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The SH2 domain protein Shep1 regulates the *in vivo* signaling function of the scaffolding protein Cas

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Abstract

The members of the p130Cas (Cas) family are important scaffolding proteins that orchestrate cell adhesion, migration and invasiveness downstream of integrin adhesion receptors and receptor tyrosine kinases by recruiting enzymes and structural molecules. Shep1, BCAR3/AND-34 and NSP1 define a recently identified family of SH2 domain-containing proteins that constitutively bind Cas proteins through a Cdc25-type nucleotide exchange factor-like domain. To gain insight into the functional interplay between Shep1 and Cas *in vivo*, we have inactivated the Shep1 gene in the mouse through Cre-mediated deletion of the exon encoding the SH2 domain. Analysis of Cas tyrosine phosphorylation in the brains of newborn mice, where Shep1 is highly expressed, revealed a strong decrease in Cas substrate domain phosphorylation in knockout compared to wild-type brains. Src family kinases bind to Cas via their SH3 and SH2 domains, which contributes to their activation, and phosphorylate multiple tyrosines in Cas substrate domain. These tyrosine phosphorylated motifs represent docking sites for the Crk adaptor, linking Cas to the downstream Rac1 and Rap1 GTPases to regulate cell adhesion and actin cytoskeleton organization. Accordingly, we detected lower Cas-Crk association and lower phosphorylation of the Src activation loop in Shep1 knockout brains compared to wild-type. Conversely, Shep1 transfection in COS cells increases Cas tyrosine phosphorylation. The SH2 domain is likely critical for the effects of Shep1 on Cas and Src signaling because the knockout mice express Shep1 fragments that lack the amino-terminal region including the SH2 domain, presumably due to aberrant translation from internal ATG codons. These fragments retain the ability to increase Cas levels in transfected cells, similar to full-length Shep1. However,

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Conflict of interest

The authors declare that they have no conflicts of interest.

Authors' contributions

SR helped with the initial characterization of Shep1 knockout mice, including expression of Shep1ΔN as well as analysis of lung and heart tissue, and discovered the *in vivo* defects of Cas and Src phosphorylation and Cas-Crk interaction in the knockout mice. YW confirmed the *in vivo* phosphorylation defects, performed the experiments in Fig. 5 and performed the analysis of feeding in newborn pups with LW. LW also performed all the histochemical analyses of embryos. VV helped with the initial characterization of Shep1 knockout mice and the RT-PCR experiments in Fig. 2C. EPB conceived the project, helped with experimental design and data interpretation, and wrote the manuscript with help from YW. All authors approved the final manuscript.

they do not affect Cas phosphorylation on their own or in the presence of co-transfected full-length Shep1. They also do not show dominant negative effects on the activity of full-length Shep1 *in vivo* because the heterozygous mice, which express the fragments, have a normal life span. This is in contrast to the homozygous knockout mice, most of which die soon after birth. These data demonstrate that Shep1 plays a critical role in the *in vivo* regulation of Src activity and Cas downstream signaling through Crk, and suggest that the SH2 domain of Shep1 is critical for these effects.

Keywords

phosphorylation; Src family kinases; integrin signaling; knockout mice

1. Introduction

The scaffolding proteins of the Cas family, including p130Cas/BCAR1, CasL/Hef1/NEDD9, EFS/Sin and HEPL/CASS4, orchestrate multiple signaling pathways that control cell adhesion, migration and invasiveness as well as cell growth and survival [1–4]. Although they lack enzymatic activity, Cas proteins play a central role in integrin signaling pathways by serving as a scaffolds for multiple protein partners that relay downstream signals in response to cell-substrate adhesion and also growth factor stimulation. Cas family proteins bind cytoplasmic tyrosine kinases, such as the focal adhesion kinase FAK and Src family kinases, and tyrosine phosphorylation is critical to regulate their scaffolding function. Motifs phosphorylated by Src kinases in the Cas substrate domain generate multiple docking sites for the SH2 domain of the adaptor protein Crk, which links Cas to the C3G and DOCK180 exchange factors [3–6]. C3G activates the Ras family GTPases R-Ras and Rap1, thus promoting integrin-mediated adhesion. DOCK180 activates the Rho family GTPase Rac1, promoting the formation of membrane ruffles, cell migration and invasiveness. Cas proteins are also highly phosphorylated on serine/threonine residues, but the role of this phosphorylation in their function is poorly understood. The founding member of the family, p130Cas (Cas), is the most widely expressed and extensively studied.

Cas interactions with many signaling proteins that bind to its amino-terminal SH3 domain, central substrate domain and Src-binding motifs have been extensively studied [3,4]. Much less is known about a recently discovered family of three proteins that form a stable complex with Cas by binding to its extreme carboxy terminus. We identified a member of this family, Shep1 (SH2 domain-containing Eph receptor-binding protein 1; gene symbol, Sh2d3c), in a screen for Eph receptor-interacting molecules [7]. Others identified the same protein as a Cas/Hef1-associated adaptor protein (Chat) [8] and a novel SH2 domain-containing protein (Nsp3) [9]. We characterized several Shep1 isoforms, which differ in their amino terminal region [10]. These isoforms all contain an SH2 domain near the amino terminus, which can bind activated Eph and ErbB receptors [7,9,11]. The carboxy-terminal portion of Shep1 contains a guanine nucleotide exchange factor (GEF)-like domain, which can bind Ras family GTPases such as R-Ras, Rap1 and Rap2 but appears to lack enzymatic activity [7,12]. The SH2 and GEF-like domains, which are separated by a proline/serine-rich region, are conserved in the two other members of the family, BCAR3 (breast cancer antiestrogen resistance locus 3)/AND-34 and NSP1 (Novel SH2-containing protein-1; gene symbol, Sh2d3a) [9,13,14]. Shep1 additionally contains a carboxy-terminal PDZ domain-binding motif that is not present in the other two family members [15]. Interestingly, Shep1 family proteins have been shown to constitutively bind members of the Cas family through their GEF-like domain [8,9,14,16]. This suggests that the functions of Cas and Shep1 family proteins are closely intertwined.

Collectively, the members of the Shep1 family are widely expressed. High levels of Shep1 are found in the brain, blood vessels and the immune system [7–10]. BCAR3/AND-34 is expressed in epithelial cells and upregulated in aggressive breast cancer cells [13,17–20]. NSP1, which has only been identified as a human gene and appears to be absent in the mouse [10], is most abundant in epithelial and cancer cells [9,10,21]. Although their tissue expression patterns are somewhat different, members of the Shep1 family may be co-expressed in at least some cell types [10,22]. To investigate the physiological role of Shep1 and its interplay with Cas, we inactivated the Shep1 gene in the mouse. Analysis of newborn brain tissue lysates from the Shep1 knockout mice revealed decreased Cas phosphorylation and association with the adaptor protein Crk as well as decreased Src phosphorylation in the activation loop. These findings indicate that Shep1 is required for proper Cas function in the brain.

2. Materials and methods

2.1. Generation of Shep1 knockout mice

The *Shep1*^{flox} knockout mice were commissioned to the company Ozgene and were generated from targeted C57BL/6 ES cells and maintained on a C57BL/6 background. The *Shep1*^{flox} mice were crossed by Ozgene with mice expressing the Cre recombinase in the germline to generate a *Shep1* knockout line in which all known Shep1 isoforms [10] (http://uswest.ensembl.org/Mus_musculus/Gene/Summary?g=ENSMUSG00000059013) are inactivated in all tissues by Cre-mediated deletion of the floxed exon 7 encoding the SH2 domain.

2.2. Antibodies and plasmids

The pcDNA3-Shep1 plasmid, encoding the mouse Shep1 β isoform [10], has been described previously [16]. The pcDNA3-Shep1 Δ N plasmid encodes a mutant form of Shep1 that lacks the N-terminus, the SH2 domain, and part of the serine/proline-rich region. This expression construct contains a cDNA mimicking that encoding the Shep1 Δ N fragments in the knockout mice, in which the first ATG corresponds to that in exon 6 and is in frame after deletion of exon 7 (Fig. 2D). Thus, pcDNA3-Shep1 Δ N encodes a protein with the N-terminal sequence MPGTTDASLV followed by amino acids 223-702 of the mouse Shep1 β isoform and/or a protein translated starting from an ATG in exon 8 and containing amino acids 240-702 (Fig. 2D).

The anti-Shep1 N terminus rabbit polyclonal antibody was generated by immunizing rabbits with a GST fusion protein containing amino acids 1-172 of the mouse β isoform [10], affinity-purified on a column containing the antigen, and absorbed on a column containing a GST fusion protein of the SH2 domain of ShcB, which is closely related to that of SHEP1, in order to remove antibodies that may recognize epitopes conserved in different SH2 domains [7]. The anti-Shep1 C terminus rabbit polyclonal antibody was generated using as the antigen a peptide comprising the 11 C-terminal amino acids of Shep1 coupled to BSA with glutaraldehyde. The immune serum was affinity purified on a column obtained by coupling the peptide to Affi-Gel 10 (BioRad) or on a column containing a GST fusion protein of the Shep1 GEF-like domain and C terminus. The Shep1 N terminus and C terminus antibodies were used for immunoprecipitations (6–10 μ g) and immunoblotting (2 μ g/ml). The following primary antibodies were also used for immunoblotting and/or immunoprecipitations: antiphosphotyrosine (4G10, 1:1,000 dilution of a 1 mg/ml stock; Millipore); Cas (1:600 dilution of a 0.25 mg/ml stock for immunoblotting and 4 μ g for immunoprecipitations; BD Transduction laboratories); phosphoCas Y165 and phosphoCas Y410 (1:1,000; Cell Signaling); phosphoSrc Y416 (1:1,000; Cell Signaling) and phosphoSrc Y418 (1:1,000; Invitrogen). The following primary antibodies were used for immunohistochemistry: Ki67 (1:25; Abcam); BrdU (1:100; Invitrogen), β III tubulin (TuJ1 antibody; 1:5,000 dilution of 1 a

mg/ml stock; Covance), CD31 (1:50; Pharmingen). Concentrations of commercial antibody stocks are indicated when available.

2.3. Immunoprecipitations and immunoblotting

Brains and lungs from P0 pups were harvested and lysed with dounce tissue grinders (Kimble Kontes) in modified RIPA buffer (150 mM NaCl, 50 mM Tris, pH 7.5 with 1% Triton X-100, 0.5% Na deoxycholate, 0.1% SDS, 2 mM EDTA, 1 mM sodium orthovanadate, 1 mM sodium fluoride, and protease inhibitors). Homogenates were sonicated 2×10 sec and centrifuged at 16,000 g for 15 min at 4°C to remove insoluble material. COS cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and transiently transfected with the indicated plasmids using Lipofectamine 2000 (Invitrogen). Two days after transfection, the cells were lysed in RIPA buffer, sonicated 2×10 sec and centrifuged at 16,000 g for 15 min at 4°C. The resulting supernatants were analyzed for protein content using the BCA Protein Assay Kit (Thermo Scientific).

For immunoprecipitations, tissue or cell lysates were incubated at 4°C with antibody for 1–2 hours, followed by incubation with Gamma-bind beads (GE Health Care Health Sciences) for 1 hour. Beads were washed 4 times with 1 ml of RIPA buffer and immunocomplexes were eluted by boiling for 5 min in SDS-containing sample buffer. Extracts were separated by SDS-PAGE and transferred to a PVDF membranes (Millipore). For immunoblotting, membranes were incubated with primary antibodies overnight at 4°C or 2 hours at room temperature followed by a 1 hour incubation with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Millipore, α M-HRP or α R-HRP 1:5000 from Millipore) or protein A-HRP (BioRad). The membranes were developed with chemiluminescence reagents (GE Health Care Health Sciences).

2.4. Histology

Phenotyping necropsy and histological analysis of two newborn wild-type and Shep1 knockout pairs was carried out by the Comparative Pathology Laboratory at UC Davis (<http://www.vetmed.ucdavis.edu/ars/cpl.html>). The report indicated no detectable abnormalities.

Hearts and lungs from newborn mice (P0) were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were stained with a rabbit polyclonal Ki67 antibody (1:50; Abcam), which was detected with a Vectastain Elite ABC Kit (Vector Laboratories), and counterstained with hematoxylin. TUNEL assays were performed using ApopTag® peroxidase *in situ* apoptosis detection kit (Millipore). As a positive control for the TUNEL assays, lung and heart sections were treated for 10 min with DNase I according to the manufacturer's recommendations.

E13.5 and E14.5 embryos were fixed in 4% formaldehyde in PBS at 4 °C and embedded in paraffin for TUNEL and H&E staining, or frozen in OCT compound (Tissue-Tek) for immunolabeling. For BrdU labeling, pregnant females were injected with BrdU (Sigma, 0.05 mg per gram of body weight) 2 hours before sacrificing. Frozen sections of embryos were treated with acid (1 M HCl for 1 hour at 37°C) prior to labeling with anti-BrdU antibodies (1:200; Invitrogen). For CD31 staining, frozen sections were labeled with rat anti-CD31 antibody (1:50; BD Biosciences/Pharmingen). For β III tubulin staining, frozen sections were labeled with TuJ1 antibody (1:5,000; Covance).

2.5. RT-PCR

Total RNA was extracted from newborn brains with the RNeasy kit (Qiagen); 1 μ g RNA was then used for RT-PCR using the GeneAmp® Gold RNA PCR Reagent Kit (Applied Biosystems).

3. Results

3.1. *Shep1* gene inactivation

Shep1^{flox} mice were generated by homologous recombination using a construct in which loxP sites for Cre recombinase flanked *Shep1* exon 7 (Fig. 1A). This exon encodes most of the SH2 domain, and corresponds to exon 8 of human *SHEP1* [10]. By crossing mice carrying the floxed *Shep1* allele with mice expressing the Cre recombinase in the germline, exon 7 was successfully deleted (Fig. 1B), allowing generation of a knockout line where the *Shep1* gene is inactivated in all tissues. Since *Shep1* is known to be highly expressed in the brain [7,8,10], we confirmed the loss of *Shep1* protein in homozygous *Shep1* knockout mice by immunoblotting *Shep1* immunoprecipitated from P0 (postnatal day 0) brain lysates using antibodies to the *Shep1* N-terminal region including the SH2 domain (Fig. 2A). However, antibodies recognizing the *Shep1* C terminus immunoprecipitated two bands of approximately 55 Kd in the knockout brain and also, at lower levels, in the heterozygous brain (Fig. 2A,B). RT-PCR analysis revealed the presence of a *Shep1* mRNA containing deletion of exon 7, due to aberrant splicing of exon 6 to exon 8 (Fig. 2C,D). Because translation from the initial ATG codons of *Shep1* [10] would shift the reading frame of exon 8 due to the deletion of exon 7, precluding generation of a protein lacking only the SH2 domain, the two bands presumably represent *Shep1* fragments generated by translation initiation from internal cryptic ATG codons present in exon 6 and exon 8, which would yield fragments of 489 and 462 amino acids (Fig. 2C). These regions contain part of the proline/serine-rich region and the GEF-like domain but not the amino terminus and SH2 domain. Thus, we collectively refer to these fragments, which were detected at variable levels in different tissues (Fig. 2A,B), as *Shep1*ΔN.

Crosses of *Shep1* heterozygous mice yielded the expected mendelian proportions of wild-type, heterozygous and knockout pups (Fig. 3A). However, the great majority of the knockout pups died in the first day after birth. Phenotyping necropsy and histological analysis of newborn pups did not reveal obvious abnormalities that could explain the perinatal death. Because defects in respiratory function or blood circulation could be responsible for perinatal death, and *Shep1* is highly expressed in the heart and the lung [8,10], we further analyzed heart and lung tissue sections from newborn mice. Hematoxylin and eosin (H&E) staining, immunostaining for the Ki67 cell proliferation marker and TUNEL staining for apoptotic cells did not show obvious differences between *Shep1* knockout and wild-type mice that would suggest defects in breathing or heart function (Suppl. Fig. 1). Similarly, H&E staining, BrdU incorporation and TUNEL staining of sections from E13-E14 embryos did not reveal anatomical abnormalities or differences in cell proliferation or apoptosis in the *Shep1* knockout mice as compared to wild-type (Suppl. Figs. 2—4). We also did not detect overall defects in developing axonal projections or blood vessels (Suppl. Figs. 5 and 6), where *Shep1* is known to be highly expressed [10,23].

Interestingly, the few *Shep1* knockout mice that survived the first several days after birth reached adulthood and did not exhibit obvious abnormalities. More detailed analysis revealed that most of the newborn *Shep1* knockout pups have little or no milk in their stomachs (Fig. 3B). This suggests suckling defects, which may be due to abnormalities in the olfactory system [23,24]. Hence, insufficient feeding due to a defective sense of smell may be responsible for the observed perinatal lethality [25,26].

3.2. *Shep1* plays an important in vivo role in Cas signaling

Shep1 and Cas are found as a complex in cells, suggesting that they have closely intertwined functions. Consistent with this, we previously reported that *Shep1* can promote Cas tyrosine phosphorylation and EGF-dependent migration in cultured cells [16]. Interestingly, immunoblots of lysates from newborn *Shep1* knockout brains revealed a marked decrease in

tyrosine-phosphorylated bands at the approximate size of Cas (arrow in Fig. 4A). We indeed detected a dramatic reduction in Cas tyrosine phosphorylation by probing Cas immunoprecipitates with anti-phosphotyrosine antibodies (Fig. 4B). In contrast, the abundance of Cas appeared similar in wild-type and knockout brains (Fig. 4A,B). Probing cell lysates with two different Cas phosphospecific antibodies showed that the decrease in Cas phosphorylation involves tyrosine phosphorylated motifs in the substrate domain (Fig. 4B). Phosphorylation at these sites links Cas to downstream signaling effectors by providing multiple docking sites for the SH2 domain of the adaptor protein Crk [5]. Consistent with a decrease in Crk binding sites, we detected reduced Cas-Crk association in the *Shep1* knockout brains (Fig. 4C). Tyrosines in the substrate domain of Cas are known to be phosphorylated by Src family kinases, which bind to Cas through their SH2 and SH3 domains thus releasing inhibitory intramolecular interactions and promoting Src kinase activity [27–30]. Accordingly, probing knockout brain lysates with a Src phosphospecific antibody showed a significant reduction in the phosphorylation of Src family proteins on a tyrosine in the activation loop, suggesting lower Src kinase activity in *Shep1* knockout compared to wild-type brain (Fig. 4D). Other proteins appearing less tyrosine phosphorylated in *Shep1* knockout brain lysates (Fig. 4A) may therefore represent other Src substrates.

Two Cas bands were detected in newborn wild-type mouse brains (Fig. 4B), as reported in other systems [4]. The upper band represents a minor proportion of the Cas immunoreactivity, but was detected as efficiently as the lower band using both general anti-phosphotyrosine antibodies and the substrate domain phosphospecific antibodies. This implies that the upper band represents a more heavily tyrosine phosphorylated form of Cas. In addition, it has been reported that the shift in the apparent size of Cas is due to hyperphosphorylation on serine residues [17,31,32]. Interestingly, this upper band was greatly reduced in the knockout brains (Fig. 4B). Altogether, the analysis of the *Shep1* knockout brain demonstrates that *in vivo* impairment of *Shep1* function leads to defects in Cas signaling.

3.3. The SH2 domain-containing amino terminus of *Shep1* is required to increase Cas phosphorylation but not Cas levels

Newborn *Shep1* knockout mice express high levels of the truncated *Shep1*ΔN fragments in the brain. To confirm that the decreased Cas phosphorylation observed in the *Shep1* knockout brain is due to the loss of full-length *Shep1* rather than the expression of the *Shep1*ΔN fragments, which retain the ability to bind Cas (data not shown), we analyzed Cas phosphorylation in COS cells. These cells, which do not express detectable levels of endogenous *Shep1*, were transfected with full-length *Shep1* (to mimic the expression in wild-type mice), *Shep1*ΔN (to mimic expression of the truncated fragments in the knockout mice), and wild-type *Shep1* together with *Shep1*ΔN (to mimic expression in the heterozygous mice). Cas tyrosine phosphorylation was increased in COS cells transfected with full-length *Shep1* and was not affected by *Shep1*ΔN (Fig. 5A). Furthermore, full-length *Shep1* caused a similar increase in Cas phosphorylation in the presence and in the absence of co-transfected *Shep1*ΔN (Fig. 5A), suggesting that *Shep1*ΔN does not decrease Cas phosphorylation on its own and does not have dominant negative effects on the activity of full-length *Shep1*. Consistent with this result, the perinatal death of the *Shep1* knockout mice also does not appear to be caused by dominant negative effects of *Shep1*ΔN. Indeed, *Shep1* heterozygous mice, which express the *Shep1*ΔN fragments in at least some tissues, are born and survive in the expected numbers (Fig. 3A), do not exhibit obvious abnormalities and breed normally. These results suggest that the SH2 domain is critical for the ability of *Shep1* to enhance the signaling function of Cas.

Interestingly, the transfection experiments also revealed higher levels of endogenous Cas protein in the COS cells expressing wild-type *Shep1* and also *Shep1*ΔN (Fig. 5B). This suggests that *Shep1* expression can increase Cas levels and that this effect does not require the SH2

domain of Shep1. Furthermore, in cells transfected with wild-type Shep1, similar levels of both the lower and the higher (hyperphosphorylated) Cas bands were detected. In contrast, in cells transfected with Shep1 Δ N the lower band was predominant, confirming the importance of the SH2 domain in inducing Cas hyperphosphorylation. Thus, the Shep1 Δ N fragments expressed in the *Shep1* knockout mice retain partial function, since they increase Cas levels but not Cas phosphorylation. This suggests that the *Shep1* knockout mice may have milder abnormalities because complete loss of Shep1 protein would be expected to not only reduce the signaling ability of Cas but also overall Cas levels.

4. Discussion

The scaffolding protein Cas has been extensively studied as a critical component of integrin and growth factor receptor signaling pathways. Cas has been recently shown to form a tight complex with members of the Shep1 family, but the *in vivo* impact of Shep1 family proteins on Cas function is poorly understood. Here we show that association with Shep1 is critical for the physiological signaling function of Cas in the newborn brain, suggesting that Shep1 and Cas function as a signaling module *in vivo*. This is consistent with our previous cell culture studies, which revealed a cooperative function of Shep1 and Cas in EGF-dependent COS cell migration [16]. Physical association between SHEP1 and Cas is also essential for Shep1-induced Cas phosphorylation, membrane ruffling and integrin-mediated adhesion [16,32]. BCAR3 and Cas (which is also known as BCAR1) were also found to both promote the development of antiestrogen resistance in breast cancer cells, further supporting the notion that the two protein families share a common signaling function [13,33,574].

We found that an important *in vivo* role of Shep1 is to promote Src and Cas phosphorylation, an activity that critically depends on the amino-terminal region of Shep1, which contains the SH2 domain. BCAR3/AND-34 has also been shown to regulate Src activity and Cas phosphorylation in cultured breast cancer cells and other cell types [17,20,34]. The SH2 domain of BCAR3 is essential for increasing Cas serine phosphorylation in breast cancer cells, whereas the ability of BCAR3 to bind Cas does not appear to be critical. In addition, the SH2 domain of Shep1 could replace that of BCAR3/AND-34 in promoting Cas phosphorylation. Shep1 and BCAR3/AND-34 might enhance activation of Src kinases by promoting binding of their SH2 and SH3 domains to Cas [20,27]. Shep1 family proteins have indeed been shown to promote Cas localization at the plasma membrane, which could facilitate interaction with Src [16,19,34]. Consistent with this, a form of Shep1 targeted to the plasma membrane by the Src myristoylation signal promotes Cas phosphorylation by Src and coupling to Crk [16]. Furthermore, the amino-terminal region of BCAR3/AND-34 is important for its membrane localization [34]. Thus, a possible model is that the SH2 domain of Shep1 family proteins – by binding to tyrosinephosphorylated cell surface receptors – promotes the recruitment of Cas to the plasma membrane, thereby favoring Cas association with Src kinases, phosphorylation in the substrate domain and downstream signaling through association with Crk.

Another interesting hypothesis is that Shep1 is part of the machinery that promotes Cas tyrosine phosphorylation in response to cell “stretching”. It has been recently shown that Cas, when artificially anchored at the amino- and carboxy-terminal ends, becomes “stretched” in cells encountering stiff extracellular matrix [35–37]. This exposes the Cas central substrate domain, facilitating its phosphorylation by Src family kinases. Shep1 family proteins bind to a 133 amino acid region at the extreme carboxy terminus of Cas located near the Src binding domain [16]. They could thus serve to physiologically anchor the Cas carboxy terminus in cells, perhaps by interacting with cell surface receptors through their SH2 domains.

Cas phosphorylation by Src controls cellular invasiveness, and indeed BCAR3 has been implicated in breast cancer cell invasiveness [17,19,20]. The crosstalk between Cas and Shep1

family proteins may therefore play a role in the malignant transformation of cancer cells by promoting not only resistance to anti-estrogens but also invasiveness. Shep1 and CasL interaction has also been shown to be essential for T-cell integrin-mediated adhesion, migration and homing to peripheral tissues [32]. In the nervous system, this could be important in developmental processes such as axon outgrowth and perhaps guidance [38,39]. We indeed found that Shep1 knockout mice have severe defects in the penetration of olfactory sensory axon across the brain basal lamina [23].

We also found that newborn brain lysates from wild-type and heterozygote mice contain substantial levels of the slow-migrating form of Cas, which has been reported to be hyperphosphorylated on serine residues [17,31,32], whereas the fast-migrating form was predominant in Shep1 knockout brains. Consistent with this, our data as well as reports by others show that Shep1 or BCAR3 expression increases the proportion of the slow-migrating hyperphosphorylated form of Cas [16,17,22,32]. How Shep1 family proteins regulate Cas serine phosphorylation and how in turn this phosphorylation affects cell migration and invasiveness also remains to be determined [17,32].

Our data suggest that another function of Shep1 is to increase Cas cellular levels. This requires the Cas-binding GEF-like domain of Shep1 and may thus involve Cas stabilization and protection from degradation [40–43]. The Shep1 Δ N fragments expressed in the *Shep1* knockout mice retain the ability to upregulate Cas abundance, suggesting that the phenotypes observed in the knockout mice might be milder than they would be if the entire Shep1 protein was absent. Nevertheless, the Shep1 Δ N fragments are not sufficient to promote Src activity and Cas phosphorylation, highlighting the importance of the SH2 domain in the physiological function of Shep1. Consistent with a functional deficiency of Shep1 Δ N, we found that a Shep1 fragment similar to those expressed in the knockout mice does not promote cell invasion through collagen in Transwell assays when ectopically expressed in HEK 293 cells [23]. Similarly, a truncated form of BCAR3/AND-34 without the N-terminus and the SH2 domain has also been recently reported to be functionally deficient in breast cancer cells [17].

Overall brain architecture appears normal in the Shep1 knockout mice (Suppl. Figs. 2–6 and data not shown). However, deficiencies in Shep1-Cas function may result in defects in axon growth or dendritic spine morphology [38, 39, 44], abnormalities that may only be revealed by a more detailed analysis. Indeed, in other studies we found that the axons of developing olfactory sensory neurons fail to connect to their synaptic targets in the olfactory bulb in Shep1 knockout mice [23]. We also detected morphological abnormalities in the dendritic spines of hippocampal pyramidal neurons of adult Shep1 knockout mice (Wang and Pasquale, unpublished data). BCAR3/AND-34 may also be co-expressed with Shep1 in some cell populations [10, 22], thus providing a redundant function. Indeed, BCAR3 knockout mice do not have obvious defects, except for the development of cataracts [22]. It will therefore be interesting to examine mice defective in both Shep1 and BCAR3, which will reveal the full extent of Cas functional impairment in the absence of Shep1 family proteins. Nevertheless, the defects in Cas phosphorylation and Src activity in the Shep1 knockout brain highlight the critical importance of Shep1 for the *in vivo* signaling function of Cas in the brain.

5. Conclusions

This work implicates Shep1 as an essential physiological binding partner for the Cas scaffolding protein. In the Shep1 knockout brain, Cas phosphorylation and association with the Crk adaptor as well as Src activity are impaired while in Shep1-transfected cells Cas phosphorylation is elevated. Our data suggest that Shep1 is a pivotal component in the Cas signaling networks that link adhesion receptors and cell surface receptor tyrosine kinases to actin cytoskeletal organization in the regulation of cell morphology, adhesion and migration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Generation of *Shep1* floxed and *Shep1* knockout mice

(A) Targeting strategy for the *Shep1* locus. The schematic drawing represents the wild-type *Shep1* allele (+), the targeting construct and the *Shep1* floxed allele resulting from homologous recombination (flox). The PGK-neo cassette is inserted downstream of exon 7 and flanked by FRT sites (white arrowheads). Expression of the Cre recombinase in the germline of mice harboring the *Shep1* floxed allele allowed recombination between the loxP sites (black arrowheads) and deletion of exon 7, generating the deleted *Shep1* knockout allele (-). (B) Southern blot analyses of ES cell clones and engineered mice. The Southern blots of BamHI- or EcoRI-digested DNAs show targeted ES cells (+/flox) using a 5' or 3' external probe, respectively. A +/-flox ES cell clone was used to derive the *Shep1* floxed mouse line. The

Southern blot of mouse tail DNAs shows examples of targeted mice, including one with a recombined knockout allele (right lane of the blot, +/-).

Figure 2. A Shep1 carboxy-terminal fragment is expressed in the *Shep1* knockout mice
(A) Immunoblot analysis of *Shep1* wild-type (+/+), heterozygous (+/-) and knockout (-/-) brain. Shep1 was immunoprecipitated from P0 brain lysates with antibodies to either the C-terminus or the N-terminus of the protein, and the immunoprecipitates were probed with anti-C-terminus antibodies. As expected, wild-type Shep1 was not detectable in knockout brain. However, two shorter fragments (Shep1ΔN), recognized only by the antibodies to the C-terminus, were detected in *Shep1* heterozygous and knockout mice. (B) Immunoblot analysis shows that Shep1ΔN is also present in P0 lung tissue, but at much lower levels than in the brain and as a single band. An asterisk marks a non-specific band recognized by the antibodies. (C) A Shep1 mRNA lacking only exon 7 is expressed in *Shep1* heterozygous and knockout mice. RT-PCR using P0 brain mRNAs demonstrates the presence of the Shep1ΔN amplification product (lacking exon 7, which contains 455 base pairs) in the heterozygous and knockout mice. The wild-type amplification product was detected in the wild-type and heterozygous mice, also as expected, although in the heterozygous mice the band is very faint and only clearly detectable in overexposed pictures. The primers used correspond to the sequences underlined in D. (D) Predicted start codons in the Shep1ΔN fragments. The DNA sequence corresponding to Shep1ΔN and amplified in C is shown. Exons are indicated by arrows below the sequence and junctions between exons are shaded. Two possible start codons are in bold and the encoded methionines (M) are enlarged. Serine 223 at the beginning of exon 7 and the second predicted initial methionine (residue 240) are numbered according to the mouse *Shep1β* isoform [10]. The first methionine is preceded by an in-frame upstream stop codon (*). Sequences corresponding to the primers used in C for RT-PCR are underlined.

A

	+/+	+/-	-/-	dead
P0	31 (23%)	72 (53%)	31 (23%)	5 (4%)
P21	36 (27%)	67 (50%)	2 (1%)	29 (22%)

B

Figure 3. Most *Shep1* knockout mice die perinatally with little milk in their stomachs

(A) The number of mice for each genotype is indicated. *Shep1* knockout (-/-) mice were born at approximately the expected frequency, but only few survived to adulthood. Most knockout mice died in the first two days after birth, whereas heterozygous mice survived and were fertile. (B) Quantification of milk in the stomachs of P0 mice reveals a feeding defect in the *Shep1* knockout mice that may account for their frequent perinatal death. The total number of mice in each group is indicated above the bars of the histogram.

Figure 4. Cas tyrosine phosphorylation is dramatically decreased in the brain of *Shep1* knockout mice

(A) P0 mouse brain lysates from *Shep1* wild-type (WT) and knockout (KO) were probed with anti-phosphotyrosine (PTyr) and anti-Cas antibodies. The arrow indicates a tyrosine phosphorylated protein band, at the size of Cas, that is decreased in knockout brains compared to wild-type. (B) Cas immunoprecipitates were probed with anti-phosphotyrosine antibodies and brain lysates and Cas immunoprecipitates were probed with two phosphospecific antibodies generated using the phosphorylated Cas Y165 or Y410 motifs in the Cas substrate domain (upper panels). The blots were then reprobed for total Cas (lower panels). The results show a significant decrease in overall Cas tyrosine phosphorylation (* $P < 0.05$, $n = 6$) and Cas substrate domain phosphorylation detected with the phospho-Cas Y165 antibody (*** $P < 0.001$, $n = 4$) or the phospho-Cas Y410 antibody (*** $P < 0.001$, $n = 6$). (C) Crk immunoprecipitates were probed with Crk and Cas antibodies. Crk/Cas complexes are decreased in knockout brain compared to wild-type (** $P < 0.01$, $n = 3$). (D) Knockout brain lysates show a decrease in phosphorylation of Src family kinases on a tyrosine in the activation loop (corresponding to tyrosine 416 of chicken Src) (** $P < 0.01$, $n = 9$), suggesting reduced Src activity. The histograms show means $\pm$ SEM. The means measured for the knockout animals are expressed as percent of wild-type. P values were calculated using a two-tailed Student's t-test.

Figure 5. The SH2 domain is crucial for the ability of Shep1 to promote Cas phosphorylation (A) Wild-type Shep1, but not a Shep1ΔN fragment lacking the SH2 domain, promotes Cas phosphorylation. COS cells were transfected with the indicated constructs, and Cas immunoprecipitates were probed with anti-phosphotyrosine antibodies and reprobed for Cas. Lysates were also probed with antibodies to the Shep1 C terminus to verify expression of the transfected constructs. A non-specific band, indicated by an asterisk, verifies equal protein loading. Bands were quantified and the histogram show means $\pm$ SEM, which are expressed as percent of tyrosine phosphorylated Cas in the vector control condition. ** $P < 0.01$ and *** $P < 0.001$ for the comparison with vector control transfected cells by one way-ANOVA followed by Dunnett's multiple comparison posthoc test ($n=6$). The upper band has been reported to represent a form of Cas hyperphosphorylated on serine residues. In contrast, Shep1ΔN appears to preferentially upregulate the lower Cas band. (B) Both Shep1 wild-type

and the Shep1 Δ N fragment lacking the SH2 domain increase endogenous Cas levels. COS cells were transfected with the indicated constructs, and lysates were probed with anti-Cas antibodies and reprobed with the Shep1 C terminus antibody. A non-specific band, indicated by an asterisk, verifies equal protein loading. Both bands in the Cas doublet were quantified and the histogram show means $\pm$ SEM, which are expressed as percent of Cas in the vector control condition. **P<0.01 and ***P<0.001 for the comparison with vector control transfected cells by one way-ANOVA followed by Dunnett's multiple comparison posthoc test (n=8 for WT, 4 for Δ N and 3 for WT + Δ N). Interestingly, the Cas blot also shows that overexpression of Shep1 wild-type upregulates both bands in the Cas doublet, whereas Shep1 Δ N upregulates preferentially the lower band. This is also apparent in the Cas immunoprecipitates probed for Cas in A.