling.

INTRODUCTION

Signaling via receptor tyrosine kinases (RTKs) is essential for many cellular processes, such as proliferation, differentiation, cell survival, and cell migration (Blume-Jensen and Hunter, 2001; van der Geer et al., 1994). Aberrant expression and activation of RTKs is causally related to human diseases such as cancer, inflammation, and angiogenesis. One of the best studied members of RTKs is the Epidermal Growth Factor Receptor (EGF receptor, also known as ErbB1). The EGF receptor is ubiquitously expressed and increased expression of the receptor is often observed in cancer (Bubil and Yarden, 2007). Inactive EGF receptor resides on the cell surface. Binding of Epidermal Growth Factor (EGF) to the extracellular amino-terminal domain of the EGF receptor leads to receptor autophosphorylation and activation and subsequently to the activation of several downstream signaling pathways, such as Erk and Akt signaling (Gan et al., 2010).

Proper signaling of the EGF receptor, followed by its downregulation, depends on correct intracellular trafficking and localization to appropriate intracellular structures. The level of EGF receptor on the cell surface is an important determinant for the outcome of downstream signaling. In resting cells, approximately 2% of the EGF receptor is constitutively internalized in the absence of ligand and most of the internalized receptor recycles back to the plasma membrane (Herbst et al., 1994). Ligand binding induces rapid internalization of the EGF receptor. This ensures efficient termination of signaling by targeting the receptor for lysosomal degradation or recycling. Upon internalization of the EGF receptor, signaling can continue from early endosomes as well. This has been shown to be important for certain signaling pathways, including the Erk pathway, as inhibition of endocytosis was found to impair pathway activation (Sigismund et al., 2008; Vieira et al., 1996). Depending on the dose of EGF, the EGF receptor can be internalized via clathrin-mediated endocytosis, which has been implicated in recycling of the receptor, and clathrin-independent endocytosis, which has been linked to receptor degradation (Sigismund et al., 2005; Sigismund et al., 2008). It is now widely accepted that internalization and trafficking is an important mode to control EGF receptor signaling (Sorkin and Goh, 2008; Wiley, 2003).

BAR-domain-containing proteins represent an important family of proteins that regulate membrane dynamics. Via their conserved BAR domain, these proteins bind to, stabilize and induce membrane curvature. As a consequence, BAR domains induce invaginations of the plasma membrane and, subsequently, vesicular-tubular structures that are involved in membrane dynamics including receptor internalization (Frost et al., 2009; Tsuji et al., 2006). Several of these BAR-proteins have been implicated in controlling EGF receptor signaling. Cbl can form a complex with the adapter protein CIN85 and the BAR-protein endophilin that initiates EGF receptor internalization thereby controlling receptor signaling and downregulation. Preventing this complex formation inhibits EGF receptor internalization and delays receptor degradation resulting in increased signaling (Soubeyran et al., 2002). Furthermore, the F-BAR (Fer-CIP4 homology-BAR; a subclass of the BAR-domain family) protein CIP4 regulates
late events in EGF receptor trafficking from endosomes towards lysosomes which results in receptor degradation. Lack of CIP4 accumulated the EGF receptor on early endosomes with prolonged signaling as a result (Hu et al., 2009).

Here we describe an additional F-BAR family member, PACSIN2, that controls growth factor signaling. PACSIN2 associates to several proteins such as Rac1, Dynamin, N-WASP, and Synaptotagmin via its C-terminal Src Homology 3 (SH3) domain (Chitu and Stanley, 2007; de Kreuk et al., 2011; Kessels and Qualmann, 2004). The PACSIN2 F-BAR domain, located in its N-terminal region, mediates membrane binding and is involved in homo- and hetero-oligomerization (Kessels and Qualmann, 2006). Due to this oligomerization, PACSIN2 can associate to multiple proteins at once, linking the actin-regulatory network with the endocytic machinery (Kessels and Qualmann, 2004).

The current study shows that PACSIN2 is a negative regulator of growth factor receptor activation and signaling. Initially we found that EGF is internalized to PACSIN2-positive vesicles and we could visualize accumulation of both total and activated EGF receptor on PACSIN2-positive endosomes upon EGF stimulation. We show that in PACSIN2 knock-down cells, as well as in cells expressing an SH3- or BAR-domain mutant of PACSIN2 (inhibiting protein-interactions and internalization respectively), EGF receptor surface levels were increased. Furthermore, EGF-mediated activation and phosphorylation of the EGF receptor as well as of its downstream targets, Erk and Akt, is enhanced in PACSIN2 knock-down cells. As a result, loss of PACSIN2 enhanced EGF-induced cell growth. Finally, we show that these effects are not specific for EGF because signaling by HGF, and in primary endothelial cells also by VEGF and TNFα, is enhanced in PACSIN2 knock-down cells. To establish whether receptor activation results in translocation of the receptor to PACSIN2-positive endosomes, we stained unstimulated or EGF-TR stimulated HeLa cells for endogenous PACSIN2. In addition, we visualized tyrosine-phosphorylated proteins using an antibody against phosphotyrosine (pTyr). Stimulation of cells with EGF-TR caused a distinct accumulation of tyrosine-phosphorylated proteins on PACSIN2-positive early endosomes (Supplementary Fig. S1). This suggests that the activated EGF receptor is targeted to PACSIN2-positive endosomes. To confirm this, we immunostained control or EGF-stimulated HeLa cells for the EGF receptor as well as specifically for the activated EGF receptor using a phospho-EGFR-specific (Tyr1068) antibody (Rush et al., 2012). We could not immunostain simultaneously for the endogenous EGF receptor and endogenous PACSIN2, since the antibodies for detection were from the same species. Because PACSIN2 colocalizes with Early Endosomal Antigen-1 (EEA1) on early endosomes (Fig. 2C; de Kreuk et al., 2011), we used EEA1 as an intermediate to analyze colocalization of the (activated) EGF receptor with PACSIN2. EGF stimulation induces accumulation of the (activated) EGF receptor on early endosomes (Fig. 2, A and B) where also PACSIN2 is localized (Fig. 2C). This data is in good agreement with what we showed for internalized EGF-TR (Fig. 1C).

Together, these data show that both internalized EGF-TR and the activated EGF receptor accumulate on PACSIN2-positive early endosomes upon EGF stimulation.

RESULTS

The activated EGF receptor and internalized EGF localize to PACSIN2-positive early endosomes

In initial live-cell imaging experiments we found that a YFP-PACSIN2 fusion protein shows extensive internalization upon stimulation of the cells with EGF. Prior to stimulation, a fraction of PACSIN2 is constitutively internalized on vesicular-tubular structures that arise from membrane ruffles (de Kreuk et al., 2011). EGF stimulation induces PACSIN2 to accumulate on peripheral structures, both small tubules as well as vesicles, with a concomitant reduction of PACSIN2 localization at the peripheral membrane (Fig. 1A, Supplementary Movie 1). To analyze in more detail the effects of EGF on PACSIN2 distribution, we documented endogenous PACSIN2 localization by confocal microscopy. Similar to the data in Fig. 1A, PACSIN2 is in resting cells partially localized on vesiculo-tubular structures in the cell periphery as well as on perinuclear vesicles which we previously identified as early endosomes (de Kreuk et al., 2011) (Fig. 1B; upper panels). Upon EGF stimulation, PACSIN2-positive peri-nuclear vesicles become slightly enlarged (Fig. 1B; bottom panels and zoomed images). To test whether EGF localizes to the PACSIN2-positive compartment we analyzed the distribution of internalized Texas Red-labelled EGF (EGF-TR) by confocal microscopy. Five minutes after its addition, a fraction of internalized EGF-TR localized to PACSIN2-positive endosomes and this fraction was significantly increased (from 34 to 76%) after 10 or 15 minutes of EGF-TR internalization (Fig. 1C). At 30 minutes after addition, we could still find EGF-TR localizing to PACSIN2-positive endosomes, although less than in earlier time points suggesting that after 30 minutes, EGF-TR leaves the PACSIN2-compartment (Fig. 1C). This fits with the notion that internalized EGF traffics through the early endosomal compartment (where PACSIN2 is present) towards the recycling or late endosomal compartment (Haglund and Dikic, 2012; Sorkin and Goh, 2008).

As demonstrated above, both internalized EGF and the EGF receptor localize to a PACSIN2-positive endocytic compartment (Fig. 1 and 2). Since PACSIN2 is an important regulator of membrane dynamics, PACSIN2 may control endocytic traffic of the EGF receptor. We therefore questioned whether PACSIN2 can regulate surface levels of the EGF receptor. To study this, we performed surface biotinylation experiments (see Material and Methods). HeLa cells, treated with either siRNA or lentiviral shRNA constructs to reduce PACSIN2 expression, were incubated with sulfo-NHS-LC-biotin at 4°C to block internalization. Following streptavidin-based pull-downs of cell lysates we analyzed protein surface expression by Western blotting. To certify that...
only surface proteins were isolated, we stained our blots for RhogDI, known to be localized to the cytosol (Olofsson, 1999). As expected, RhogDI was not detected in the pull-down fraction (Fig. 3). Interestingly, EGF receptor surface expression showed a marked increase in HeLa cells treated with either PACSIN2-specific siRNA (Fig. 3A) or lentiviral shRNA directed against PACSIN2 (Fig. 3B). Surface levels of β1-integrin were comparable in PACSIN2 knock-down cells versus control cells (Fig. 3, A and B) indicating that the loss of PACSIN2 does not in general affect surface proteins. These experiments indicate that, in the absence of EGF, PACSIN2 controls the surface levels of the EGF receptor.

We next questioned whether PACSIN2 can regulate EGF-mediated internalization of the EGF receptor. HeLa cells, treated with control and PACSIN2 siRNA oligos, were incubated with EGF for the indicated time points. We then performed a surface biotinylation experiment as described above and analyzed EGF receptor surface expression by Western blotting. As expected, EGF stimulation downregulated EGF receptor surface levels in control cells (Fig. 4A; upper and middle panel). Although we observed higher EGF receptor surface levels in PACSIN2 knock-down cells upon EGF stimulation compared to control cells, still an EGF-mediated downregulation of the receptor was observed (Fig. 4A; upper and middle panel). This could be explained by the fact that in resting cells, EGF receptor surface levels are already higher in PACSIN2 knock-down cells compared to control cells. These experiments indicate that, although PACSIN2 knock-down cells consistently show higher EGF receptor surface expression, EGF-mediated internalization of the receptor is not impaired.

We then questioned what the effects were on EGF receptor surface expression of the ectopic expression of wild-type PACSIN2 (P2-WT). In addition, we also expressed an SH3-domain mutant of PACSIN2 (P2-Y435E/P478L), which is impaired in binding to several proteins regulating membrane and cytoskeletal dynamics such as Dynamin and N-WASP (Chitu and Stanley, 2007; Kessels and Qualmann, 2004). Finally, we also expressed a BAR-domain mutant (P2-R50D), that, because of an arginine to aspartic

Figure 1: EGF stimulation alters PACSIN2 distribution and is internalized to PACSIN2-positive endosomes. (A) Live-cell imaging of HeLa cells, transfected with YFP-PACSIN2, shows that PACSIN2 is associated with vesicular-tubular structures upon constitutive internalization in unstimulated cells. EGF (100 ng/ml) stimulation transiently accumulated internalized PACSIN2 in peripheral structures, both small tubules as well as vesicles. (See also supplementary movie 1). (B) Intracellular localization of endogenous PACSIN2 in response to EGF (5 minutes; 100 ng/ml) was studied by confocal microscopy using HeLa cells. In resting cells, PACSIN2 is localized at membrane ruffles, partially on vesicular-tubular structures, and on peri-nuclear vesicles. Upon EGF stimulation, a reduction of peripheral tubules was observed together with enlarged PACSIN2-positive peri-nuclear vesicles. (C) EGF-Texas-Red (100 ng/ml) transiently translocates upon internalization to PACSIN2-positive endosomes with a peak at 10-15 minutes. At 30 minutes, some EGF-Texas-Red can still be observed on PACSIN2-positive vesicles. Colocalization images show the amount of EGF-Texas-Red colocalized to PACSIN2 structures in white. Numbers depict mean colocalization ± SEM measured using Zen 2009 software. Scale bars, 10 μm. *** P<0.001 in comparison to 5 minute time point. # P<0.01 in comparison to 15 minute time point.
Figure 2: The activated EGF receptor accumulates on PACSIN2-positive early endosomes upon EGF stimulation. (A-C) Using confocal microscopy, colocalization of the EGF receptor (A), phosphorylated EGF receptor at Tyr1068 (B), and PACSIN2 (C) with Early Endosomal Antigen-1 (EEA1) was analyzed. Endogenous proteins were detected by immunostaining. EGF (5 minutes; 100 ng/ml) stimulation accumulates the (phosphorylated) EGF receptor on early endosomes (A-B) where PACSIN2 is also present (C). Colocalization plots show the amount of EGF receptor (A), phospho-EGF receptor (B), and PACSIN2 (C) localized to EEA1-positive endosomes in white. Bar diagrams show mean colocalization ± SEM. Scale bars, 10 μm. *** P<0.001.

Figure 3: Knock-down of PACSIN2 increases EGF receptor surface levels in resting cells. (A and B) Left panels: Surface biotinylation experiments, using HeLa cells, were performed to isolate all surface proteins. Endogenous PACSIN2, EGF receptor, and β1-integrin were detected by immunoblotting (IB). To certify that only surface proteins are isolated, RhoGDI, known to be localized to the cytosol, was detected by immunoblotting in conjunction with actin which, together with RhoGDI, served as a loading control for the PACSIN2 knock-down samples. EGF receptor surface expression showed a marked increase in HeLa cells treated with either PACSIN2-specific siRNA (A) or lentiviral shRNA directed against PACSIN2 (B). Surface levels of β1-integrin were comparable in PACSIN2 knock-down cells versus control cells. Right panels: Quantification of protein surface expression. Values are relative to control cells. Data are mean values ± SEM of three independent experiments. * P<0.05. TCL, Total Cell Lysate. PD, Pull-Down
Acid mutation at position 50, can no longer bind to membranes and induce vesicular-tubular structures required for proper PACSIN2-mediated membrane dynamics (Shimada et al., 2010; Wang et al., 2009). Compared to control cells, expression of wild-type PACSIN2 did not affect EGF receptor surface expression, neither in resting nor in EGF-stimulated cells (Fig. 4B; upper and middle panel). Interestingly, similar to the knock-down experiments, ectopic expression of both the SH3-domain and the BAR-domain mutants of PACSIN2, increased the EGF receptor surface levels in unstimulated cells. In addition, although higher surface levels of the EGF receptor were observed after EGF stimulation in cells expressing both PACSIN2 mutants, EGF still induced internalization of EGF receptor (Fig. 4B; upper and middle panel). Interestingly, we observed a slight increase in total EGF receptor levels as well (Fig. 4, A and B; bottom panels), suggesting that functional inhibition of PACSIN2 results in reduced receptor degradation. Moreover, we could (partially) prevent this in cells where proteosomal and lysosomal degradation was blocked using MG132 and Chloroquine respectively (data not shown).

Next, we analyzed internalization of the EGF receptor upon stimulation with EGF-TR by confocal microscopy. We allowed EGF-TR to internalize in HeLa cells, transfected with control or PACSIN2 siRNA, for several time points and analyzed colocalization of EGF-TR as well as the EGF receptor itself with early endosomes, visualized by EEA1 staining. After 15 minutes of internalization we did not find a statistically significant reduction in internalized EGF-TR and EGF receptor to early endosomes in PACSIN2 knock-down cells (Supplementary Fig. S2). In addition, after 30 or 90 minutes following addition of EGF-TR no major difference was observed either, with the exception of a small difference at t = 60 min, indicating that internalization of the EGF receptor upon stimulation with EGF-TR is not impaired in PACSIN2 knock-down cells (Supplementary Fig. S2).

Together, these data indicate that inhibiting PACSIN2 function, either by knockdown or by ectopic expression of dominant-negative mutants, resulted in increased EGF receptor expression at the plasma membrane. Under these conditions, ligand-induced loss of surface EGF receptor levels remain largely unaffected. Furthermore, internalization of EGF-TR is similar in control cells versus PACSIN2-depleted cells, suggesting that PACSIN2 is not required for ligand-induced EGF receptor internalization but may regulate ligand-independent receptor internalization, receptor degradation and/or recycling.

Knock-down of PACSIN2 increases EGF-mediated phosphorylation of the EGF receptor
To determine whether the increased surface levels of the EGF receptor upon loss of PACSIN2 affects cellular responses to EGF, we first examined EGF receptor phosphorylation in control or PACSIN2 knock-down cells. Initially, to visualize EGF receptor phosphorylation we used an antibody against tyrosine phosphorylated EGF receptor.
proteins (pTyr) and focused on the 175 kD activated EGF receptor band. siRNA-mediated knock-down of PACSIN2 clearly increased EGF-mediated phosphorylation of the 175 kD band (Fig. 5A). Subsequently, we visualized EGF receptor phosphorylation with an antibody specific for phosphorylation on Tyr1068. Similar to what we observed in Fig. 5A, siRNA-mediated knock-down of PACSIN2 increased EGF receptor phosphorylation on Tyr1068 in response to EGF (Fig. 5B). To exclude off-target effects of the PACSIN2 siRNA, we examined EGF receptor phosphorylation in HeLa cells treated with a lentiviral shRNA targeting PACSIN2. In line with the results in Fig. 5B, lentiviral knock-down of PACSIN2 results in increased phosphorylation of the EGF receptor upon EGF stimulation (Fig. 5C). Interestingly, the effect of loss of PACSIN2 on EGF receptor phosphorylation is apparent mainly in the first 20 minutes because at later time points, EGF receptor phosphorylation declined to control levels (Fig. 5). In contrast, HeLa cells that ectopically express PACSIN2 show a slight reduction in EGF receptor phosphorylation in response to EGF (data not shown). However, this effect is limited, compared to the effects seen in the siRNA experiments, indicating that levels of endogenous PACSIN2 in HeLa cells are sufficient to regulate EGF receptor activation and phosphorylation.

Together, these data show that PACSIN2 acts as a negative regulator of EGF receptor activation and phosphorylation. The marked increase in EGF-mediated receptor phosphorylation observed in PACSIN2 knock-down cells reveals an important, previously unnoticed, role for PACSIN2 in regulating EGF receptor activation.

PACSIN2 negatively regulates signaling downstream of the EGF receptor

EGF engagement leads to activation of the EGF receptor and of several downstream intracellular signaling pathways, such as the Erk and Akt pathways (Grant et al., 2002; Wells, 1999). As we observed a marked increase in EGF receptor phosphorylation in PACSIN2 knock-down cells, we decided to study whether loss of PACSIN2 affects signaling downstream of the EGF receptor as well. Therefore, we analyzed EGF-mediated Erk phosphorylation in HeLa cells transfected with the PACSIN2 siRNA. Interestingly, in resting cells, loss of PACSIN2 results in a slight decrease in the levels of phosphorylated Erk1/2. In contrast, upon EGF stimulation we observed an increase in Erk phosphorylation in PACSIN2 knock-down cells compared to control cells (Supplementary Fig. S3, A and B). In combination, these changes result in enhanced Erk1/2 phosphorylation in response to EGF in PACSIN2 knock-down cells (Supplementary Fig. S3B; bottom panel).

To study Erk phosphorylation in a more quantitative manner, we used a NanoPro Assay (see Materials and Methods). Here, levels of (un)-phosphorylated Erk are quantitatively measured by separation of proteins based on their pI, followed by immunodetection. As shown in Fig. 6A, for each Erk isoform, both the unphosphorylated and phosphorylated species, a peak is generated and the size of the peak (measured by the area under the peak) represents the (phospho) protein levels. Using this approach, it is possible to study in a quantitative manner, the amount of Erk phosphorylation for both isoforms separately but in the same samples. Fig. 6A (upper panel) shows that in resting cells, the majority of Erk is in its unphosphorylated state. However, upon EGF stimulation, a shift towards the phosphorylated state can be clearly observed (Fig. 6A; bottom panel). Using this technique, we analyzed the effects of loss of PACSIN2 on EGF-mediated Erk phosphorylation and activation.
In addition to Erk phosphorylation, we analyzed whether loss of PACSIN2 affects EGF-induced Akt phosphorylation as well. Western blot analysis shows that upon knock-down of PACSIN2, levels of phosphorylated Akt are slightly decreased. However, upon stimulation with EGF, Akt phosphorylation is increased in PACSIN2 knock-down cells compared to control cells (Supplementary Fig. S4, A and B) resulting in enhanced EGF-mediated Akt phosphorylation in PACSIN2 knock-down cells. These findings are similar to what we observed for Erk phosphorylation. Next we analyzed Akt phosphorylation using the NanoPro Assay. In contrast to Erk, the peak pattern for Akt phosphorylation comprises a series of different Akt phospho-species. Therefore, we determined Akt phosphorylation based on the combined signal generated upon detection with a phospho-specific Akt antibody. The NanoPro 1000 system is capable of generating a Western blot-like representation of the data (Supplementary Fig. S4C; left panel) which gives an indication of the level of Akt phosphorylation. Similar to our Western blotting results, it is clear that loss of PACSIN2 results in increased Akt phosphorylation in response to EGF (Supplementary Fig. S4C; right panel). These experiments show that PACSIN2 is an important regulator of EGF-mediated signaling as knock-down of PACSIN2 results in enhanced phosphorylation of Erk and Akt in response to EGF.

EGF receptor signaling is involved in various processes including cell growth. Therefore, we analyzed the role of PACSIN2 in EGF-mediated cell growth. HeLa cells, treated with control or PACSIN2-specific siRNA oligos, were stimulated with EGF. 24 hours after addition of EGF, cell numbers were determined in the different conditions. Loss of PACSIN2 has no effect on proliferation of cells that were not stimulated with EGF. However, EGF stimulation did lead to a significant increase in cell number in PACSIN2 knock-down cells compared to control cells (Fig. 6D).

Together, these data show that PACSIN2 is a key player in regulating the activation and downstream signaling of the EGF receptor. EGF-mediated activation and phosphorylation of the EGF receptor as well as of its downstream targets, Erk and Akt, is enhanced in PACSIN2 knock-down cells. As a result, loss of PACSIN2 enhanced EGF-induced cell growth.

PACSIN2 is a generic regulator of growth factor signaling

So far, we have shown that PACSIN2 is an important regulator of EGF receptor signaling. We questioned whether the regulatory role of PACSIN2 is specific for the EGF receptor or that PACSIN2 regulates growth factor receptor signaling in general. To study this we investigated whether PACSIN2 can regulate signaling downstream of the Hepatocyte Growth Factor (HGF) receptor, which is known to activate the MAPK pathway (Liu et al., 2002). HeLa cells, treated with control or PACSIN2-specific siRNA, were stimulated with HGF. Using the NanoPro Assay, we analyzed Erk phosphorylation and we observed, similar to what we have shown for EGF, enhanced phosphorylation of Erk in response to HGF (Fig. 7A). Next we questioned whether the role for PACSIN2 is cell-type specific. Therefore we analyzed Erk phosphorylation...
in primary human endothelial cells in response to VEGF and TNFα, both known to activate the MAPK pathway (Mechtcheriakova et al., 2001). Human Umbilical Vein Endothelial Cells (HUVEC), treated with control or PACSIN2-specific lentiviral shRNA constructs, were stimulated with either TNFα or VEGF. Subsequently, Erk phosphorylation was measured using the NanoPro Assay. Phosphorylation of Erk, induced by TNFα (Fig. 7B), showed a marked increase in cells transduced with the PACSIN2 shRNA, compared to control cells. Moreover, although less prominent, loss of PACSIN2 also increased Erk activation downstream of VEGF (Fig. 7C).

Collectively, these data show that PACSIN2 regulates growth factor signaling. Loss of PACSIN2 results in an increase in Erk activation downstream of both EGF and HGF in epithelial cells and of TNFα and VEGF in endothelial cells indicating that PACSIN2 plays a key role in regulating growth factor receptor output, regulating various growth factors in different types of cell.

**DISCUSSION**

BAR-domains are found in proteins that are important regulators of membrane dynamics and vesicular traffic. Several BAR-domain proteins have been implicated in regulating growth factor signaling. The BAR-domain protein endophilin regulates, in conjunction with Cbl and CIN85, EGF receptor internalization. Inhibition of this complex, e.g. via Alix/AIP1, impairs proper receptor endocytosis thereby preventing signal termination with increased signaling as a result (Schmidt et al., 2004; Soubeiran et al., 2002). These studies underscored the importance of receptor internalization in controlling signaling output. In addition to internalization, receptor sorting towards the lysosomal pathway, with receptor degradation as a consequence, represents an alternative mechanism to terminate growth factor signaling. Several BAR-domain proteins have been shown to regulate the sorting of the EGF receptor. Sorting Nexins (SNXs), of which several comprise a BAR domain, have emerged as a group of proteins, associated with endosomal compartments, that regulate receptor trafficking (van Weering et al., 2010; Worby and Dixon, 2002). SNX1 and SNX5 associate with each other via their BAR domain. Interestingly, whereas SNX5 prevents EGF receptor degradation, SNX1 promotes degradation of the EGF receptor and attenuates the effect of SNX5 (Kurten et al., 1996; Liu et al., 2006).

To avoid aberrant growth factor signaling, with severe diseases as potential outcome, efficient signal termination is of great importance. Upon growth factor receptor activation, the receptor is internalized and subsequent post-endocytic traffic targets the receptor either for degradation or for recycling back to the plasma membrane. The increased surface EGF receptor levels in unstimulated, PACSIN2 knock-down cells could be due to decreased internalization or increased receptor recycling. Although we could not find clear evidence that PACSIN2 regulates ligand-dependent internalization of the EGF receptor, because EGF stimulation still reduced surface EGF receptor levels, it could well be that PACSIN2 is involved in ligand-independent internalization of the EGF receptor. Ligand-independent, constitutive receptor internalization is not well understood, as most studies focused on ligand-induced downregulation of growth factor receptors. However, it was shown that expression of dominant active Rab5 (Q79L) caused ligand-independent internalization of the EGF receptor thereby decreasing surface EGF receptor levels. As a result, upon EGF stimulation, less receptor activation and downstream signaling was observed (Dinneen and Ceresa, 2004). Interestingly, we have previously shown that PACSIN2 is localized to Rab5-positive endosomes (de Kreuk et al., 2011). Since Rab5Q79L accumulates PACSIN2 on these endosomes (unpublished data) it could well be that PACSIN2 functions in the same pathway regulating ligand-independent internalization of the EGF receptor. In accordance, we show that loss of PACSIN2 results in increased EGF receptor surface levels and subsequently increased EGF-mediated signaling.

![Figure 7: PACSIN2 is a generic regulator of growth factor signaling.](image-url)

(A-C). Using the NanoPro 1000 Assay, Erk phosphorylation was measured downstream of HGF in HeLa cells, and downstream of TNFα and VEGF in HUVECs. Upper and middle panels: Knock-down of PACSIN2 enhanced Erk1 (upper panels) and Erk2 (middle panels) phosphorylation downstream of hHGF (A), TNFα (B), and VEGF (C) compared to control cells. Bottom panels: Immunoblotting (IB) was performed to assess siRNA mediated knock-down of PACSIN2 in HeLa cells (A) and shRNA-mediated knock-down of PACSIN2 in HUVECs (B and C). Graphs are representative for three independent experiments. Data are normalized to basal phospho-Erk levels. Error bars indicate SEM.
Recent studies have revealed distinct internalization pathways depending on EGF concentrations. High (100 ng/ml) concentrations of EGF lead to clathrin-independent/lipid raft-dependent internalization of the EGF receptor while low (1 ng/ml) EGF concentrations lead to clathrin dependent internalization (Sigismund et al., 2005). Interestingly, some studies showed that this raft-mediated internalization is associated with EGF receptor degradation while clathrin-mediated internalization is associated with sustained signaling (Sigismund et al., 2008). We previously found evidence that PACSIN2 could function in raft-mediated endocytosis as we found Cholera Toxin B to be internalized via PACSIN2-positive tubular structures (de Kreuk et al., 2011) so it could well be that depletion of PACSIN2 shifts internalization of the EGF receptor to a clathrin-dependent pathway with increased signaling as a result. In line with this, we found no effect of PACSIN2 depletion on EGF receptor internalization when using low EGF concentrations (unpublished data). However, this topic requires further investigation as some studies have implicated PACSIN proteins in clathrin-mediated internalization as well (Modregger et al., 2000; Qualmann and Kelly, 2000).

In addition to internalization, an alternative mechanism that controls receptor surface levels is recycling. Similar to the F-BAR protein CIP4 (Hu et al., 2009), PACSIN2 could regulate receptor surface expression by regulating receptor sorting. Upon ligand-(in)dependent internalization, part of the receptor is targeted for degradation while the remainder recycles back to the plasma membrane (Sorkin and Goh, 2008; Wiley, 2003). Receptors destined for degradation travel via early endosomes to multivesicular endosomes/bodies (MVBs) and are eventually degraded in lysosomes. The ESCRT protein Tsg101 is involved in the formation of these MVBs (Razi and Futter, 2006). Upon depletion of Tsg101, EGF receptor degradation was impaired and consequently increased EGF-mediated receptor phosphorylation was observed (Rush et al., 2006). Similarly, preventing EGF receptor degradation by the pharmacological inhibitor monensin, which blocks acidification of early endosomes and thereby formation of lysosomes (King, 1984), resulted in accumulation of the EGF receptor on early endosomes and enhanced receptor phosphorylation (Rush et al., 2012). In line with these studies, we observe less degradation and increased MAP kinase activation when PACSIN2 is depleted. However, in these published studies, the EGF receptor is retained on early endosomes causing increased signaling. In contrast, we did not find clear evidence that PACSIN2 depletion retains the EGF receptor on early endosomes. Thus, both inhibition of ligand-independent internalization and a shift towards recycling would result in less receptor degradation and an increase in surface expression, which is what we observed in PACSIN2 knock-down cells or cells expressing dominant-negative PACSIN2 mutants.

An interesting additional finding of this study was that the regulatory role of PACSIN2 is not specific for the EGF receptor. Depletion of PACSIN2 enhanced Erk activation downstream of EGF and HGF in epithelial cells but also downstream of VEGF and TNFα in endothelial cells indicating a generic role for PACSIN2 in growth factor receptor signaling.

In conclusion, our data suggest the following model (Fig. 8). In control cells, constitutive internalization of the growth factor receptor takes place (a.). Subsequently, the receptor gets partially degraded (b.) but the main fraction recycles back to the plasma membrane (c.). When PACSIN2 is depleted, growth factor receptor at the surface accumulates, consequent to either inhibition of ligand-independent internalization (1.) or increased receptor recycling (2.), or both. As a result of increased surface receptor levels in PACSIN2-depleted cells, growth factor stimulation increases activation of the receptor and downstream signaling, possibly also from sustained endosomal signaling. In summary, this study identifies a novel regulatory role for the F-BAR-domain protein PACSIN2 in regulating growth factor receptor surface levels and signaling output.

Figure 8: PACSIN2 regulates growth factor receptor surface expression thereby controlling receptor output. In control cells, constitutive internalization (a.) of the growth factor receptor occurs. Upon internalization, a fraction is targeted for degradation (b.) while the remainder recycles back to the plasma membrane (c.). In PACSIN2-depleted cells, increased growth factor receptor surface levels are observed either by inhibiting ligand-independent endocytosis (1.) or by increased receptor recycling (2.). As a result of increased surface receptor levels (and possibly sustained endosomal signaling), in cells depleted for PACSIN2, growth factor stimulation enhanced receptor activation and downstream signaling (indicated by the blue arrows).
MATERIALS AND METHODS
Antibodies, Reagents, and Expression constructs
Antibodies: Anti-PACSIN2 (AP8088b) was from Abgent. Anti-Actin (A3853) was from Sigma. Anti-EEA1 (610457) was from BD Transduction Laboratories. Anti-pYtrp (pY120; 03-7720), anti-Transferrin Receptor (13-6800), and anti-c-myc (13-2500) were from Invitrogen. Anti-β1-integrin (610468) and anti-RhoGD1 (610255) were from BD Transduction Laboratories. Anti-EGFR (4267), anti-pEGFR-Y1068 (3777), anti-Akt (9272), and anti-p-Akt 5473 (37875) were from Cell Signaling. For Western blot, anti-Erk (SC-153) and anti-p-Erk (SC-7383) were from Santa Cruz. For the NanoPro assays, anti-Erk1/2 (06-162) was from Millipore and anti-p-Erk (9101) was from Cell Signaling. For detection in NanoPro, secondary HRP-labelled antibodies were from ProteinSimple. Secondary HRP-labelled antibodies for Western blot were from Pierce. Secondary Alexa-labelled antibodies for immunofluorescence were from Invitrogen. F-A CT was detected using Texas-Red- or Alexa-633-labelled Phalloidin (Invitrogen). Nuclei were stained with Hoechst (H-3569; Invitrogen).

Reagents: Recombinant Human Epidermal Growth Factor (hEGF; Cyt-217) and recombinant human Hepatocyte Growth Factor (hHGF; Cyt-244) were obtained from Prospec and used at a concentration of 100 ng/ml for the indicated time points. EGF-Texas Red was obtained from Molecular Probes (E-3480) and used at a concentration of 100 ng/ml for the indicated time periods. Recombinant human Vascular Endothelial Growth Factor (hVEGF) was obtained from R&D Systems (293-VE). Recombinant human Tumor Necrosis Factor-α (hTNF-α) was obtained from Peprotech (300-01A).

Expression constructs: pEYFP-PACSIN WT and myc-tagged PACSIN2 R50D were described previously (Ste Keuk et al., 2011). Myc-tagged PACSIN2 WT and myc-tagged PACSIN2 Y435E;P478L were a kind gift from Markus Plomann (University of Cologne, Germany).

Cell culture and transfections
HeLa cells are maintained in a humidified atmosphere at 37°C and 5% CO2 in Iscove’s Modified Dulbecco’s Medium (IMDM, Biowhittaker) supplemented with 10% heat-inactivated Fetal Calf Serum (Life Technologies, Breda, The Netherlands), 300 μg/ml glutamine, 100 units/ml penicillin and streptomycin. Primary Human Umbilical Vein Endothelial Cells (HUVEC) were purchased from Lonza (Baltimore M.D) and cultured in EGM2 medium, containing singlequots (Lonza Baltimore M.D). For ectopic expression, HeLa cells were transiently transfected with TransIT (Mirus) according to the manufacturers’ recommendations.

Lentiviral shRNAi and siRNA silencing
Lentiviral shRNA constructs for PACSIN2 from the TRC/Sigma Mission library were obtained from Sigma-Aldrich (St. Louis, MI, USA). The SHC002 scrambled shRNA construct (Sigma-Aldrich) was used as a negative control. All shRNA constructs were in the pLKO.1 vector backbone. shRNA-expressing lentiviral particles were prepared using HEK293T cells and virus was transduced as described previously (Nethe et al., 2010).

The sequence for control siRNA was: 5’-CUAGCCGGAACCUUCUGAAtt-3’ (Eurogentec). PACSIN2 siRNA was described before (de Kreuk et al., 2011). Transfections of siRNA were performed with INTERFERin (Polyplus transfection) according to the manufacturers’ recommendations.

SDS-PAGE and Western blot analysis
Proteins were separated on SDS-PAGE gels and transferred onto nitrocellulose transfer membrane using the iBLOT Dry Blotting System (Invitrogen) according to the manufacturers’ recommendations. Following blocking in 5% low fat milk in TBST (Tris-Buffered Saline Tween-20) for 30 minutes, the blots were incubated with the primary antibody overnight at 4°C. Next, the blots were washed 5 times for 10 minutes in TBST and subsequently incubated with HRP-conjugated secondary antibodies (dilution 1:5000) in TBST for 1 hour at RT. Finally, blots were washed 3 times with TBST for 30 minutes each and subsequently developed by ECL (GE Healthcare, Hoevelaken, The Netherlands).

Confocal Laser Scanning Microscopy
Cells, seeded on fibronectin-coated glass coverslips, were transfected with the indicated plasmids or siRNA, and after 24-48 hours fixed by 3.7% formaldehyde (Merck) in PBS for 10 minutes followed by permeabilization with 0.5% Triton X-100 in PBS (5 minutes; RT). Coverslips were then incubated for 15 minutes with 2% BSA in PBS at 37°C to block aspecific binding. Immunostainings were performed at RT for 1 hour with the indicated antibodies. Fluorescent imaging was performed with a confocal laser scanning microscope (LSM510/Meta; Carl Zeiss Microlmaging, Inc.) using a 63X/NA 1.40 (Carl Zeiss Microlmaging, Inc.). Image acquisition was performed with Zen 2009 software (Carl Zeiss Microlmaging, Inc.). For live-cell imaging, cells, seeded on fibronectin-coated glass coverslips, were transfected with the indicated plasmids. After 24 hours, fluorescent imaging was performed. Colocalization was analyzed using the Zen 2009 software (Carl Zeiss Microlmaging, Inc.).

Surface Biotinylation Pull-Down assay
Surface protein labeling studies were performed as follows. 24 Hours after seeding, cells were transfected with siRNA as indicated. 48 Hours after siRNA transfection, resting cells or cells, treated with hEGF for the indicated time periods were washed three times with cold PBS supplemented with 0.5 mM MgCl2 and 1 mM CaCl2. Cells were then incubated with 0.5 mg/mL sulfo-NHS-LC-biotin (21335, Thermo Scientific) in PBS for 30 minutes at 4°C. After biotinylation all unbound biotin was removed by quenching with PBS containing 100 mM glycine for 15 minutes at 4°C and subsequently washed three times with cold PBS supplemented with 0.5 mM MgCl2 and 1 mM CaCl2. Cells were then lysed in NP-40 lysis buffer (50mM TRIS/HCl pH 7.5, 100mM NaCl,
10 mM MgCl₂, 10% glycerol and 1% NP40) supplemented with protease inhibitors (Complete mini EDTA, Roche, Almere, The Netherlands) and centrifuged at 20,000 x g for 10 minutes at 4°C. The supernatant, containing biotinylated surface proteins, was then incubated in the presence of streptavidin-coated beads (Sigma) at 4°C for 1 hour while rotating. Surface protein levels were assayed by Western blot analysis.

NanoPro Assay
Erk and Akt phosphorylation were measured by the NanoPro 1000 System (Protein Simple) according to the manufacturers’ recommendations. In short, 48 hours after transfection with the indicated siRNA oligos, cells were stimulated with hEGF (100 ng/ml) and subsequently lysed in Bicine/CHAPS lysis buffer (Protein Simple; 040-327) supplemented with 1x DMSO Inhibitor Mix (Protein Simple; 040-510) and 1x Aqueous Inhibitor Mix (Protein Simple; 040-482). Lysates were centrifuged at 20,000 x g for 10 minutes at 4°C. Supernatant was loaded in small capillaries (ProteinSimple) together with Ampholyte premix G2 (ProteinSimple; 040-973) and pI Standard Ladder 3 (ProteinSimple; 040-646). Isoelectric focusing of proteins was performed by applying 21000 µW for 40 minutes. After focusing, UV light was used to cross-link proteins to the inner capillary wall. After that, the capillary was washed and immunoprobed for the indicated proteins followed by washing to remove unbound antibodies. Finally Luminol and peroxide were added to generate chemiluminescence which was captured by a CCD camera. Results were analyzed by software (Compass; ProteinSimple).

Peaks, generated using a total anti-Erk antibody, representing phospho-Erk isoforms were validated with phospho-specific antibodies against Erk. Percentage phosphorylation of Erk, using a total anti-Erk antibody, was measured by calculating the phospho-peak area as a percentage of total phospho- and non-phospho-peak areas.

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REFERENCE LIST

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Supplementary Figure S1: EGF stimulation induces translocation of tyrosine phosphorylated proteins to PACSIN2-positive early endosomes. Control HeLa cells, or HeLa cells stimulated with EGF-Texas-red (100 ng/ml; 5 min) were analyzed by confocal microscopy. PACSIN2 and tyrosine phosphorylated proteins (pTyr) were detected by immunostaining. In resting cells, hardly any tyrosine phosphorylated proteins are localized to PACSIN2-positive early endosomes (upper panels). In contrast, EGF-Texas-Red induces accumulation of tyrosine phosphorylated proteins to these PACSIN2-positive vesicles (bottom panels). Colocalization plots show the amount of tyrosine phosphorylated proteins localized to PACSIN2-positive endosomes in white. Bar diagram shows mean colocalization ± SEM. *** P<0.001. Scale bars, 10 μm.
Supplementary Figure S2: Internalization of EGF-Texas-Red or EGF receptor is similar in control cells versus PACSIN2-depleted cells. (A and B) HeLa cells, treated with control or PACSIN2-specific siRNA, were stimulated with EGF-Texas-Red (100 ng/ml) for the indicated time points. Endogenous EEA1 and EGF receptor, in conjunction with EGF-Texas-Red, were visualized by immunostainings (Right panels). Colocalization of EGF-Texas-Red (A) or EGF receptor (B) with EEA1 was analyzed, using Zen 2009 software, as a measure for internalization (Left panels). In general, no significant differences were observed between control and PACSIN2-depleted cells. However, at 60 minutes of internalization, a small increase in early endosomal localization of EGF-Texas-Red was observed in PACSIN2-depleted cells. Bar diagram shows mean colocalization ± SEM. * P<0.05. Scale bars, 10 μm.

Supplementary Figure S3: PACSIN2 negatively regulates Erk phosphorylation downstream of EGF. (A) HeLa cells, treated with control or PACSIN2-specific siRNA, were stimulated with EGF (100 ng/ml) for the indicated time points. Endogenous PACSIN2, Erk1/2, and phospho-Erk1/2 were detected by immunoblotting (IB). PACSIN2 depletion enhances EGF-mediated Erk1/2 phosphorylation compared to control cells within the initial 20 minutes. After 30-60 minutes, phospho-Erk1/2 levels declined to control levels. (B) Upper panel: Phospho-Erk1/2 levels relative to total Erk for each time point are shown. Data are normalized to basal levels of phospho-Erk1/2 in control cells. Mean values of three independent experiments are shown. Error bars indicate SEM. Bottom panel: EGF-mediated induction of Erk1/2 phosphorylation in control versus PACSIN2-depleted cells is shown. Data are relative to total Erk1/2 levels and normalized to basal levels of phospho-Erk1/2. Mean values of three independent experiments. Error bars indicate SEM.
SUPPLEMENTARY MOVIES

Supplementary Movie 1: EGF stimulation induces internalization of YFP-PACSIN2. HeLa cells were transfected with YFP-PACSIN2 WT. After 2 minutes EGF was added (100 ng/ml). Live-imaging analysis was performed with a laser-scanning confocal microscope using a 63X/NA 1.40 oil lens (LSM510/Meta, Carl Zeiss Microimaging, Inc.). Frames were taken every 8 seconds for 20 minutes. Video runs at 7 frames/sec. Still images are in Fig. 1A.

**Supplementary Figure S4: PACSIN2 negatively regulates Akt phosphorylation downstream of EGF.** (A) HeLa cells, treated with control or PACSIN2-specific siRNA, were stimulated with EGF (100 ng/ml) for the indicated time points. Endogenous PACSIN2, Akt, and phospho-Akt (S473) were detected by immunoblotting (IB). Knock-down of PACSIN2 increased Akt phosphorylation downstream of EGF compared to control cells within the initial 20 minutes. After 30-60 minutes, phospho-Akt levels declined to control levels. (B) Left panel: Phospho-Akt (S473) levels relative to total Akt for each time point are shown. Data are normalized to basal levels of phospho-Akt in control cells. Mean values of two independent experiments are shown. Right panel: EGF-mediated induction of Akt phosphorylation in control versus PACSIN2-depleted cells is shown. Data are relative to total Akt levels and normalized to basal levels of phospho-Akt (S473). Mean values of two independent experiments are shown. (C) Akt phosphorylation measured by NanoPro Assay. Left panel: Control or PACSIN2 siRNA treated HeLa cells were analyzed in triplicate for Akt phosphorylation using the NanoPro Assay. Peaks generated are transformed in a Western blot-like representation of the data (Compass Software; ProteinSimple). All peaks are represented as bands and band intensity indicates phospho-Akt protein levels. PACSIN2 depletion decreased basal phospho-Akt levels. Upon EGF stimulation, enhanced Akt phosphorylation is observed in PACSIN2-depleted cells compared to control cells. Right panel: Quantification of Akt phosphorylation shows increased phospho-Akt levels in PACSIN2-depleted cells in response to EGF compared to control cells. Bar diagram shows levels of phospho-Akt normalized to levels in unstimulated cells. Mean values of Akt phosphorylation are shown. Error bars indicate SEM. *** P<0.001.