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Characterization of EVL-I as a protein kinase D substrate

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Abstract

EVL-I is a splice variant of EVL (Ena/VASP like protein), whose *in vivo* function and regulation are still poorly understood. We found that Protein Kinase D (PKD) interacts *in vitro* and *in vivo* with EVL-I and phosphorylates EVL-I in a 21 amino acid alternately-included insert in the EVH2 domain. Following knockdown of the capping protein CP β and spreading on laminin, phosphorylated EVL-I can support filopodia formation and the phosphorylated EVL-I is localized at filopodial tips. Furthermore, we found that the lamellipodial localization of EVL-I is unaffected by phosphorylation, but that impairment of EVL-I phosphorylation is associated with ruffling of lamellipodia upon PDBu stimulation. Besides the lamellipodial and filopodial localization of phosphorylated EVL-I in fibroblasts, we determined that EVL-I is hyperphosphorylated and localized in the cell–cell contacts of certain breast cancer cells and mouse embryo keratinocytes. Taken together, our results show that phosphorylated EVL-I is present in lamellipodia, filopodia and cell–cell contacts and suggest the existence of signaling pathways that may affect EVL-I via phosphorylation of its EVH2 domain.

Keywords

Protein kinases; Protein phosphorylation; DAG signalling; Protein kinase D; Ena/VASP proteins; Cytoskeleton; Filopodia; Lamellipodia; Cell–cell contacts

1. Introduction

The actin cytoskeleton is a highly dynamic structure, involved in a wide variety of cell motility processes, and a growing number of key regulatory actin binding proteins have been identified [1,2]. The availability of free barbed ends limits actin assembly *in vivo*; barbed ends can be generated by severing capped filaments or by de novo nucleation by a number of nucleation promoting factors such as the Arp2/3 complex or formins. Barbed end elongation can be regulated by a number of proteins including capping proteins, which terminate filament growth or by Ena/VASP proteins which antagonize capping activities and promote the formation of longer, sparsely branched filament networks [3–5].

Ena/VASP proteins are actin-binding proteins that are present along actin stress fibers, the lamellipodial leading edge, and the tips of filopodia on growth cones, sites where actin

polymerization occurs [6]. Therefore, this protein family is well suited to be involved in early rearrangement of the actin cytoskeleton in response to guidance cues. *Drosophila* contains a single Ena/VASP ortholog, *Enabled* (*Ena*) protein, which was identified through its genetic interactions with the *D-Abl* tyrosine kinase gene and was later found to be involved in signaling pathways that control axon guidance in the developing nervous system [7–9]. Vertebrates have 3 Ena-related genes: Mena (“mammalian *Enabled*”), VASP (vasodilator stimulated phosphoprotein) and EVL (*Ena/VASP* like). Mena deficient mice display defects in the formation of nerve fiber tracts in the brain [10]. VASP mutant mice exhibit increased “inside-out” activation of integrins [11,12] and defects in spectrin II α -dependent cytoskeletal assembly at endothelial junctions [13].

EVL (which has a splice variant called EVL-I [14]) has been implicated in a variety of processes. First, in studies on the intracellular movement of *Listeria monocytogenes*, EVL (and also Mena and VASP) stimulate the actin-based intracellular motility of the pathogen [15,16]. In T-cells, EVL was found in F-actin-rich patches and at the distal tips of the microspikes that form on the activated side of the T-cells [14]. When expressed in fibroblasts EVL localized to focal adhesions and to the leading edge of lamellipodia [14]. Mice deficient for EVL alone are viable and fertile while combined deletion of all Ena/VASP proteins (e.g. Mena/VASP/EVL triple mutants) causes a range of defects including: failure to form filopodia on neurons [17], defects in cortical neurite initiation [18] and hemorrhaging arising from defective endothelial barrier function [19].

Ena/VASP family members share a similar modular structure, consisting of an N-terminal Ena/VASP homology domain-1 (EVH1), a central proline rich domain, and a C-terminal Ena/VASP homology domain-2 (EVH2) [20]. The EVH1 domain plays a key role in subcellular targeting of Ena/VASP proteins by binding specific proline-rich motifs found in a number of cellular proteins such as zyxin and vinculin [21]. The middle portion of the protein consists of a proline-rich domain that binds SH3 and WW domain-containing proteins and the actin monomer binding protein profilin [22–24]. The C-terminal EVH2 domain mediates tetramerization and binds both G- and F-actin [25]. The EVH2 domain captures the growing ends of actin filaments [26] and is required for Ena/VASP targeting to lamellipodia and filopodia [27].

The vertebrate Ena/VASP proteins contain several conserved phosphorylation sites: an amino terminal site found in all three that is phosphorylated by PKA (and PKC, for VASP), a C-terminal site phosphorylated by PKG found in Mena and VASP and another C-terminal site found in VASP only phosphorylated by AMPK [14,28–32]. Phosphorylation of VASP in the EVH2 domain interferes with its ability to regulate actin dynamics [33] and displaces it from lamellipodia [34]. Interestingly, we observed that the 21 amino acid insert within the EVH2 domain of EVL-I contains a consensus sequence for phosphorylation by protein kinase D (PKD).

The protein kinase D family comprises three isoforms: PKD1 (also named PKC μ), PKD2 and PKD3 (also called PKC ν) [35–37]. All three are multi-domain proteins, consisting of an N-terminal regulatory region (containing two cysteine-rich zinc-fingers and a pleckstrin-homology domain) and a C-terminal Ser/Thr kinase catalytic domain [38]. The entire regulatory domain of the protein inhibits the catalytic activity of the kinase. PKD can be activated by a multitude of stimuli such as diacylglycerol and analogues (phorbol esters), neuropeptides such as bombesin, vasopressin, endothelin, bradykinin and angiotensin II, platelet derived growth factor, IGF-1, Wnt, and T- and B-cell receptor signaling events [39–42]. All of these stimuli activate PKD in a PKC-dependent way, by activation loop phosphorylation. PKD has been implicated in a multitude of normal as well as pathological processes including Golgi function, cell proliferation, apoptosis, conditions of oxidative

stress, regulation of the immune system, gene regulatory processes, angiogenesis and cardiac hypertrophy (for reviews, see: [38,43–48]).

Recently, a lot of attention has focused on PKD for its potential role in cancer cell invasion. In invasive breast cancer cells, PKD is found in invadopodia as a complex with cortactin and paxillin, whereas this complex is absent in non-invasive breast cancer cells [49]. In myeloma cells, the pro-invasive factors IGF-I and Wnt both activate PKD [50,51]. Wnt stimulation causes association of PKD with Rho and several PKCs, and blocking Rho kinase leads to PKD inhibition, suggesting that Wnt activates PKD via a Rho-ROCK-PKC pathway [51]. The exact role of PKD in cancer cell invasion is entirely unknown. Furthermore, as mentioned above, endothelial cell migration is critically dependent on PKD activity [52].

At the leading edge of migrating cells, PKD colocalizes with F-actin, Arp2/3 and cortactin [53]. Ena/VASP proteins also localize to lamellipodia of migrating cells and are known to be regulated by phosphorylation. The predicted PKD phosphorylation site in EVL-I could provide a means for differential regulation of EVL and its splice variant EVL-I. In this study we have identified Ser-345 in EVL-I as a “bona fide” PKD phosphorylation site, and have addressed the functional consequences of this phosphorylation.

2. Materials and methods

2.1. Plasmids

The rat EVL-I cDNA in pDONR 201 (entry clone, Invitrogen) was a kind gift of Prof. D. Dhermy (INSERM, Paris, France). This construct was subcloned into a myc-destination vector (gift of Dr T. Johansen, University of Tromsø, Norway) and a HIS-destination vector (gift of Dr. D. Busso, University of Strasbourg, France) according to the manufacturer's protocol (gateway system, Invitrogen).

The Quikchange II site-directed mutagenesis kit was used, according to the manufacturer's protocol (Stratagene), to produce mutant Ser345Ala and Ser345Glu rat EVL-I cDNA constructs. The sequence of the mutated vectors was verified by DNA sequencing. The human PKD1 constructs were a gift from Dr. P. Storz (Mayo Clinic, Jacksonville, USA) and the mouse kinase dead (KD) PKD1 (K618N) used for the co-immunoprecipitation assay was cloned into a pcDNA3 vector.

The mouse EVL-I cDNA was cloned into the pMSCV-EGFP retroviral plasmid using standard techniques. Ser345Ala and Ser345Glu mutants were made using the Quikchange II site-directed mutagenesis kit.

2.2. Antibodies

Anti-Myc-tag monoclonal antibody 9E10 was prepared from hybridomas and purified on protein A -Sepharose beads (GE Healthcare). EVL-I-P antibody and PKD antibody are rabbit polyclonal antibodies, purified through phospho- and dephosphopeptide columns (SulfoLink coupling Gel, Pierce). ZO-1 was purchased from BD Biosciences. Anti-mouse IgG and anti-rabbit IgG (horseradish peroxidase-conjugated) were purchased from Cell Signaling.

2.3. Cell culture and transfection

Human embryonic kidney (HEK) 293T cells (ATCC) and SKBr3 cells (ATCC) were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone), penicillin (100 U/ml) and 100 µg/ml streptomycin (Invitrogen) in a humidified atmosphere at 37 °C under 5% CO₂. Transfection of 293T cells

was performed with polyethyleneimine (PEI, linear, MW ~25000, Polysciences Inc., stock concentration of 1 mg/ml) with a ratio of 1:7.5 ($\mu\text{g DNA}:\mu\text{l PEI}$). Stimulation of cells with PDBu (Sigma) was done for 10 min at a 400 nM final concentration.

MV^{D7} EGFP-EVL-I derivative cell lines including EGFP-EVL-I wt, EGFP-EVL-I Ser345Ala, EGFP-EVL-I Ser345Glu were generated and cultured as previously described in [54,55].

To analyze phosphorylation of EVL-I in breast cancer cells, we used the following cell lines: T47D, MDA-MB-453, MDA-MB-231, BT549, MTLn3, SKBr3 and MCF-7 (ATCC).

2.4. Immunoprecipitation and immunoblotting

HEK 293T cells were transfected and harvested 48 h later. Cells were lysed in 0.75 ml of lysis buffer containing 50 mM Tris, pH 7.4, 2 mM EDTA, 2 mM EGTA, 1% Triton, 50 mM NaF, 1 mM Na_3VO_4 , pepstatin and protease inhibitors (Roche). 1 mg of cell lysates for each condition was incubated with protein A-Sepharose beads (GE Healthcare) with rotation at 4 °C for 1 h (preclearing). We re-incubated the precleared lysates with antibody (rotation, 2 h, 4 °C). The immune complexes were immobilized on protein A-Sepharose beads for 1 h (rotation, 4 °C), washed with ice-cold lysis buffer containing 150 mM NaCl and boiled in SDS-sample buffer. We separated the samples by Tris-acetate separating gels (Biorad) and transferred them onto a Protran nitrocellulose membrane (Schleicher and Schuell, Bioscience). We immunoblotted by first blocking the membranes in 20 mM Tris, pH 7.5, 500 mM NaCl, and 0.1% Tween 20 (TBST buffer) containing 5% non-fat milk or 5% BSA. The membranes were incubated with primary antibodies overnight at 4 °C. Membranes were washed with TBST buffer and then incubated for 2 h at 4 °C with HRP-conjugated secondary antibody. Immunoreactive protein bands were detected by an enhanced chemiluminescence detection system (Pierce).

2.5. Purification of activated PKD1 and HIS-EVL-I

Activated PKD1 was purified as described before [56]. HIS-tagged EVL-I proteins were purified from *Escherichia coli* BL21 extracts. BL21 cells were grown till $\text{OD}_{600} = 1$ and induced with 1 mM IPTG (isopropyl β -D-1-thiogalactopyranoside) for 5 h at 37 °C. After centrifugation, bacterial pellets were stored in -80 °C and purified with Ni-NTA resin following manufacturer's protocol (Qiagen).

2.6. Kinase reactions

2 μg purified HIS-EVL-I protein was incubated with phosphorylation mix (containing a final concentration of 100 μM ATP, 2 μCi [γ - ^{32}P] ATP, 12.5 mM Tris pH7.4, 12.5 mM MgCl_2). Activated PKD was added and the mixture was incubated 5 min at 30 °C. Reaction was then terminated by adding SDS sample buffer.

For determination of the K_m value, a dilution was made of purified His-EVL-I protein with final concentration between 10 μM and 25 nM. These different dilutions were phosphorylated with PKD (as previously described). After drying the SDS-PAGE gel, the bands of the phosphorylated His-EVL-I proteins were cut out and counted in a scintillation counter. The K_m value was determined via a computational program (SigmaPlot).

2.7. GST-binding assay

Purified HIS-EVL-I was precleared with glutathione Sepharose 4-B beads by rotating for 1 h at 4 °C. GST-PKD coupled glutathione sepharose 4-B beads were incubated with the precleared HIS-EVL-I (rotation for 1 h at 4 °C). After extensive washing (50 mM Tris pH 7.4, 1% triton, 10% glycerol, protease inhibitors (Roche)) SDS-sample buffer was added.

Samples were analyzed by western blotting with EVL polyclonal antibody (kind gift of Prof. D. Dhermy).

2.8. Immunofluorescence microscopy

SKBr3 cells were plated on coverslips coated with collagen (100 µg/ml) and grown until cell–cell contacts were formed. Cells were permeabilized on ice for 3 min with CSK buffer (100 mM NaCl, 300 mM sucrose, 10 mM PIPES pH 6.8, 3 mM MgCl₂, 1 mM EGTA) containing 0.5% Triton X-100. Cells were fixed on ice in CSK buffer with 4% paraformaldehyde. Cells were washed with PBS and blocked for 1 h with 10% BSA in PBS. Coverslips were washed and incubated with primary antibody (P-EVL-I, ZO-1) for 1 h at room temperature (RT) in 1% BSA in PBS solution. Coverslips were washed with PBS and incubated with the Alexa dye labeled secondary antibody (Molecular Probes) and phalloidin (Alexa fluor 647, Molecular Probes) for 1 h at RT. Coverslips were washed and mounted on slides.

MV^{D7} cells were trypsinized and replated on laminin (20 µg/ml) or fibronectin (0.1% from Bovine plasma, 1/100) coated coverslips. The MV^{D7} cells on the laminin coated coverslips were fixed after 30 min, the MV^{D7} cells on the fibronectin coated coverslips were fixed after 15 h. Cells were permeabilized, washed and blocked for 1 h with 10% BSA in PBS at RT. Coverslips were washed and incubated with P-EVL-I antibody for 1 h at RT in 1% BSA in PBS solution. Coverslips were washed with PBS and incubated with the secondary antibody and phalloidin for 1 h at RT. Coverslips were washed and mounted on slides.

Localization was visualized using a Deltavision microscope (Applied Precision, Olympus IX71, 60×/1.4NA Plan Apon Nikon objective) and processed using Softworx software (SGI, Mountain View, CA).

2.9. Spreading assay

MV^{D7} EVL-I derivative cell lines were transfected with the shRNA vector (described in [57]) targeted against CPβ with Amaxa Nucleofector. Four days after transfection cells were trypsinized and replated on laminin (20 µg/ml) coated coverslips. Cells were allowed to spread for 30 min and fixed with 4% paraformaldehyde for 15 min at RT. Coverslips were washed 2 times with PBS. Afterwards cells were permeabilized 3 min at RT with 0.2% Triton-X 100 in PBS, washed 2 times with PBS and incubated with phalloidin. Coverslips were washed with PBS, mounted on slides. Transfected cells (containing the shRNA vector targeted against CPβ) were identified by DsRed2-soluble marker by epifluorescence. Cell phenotype was analyzed and categorized into the classes with and without filopodia.

2.10. Confocal microscopy

Newborn mice were euthanized and organs were fixed in 4% paraformaldehyde overnight at 4 °C. The day after, the organs were washed 3 times with PBS and embedded in PBS-20% gelatin. Sectioning was performed using a vibrotome (100 µm sections). Following sectioning, tissue sections were blocked overnight in PBS containing 0.1% triton X-100 (PBT) containing 10% goat serum. Tissues were washed 1 h in PBT containing 1% goat serum and stained with primary antibody in PBT containing 1% goat serum at RT for 2 h. Tissues were washed 5 times for 10 min in PBT containing 1% goat serum and then stained for 2 h at RT with the secondary antibody in PBT containing 1% goat serum. Again tissues were washed 5 times for 10 min in PBT containing 1% goat serum. Tissues were then washed for 30 min in PBS, and mounted on microscopy slides. Tissues were imaged with an ORCA-ER camera (Hamamatsu) attached to a Nikon TE2000 microscope with dual Sutter filter wheels, a spinning disk confocal head (Yokagawa), a mercury light source and a coherent 70C two watt multi-line laser using a 20×/0.75NA Plan Apo Nikon objective. All

images were collected, measured and compiled with the aid of Metamorph imaging software (Molecular Devices) and Adobe Photoshop.

3. Results

3.1. PKD phosphorylates EVL-I *in vitro*

EVL-I is regulated by phosphorylation and localizes to lamellipodia, and PKD is present in the lamellipodia of migrating cells [53], therefore we investigated whether EVL-I could be a target for phosphorylation by PKD. Examination of the EVL-I protein sequence for potential PKD phosphorylation sites fitting the consensus sequence [L/V/I]-X-[R/K]-X-X-[S/T] [58] revealed one potential PKD phosphorylation site at Ser-345: L³⁴⁰SRTPS³⁴⁵ (see Fig. 1A, right). This consensus is conserved in all known PKD substrates such as Kidins-220 [59], RIN1 [60], Hsp27 [61], HPK1 [62], HDAC7 [63], CREB [64], PI4-kinase [65], Troponin-I [66], and the vanilloid receptor [67]. HIS-tagged recombinant EVL-I was purified from bacteria and incubated *in vitro* with active PKD, followed by SDS-PAGE and autoradiography. EVL-I was phosphorylated by protein kinase D (Fig. 1A, left), and a Ser345Ala or a Ser345Glu mutation completely abolished this phosphorylation, indicating that Ser345 is the only target in EVL-I for phosphorylation by PKD. Further analysis showed that PKD phosphorylates EVL-I with favorable kinetics (Fig. 1B). The phosphorylation stoichiometry was 0.5 mol Pi per mol EVL-I. Furthermore, PKD phosphorylated EVL-I with a $K_m = 0.33 \mu M$, indicating a high affinity, well within the range of affinities found for other physiological kinase substrates.

3.2. PKD phosphorylates EVL-I *in vivo*

We raised phosphospecific antibodies against the peptide LSRTPSVAKSPE to investigate whether EVL-I is also phosphorylated by PKD in intact cells. After sequential purification on a phosphopeptide column and a dephosphopeptide column, the antibody displayed a high specificity for phospho-Ser345-EVL-I. We cotransfected myc-EVL-I (wild type, Ser345Ala or Ser345Glu) with PKD1 (wild type, wild type stimulated with the PKD activator PDBu, kinase-dead or constitutively active), and as shown in Fig. 1C, wild type PKD1 phosphorylated myc-EVL-I and this phosphorylation was stimulated by PDBu. Furthermore, constitutively active PKD1 phosphorylated myc-EVL-I, while kinase-dead PKD1 did not.

3.3. PKD and EVL-I interact directly *in vitro* and *in vivo*

We investigated whether PKD and EVL-I could bind directly. For this purpose, we incubated a range of concentrations of His-tagged EVL-I with GST-PKD1 or GST (as a control). As shown in Fig. 2A, an amount as low as 200 ng His-EVL-I could be specifically pulled down by GST-PKD1 and not by GST alone. These data indicate that PKD1 and EVL-I can directly interact with each other *in vitro*, in the absence of ATP and actin. We have also produced the isolated EVH2 domain of EVL-I, and this domain binds PKD. This also fits with the fact that the PKD phosphorylation site in EVL-I is located in an insert in the EVH2 domain (data not shown).

To find out whether PKD1 and EVL-I also form a complex *in vivo*, we co-transfected 293 T cells with kinase-dead PKD1 and myc-EVL-I. As shown in Fig. 2B, lane 7, kinase-dead PKD co-immunoprecipitated with myc-EVL-I upon immunoprecipitation with anti-myc antibodies. Furthermore, as shown in Fig. 2B, lane 8, myc-EVL-I co-immunoprecipitates with kinase-dead PKD1 upon immunoprecipitation with anti-PKD antibodies. As a control, the myc antibodies did not immunoprecipitate PKD in cells that were transfected with PKD alone, and PKD antibodies did not immunoprecipitate myc-EVL-I in cells that were transfected with myc-EVL-I alone.

It should be pointed out that although 293T cells contain both PKD1 and PKD2 (see Fig. 2B lanes 2 and 4), these proteins do not co-immunoprecipitate with transfected myc-EVL-I. In contrast, the transfected kinase-dead PKD does co-immunoprecipitate with myc-EVL-I. The underlying reason for this may be that active kinases often only transiently bind their substrates, and release them after the phosphorylation event.

3.4. Phosphorylation of EVL-I does not affect filopodia formation in fibroblasts

Since Ena/VASP proteins have a critical role in filopodia formation, we investigated whether phosphorylation of EVL-I has an effect on this process. For this purpose, we used the MV^{D7} cell system. These MV^{D7} cells are immortalized fibroblasts from VASP/Mena double knockout mice, that express only trace amounts of EVL. This is the only system currently available to address the functions of Ena/VASP proteins separately (the three Ena/VASP proteins, VASP, Mena and EVL, have overlapping functions in several cell systems, and hence the function of the individual proteins can only be addressed fully in the absence of the other family members).

Ena/VASP proteins function in filopodia formation in at least two ways: they antagonize filament capping and branching and they cluster growing filaments at their barbed ends [68–70]. Cells in which a subunit of heterodimeric capping proteins (“CP”) is depleted by RNAi exhibit a robust Ena/VASP-dependent filopodia phenotype [71] while CP knockdown in the absence of Ena/VASP leads to a ruffling phenotype. We generated stable MV^{D7} cell lines expressing EGFP-EVL, EGFP-EVL-I, EGFP-EVL-I-Ser345Ala or EGFP-EVL-I-Ser345Glu and tested the ability of these proteins to support filopodia after CP knockdown. As shown in Fig. 3, we observed no difference in the extent of filopodia formation between the different EVL mutants/isoforms. Therefore, PKD mediated EVL-I phosphorylation does not affect filopodia formation.

3.5. Phosphorylation of EVL-I does not affect its localization in filopodial tips

We investigated whether EVL-I localization in filopodial tips would be affected by phosphorylation. For this purpose, MV^{D7} cell lines expressing EGFP-EVL-I, EGFP-EVL-I-Ser345Ala or EGFP-EVL-I-Ser345Glu were analyzed. As shown in Fig. 4, each of these stable cell lines is able to form filopodia, as visualized by phalloidin staining. Moreover, the localization pattern of EGFP-EVL-I (as visualized with the above mentioned EGFP-tagged constructs), was identical for all three cell lines: EGFP-EVL-I is present in the filopodia and in the filopodial tips (as indicated by the arrows, and magnified in the lower half of panel A and in the insets of panel B). In addition, phosphorylated EGFP-EVL-I was detected by staining with the phosphospecific antibody in the EGFP-EVL-I wild type cells (in the EGFP-EVL-I-Ser345Ala or EGFP-EVL-I-Ser345Glu recognition by the antibody is impossible, because of the absence of a phosphorylated Ser-345) and found in filopodia and filopodial tips (Fig. 4, see arrows).

3.6. Impairment of EVL-I phosphorylation is associated with ruffling of lamellipodia upon PDBu stimulation

Ena/VASP proteins are also localized in lamellipodia. For this purpose, we also used the MV^{D7} cell system. When MV^{D7} cells are plated on fibronectin coated coverslips, they will spontaneously form lamellipodia, a process which is further enhanced by phorbol ester stimulation. Hence, we used our MV^{D7} cell lines expressing EGFP-EVL-I, EGFP-EVL-I-Ser345Ala or EGFP-EVL-I-Ser345Glu to test 1) whether this phosphorylation would affect EGFP-EVL-I *localization* to lamellipodia, 2) whether phosphorylation of EGFP-EVL-I had any effect on the *spontaneous or phorbol ester enhanced formation* of lamellipodia.

As shown in Fig. 5, we found that MV^{D7} cells expressing EGFP-tagged EVL-I wild type, or the Ser345Ala or Ser345Glu mutants of EVL-I are equally capable of forming lamellipodia.

When we examined the localization of EGFP-tagged EVL-I wild type, or the Ser345Ala or Ser345Glu mutants of EVL-I, we noticed several remarkable differences.

3.6.1. Basally phosphorylated EVL-I resides in a punctate pattern in the lamellipodia—When we looked at unstimulated EGFP-EVL-I-wt cells via the EGFP fluorescence (Fig. 5A, panels E and F), we found that the EGFP-EVL-I displayed a diffuse cytoplasmic fluorescence pattern and, importantly, a lamellipodial fluorescence pattern in three distinct sublocations: in the lamellipodial leading edge, along lamellipodial actin filaments and in a (less pronounced) granular pattern.

In contrast, when we then specifically looked at the localization of phosphorylated EGFP-EVL-I-wt (indicated as “P-EVL-I”, Fig. 5A, panels I and J) via staining with phospho-Ser345 specific antibodies, we noticed that the phosphorylated EGFP-EVL-I was present in a distinct punctate pattern.

3.6.2. Phorbol ester stimulation recruits more phosphorylated EVL-I into the lamellipodia—We then stimulated EGFP-EVL-I-wt cells with phorbol dibutyrate (PDBu). The majority of EGFP-EVL-I was still in a major diffuse cytoplasmic staining pattern and in the lamellipodial leading edge (Fig. 5A, panels G and H). In contrast, the phospho-EGFP-EVL-I was also cytoplasmic but relatively more concentrated in the lamellipodia and the lamellipodial leading edge (Fig. 5A, panels K and L) as compared with the EGFP-EVL-I-wt fluorescence. This would suggest that a subpopulation of EVL-I is recruited to the lamellipodia upon phosphorylation. Furthermore, some degree of ruffling of the lamellipodial membrane was induced by phorbol ester stimulation (e.g. Fig. 5A compare rows 2 and 3).

3.6.3. Impairment of EVL-I phosphorylation potentiates phorbol ester induced membrane ruffling—When we looked at the localization of EGFP-EVL-I-Ser345Ala (Fig. 5B, panels E and F), we noticed that in non-stimulated cells, EGFP-EVL-I-Ser345Ala (just like EGFP-EVL-I-wt) is localized in a diffuse cytoplasmic staining (Fig. 5B, panel E) and in the lamellipodial leading edge and along lamellipodial actin filaments (Fig. 5B, panel F). Because of the Ser345Ala mutation, P-EVL-I staining of the Ser345Ala mutant is not possible in these cells.

When we stimulated these EGFP-EVL-I-Ser345A cells with PDBu we noticed a striking difference as compared to EGFP-EVL-I-wt (compare panels G and H in Fig. 5A and B). Indeed, when these cells were stimulated with PDBu, we observed a very strong increase in membrane ruffling (Fig. 5B, panels G and H). Also, these ruffles were much longer as compared with those that were induced by PDBu in the EGFP-EVL-I-wt cells (compare panels H in Fig. 5A and B).

3.6.4. An EVL-I mutant mimicking constitutive phosphorylation is recruited to the lamellipodial leading edge, focal adhesions and the end of stress fibers—We created a Ser345Glu mutant of EGFP-EVL-I, which effectively mimicks phosphorylation at Ser-345. Indeed, comparison of the localization of phosphorylated wildtype EVL-I (via staining with phospho-EVL-I antibodies, indicated by P-EVL-I in Fig. 5A) with the localization of the Ser345Glu mutant of EVL-I (via EGFP fluorescence in Fig. 5C) shows that both localization patterns are very similar.

When we looked at the localization of EGFP-EVL-I-Ser345Glu (Fig. 5C), we noticed that in non-stimulated cells, EGFP-EVL-I-Ser345Glu is localized at the end of stress fibers, focal adhesions and in the lamellipodial leading edge (Fig. 5C, panels E and F). Because of the Ser345Glu mutation, P-EVL-I staining of the Ser345Glu mutant is not possible in these cells.

When we stimulated these EGFP-EVL-I-Ser345Glu cells with PDBu, EGFP-EVL-I-Ser345Glu was no longer localized at the end of stress fibers or focal adhesions but was more localized at the leading edge of the lamellipodia (Fig. 5C, panels G and H). No ruffles were observed.

3.7. EVL-I is hyperphosphorylated and located in cell–cell contacts of breast cancer cells

We next investigated whether EVL-I phosphorylation occurs in breast cancer cells, since the EVL gene (coding for EVL and its splice variant EVL-I) is upregulated in certain breast cancer cell lines [72]. Furthermore, Mena11a+, a splice variant of Mena which like EVL-I contains an alternately-included sequence in the same location in the EVH2 domain, is strongly phosphorylated in breast cancer cells [73]. Western blot analysis of a set of breast cancer cell lines (MTLn3, SKBr3, MCF-7, T47D, MDA-MB-453, MDA-MB-231 and BT549) with the phospho-EVL-I antibodies revealed a strong phosphorylation of EVL-I in the SKBr3, T47D and MDA-MB-453 cell lines (Fig. 6A). We focused on the SKBr3 cell line, to examine the subcellular distribution of phospho-EVL-I. We stained SKBr3 cells with antibodies to phospho-EVL-I and ZO-1 (zonula occludens-1, a tight junction marker) and found that phospho-EVL-I localized to cell–cell contacts in these cells (Fig. 6B, upper row). Phospho-EVL-I appeared to be enriched in areas containing ZO-1, pointing to a localization of phospho-EVL-I in the tight junctions (Fig. 6B, lower row).

3.8. Phospho EVL-I is enriched in basal keratinocytes

Because of the important developmental roles of Ena/VASP proteins, we stained sections of neonatal mice to determine the tissue distribution of phospho-EVL-I (Fig. 6C). A strong phospho-EVL-I signal was detected in the skin, and in particular in the basal keratinocyte layers. A closer examination of the location of EVL-I indicated that the phosphorylated EVL-I appeared to be enriched in cell–cell contacts of the keratinocytes (Fig. 6C, inset).

4. Discussion

The functions of many key actin binding proteins are regulated by phosphorylation. In this study, we set out to investigate whether EVL-I was a target for regulation by PKD based on bioinformatics prediction of a candidate phosphorylation site within the alternately-included, “I” sequence.

4.1. Phosphorylation site location

EVL-I is a splice variant that only differs from EVL by an exon that encodes a 21 amino acid insertion in the EVH2 domain of EVL-I. PKD phosphorylates EVL-I at a single site within the “I” sequence, Ser-345: mutation of this site completely abrogated PKD mediated phosphorylation. The specific phosphorylation of EVL-I (and not of EVL) could provide a means for differential regulation of EVL-I and EVL. Moreover, the phosphorylation of EVL-I on a site inserted in the EVH2 domain, a domain that contains binding sites for F- and G- actin [74], could potentially affect the interaction of EVL-I with the actin cytoskeleton. Interestingly, phosphorylation of VASP within its EVH2 domain impairs its binding to F- and G- actin and inhibits the F-actin bundling and anti-capping activities of VASP [75,76].

4.2. Complex formation of EVL-I and PKD

Purified PKD and EVL-I can bind directly *in vitro*, in the absence of ATP. *In vivo*, the formation of a PKD1:EVL-I complex was only observed with a kinase dead PKD1, likely because the interaction between a protein kinase and its substrate is often transient (since substrates are released upon phosphorylation).

4.3. Phosphorylation of EVL-I does not affect filopodia formation or its localization within filopodial tips

Because of the location of the PKD phosphorylation site in an insert in the EVH2 domain of EVL-I, we reasoned that the ability of EVL-I to bind actin and regulate actin dynamics would be affected by phosphorylation of this site. However, there was no obvious difference in localization of either the phosphomimetic EVL-I-Ser345Glu mutant or the non-phosphorylatable EVL-I-Ser345Ala mutant as compared with wild type EVL-I: all three proteins localized to filopodial tips when expressed in the Ena/VASP-deficient MV^{D7} cell line. Furthermore, EVL-I-Ser345Glu was able to support efficient filopodia formation in capping protein-depleted cells, indicating that the EVL-I-Ser345Glu could still bind to and cluster filament barbed ends *in vivo* as these activities are required for Ena/VASP-mediated filopodia formation [77]. Together, these data indicate that phosphorylation of EVL-I by PKD does not affect its localization to filopodial tips or the ability of the protein to support filopodia formation.

4.4. Impairment of EVL-I phosphorylation may increase lamellipodial ruffling

In contrast to the apparent lack of role for phosphorylation of EVL-I in filopodial localization/formation, phosphorylation of EVL-I appeared to affect its localization in lamellipodia.

Phospho-EVL-I localizes in a speckled/punctate pattern in resting cells, decorating actin bundles and lamellipodia. In PDBu stimulated cells, phospho-EVL-I appeared to be more enriched in lamellipodia than in resting cells, though this may arise as a secondary consequence of increased lamellipodial activity in the stimulated cells. The nature of the speckles/punctate pattern is unclear at present, but the comparison of the localization pattern of total EVL-I and phospho-EVL-I indicates that phosphorylated EVL-I is recruited to only a subset of the distribution of EVL-I.

Impairment of EVL-I phosphorylation in the Ser345Ala mutant does not affect lamellipodial leading edge localization of EVL-I, but it appears to increase lamellipodial ruffling; therefore, it is possible that phosphorylation of EVL-I at Ser 345 could act to fine tune lamellipodial dynamics. It is known that phorbol esters promote ruffling in many cell types, and mechanistically this represents the end result of a delicate regulation of several PKC isozymes. However, specific PKC isoenzymes may very well have different roles in the regulation of membrane ruffling. For example, PKC-delta negatively regulates Rac1 induced ruffling [78]. Since several PKC-isoforms, including PKC-delta, are known as upstream activators of PKD, this regulation of Rac1 induced ruffling may involve EVL-I phosphorylation by PKD.

4.5. EVL-I is phosphorylated and located in cell–cell contacts of some breast cancer cell types

EVL is known to localize mainly to actin cytoskeleton of filopodia, lamellipodia, focal adhesions, and cell–cell contacts. In contrast very little is known about the specific localization of EVL-I. Lambrechts et al. [14] found that, upon expression in Rat-2 cells, EVL-I was localized in focal adhesions and at the end of stress fibers. We found that phosphorylated EVL-I is located in cell–cell contacts in a subset of breast cancer cell lines.

The functional impact of this phosphorylation of EVL-I in these cell–cell contacts is at present unknown. To characterize the localization of this phospho-EVL-I, we co-stained these cells with anti-phospho-EVL-I and phalloidin (to label F-actin) and found that phosphorylated EVL-I localizes to cell–cell contacts in a patched pattern. This could suggest a role for EVL-I phosphorylation in the regulation of actin dynamics in cell–cell contacts. The fact that phosphorylated EVL-I accumulates in areas rich in ZO-1, suggests that EVL-I may be a component of tight junctions. Tight junctions may have an important role in breast cancer: accumulating data indicating that the loss of tight junctional proteins (or their function) is correlated with a more malignant and less differentiated nature of certain breast cancers [79–82]. Interestingly, EVL is highly upregulated in estrogen receptor-positive breast cancers [83]; thus it is possible that EVL-I plays a role in the maintenance of the more differentiated status of estrogen receptor-positive breast cancers. Furthermore Ena/VASP proteins also regulate endothelial cells by modulating their tight junctions [84–86]. Interestingly, the same phospho-EVL-I was found in the cell–cell contacts of the keratinocytes in the basal layer of neonatal mouse skin. It is therefore possible that phosphoregulation of EVL-I could play an important role in regulating barrier function in epithelial cells.

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Fig. 1.

In vitro and *in vivo* phosphorylation of EVL-I by PKD. Panel A: MBP (Myelin Basic Protein, a positive control for phosphorylation by PKD) or bacterially produced His-EVL-I wt, His-EVL-I Ser345Ala or His-EVL-I Ser345Glu was incubated with [γ - 32 P]ATP with and without GST-PKD, followed by SDS-PAGE and autoradiography. To the right: alignment of known PKD phosphorylation sites. Numbers between brackets indicate the amino acid numbers of the phosphorylated residues in the respective proteins. Panel B: Left: the extent of incorporation of phosphate in EVL-I by PKD was measured for the indicated incubation times via SDS-PAGE followed by scintillation counting of the excised bands. Right: Kinetic parameters for the phosphorylation of EVL-I by PKD were deduced from a Lineweaver–Burk plot of $1/v$ versus $1/[S]$. Panel C: 293T cells were transfected with myc-tagged EVL-I wt, EVL-I Ser345Ala or EVL-I Ser345Glu together with PKD1 wild type (wt), PKD1 kinase dead (kd) or PKD1 constitutively active (ca). Cell lysates were analyzed via Western blotting with anti-phospho EVL-I antibody (P-EVL-I) or anti-myc antibody. The faint band in lane 3 is due to background phosphorylation by the low level of endogenous PKD1 present in 293T cells.

Fig. 2.

Interaction of EVL-I with PKD. Panel A: GST-PKD1 or GST was incubated with HIS-tagged EVL-I. Glutathione beads were added, and the GST-pulldowns were analyzed via SDS-PAGE and Western blotting with anti-EVL antibody. Panel B: Cells were transfected with control vector, myc-EVL-I wt, PKD1 kd, or both (as indicated above the blots of panel B). The corresponding lysates were immunoprecipitated with anti-myc antibody or anti-PKD antibody (as indicated below the blots of panel B). Immunoprecipitates were then analyzed via SDS-PAGE and Western blotting with anti-PKD antibody (upper blot) or with anti-myc antibody (lower blot).

Fig. 3.

Effect of EVL-I phosphorylation on filopodia formation. MV^{D7} cells were stably transfected with EGFP-EVL, EGFP-EVL-I Ser345Glu, EGFP-EVL-I Ser345Ala or EGFP-EVL-I. CP β was then knocked down, and cells were plated on laminin coated coverslips. The percentage of cells having no filopodia (black), few filopodia (dark grey) or a lot of filopodia (light grey) is indicated by the bars. Results were analyzed with a χ^2 -test, giving a χ^2 -value of 3.94 and a two-tailed P -value of 0.55, indicating that there are no statistically significant differences for filopodia formation among MV^{D7} EGFP-EVL-I and MV^{D7} EGFP-EVL-I-Ser345Ala or MV^{D7} EGFP-EVL-I-Ser345Glu.

Fig. 4.

Filopodial tip localization of EVL-I is unaffected by phosphorylation by PKD. Panel A: MV^{D7} cells were stably transfected with EGFP-EVL-I and were plated on laminin, and visualized with phalloidin staining (red), EGFP fluorescence (blue) and anti-phospho-EVL-I antibody (P-EVL-I, green). Panel B: MV^{D7} cells were stably transfected with EGFP-EVL-I Ser345Ala or EGFP-EVL-I Ser345Glu and were plated on laminin, and visualized with phalloidin staining (red) or EGFP fluorescence (green). Panel C: Expression of EGFP-EVL-I wt, EGFP-EVL-I Ser345Ala and EGFP-EVL-I Ser345Glu was verified via Western blotting with anti-EGFP-antibody. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 5.

Impairment of EVL-I phosphorylation of EVL-I increases PDBu induced ruffling of lamellipodia. Part A: MV^{D7} cells stably transfected with EGFP-EVL-I wt were plated on fibronectin coated coverslips, receiving either no stimulation (row 1 and row 2 for higher magnification), or 400 nM PDBu (row 3 and row 4 for higher magnification). Cells were visualized via phalloidin staining for the actin cytoskeleton (red, A–D), EGFP fluorescence for the localization of EGFP-EVL-I wt (blue, E–H) and anti-phospho-EVL-I antibody for the localization of the *phosphorylated* subfraction of EGFP-EVL-I wt (P-EVL-I, green, I–L). The far right column shows the overlay of all three images (overlay, M–P). Part B: MV^{D7} cells stably transfected with EGFP-EVL-I Ser345Ala were plated on fibronectin coated coverslips, receiving either no stimulation (row 1 and row 2 for higher magnification), or 400 nM PDBu (row 3 and row 4 for higher magnification). Cells were visualized via phalloidin staining for the actin cytoskeleton (red, A–D), or EGFP fluorescence for the localization of EGFP-EVL-I Ser345Ala (green, E–H). The far right column shows the overlay of both images (overlay, I–L). Part C: MV^{D7} cells stably transfected with EGFP-EVL-I Ser345Glu were plated on fibronectin coated coverslips, receiving either no stimulation (row 1 and row 2 for higher magnification), or 400 nM PDBu (row 3 and row 4 for higher magnification). Cells were visualized via phalloidin staining for the actin cytoskeleton (red, A–D), or EGFP fluorescence for the localization of EGFP-EVL-I Ser345Glu (green, E–H). The far right column shows the overlay of both images (overlay, I–L). Note: EGFP signals are green in B and C, but blue in A. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 6.

EVL-I is strongly phosphorylated in cell–cell contacts of several breast cancer cell lines and mouse embryo keratinocytes. Panel A: Lysates of breast cancer cells (SS: serum starved, 10%: treated with 10% fetal bovine serum) were separated on SDS-PAGE and Western blotted with anti active PKD antibodies (detecting activation loop phosphorylation), anti-phospho EVL-I (P-EVL-I) or tubulin (loading control). Panel B: Upper row: SKBr3 breast cancer cells were stained with phalloidin (red) or with anti-phospho EVL-I (P-EVL-I, green). Lower row: SKBr3 cells were stained with anti-phospho EVL-I (P-EVL-I, red) or anti-ZO-1 antibodies (green). Cell–cell contacts are shown in higher magnification in the insets. Panel C: Mouse embryo skin was stained with anti-phospho EVL-I. A higher magnification of the area indicated by the arrow is shown in the inset. (For interpretation of

the references to colour in this figure legend, the reader is referred to the web version of this article.)