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PDZ domain from Dishevelled — a specificity study

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Intracellular signaling cascades induced by Wnt proteins play a key role in developmental processes and are implicated in cancerogenesis. It is still unclear how the cell determines which of the three possible Wnt response mechanisms should be activated, but the decision process is most likely dependent on Dishevelled proteins. Dishevelled family members interact with many diverse targets, however, molecular mechanisms underlying these binding events have not been comprehensively described so far. Here, we investigated the specificity of the PDZ domain from human Dishevelled-2 using C-terminal phage display, which led us to identification of a leucine-rich binding motif strongly resembling the consensus sequence of a nuclear export signal. PDZ interactions with several peptide and protein motifs (including the nuclear export signal sequence from Dishevelled-2 protein) were investigated in detail using fluorescence spectroscopy, mutational analysis and immunoenzymatic assays. The experiments showed that the PDZ domain can bind the nuclear export signal sequence of the Dishevelled-2 protein. Since the intracellular localization of Dishevelled is governed by nuclear localization and nuclear export signal sequences, it is possible that the intramolecular interaction between PDZ domain and the export signal could modulate the balance between nuclear and cytoplasmic pool of the Dishevelled protein. Such a regulatory mechanism would be of utmost importance for the differential activation of Wnt signaling cascades, leading to selective promotion of the nucleus-dependent Wnt β-catenin pathway at the expense of non-canonical Wnt signaling.

Keywords: Dishevelled, Cl2FlAsH-EDT$_2$, NES, nuclear export signal, PDZ, Wnt

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INTRODUCTION

Wnt signaling is a complex assembly of three transduction pathways induced by Wnt glycoproteins (Willert *et al.*, 2003) that regulate proliferation, apoptosis and cell fate determination (Logan & Nusse, 2004; Malbon, 2005). The activation of Wnt receptor proteins leads either to the canonical/β-catenin signaling or to one of the non-canonical cascades, i.e., planar cell polarity (PCP) or Ca^{2+}/cyclic guanosine monophosphate (cGMP) pathway. The activation of different downstream effectors in these signaling cascades results in distinct cellular responses ranging from the activation of genes involved in development to the regulation of planar cell polarity and cell movements (Logan & Nusse, 2004; Malbon, 2005).

It is still unclear what mechanism governs the activation of the correct Wnt signaling branch. Dishevelled (Dvl) proteins, the last shared link of the canonical and non-canonical pathways, most likely play a key regulatory role in the signal distribution process (Habas & Dawid, 2005; Itoh *et al.*, 2005; Leonard & Ettensohn, 2007). This notion is further supported by the fact that the multitude of Dvl binding partners includes proteins that relay signaling to β-catenin pathways (axin (Li *et al.*, 1999; Wharton, 2003), GBP/Frat (Li *et al.*, 1999)), PCP (Rac1 (Fanto *et al.*, 2000), Daam1 (Habas *et al.*, 2001), strabismus (Bastock *et al.*, 2003)) and Ca^{2+} ($G\alpha_o$/$G\alpha_t$ (Liu *et al.*, 1999)). Dvl can bind several cellular partners simultaneously *via* its three protein–protein interaction domains, i.e., DIX (Dishevelled and Axin), PDZ (PSD-95, Dlg, ZO-1) and DEP (Dishevelled, Egl-10, Pleckstrin). Out of these, the PDZ module can interact with the highest number of different ligands (see Table 1). This versatility creates vast regulatory possibilities and suggests the presence of a control mechanism involving interactions of Dvl PDZ domain with partner proteins (supported by functional studies (Strutt *et al.*, 2006; Wu *et al.*, 2008)).

Despite the multitude of identified protein binders, the specificity of Dvl PDZ domain remains elusive. For most natural ligands the exact interacting sequence fragment has not been identified due to the applied methodology (mainly two-hybrid screens and co-immunoprecipitation) (Peters *et al.*, 1999; Willert *et al.*, 1997; Bastock *et al.*, 2003; Ossipova *et al.*, 2007; Strovel *et al.*, 2000; Jenny *et al.*, 2005; Li *et al.*, 1999; Habas *et al.*, 2001; Inobe *et al.*, 1999; Warner *et al.*, 2005). Moreover, in several binding events the PDZ domain was proven important but not sufficient for both biochemical and functional interaction to occur (Chen *et al.*, 2003; Tanaka *et al.*, 2003; Kinoshita *et al.*, 2003; Miyakoshi *et al.*, 2004). This motivated us to investigate the specificity of this module employing C-terminal phage display (CTPD), a combinatorial method designed and successfully applied to determining the binding preferences of various PDZ domains (Fuh *et al.*, 2000; Laura *et al.*, 2002; Zhang *et al.*, 2006; Vaccaro *et al.*, 2001; van den Berk *et al.*, 2007; Runyon *et al.*, 2007; Tonikian *et al.*, 2007; van den Berk *et al.*, 2005; Zhang *et al.*, 2007; Smietana *et al.*, 2008).

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Abbreviations: CTPD, C-terminal phage display; DEP, Dishevelled, Egl-10, Pleckstrin; DIX, Dishevelled and Axin; Dvl, Dishevelled; Dns, dansyl moiety; Cl2FlAsH-EDT$_2$, nonfluorescent biarsenical probed capped with 1,2-ethanedithiol; Cl2FlAsH-TC, complex of tetracysteine motif with Cl2FlAsH moiety; NES, nuclear export signal; NLS, nuclear localization signal; PDZ, PSD-95, Dlg and ZO-1; PDZ^{Dvl}, PDZ domain from human Dishevelled-2 protein

Table 1. Biological partners of PDZ domain from Dishevelled

Partner name	Function[1]	Reference
Casein Kinase I family proteins Casein Kinase II family proteins	Positive regulators of Wnt signaling	Peters *et al.*, 1999 Willert *et al.*, 1997
Daam1 (Dishevelled-associated activator of morphogenesis 1)	Mediates Dvl-Rho complex formation	Habas *et al.*, 2001
Daple (Dvl-associating protein with a high frequency of leucine residues)	Positive regulator of the Wnt/β-catenin pathway	Oshita *et al.*, 2003
Dapper/Frodo	Dvl antagonist	Cheyette *et al.*, 2002
Diego	Effector in planar polarization pathway	Cheyette *et al.*, 2002
Eps8	Actin cytoskeleton regulator	Cheyette *et al.*, 2002
GBP/Frat (GSK3-binding protein)	Blocks β-catenin degradation by inactivating GSK3	Li *et al.*, 1999
Idax (Inhibitor of the Dvl and Axin complex)	Wnt signaling inhibitor	London *et al.*, 2004
Naked cuticle	Dvl inducible antagonist	Rousset *et al.*, 2001
Notch	Receptor initiating Notch signaling pathway	Axelrod *et al.*, 1996
Par1 (Partitioning-defective 1 protein)	Serine/threonine protein kinase implicated in the establishment of cell polarity	Ossipova *et al.*, 2007
Prikle	Wnt pathway modulator	Tree *et al.*, 2002
PTP 2Cα (Protein Phosphatase 2Cα)	Phosphatase (dephosphorylates axin)	Strovel *et al.*, 2000
Smad3	Transcription factor	Warner *et al.*, 2005
Strabismus	Wnt pathway modulator	Bastock *et al.*, 2003

[1]Not exhaustive

MATERIALS AND METHODS

Materials and reagents. *Escherichia coli* XL1-Blue, *E. coli* BL21-RIL, and VCSM13 helper phage were from Stratagene. *E. coli* ER2738 strain was from New England Biolabs. Gateway cloning system was from Invitrogen. The coding sequence of human Dvl2 protein was obtained from ImaGenes GmbH. Maxisorp 96-well immunoplates were from Nalge NUNC International. Horseradish peroxidase/anti-M13 antibody conjugate was from Amersham Biosciences, mouse anti-glutathione S-transferase (GST) antibody was from Sigma Chemical company and alkaline phosphatase/anti-mouse IgG antibody conjugate was from AnaSpec. The TEV (tobacco etch virus) protease expression plasmid was kindly provided by J. A. Doudna (Lucast *et al.*, 2001).

Synthetic peptides. Peptides used in binding assays were synthesized by Pepscan Presto BV. Synthetic crudes were purified on a reversed-phase C-18 Vydac column (218TP510) with a linear gradient (1 ml/min, 0.1% TFA – 90% acetonitrile/H_2O, v/v), converted to acetate forms and lyophilized. The peptides were dissolved in water and their concentration was estimated from either molar absorption coefficient of dansyl group at 334 nm or of the aromatic residues in the sequence. Peptide identities and purity were verified by ESI-MS.

Cloning, mutagenesis, expression and purification of protein. Nucleotide sequences encoding the PDZ domain (residues 264–353) (PDZ^{Dvl}) and DEP domain followed by NES-containing linker sequence (residues 434–526) (DEP-NES) of human Dvl2 protein were amplified by two-step PCR and introduced into the entry vector pDONR-201 (Invitrogen) together with TEV protease recognition site. Correct clones, verified by restriction site mapping and sequencing analysis, were used for recombination into the expression vector pDEST15 (Invitrogen). DEP-NES alanine-substitution variants, truncated DEP construct and PDZ^{Dvl} elongated with C-terminal CCPGCC sequence (tetracysteine tag — TC) for labeling with biarsenical probe (Cl2FlAsH-EDT_2) were obtained using QuikChange™ site-directed mutagenesis protocol (Stratagene) and mutagenic oligonucleotides (Sigma).

PDZ^{Dvl} and DEP-NES constructs were expressed in fusion with 26 kDa glutathione *S*-transferase (GST) in the BL21(DE3)-RIL *E. coli* strain. Cells were grown at 37 °C in LB medium with 100 μg/ml ampicillin to OD_{600} of 0.8–1.0 and induced with 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG). Protein expression continued for 16 h at 20 °C. Cells were harvested by centrifugation, resuspended in lysis buffer (50 mM Tris/HCl, 300 mM NaCl, pH 8.0) and lysed by sonication. N-terminally tagged proteins were purified using glutathione-Sepharose 4B (GE Healthcare). In the case of DEP-NES constructs, the proteins were dialysed against the lysis buffer and concentrated. Their purification was concluded at this point, resulting in a homogeneity level sufficient for immunoenzymatic assays. The PDZ^{Dvl} and PDZ^{Dvl}-CCPGCC proteins were separated from the GST tag by TEV protease cleavage and ion exchange chromatography on HiTrap Q Sepharose HP (GE Healthcare) was employed to remove uncut full-length fusion protein and GST. High molecular mass impurities and TEV protease were removed by gel filtration chromatography on Superdex 75 (HR 10/30; GE Healthcare) equilibrated with the lysis buffer. The protein was estimated by SDS/PAGE to be at least 95% pure. All purified Dvl fragments contain additional Gly-Ala-Ser residues at the N-terminus, which originate from the TEV recognition sequence.

C-terminal phage display. A random hexapeptide library was fused to the C-terminus of a modified M13 major phage coat protein using a linker sequence designed by Sidhu and coworkers (Fuh & Sidhu, 2000), as described (Smietana *et al.*, 2008). Panning experiments were performed in Tris-buffered saline (TBS; 50 mM Tris/HCl, 150 mM NaCl, pH 7.4) containing 0.1% BSA (bovine serum albumin) on a homogenous PDZ^{Dvl} protein (10 μg) immobilized on 96-well Maxisorp immunoplates. BSA was used as a blocking agent, wells were washed 10 times with TBS containing 0.05% Tween 20,

and bound phage were eluted at pH 2.2. Eluted phages were propagated in *E. coli* ER2738 with VCSM13 helper phage (without IPTG) and isolated by double precipitation with 20% polyethylene glycol 8000 in 15% NaCl. Enrichment ratio was monitored after each round of panning as the proportion of phage titers of phage bound to PDZ-coated well to those eluted from BSA-coated control well. The enrichment ratio during the panning was 1.5× in the first round, and 3.875×, 10.64× and 19× in the second, third and fourth round, respectively. The content of different amino-acid residues in the pool of selected peptide sequences was calculated and compared with the bias resulting from the usage of an NNS randomization scheme to assess the reliability of experiment results.

Binding assays. Individual phage clones specifically interacting with the target protein were identified in phage ELISA, as described (Smietana *et al.*, 2008). Nine phage clones characterized by highest signal/control ratio (out of 96 clones examined) were chosen for further characterization. ELISA and dot-blot experiments were used to verify the interaction between PDZ^{Dvl} and GST-DEP-NES constructs. For ELISA and dot-blot, PDZ^{Dvl} protein was immobilized on 96-well Maxisorp immunoplate or PVDF microporous membrane (pore size of 0.2 μm) (Roche), respectively. Both experiments were performed in TBS at room temperature. Immobilized BSA was used as a negative control, and GST as a positive one. After blocking with 1% BSA, GST-DEP-peptide solutions were added to the protein-coated wells/dots. Subsequently, mouse anti-GST antibody and alkaline phosphatase/anti-mouse IgG antibody conjugate were added. Nonspecifically bound proteins were removed after each step by washing 5 times with TBS containing 0.05% Tween 20. The interaction was detected using pNPP (*p*-nitrophenyl phosphate) and TMB (3,3',5,5'-tetramethylbenzidine) substrates (Sigma).

Fluorescence binding measurements. Changes in the fluorescence of 1 μM buffered peptide solution upon titration with concentrated PDZ^{Dvl} protein were monitored to determine the equilibrium dissociation constants (K_d) of the interactions between synthetic peptides and the PDZ domain (Harris *et al.*, 2001). To increase solubility and avoid precipitation, the highly hydrophobic CTPD-derived sequences were elongated with an EESG tetrapeptide at the N-terminus. Such an addition had been shown earlier not to influence the PDZ-peptide binding (Smietana *et al.*, 2008). Experiments were performed at 21 °C in near-physiological conditions (25 mM Tris/HCl, 150 mM NaCl, pH 7.5) using an FP-750 spectrofluorimeter (Jasco) equipped with an ETC 272T Peltier accessory at the following conditions: excitation wavelength of 334 nm for N-terminally dansylated peptides (295 nm in the case of EESGVSDVVW, where Trp fluorescence was monitored), emission wavelength of 540 nm for Dns (361 nm for Trp), excitation and emission slit widths of 5 nm. Data were collected at medium sensitivity and averaged for 500 seconds for each data point. Three measurements were conducted for each PDZ-peptide combination. After the initial fit using the equation:

$$y = F_0 + ((F_{max} - F_0) \times c / K_d) / (1 + c / K_d)$$

(where y is the fluorescence signal, c is the concentration of protein, K_d is the dissociation constant, F_0 is the initial fluorescence value, and F_{max} is the maximal fluorescence value at saturation) (Harris *et al.*, 2001) data were normalized and a nonlinear regression analysis including an F-test comparison was performed to calculate K_d, as previously described (Smietana *et al.*, 2008).

FRET experiments. $Cl2FlAsH\text{-}EDT_2$ (4',5'-bis(1,-2,3-dithioarsolan-2-yl)-2',7'-dichlorofluorescein-(1,2-ethanedithiol)$_2$) was synthesized according to recently published protocol with slight modifications (Pomorski *et al.*, 2010). Cl2FlAsH-labeled PDZ^{Dvl}-CCPGCC protein at 0.5 μM was titrated with N-terminally dansylated peptides corresponding to the Dvl NES signal sequence and NES with the three leucine residues substituted with alanines. Experiments were performed at 21 °C in 25 mM Tris/HCl, 150 mM NaCl, pH 7.5 using an FP-750 spectrofluorimeter (Jasco) equipped with an ETC 272T Peltier accessory. Data were recorded at emission wavelength range of 450–650 nm at medium sensitivity, using excitation wavelength of 334 nm (Dns excitation wavelength), and excitation and emission slit widths of 5 nm. Dns group when excited at 334 nm displays a broad emission spectrum with a maximum at 552 nm. The Cl-2FlAsH–TC–PDZ complex (fluorescent biarsenical complex of PDZ tagged with TC motif) emits light with a maximum at 539 nm when excited at 520 nm (which is within the Dns emission spectrum), and does not emit at all when excited at 334 nm. FRET from Dns-peptide to Cl2FlAsH-TC-PDZ complex upon PDZ-peptide interaction results in maximum emission wavelength shift from 552 nm towards 539 nm. Maximum fluorescence transfer was observed at the emission wavelength of 547 nm (fluorescence of the Dns-peptide solution alone was subtracted to compensate for the overlap in Dns and Cl2FlAsH–TC complex emission spectra). The control measurements were done in the same manner as described above, except the protein solution was replaced with Tris buffer (25 mM Tris/HCl, 150 mM NaCl, pH 7.5). Data analysis was performed as described above.

RESULTS AND DISCUSSION

We used C-terminal phage display library selection to define PDZ^{Dvl} binding specificity profile and identify hexapeptides interacting with this domain. The phages were propagated in the absence of IPTG to obtain medium peptide display level and allow the selection of a wider range of interacting sequences. Gradual increase of the enrichment ratio was observed with a maximum at 4-th round of panning. Individual phage clones from this round were propagated and tested for the PDZ affinity and specificity in a phage ELISA experiment. Variants characterized by the highest signal-to-control ratios were sequenced, providing an array of Dvl-binding peptides (Table 2).

The sequences were rather diverse, which is not unusual for PDZ binding profiles (Vaccaro *et al.*, 2001; van den Berk *et al.*, 2005; Runyon *et al.*, 2007) and reflects high sequence variability observed among binding sequences of Dvl cellular ligands. The exact amino-acid sequence recognized by PDZ^{Dvl} has not been identified for many of Dvl natural partner proteins. The best characterized PDZ^{Dvl} ligands represent several classes of PDZ binding sequences according to the most common classification method (Harris & Lim, 2001; Jelen *et al.*, 2003). Class I, characterized by a hydrophobic residue in the C-terminal position (P0) and serine or threonine as the second residue from P0 (P-2), is represented in

Table 2. Hexapeptide sequences selected on PDZ^{Dvl} from C-terminal phage library

Sequence[1]						Specificity class[2]	ELISA signal/ control ratio
P-5	P-4	P-3	P-2	P-1	P0		
L	L	H	L	R	L	class II	2.89
L	A	L	F	L	A	class II	3.74
L	R	Q	V	W	L	class II	3.5
P	L	A	L	L	H	class IV-like	3.14
Q	L	G	L	N	P	class II	3.89
T	N	F	M	L	P	class II	3.69
T	W	L	G	Q	G	class II-like	3.56
S	P	L	T	S	M	class I	2.53
V	S	D	V	V	W	class II	3.73

[1]P-5 to P0 indicate distance from peptide C-terminus. [2]Specificity classes according to the most common classification (Harris & Lim, 2001; Jelen *et al.*, 2003).

the selection results by -SPLTSM peptide, resembling the -KLMTTV Dapper/Frodo-derived Dvl-binding fragment (Cheyette *et al.*, 2002). One peptide similar to class IV (charged amino acid in P0 and aromatic in P-1) was also selected (-PLALLH), however, it differs from the Dvl-interacting class IV Notch C-terminus (-IPEAFK) (Munoz-Descalzo *et al.*, 2010). Class II peptides, with hydrophobic amino-acid residues in both P0 and P-2 were the most prevalent in the CTPD experiment results.

Since the selected peptide variants were clearly dominated by class II sequences, three of the CTPD-derived peptides belonging to this group (VSDVVW, LLHLRL, LALFLA) were synthesized and assayed for PDZ^{Dvl} binding. They interacted with the PDZ domain with similar micromolar K_ds (Table 3, Fig. 1), confirming the reliability of the phage display selection. Such affinities are in the range of typical PDZ-ligand interactions (Jelen *et al.*, 2003). Other reports confirm that PDZ^{Dvl} readily interacts with class II sequences characterized by high hydrophobicity (Zhang *et al.*, 2009; Lee *et al.*, 2009). The best interacting peptide (VSDVVW) bears similarity to the Dvl internal binding motif from Frizzled and Idax proteins, KTXXXW/I (London *et al.*, 2004; Wong *et al.*, 2003), suggesting a close resemblance between the determinants of C-terminal and internal motif recognition in the case of PDZ^{Dvl}. This notion is supported by the recent study describing the identification of N- and C-terminal PDZ^{Dvl}-binding peptides, where similarities can be observed within the consensus sequences obtained by selection from different phage display libraries (Zhang *et al.*, 2009). Our selection resulted in a more diverse set of peptide variants, however, several sequences observed in our study are similar to the ones obtained by Zhang and others, and the general characteristics of the selected peptides is similar between the two studies (Zhang *et al.*, 2009).

Interestingly, the selected sequences were generally characterized by high hydrophobicity and high leucine content. Several peptide variants bore resemblance to the NES consensus LXXLXL (LLHLRL, LALFLA, LRQVWL, PLALLH, QLGLNP), which led us to hypothesize that PDZ^{Dvl} may specifically recognize and bind NES sequences in target proteins. This notion is further supported by the Dvl ability to interact with internal protein sequences (e.g., the -KTXXXW/I-motif in Frizzled (Wong *et al.*, 2003) and Idax (London *et al.*, 2004), and unidentified fragments of strabismus (Bastock *et al.*, 2003) and Smad3 proteins (Warner *et al.*, 2005)), similarly to the typical intraprotein NES localization. This notion is further supported by N-terminal phage display studies showing that PDZ^{Dvl}-binding sequences are not limited to C-terminal peptides (Zhang *et al.*, 2009).

Recent studies suggest that the direction of Wnt signal can be influenced by modulating Dvl intracellular localization (Habas & Dawid, 2005; Itoh *et al.*, 2005; Leonard & Ettensohn, 2007). The presence of both nuclear localization signal (NLS) and nuclear export signal (NES) in Dishevelled sequence enables the protein to be transported into and out of the nucleus, and nuclear localization of Dvl is critical for the β-catenin pathway but is not required for non-canonical Wnt signaling (Itoh *et al.*, 2005). It seems feasible that changes in the cellular Dvl distribution are crucial for the type of the cellular response elicited by Wnt stimulation, however, no mechanism of Dvl nuclear shuttling regulation has been identified to date. An intramolecular PDZ–NES interaction, modulated by the presence of other PDZ^{Dvl} ligands, could trigger Dvl transition from an "open" to a "closed" conformation, causing the signal sequence to become inaccessible. This would promote the canonical, nucleus-dependent Wnt signaling pathway, and down-regulate PCP and Ca^{2+} cascades. Since Dishevelled proteins have been shown to form homo- and heterodimers through DIX–DIX interaction (Kishida *et al.*, 1999; Zhang *et al.*, 2000), it is likely that in dimeric Dvl the binding between the PDZ module of one protein and the NES motif present in the second Dvl molecule could be achieved more easily. Moreover, the PDZ-NES binding could influence dimer formation, acting as another interaction interface in addition to DIX domains.

Table 3. Dissociation constants for PDZ-peptide complexes.
Each constant was calculated based on data sets from three independent measurements, analyzed as described in Materials and Methods section.

Peptide sequence	Source	K_d [mM]	Type of measurement
EESGVSDVVW	CTPD experiment	0.78 ± 0.03	Fluorescence saturation
Dns-EESGLLHLRL	CTPD experiment	1.04 ± 0.07	Fluorescence saturation
Dns-EESGLALFLA	CTPD experiment	5.36 ± 0.21	Fluorescence saturation
Dns-ESYLVNLSLNDND	Dvl NES motif	4.39 ± 0.23	Fluorescence saturation
		3.55 ± 0.31	FRET[1]
Dns-ESYLVNASANDND	Dvl NES motif with two Leu residues substituted by Ala	no interaction	Fluorescence saturation and FRET[1]

[1]FRET between Dns moiety of the peptide and Cl2FlAsH moiety from PDZ (Pomorski *et al.*, 2010)

Fluorescence binding experiments with a synthetic peptide correspond-

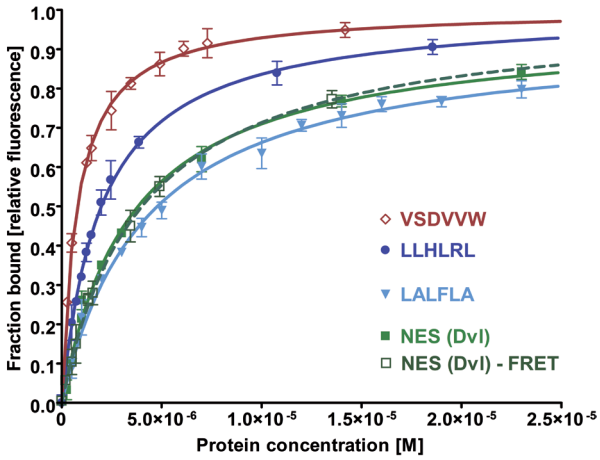

Figure 1. Binding curves for interactions between representative peptide variants and PDZ^{Dvl}
Changes in relative fluorescence of a peptide were monitored upon the addition of increasing amounts of concentrated protein. Fluorescence data representing single measurements were normalized and presented as the fraction of bound peptide *versus* protein concentration.

ing to the Dvl2 fragment including NES sequence in the middle (to obtain "internal" binding motif, ESYLVNLSLNDND) and a control peptide (NES motif with two leucine residues in positions corresponding to P0 and P-2 substituted by alanines, ESYLVNASANDND, to exclude the possibility of C-terminal binding) were performed to address the possibility of a PDZ–NES interaction. Although the available crystal (Cheyette *et al.*, 2002) and solution (Wong *et al.*, 2003) structures of Dvl PDZ domain show that the binding pocket is large allowing internal protein fragments to be easily accommodated, it has not yet been determined whether C-terminal and intraprotein sequences are bound with comparable affinities. The affinity of NES "internal" peptide for the PDZ domain was very close to those of the "NES-like" selected peptides (LLHLRL and LALFLA) (Table 3, Fig. 1). The fact that these three peptides share only the leucine arrangement stands in agreement with the bias towards leucines observed in the selection experiment, suggesting that these residues play a crucial role for the whole set of PDZ^{Dvl}–peptide interactions. The lack of binding of the NES alanine-substituted peptide supports the importance of leucine residues and confirms that the NES peptide–PDZ interaction is mediated by an internal sequence instead of a canonical C-terminal binding. The NES peptide–PDZ binding was additionally confirmed using an innovative fluorescence method involving labeling of tetracysteine sequence, CCPGCC, genetically fused to the C-terminal part of the PDZ^{Dvl} protein with biarsenical probes (Adams *et al.*, 2002). Those probes, practically nonluminescent, become highly fluorescent upon formation of a stable complex with the tetracysteine motif (Griffin *et al.*, 1998). This labeling method has many advantages comparing to single cysteine residue labeling with a typical fluorescent sensor. One of them is selective recognition of the tetracysteine sequence and being nonluminescent when a single residue or other cysteine sequences are present (Pomorski & Krężel, 2011). The sterically stable hairpin complex can easily be used for FRET studies (Spagnuolo *et al.*, 2006; Madani *et al.*, 2009). In this case we used the recently synthesized and characterized Cl2FlAsH-EDT$_2$ probe that selectively bound to the tetracysteine tag emitting light at 539 nm when excited at 520 nm (Pomorski *et al.*, 2010). A peptide modified with the Dns moiety when excited at 334 nm displays emission maximum at 552 nm and 547 nm in the case of TC-complex. FRET was observed between the Dns moiety bound to the NES peptide and the Cl2FlAsH-EDT$_2$ probe attached to PDZ^{Dvl}–CCPGCC, and the K_d value calculated based on this experiment was very similar to the one obtained from fluorescence saturation binding studies (Table 3, Fig. 1).

PDZ domains interact only with short peptide fragments of the ligand protein and thus PDZ–peptide assays are often assumed a good approximation of a protein–protein binding event (Kang *et al.*, 2003; Appleton *et al.*, 2006; Jelen *et al.*, 2003; Lee *et al.*, 2009). Nevertheless, the protein–protein interaction was additionally confirmed using several Dvl2 protein constructs. To avoid dimerization *via* other interaction domains or motifs in the Dvl2 sequence, a fragment including only the DEP domain followed by a short linker, NES motif and second linker (ensuring internal binding mode) was chosen. Six mutants were constructed: the GST-DEP construct with a stop codon inserted before the NES sequence to investigate the possibility of a PDZ–DEP

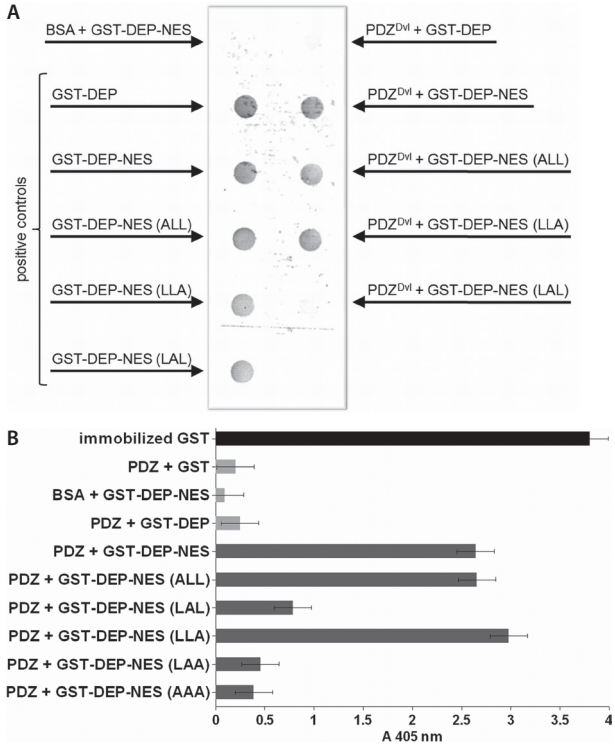

Figure 2. Binding of GST-DEP-NES protein constructs to PDZ^{Dvl}
(A) Dot-blot experiment. Top arrows represent negative controls (GST-DEP-NES binding to uncoated membrane and interaction between the PDZ domain and GST-DEP), five arrows on the bottom left are positive controls (immobilized GST-DEP, GST-DEP-NES and its alanine mutants, proving correct GST recognition by the antibody used in experiments), four arrows on the bottom right show interactions between PDZ^{Dvl} and GST-DEP-NES and its single Leu-to-Ala substitutions. **(B)** ELISA assay. Black bar represents positive control (immobilized GST), light gray are negative controls (excluding the interaction between PDZ and GST or GST-DEP and GST-DEP-NES-binding to PDZ-uncoated well). The binding of GST-DEP-NES and its single and multiple substitution mutants to PDZ^{Dvl} is shown in dark gray. The experiment was done in triplicate.

interaction, and leucine to alanine substitutions (three single mutants: ALL, LAL, LLA, one double (LAA) and one triple (AAA)) to assess the importance of particular leucines for the binding affinity. The binding between PDZ^{Dvl} and the GST-DEP-NES protein construct was confirmed using ELISA and dot blot methods (Fig. 2). The truncated GST-DEP fragment did not interact with the PDZ domain. The substitution of the first or third leucine in the NES motif did not affect binding significantly, while the removal of the second leucine had a significant effect, which is consistent with its role as a P-2 position residue crucial for ligand binding in a canonical model of PDZ interactions. Double and triple NES mutants did not interact with the PDZ domain.

In cellular conditions the PDZ–NES interaction may prevent the exposition of the nuclear export signal, thus promoting Dvl retention inside the nucleus. This, in turn, could influence the Wnt response, shifting the equilibrium towards the β-catenin pathway. Also, the enhanced Dvl dimer formation might have some effect on the protein function, although significant impact seems unlikely (Zhang *et al.*, 2000). In abundance of other Dvl PDZ binding partners, the NES motif would be released making the protein more susceptible to nuclear export and resulting in an increased induction of PCP and/or calcium signaling cascade. So far, our understanding of the Wnt signaling network is very limited, and we are still discovering new possible regulatory mechanisms (Yokoyama *et al.*, 2007; Chan *et al.*, 2008; Wu *et al.*, 2008) and links to other cellular processes and pathways (Luyten *et al.*, 2008) to broaden our comprehension of the molecular basis of development.

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