Protease-activated Receptor-1 (PAR1) Acts via a Novel Gα₁₃–Dishevelled Axis to Stabilize β-Catenin Levels*

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We have previously shown a novel link between hPar-1 (human protease-activated receptor-1) and β-catenin stabilization. Although it is well recognized that Wnt signaling leads to β-catenin accumulation, the role of PAR1 in the process is unknown. We provide here evidence that PAR1 induces β-catenin stabilization independent of Wnt, Fz (Frizzled), and the coreceptor LRP5/6 (low density lipoprotein-related protein 5/6) and identify selective mediators of the PAR1–β-catenin axis. Immunohistological analyses of hPar1-transgenic (TG) mouse mammary tissues show the expression of both Gα₁₂ and Gα₁₃ compared with age-matched control counterparts. However, only Gα₁₃ was found to be actively involved in PAR1-induced β-catenin stabilization. Indeed, a dominant negative form of Gα₁₃ inhibited both PAR1-induced Matrigel invasion and Lef/Tcf (lymphoid enhancer factor/T cell factor) transcription activity. PAR1–Gα₁₃ association is followed by the recruitment of DVL (Dishevelled), an upstream Wnt signaling protein via the DIX domain. Small interfering RNA-Dvl silencing leads to a reduction in PAR1-induced Matrigel invasion, inhibition of Lef/Tcf transcription activity, and decreased β-catenin accumulation. It is of note that PAR1 also promotes the binding of β-arrestin-2 to DVL, suggesting a role for β-arrestin-2 in PAR1-induced DVL phosphorylation dynamics. Although infection of small interfering RNA-LRP5/6 or the use of the Wnt antagonists, SFRP2 (soluble Frizzled-related protein 2) or SFRP5 potently reduced Wnt3a-mediated β-catenin accumulation, no effect was observed on PAR1-induced β-catenin stabilization. Collectively, our data show that PAR1 mediates β-catenin stabilization independent of Wnt. We propose here a novel cascade of PAR1-induced Gα₁₃–Dvl axis in cancer and β-catenin stabilization.

PAR1 (protease-activated receptor-1) is the first identified and prototype member of an established protease-activated receptor family. In addition to the traditional role of PAR1 in thrombosis, hemostasis, and vascular biology, its role in tumor biology is currently emerging (1–3). We have established a novel link between hPar1 and β-catenin stabilization, both in transgenic mouse mammary glands and in a wide spectrum of tumor cell lines (4). Protease-activated receptors are part of a large seven-transmembrane-spanning G protein-coupled receptor (GPCR) family (5, 6), shown to couple to Gα₁₂, Gα₁₃, or Gα₁₂/1₃ within the same cell type (7). Of the 16 Gα genes found in the mammalian genome, the Gα₁₂ subfamily is of particular interest to cancer biologists. Gα₁₂ and its sister family member, Gα₁₃, are the only heterotrimeric G proteins that are capable of transforming fibroblasts when overexpressed in their wild-type (WT) form (8–10). Recent studies have demonstrated that Gα₁₃ is markedly up-regulated in adenocarcinoma of the breast and have identified Gα₁₃ protein as an important regulator of breast and prostate cancer invasion (11, 12). The Gα₁₂ protein subunit also plays a role in disrupting cadherin–β-catenin interaction and in the down-regulation of the extracellular cell-cell adhesive function of cadherins (13).

Although an association between PAR1 and β-catenin stabilization has been demonstrated (4), the path connecting PAR1 to β-catenin is unknown. Here we set out to elucidate the molecular mechanism and identify selective partners that mediate this association. β-Catenin accumulation is a well-established core process of the Wnt signaling pathway. Wnt proteins are secreted glycoprotein ligands that bind to members of the Fz (Frizzled) class of heptahelical GPCR and low density lipoprotein receptor-related protein (LRP5/6) (14, 15), known to regulate cell fate and play a central role in both embryonic development (16) and oncogenicity (17, 18). The canonical Wnt signaling pathway has been shown to require Gα₁₂ and Gα₁₃, which centrally regulate the stability of intracellular β-catenin (19–21). In the absence of Wnt signaling, a complex of proteins comprising the scaffold protein axin, the APC (adenomatous polyposis coli) tumor suppressor protein, GSK-3β (glycogen synthase kinase 3β), and CK1 (casein kinase I) assemble to comprise the destruction machinery of β-catenin. Once the complex is assembled, GSK-3β phosphorylates β-catenin that is now being tagged for ubiquitination and degradation by the proteasomal system (22, 23). Upon Wnt activation, the targeting is eliminated, and hence, β-catenin is no longer detected for degradation. Accumulation of β-catenin in the cytoplasm further leads to its translocation to the nucleus, where it acts as a co-transcription factor with Lef/Tcf (lymphoid enhancer factor/T-cell factor family) and activates wnt target genes downstream (22, 23). DVL (Dishevelled) is an upstream Wnt signaling protein that mediates all known Wnt pathways, including the canonical, non-canonical, and planar cell polarity pathways

1 The abbreviations used are: GPCR, G protein-coupled receptor; WT, wild type; MEF, mouse embryo fibroblast; MMTV, murine mammary tumor virus; siRNA, small interfering RNA; DN, dominant negative; IP, immunoprecipitation; LPA, lysophosphatidic acid; TG, transgenic.

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(24–26). Three DVL isoforms are found in mammalian species (e.g. DVL1 to -3) (27, 28, 29). High DVL1 protein expression is detected in breast and cervical squamous cell carcinoma as well as in prostate cancer (30, 31). Similarly, elicited levels of DVL3 have been demonstrated in malignant mesotheliomas and lung cancer (32, 33). DVL is composed of several structural domains: a DIX binding domain located at the N terminus, a PDZ domain in the midregion, and a DEP domain located about midway between the PDZ domain and the C terminus of DVL (34, 35). Although the DIX domain enables the dimerization of DVL with other members of the family as well as with axin (36), the PDZ domain provides a docking site for the C terminus of Fz receptors (37). The linker between DIX and PDZ provides an essential phosphorylation site for CKI (38). The DEP domain regulates Rho GTPases in the planar polarity pathway (39) and is also involved in Fz-induced translocation of DVL1 to the plasma membrane in the canonical pathway (40). Upon Wnt signaling, Fz, together with its co-receptor LRP5/6, recruits DVL via the PDZ domain, leaving the DIX domain free to bind axin-GSK3β proteins (41).

In the present study, we identified the selective involvement of Gα13 and DIX-DVL as mediators of PAR1-induced β-catenin stabilization. Our data point to a novel Gα13-DVL axis in cancer, independent (at least upstream of DVL) of Wnt-elicited β-catenin phosphorylation site for CKI (38). The DEP domain regulates Rho GTPases in the planar polarity pathway (39) and is also involved in Fz-induced translocation of DVL1 to the plasma membrane in the canonical pathway (40). Upon Wnt signaling, Fz, together with its co-receptor LRP5/6, recruits DVL via the PDZ domain, leaving the DIX domain free to bind axin-GSK3β proteins (41).

In the present study, we identified the selective involvement of Gα13 and DIX-DVL as mediators of PAR1-induced β-catenin stabilization. Our data point to a novel Gα13-DVL axis in cancer, independent (at least upstream of DVL) of Wnt-elicited β-catenin stabilization.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Reagents**—The cDNA encoding Gα12L, Gα13L, and Gαq, constructs, WT, and constitutively active and dominant negative forms were prepared as previously described (42). cDNAs encoding mouse FLAG-Dvl1, FLAG-ΔDIX-Dvl1, FLAG-ΔPDx-Dvl1, and FLAG-human β-catenin were kindly provided by Dr. Y. Ben-Neria (The Hebrew University, Jerusalem, Israel). cDNAs encoding mouse FLAG-Dvl1/DIX or FLAG-Dvl1/ΔPDx were kindly provided by Lin Li (Chinese Academy of Sciences, Shanghai, China). β-Arestrin-2 was kindly provided by Dr. R. Seger (The Weizmann Institute, Rehovot, Israel). The PAR-1 agonist peptides, SFLRN and TFLLPNPDK, were obtained from PEPTRID 2.0 Inc. (Chantilly, VA). Thrombin was obtained from OMRIX Bio Pharmaceutical (Ramat Gan, Israel). U-73122, a known inhibitor of PLC-β, a component of the Gαq pathway, and Y-27632, an inhibitor of Rho-kinase downstream of Gαq, were obtained from Calbiochem (Darmstadt, Germany). The PAR-1 antagonist, SCH79797, was obtained from Tocris Bioscience (Ellisville, MO).

**Cells**—HCT116, HT-29, RKO, and HEK-293 cells as well as mouse embryob fibroblasts (MEFs) (Gα12L−/−/Gα13L−/− knock-out or WT cells), were grown in 10% fetal calf serum-Dulbecco’s modified Eagle’s medium. The medium was supplemented with 50 units/ml penicillin and streptomycin (Invitrogen) and maintained in a humidified incubator with 8% CO2 at 37 °C.

**Mammot-Hpar1-TG Mice**—Transgenic (TG) mammary-directed hPar1 mice were generated as previously described (4). Briefly, the MMTV-hPar1 construct (full-length hPar1 gene from pcDNA3, subcloned into MMTV-SV40-BSSK, subsequent to MMTV-LTR) was used to generate CB6/F1 mice targeted to overexpress hPar1 in the mammary glands. Ten transgenic mice were obtained and maintained in the Animal Facilities House of Hadassah-Hebrew University Medical School (Jerusalem, Israel).

**PAR1 Activation**—PAR1 is activated by either the SFLRN (H-Ser-Phe-Leu-Leu-Arg-Asn-NH2) peptide, the TFLLPNPDK peptide, a selective PAR1 agonist, or thrombin (1 unit/ml).

**Immunohistological Staining**—Tissue samples derived from mammary glands of either WT or hPar1-TG mice were fixed with 4% formaldehyde in phosphate-buffered saline, embedded in paraffin, and sectioned (3–μm sections). After deparaffinization and rehydration, sections were stained with hematoxylin and eosin or subjected to immunohistochemistry using anti-Gα12 (SC-409 rabbit polyclonal IgG) and anti-Gα13 (SC-410 rabbit polyclonal IgG) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

**Cell Transfections**—Cells grown to 80% confluence were transfected with 0.5–2 μg/ml of plasmid DNA in Fugene 6 transfection reagent (Roche Applied Science) according to the manufacturer’s instructions.

**Lef/Tcf Luciferase Reporter Assay**—HEK-293 cells (0.5 × 10^5) were plated in 12-well plates to form 60–80% confluent cultures. Transfection of Lef/Tcf luciferase reporter plasmids in combination with the relevant assay plasmids (e.g. Gα13LQ, Gα13GA, or siRNA-DVL) was carried out. We used TOPflash (Tcf optimal promoter and luciferase), which contains three optimal copies of the Lef/Tcf binding site, and FOPflash, which contains mutated copies of the Lef/Tcf binding sites upstream of a minimal thymidine kinase promoter directing transcription of a luciferase gene.

**siRNA Constructs and Lentiviral Vector Production**—We used a U6 promoter-driven and lentivirus (pLentilox 3.7)-mediated delivery cassette of siRNA, specific for the target genes DVL1, LRP5, and LRP6, to prepare siRNA constructs. For this, a sequence of 19 nucleotides of the target gene coding region was selected for stem-and-loop oligonucleotide siRNA. Appropriate DNA oligonucleotides were synthesized to generate the hairpin stem-and-loop siRNA expression cassette. The oligonucleotides comprised the following: 19 bases of the target gene coding sequence, the loop sequence linker (9 bases), reverse complement of 19 bases of the target gene coding region, and a terminator sequence poly(T). The sticky end of the XhoI site was added to the antisense strand oligonucleotides. Both sense and antisense sequences were phosphorylated at the 5’-ends. The sense sequence oligonucleotides were annealed to their respective antisense oligonucleotides. siRNA cassette sequences were then ligated into pLentilox 3.7 vector (the Van Parij Laboratory, Massachusetts Institute of Technology, Cambridge, MA). The siRNA sequences for the target genes were as follows: AACAAGATCACCTTCTCCGAG (DVL1), CATGATCGAGTCGTCCAAC (LRP5), and CCGCATGTGTCATTATGTA (LRP6).

**Western Blot Analysis**—Cells were solubilized in lysis buffer containing 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, protease inhibitor mixture, including 5 μg/ml aproriel, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate (Sigma), for 30 min at 4 °C. After centri-

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fugation at 10,000 \times g for 20 min at 4 °C, the supernatants were transferred, and the protein content was measured. Lysates (50 μg) were separated by 10% SDS-PAGE, followed by transfer to Immobilon-P membrane (Millipore, MA). Membranes were blocked and probed with the appropriate antibodies at a concentration of 1 μg/ml. Anti-β-catenin, anti-GAK3β (BD Transduction Laboratories), anti-phospho-GSK3β (Cell Signaling), anti-Myc, anti-Gα13, or anti-Gα13 antibody (Santa Cruz Biotechnology, Inc.), or anti-FLAG (Sigma) was suspended in 3% bovine serum albumin in 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.1% Tween 20. After washes, blots were incubated with secondary antibodies conjugated to horseradish peroxidase. Immunoreactive bands were detected by the enhanced chemiluminescence reagent (Pierce).

Nuclear Extract—Cells were solubilized in lysis buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, and 1 mM dithiothreitol), a protease inhibitor mixture (1:100), 1 mM phenethylsulfonyl fluoride, and 1 mM sodium orthovanadate (Sigma) for 15 min at 4 °C. After adding 10% Nonidet P-40 solution, centrifugation at 10,000 \times g was performed. The pellet was incubated on ice with buffer C (20 mM HEPES, pH 7.9, 420 mM KCl, 1 mM EDTA and 1 mM dithiothreitol), a protease inhibitor mixture, 1 mM phenethylsulfonyl fluoride, and 1 mM sodium orthovanadate. After centrifugation at 12,000 \times g for 15 min at 4 °C, the supernatants were collected, and the protein content was evaluated.

Membrane and Cytoplasmic Extracts—Cells were solubilized in lysis buffer containing 10 mM Tris-HCl, pH 8.2, 2.5 mM MgCl2, 5 mM KCl, 1 mM dithiothreitol, protease inhibitor mixture (1:100), 1 mM phenethylsulfonyl fluoride, and 1 mM sodium orthovanadate (Sigma) for 30 min at 4 °C. After centrifugation at 12,000 \times g, the supernatant containing the cytoplasmic fraction was collected, and the pellet was resuspended with 1% Triton X-100, 150 mM NaCl, and 50 mM Tris acetate, pH 8.2. The supernatants obtained after centrifugation at 12,000 \times g contained the membrane fraction.

Immunoprecipitation—Protein cell lysates (400 μg) were used for immunoprecipitation analysis. Mouse anti-Gα13, anti-Gα13, or anti-FLAG antibodies were added to the cell lysates. After overnight incubation, protein A-Sepharose beads were added to the suspension, which was subsequently rotated at 4 °C for 1 h. Elution of the reactive proteins was performed by resuspending the beads in protein sample buffer, followed by boiling for 5 min. The supernatant was then resolved on a 10% SDS-polyacrylamide gel and treated as indicated above for Western blotting.

Fluorescent Immunostaining—Either HCT-116 colon cancer or HEK-293 cells were plated on fibronectin (5 μg/ml)-coated coverslips and transfected with Gα13 and FLAG-Dvl1. After overnight serum deprivation (0.2% bovine serum albumin), the cells were activated by thrombin for various periods of time. Samples were then fixed with 4% paraformaldehyde in phosphate-buffered saline containing 5% sucrose for 10 min and permeabilized with 0.5% Triton X-100 in phosphate-buffered saline for 3 min. Samples were fixed and stained with anti-FLAG antibodies (Sigma). Cy3-conjugated anti-mouse IgG (Jackson Immunoresearch Laboratories) was used as a secondary antibody. Cells were examined by confocal microscopy (Zeiss laser confocal microscope, model 410).

Matrigel Invasion Assay—Transwell Boyden chemotaxis chambers and 13-mm diameter filters were used for this assay. Polyvinylpyrrolidone-free polycarbonate filters, 8-mm pore size (Costar Scientific Co., Cambridge, MA), were coated with basement membrane Matrigel (200 ng/ml/g filter), as described earlier (43). Briefly, cells (2 \times 105) were applied to the upper chamber compartment. Conditioned medium from 3T3 fibroblasts was used as a chemoattractant and placed in the lower compartment. At the end of the incubation period, the cells on the upper surface of the filter were removed by wiping with a cotton swab. Filters were fixed, stained with the DiQuick System (Dade Behring Inc., Newark, NY) and counted to determine the number of cells on the lower surface of the filter.

Statistical Analysis of the Data—The data are presented as means ± S.D. of ≥3–5 separate experiments carried out in triplicate. Statistical analysis was performed using paired Student’s t test (two-tailed). A p value of <0.05 was considered significant.

RESULTS

Gα1,3 but Not Gα12,13 Is Involved in PAR1-mediated β-Catenin Stabilization—We have recently established a novel link between PAR1 and β-catenin stabilization, shown both in TG mouse mammary gland tissues and transformed epithelial cells (4). Because only 1 of 15 breast cancer cell lines analyzed showed Tcf-mediated β-catenin transcription activity (44), we chose colorectal cancer lines, widely used for studies of the Wnt/β-catenin pathway. It was demonstrated that in both HCT116 colon cancer (e.g. of mutated β-catenin at residue 45) and HT-29 cell lines (e.g. of truncated APC), the activation of PAR1 leads to marked nuclear β-catenin localization (4). We show in HT-29 cells that marked nuclear β-catenin levels are observed following pretreatment (e.g. 2 h) with LiCl (a known glycogen synthethase kinase 3β inhibitor) and activation of PAR1 (Fig. 1ai). This was visualized by fluorescent image detection of the tissue sections grown on glass slides as well as Western blot analysis (Fig. 1aii). Similar results were obtained in RKO cells, a colorectal cell line transformed on the background of microsatellite instability, leading to hypermethylation of the hMLH1 promoter but with intact β-catenin machinery (Fig. 1b). RKO cells express WT APC, intact β-catenin, and p53 (45, 46). We demonstrate that activation of PAR1 elicits β-catenin stabilization in RKO cells, regardless of whether they have been pretreated with LiCl or MG132 (an inhibitor of the proteasomal system, as shown in Ref. 4).

Although Fz receptors are members of the GPCR superfAMILY that couples via Gαq, the canonical β-catenin pathway, the path of PAR1-induced β-catenin stabilization is unknown. We set out to analyze the association of discrete Gα13 family members with PAR1-induced β-catenin stabilization. For this purpose, we immunostained tissue sections of TG-hPar1 mouse mammary glands (exhibiting elevated levels of β-catenin) (4) and compared them with WT tissues. Massive staining of Gα13, as well as Gα12, is observed in the TG mice compared with WT age-matched tissue sections (Fig. 1c). Thus, both Gα12/13 family members are abundantly overexpressed in the hPar1-TG tissue.
**Gα\textsubscript{13} and DVL Regulate PAR1-induced β-Catenin Stabilization**

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\begin{array}{c|c|c|c|c|c}
\text{LiCl} & - & - & + & + & + \\
\text{SFLRN} & - & + & - & - & - \\
\hline
\end{array}
\]

\text{Bar=25μm}

*bi.*

\text{Thrombin (1U/ml), Time (h):} \quad - \quad 4h \quad 5h \quad - \quad 4h \quad 5h

\text{LiCl} \quad - \quad + \quad + \quad + \quad + \\
\text{β-catenin} \quad + \quad - \quad - \quad + \quad + \\
\text{lamin} \quad + \quad - \quad - \quad + \quad + \\

\text{Bar=50μm}

_sues._ In order to dissect the relative involvement of Gα\textsubscript{12} and/or Gα\textsubscript{13} components, constitutively active and dominant negative (DN) forms (47) as well as WT Gα\textsubscript{13} and Gα\textsubscript{12} constructs were utilized. We transiently transfected HEK-293 or HCT-116 colon cancer cells (data not shown) with FLAG-β-catenin and constitutively active Gα\textsubscript{12}QL, Gα\textsubscript{13}QL mutants. After activation of PAR1, cell lysates were prepared, and the immunoblots were further evaluated by applying anti-FLAG (for detecting levels of β-catenin) or anti-Gα\textsubscript{12} and -Gα\textsubscript{13} antibodies for the detection of G-protein levels. We found that overexpression of constitutively active Gα\textsubscript{13} as well as Gα\textsubscript{q} (data not shown) leads to PAR1-induced β-catenin stabilization (Fig. 2a).

The increased expression of Gα\textsubscript{13}GA inhibited Gα\textsubscript{13}QL-mediated β-catenin stabilization in a dose-dependent manner (Fig. 2b). When a similar approach was taken for Gα\textsubscript{12}, no effect on PAR1-induced β-catenin levels was observed (Fig. 2c). Furthermore, upon increasing concentrations of the DN Gα\textsubscript{12} applied to the system (e.g. cells expressing active Gα\textsubscript{13}; Gα\textsubscript{13}QL), no effect on β-catenin levels was observed. Although Gα\textsubscript{13}QL potently induced β-catenin stabilization, the DN Gα\textsubscript{13}GA construct inhibited the induction of β-catenin levels by Gα\textsubscript{13}QL (Fig. 2c, lane 7), but when transfected alone, it had no effect on β-catenin levels. We thus conclude that there is no overlap in PAR1-induced β-catenin stabilization with regard to the part played by Gα\textsubscript{12} and Gα\textsubscript{13}. The central participation of Gα\textsubscript{13} is also demonstrated following co-transfection with both increasing concentrations of the DN form and a constant concentration of the constitutively active Gα\textsubscript{13}. Moreover, when MEFs derived from Gα\textsubscript{12-/-}/Gα\textsubscript{13-/-} mice were analyzed for PAR1-enhanced β-catenin levels, no induction was seen. This stands in contrast to the outcome obtained following the activation of WT MEFs, where a markedly increased level of β-catenin was observed (Fig. 2d). Altogether, these data point to the involvement of Gα\textsubscript{13} in PAR1-induced β-catenin stabilization. In addition, in the presence of U73122, a selective Gq inhibitor (known to inhibit PLC-β, a component of the Gq pathway), no effect on PAR1-induced β-catenin stabilization was observed. This inhibitor is effective when Wnt3A is added to initiate β-catenin stabilization. In contrast, an effective dose-dependent inhibition was seen in the presence of Y-27662, which inhibits Rho-kinase downstream of Gα\textsubscript{12/13} (Fig. 3a). No effect on Wnt3A-induced β-catenin levels was observed in the presence of Y-27662 inhibitor (Fig. 3b). The functional outcome of Gα\textsubscript{13} in PAR1-induced β-catenin stabilization was demonstrated by the Lef/Tcf luciferase assay for β-catenin transcription activity. As shown in Fig. 4a, elicited Lef/Tcf promoter activity was seen following PAR1 activation. Low levels of luciferase promoter activity are observed with a DN form of Gα\textsubscript{13}, compared with a markedly induced level in the presence of the constitutively active Gα\textsubscript{13} construct. The system is active in the presence of a TOPflash promoter compared with a control FOPflash promoter, exhibiting base-line activity of the system.
**Gα₁₃ and DVL Regulate PAR1-induced β-Catenin Stabilization**

To underscore the contribution of Gα₁₃ in PAR1 signaling, we transiently transfected HEK-293 cells with the DN Gα₁₃GA construct and analyzed its impact on the ability of the cells to invade Matrigel-coated filters following PAR1 activation. Although activation of PAR1 enhanced cell invasion, strong inhibition was observed in the presence of a DN form of Gα₁₃ (Fig. 4b).

**Gα₁₃ Specifically Interacts with DVL following PAR1 Activation**—In canonical Wnt signaling, the DVL protein is considered to be a critical cytoplasmic intersection of the Wnt signal traffic. It is regarded as a transducer of Fz receptors following activation by Wnts and ultimately releases β-catenin from the on-going, active degradation process that takes place under normal conditions. We analyzed the specific interaction between Gα₁₃ and DVL following PAR1 activation. For this purpose, we co-transfected HEK-293 cells with both FLAG-Dvl and WT Gα₁₃ plasmids. After PAR1 activation and immunoprecipitation, a specific and transient interaction between Gα₁₃ and DVL was observed. Maximal interaction was seen at 15 min after PAR1 activation and was markedly decreased by 30 min and terminated after 1 h of PAR1 activation (Fig. 5, a and b). The interaction between Gα₁₃ and DVL was highly dependent on PAR1 activation because SCH77977, a selective PAR1 antagonist, blocked PAR1-induced association between Gα₁₃ and DVL (Fig. 5b). Furthermore, activation of PAR1 (thrombin, 1 unit/ml) leads to phosphorylation of the DVL1 protein, as shown by the mobility shift of DVL separated on SDS-PAGE (Fig. 5c). The first visible signal of DVL phosphorylation was obtained at 0.5 h of PAR1 activation and remained elevated up to 3 h. This effect is similarly observed after Wnt3A treatment (for 2 h; Fig. 5c).

Cellular localization of DVL was further examined following ectopic introduction into HCT-116 colon cancer cells of both FLAG-Dvl and Gα₁₃ constructs. Although initially abundant levels of DVL are observed within the cytoplasmic pool of non-treated control cells, a massive relocation of DVL to the cell surface membrane compartment was seen as soon as 15 min after PAR1 activation. DVL relocation is also noticed 30 min after activation, demonstrating a confined localization of DVL to the cell membrane (Fig. 5di). This is in contrast to the predominantly cytoplasmic localization of DVL in the untreated control cells. Translocation of DVL to the membrane fits well with the association between Gα₁₃ and DVL (Fig. 5, a and b), whereby a specific complex is formed 15 min after PAR1 activation. Immunohistological cell distribution of DVL was accompanied by quantitative Western blot analysis of both the membrane and cytoplasmic compartments for DVL (Fig. 5b). A time-dependent increase in the level of DVL1 protein, as shown by the mobility shift of DVL separated on SDS-PAGE (Fig. 5c). The first visible signal of DVL phosphorylation was obtained at 0.5 h of PAR1 activation and remained elevated up to 3 h. This effect is similarly observed after Wnt3A treatment (for 2 h; Fig. 5c).

**FIGURE 2. Gα₁₃, but not Gα₁₃QL, is involved in PAR1-mediated β-catenin stabilization**. a, level of β-catenin. HEK-293 cells were transiently transfected with FLAG-β-catenin and either empty vector or constitutively active (Gα₁₃QL and Gα₁₃QL) plasmids. Following TFLRNDK PAR1 activation, immunobots were analyzed by using anti-FLAG (for FLAG-β-catenin) or anti-β-actin antibodies. Gα₁₃, but not Gα₁₃QL, potently induced β-catenin stabilization. b, constitutively active Gα₁₃, induced β-catenin stabilization is inhibited by a DN Gα₁₃GA plasmid. HEK-293 cells were transiently transfected with FLAG-β-catenin and either constitutively active (Gα₁₃QL alone or with DN (Gα₁₃GA) Gα₁₃QL plasmids. The DN Gα₁₃GA form inhibited β-catenin stabilization induced by Gα₁₃QL in a dose-dependent manner. c, the Gα₁₃ constructs, constitutively active (Gα₁₃QL) and DN (Gα₁₃GA), do not affect β-catenin stabilization. HEK-293 cells were transiently transfected with DVL localization of DVL to the cell membrane (Fig. 5b). This is in contrast to the predominantly cytoplasmic localization of DVL in the untreated control cells. Translocation of DVL to the membrane fits well with the association between Gα₁₃ and DVL (Fig. 5, a and b), whereby a specific complex is formed 15 min after PAR1 activation. Immunohistological cell distribution of DVL was accompanied by quantitative Western blot analysis of both the membrane and cytoplasmic compartments for DVL (Fig. 5b). A time-dependent increase in the level of DVL1 protein, as shown by the mobility shift of DVL separated on SDS-PAGE (Fig. 5c). The first visible signal of DVL phosphorylation was obtained at 0.5 h of PAR1 activation and remained elevated up to 3 h. This effect is similarly observed after Wnt3A treatment (for 2 h; Fig. 5c).

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60 min of PAR1 activation. We thus propose that DVL translocates to the cell membrane upon PAR1 activation due to the binding association with activated G\(_{\alpha_{13}}\).

To decisively demonstrate the impact of DVL in PAR1-induced \(\beta\)-catenin stabilization, we prepared a construct expressing siRNA against DVL and inserted it into a lentiviral vector. When HCT-116 colon cancer cells were infected with siRNA-DVL (e.g. directed against DVL1 and DVL3), a strong inhibition of \(\beta\)-catenin stabilization was observed (Fig. 6a). In parallel, a much lesser extent of inhibition was seen in phospho-GSK3\(\beta\), as compared with the otherwise markedly induced \(\beta\)-catenin levels following PAR1 activation (when DVL is well expressed; Fig. 6a). Our siRNA construct of DVL is potent in affecting \(\beta\)-catenin although directed to DVL1 and -3 but not DVL2. These data are in agreement with a previous study (48), which showed that, in fact, each of the DVL isoforms (e.g. 1–3) is capable of potently inhibiting \(\beta\)-catenin stabilization and Lef/Tcf transcriptional activity. Thus, to obtain an inhibitory effect on the \(\beta\)-catenin machinery system, it is sufficient to silence one of the DVL isoforms. The silencing of DVL, which abrogates PAR1-induced \(\beta\)-catenin stabilization as a consequence, clearly points to the fact that DVL is a key determinant linking PAR1 to \(\beta\)-catenin. The importance of DVL1 in PAR1 signaling is highlighted by the decreased PAR1-induced invasion in the presence of silenced siRNA-DVL-infected cells (Fig. 6, ci and cii).

To firmly demonstrate that endogenous DVL is linked with PAR1-induced \(\beta\)-catenin stabilization, we analyzed levels of PAR1-induced TOPflash Lef/Tcf luciferase activity in the presence and absence of DVL-siRNA. Although activation of PAR1 increases Lef/Tcf luciferase activity, under conditions where DVL levels are attenuated, dramatic inhibition is observed when a comparison is made with control cells expressing DVL and FOPflash control luciferase activity (Fig. 6a). Collectively, in the presence of siRNA-knocked down DVL, PAR1-induced \(\beta\)-catenin levels, Matrigel invasion, and Lef/Tcf \(\beta\)-catenin transcription activities are significantly inhibited.

The DVL protein comprises an N-terminal DIX domain, a central PDZ domain, and a C-terminal DEP domain (see scheme in Fig. 7a). We wished to identify the interacting region responsible for the association between DVL and G\(_{\alpha_{13}}\). We performed co-immunoprecipitation (co-IP) analyses of cell lysates expressing the Dvl constructs of either deleted domains of DIX- or PDZ-plasmids (deleted for DIX or for PDZ) or the WT Dvl form. In parallel, we performed IP analyses utilizing constructs exclusively exhibiting each of the three distinct domains. Transfection with FLAG-WT Dvl, FLAG-Dvl-DIX, Myc-Dvl-PDZ, or Myc-Dvl-DEP,
**Gα<sub>13</sub> and DVL Regulate PAR1-induced β-Catenin Stabilization**

![Diagram](https://via.placeholder.com/150)

**a.** Time (min): 5, 15, 30, 60
- Thrombin
- DVL
- IP: flg-DVL

**b.** Thrombin, Time (min): +, −
- G<sub>α13</sub>
- IP: G<sub>α13</sub>

**c.** Time (h): 0.5, 1, 3, 2
- Wnt 3A
- Thrombin 1U/ml
- G<sup>C</sup>/H9251
- G<sub>α13</sub>
- flg-DVL
- β-actin

**dii.** Membrane
- Thrombin, Time (min): −, −, +, +
- flg-DVL1
- G<sub>α13</sub>

**diii.** Cytoplasm
- Thrombin, Time (min): −, +, +, +
- flg-DVL1
- β-actin

**FIGURE 5. Gα<sub>13</sub> specifically interacts with DVL following PAR1 activation.** a. Activation of PAR1 induces Gα<sub>13</sub>-DVL interactions. HEK-293 cells were transiently transfected with FLAG-Dvl and WT Gα<sub>13</sub>. Immunoprecipitation analysis using anti-DVL antibodies was performed after PAR1 activation. Specific interaction between DVL and Gα<sub>13</sub> is observed, reaching a maximum by 15 min of PAR1 activation. b. Interaction between Gα<sub>13</sub> and DVL is inhibited by PAR1-specific antagonist. HEK-293 cells were transiently transfected with FLAG-Dvl and WT Gα<sub>13</sub>. After PAR1 activation, SCH79797 immunoprecipitation by anti-Gα<sub>13</sub> antibodies was performed in the presence or absence of a potent PAR1 antagonist. Specific association between DVL and Gα<sub>13</sub> was observed. Maximal interaction was observed 15 min following PAR1 activation. Detection using anti-DVL antibodies showed a potent inhibition of the association between DVL and Gα<sub>13</sub> by the PAR1 antagonist. c. PAR1 activation induces DVL phosphorylation. The phosphorylation state of the DVL1 isoform was detected as a phosphorylation-dependent mobility shift of the DVL1 isoform. HEK-293 cells were transfected with FLAG-Dvl1 and either activated (e.g. thrombin) for the indicated periods of time or treated with Wnt3A (e.g. conditioned medium, 2 h). The levels of the phosphorylated form of DVL1 were increased after 0.5 h of PAR1 activation. d. Cytosolic DVL1 is transferred to the plasma membrane following PAR1 activation. HCT-116 colon cancer cells were transfected with both FLAG-Dvl1 and WT Gα<sub>13</sub>, followed by thrombin treatment for 15 and 30 min. Detection was carried out using anti-FLAG antibodies and fluorescent Cy3-conjugated anti-mouse antibodies. The abundant DVL present in the cytoplasm is relocated to the cell membrane following 15 min of stimulation. d. DVL-Western blot analysis of membrane and cytoplasmic fractions. Cells were transiently transfected with FLAG-Dvl1 and WT Gα<sub>13</sub>. Both nuclear and cytoplasmic fractions were prepared after PAR1 activation, and immunoblots were analyzed using anti-FLAG (for DVL). A pattern of increased levels of DVL in the membrane fraction is observed following PAR1 activation. Accordingly, reduced levels in the cytoplasmic DVL are seen with maximal reduction at 60 min.

Along with the constitutively active Gα<sub>13</sub> (Gα<sub>13</sub>QL) plasmid, showed the following outcome. Specific interaction of Gα<sub>13</sub>QL was observed with both WT DVL and the DVL-DIX domain but not with either the PDZ or the DEP domain alone (Fig. 7c). The results indicate that activated Gα<sub>13</sub> interacts rather specifically with DVL via the DIX domain. This interaction did not take place in the presence of WT Gα<sub>13</sub> prior to activation (Fig. 6d). In fact, immunoprecipitation analyses utilizing the deleted constructs revealed specific interaction only with the WT DVL form. No interaction was observed when deleted DIX or PDZ domain constructs were employed (Fig. 7b). It is unclear why the PDZ domain-deficient construct did not interact with Gα<sub>13</sub> specifically. It is possible, however, that deleting PDZ leads to interruption of a critical folding site of the protein that is necessary for the appropriate binding of the DIX domain to activated Gα<sub>13</sub>. No specific DVL interaction was observed when the constitutively active Gα<sub>12</sub> (Gα<sub>12</sub>QL) protein was used (Fig. 7b). The above data are supported by results obtained by applying cell lysates on a glutathione S-transferase-DIX column. Upon loading of cell lysates, before and after PAR1 thrombin activation, a marked endogenous WT Gα<sub>13</sub> protein band was seen 15 min after PAR1 activation. This protein band declined dramatically after 30 min and disappeared following 60 min of PAR1 activation (Fig. 7d). Collectively, our data indicate that PAR1 activation leads to a specific Gα<sub>13</sub> association, followed by the binding of the DVL-DIX domain.

**Activation of PAR1 Stimulates β-Arrestin Association with DVL—** A well known role for the β-arrestin isomers 1 and 2 is their function as protein adaptors, mediating the desensitization and internalization of phosphorylated GPCRs (49). Recently, an additional signaling function for β-arrestin has been described in the Wnt pathway (50). DVL interacts with the multifunc-
Go\textsubscript{13} and DVL Regulate PAR1-induced β-Catenin Stabilization

![Diagram of β-catenin stabilization and Matrigel invasion](image_url)

**FIGURE 6.** DVL-siRNA inhibits PAR1-induced β-catenin stabilization, Matrigel invasion, and Lef/Tcf transcription activity. 

- **a.** DVL-siRNA inhibits PAR1-induced β-catenin stabilization. HCT-116 colon cancer cells were infected with either DVL-siRNA lentiviral vector or an empty vector and transfected with the FLAG-β-catenin plasmid. Immunoprecipitation analysis was carried out following 3 or 4 h of PAR1 activation using either anti-FLAG (for FLAG-β-catenin plasmid) or anti-pGSK-3β antibodies. The data obtained show that DVL-siRNA potently inhibits PAR1-induced β-catenin stabilization. The phosphorylation of GSK-3β, a downstream DVL protein that participates in β-catenin degradation, is inhibited to a much lesser extent by DVL-siRNA. 

- **b.** DVL-siRNA inhibits DVL levels as shown by Western blot analysis for protein levels (top) and DVL-RNA levels (bottom). ci, PAR1-induced Matrigel invasion is inhibited by DVL-siRNA. HEK-293 cells infected with DVL-siRNA exhibits markedly reduced Matrigel invasion properties in response to thrombin PAR1 activation. cii, representative histogram summarizes the number of invading cells. The data are representative of five independent experiments. S.D. was determined by Student's t test; **, p < 0.005.

- **d.** Lef/Tcf transcription activity

Knocking Down Cell Surface Co-receptors LRP5/6 or Antagonizing Extracellular Wnt Does Not Affect PAR1-induced β-Catenin Stabilization—The canonical Wnt signaling pathway involves the binding of both Fz and the co-receptor LRP5/6. In order to dissect the role of PAR1 activation from the Wnt pathway, we prepared selective siRNA to LRP5 and LRP6. Cells were infected with both siRNA-LRP5 and -LRP6 and activated with thrombin (5 h) or Wnt3A-conditioned medium (2 h). The prepared immunoblots were further analyzed using both anti-FLAG antibodies to detect FLAG-β-catenin and anti-β-actin for protein loading. The results showed unequivocally that although Wnt3A-induced β-catenin was potently inhibited after the selective knockdown of LRP5 and -6 by siRNA infection, the PAR1-mediated effect was not affected (Fig. 9, ai and ii). RT-PCR analysis for the levels of LRP5/6 before and after siRNA infection shows a marked reduction of their selective targeted kinases as well. It was therefore hypothesized that β-arrestin plays a role in the dynamics of phosphoserine-DVL formation, ultimately affecting β-catenin stabilization. It was also shown that β-arrestin-2 plays a more pronounced part than β-arrestin-1, at least in the response to Wnt3A stimulation. When we overexpressed Go\textsubscript{13Ql} in HEK-293 cells, along with the ectopic expression of FLAG-Dvl and GFP-β-arrestin-2, we found increased association of β-arrestin-2 in the pull-down complex formed between PAR1 and Go\textsubscript{13} following TFFLRNPNDK PAR1 activation. As shown in Fig. 8b, increased levels of β-arrestin-2 were seen with DVL 15 min following PAR1 activation, declining by 30 min and 1 h and completely disappearing by 2 h. Our data thus demonstrate that, similar to Wnt stimulation, the activation of PAR1 and the Go\textsubscript{13}-DVL axis specifically recruits β-arrestin-2 in a time-dependent manner.
PAR1 activation. However, these experiments were conducted for a much longer period of PAR1 stimulation (12 h and beyond), whereas here we evaluated TFLLRNPDNK PAR1 activation for the early 5-h time period. It is possible that long periods of PAR1 stimulation may ultimately also increase Wnt levels, consequently affecting (indirectly) β-catenin stabilization. It should be pointed out that for a shorter time period, we unequivocally observed no effect of SFRP2 and -5 on PAR1-induced β-catenin stabilization. These results underscore the idea that PAR1 acts independently of Wnt. Activation of PAR1 leads to β-catenin stabilization in a manner similar to that of Wnt-induced β-catenin levels. It has previously been shown that Wnt signaling via Goq disrupts GSK3β-axin complexes, thereby stabilizing β-catenin levels (20). We therefore wished to examine whether the same effect appears following PAR1 activation. To this end, we transiently transfected HCT-116 colon cancer cells with the FLAG-axin construct. After PAR1 activation or ectopic application of Wnt3A, immunoprecipitation analyses using anti-FLAG antibodies were performed, and Western blot detection was carried out with anti-GSK3β antibodies. Although GSK3β is notably associated with axin in the untreated control HCT-116 cells, a pronounced reduction in GSK3β association to axin is seen after PAR1 activation (Fig. 9c). This is similar to the effect obtained with Wnt3A. We thus assume that PAR1 leads to downstream β-catenin stabilization by disconnecting GSK3β from the degradation complex, thereby elevating β-catenin levels.

**DISCUSSION**

In the present paper, we identified a novel pathway of PAR1-induced β-catenin stabilization. Although both Gα12/13 G-protein family members are abundantly overexpressed in hPar1-TG mouse mammary glands as compared with age-matched WT counterparts, dissection between the family members demonstrates the selective functional involvement of activated Gα13. DVL, an upstream junction protein of Wnt signaling, is recruited to Gα13. Once associated with activated Gα13, DVL is dislocated from the cytoplasmic pools and becomes connected with the cell membrane compartment.
DVL association with activated G\textsubscript{\(\alpha_{13}\)} is shown to be mediated specifically via the DIX domain. The fact that DVL binds to the activated form of G\textsubscript{\(\alpha_{13}\)} but not to the inactive form suggests a high affinity interaction of DIX-DVL with the GTP-bound form of G\textsubscript{\(\alpha_{13}\)} as to axin. Although we found that PAR1 interacts via G\textsubscript{\(\alpha_{13}\)} or siRNA-LRP5/6 inhibits Wnt3A but not PAR1-induced \(\beta\)-catenin stabilization. HEK-293 cells were transiently transfected with WT G\textsubscript{\(\alpha_{13}\)}, DVL1, and G\textsubscript{\(\alpha_{13}\)}-beta-actin. After PAR1 activation, immunoprecipitation using anti-G\textsubscript{\(\alpha_{13}\)} antibodies was performed. Maximal interaction between G\textsubscript{\(\alpha_{13}\)} and beta-actin is observed by 15 min and declines after 2 h of PAR1 activation.

Other GPCRs have been reported to stimulate \(\beta\)-catenin stabilization and proliferation of colon cancer cells. Most notable of these are the lysophosphatidic acid (LPA) receptors (e.g. LPA\textsubscript{1}, LPA\textsubscript{2}, and LPA\textsubscript{3}) (51) and EP2 receptor activated by the cyclooxygenase (COX-2) proinflammatory metabolite prostaglandin E2 (52, 53). The LPA\textsubscript{2} receptor couples to G\textsubscript{\(\alpha_{12/13}\)} and LPA\textsubscript{3} couples to G\textsubscript{\(\alpha_{12}\)}; both receptors function to trigger massive \(\beta\)-catenin stabilization and cell proliferation via protein kinase C. The signaling route of COX-2 and EP2 is suggested to involve PI3K and the protein kinase Akt, initiated by the direct association of Go\textsubscript{\(\alpha_{12}\)} with the "regulator of G-protein signaling" (RGS) domain of axin. Altogether, these pathways were shown to converge on the Wnt signaling route to induce cytoplasmic \(\beta\)-catenin accumulation, nuclear localization, and enhanced Lef/Tcf transcriptional activities.

Each of the G\textsubscript{\(\alpha_{12/13}\)} subunits exhibits a different path. Knock-out of the G\textsubscript{\(\alpha_{13}\)} gene causes lethality in mouse embryos at midgestation, a phenotype that largely resembles that of the PAR1 knock-out mice (54). In contrast, G\textsubscript{\(\alpha_{12}\)} \(-/-\) mice appear viable and grow normally. In addition, during vascular development, a selective and critical role for G\textsubscript{\(\alpha_{13}\)} in endothelial cells has been demonstrated (55). It therefore appears that Go\textsubscript{\(\alpha_{13}\)} plays a significant role in PAR1-induced developmental processes. It remains to be determined whether a similar role is played by Go\textsubscript{\(\alpha_{13}\)} in PAR1-induced tumor biology. Here, we assign a prime function for Go\textsubscript{\(\alpha_{13}\)} in PAR1-mediated \(\beta\)-catenin stabilization. Recent studies have underscored the role of G\textsubscript{\(\alpha_{13}\)} family signaling in both breast and prostate cancer progression (11, 12). Unexpectedly, we found direct binding of DVL with activated PAR1. Immunoprecipitation was carried out using anti-PAR1 antibodies, and Western blot detection using anti-PAR1 antibodies was performed. Maximal interaction between PAR1 and beta-actin is observed by 15 min and declines after 2 h of PAR1 activation.

**FIGURE 8.** \(\beta\)-arrestin-2 specifically binds DVL following PAR1 activation. a, interaction between Go\textsubscript{\(\alpha_{13}\)}-GTP and \(\beta\)-arrestin-2 is DVL-dependent. HEK-293 cells were transiently transfected with Go\textsubscript{\(\alpha_{13}\)}-GTP and DVL-2 or without FLAG-DVL. After immunoprecipitation with anti-G\textsubscript{\(\alpha_{13}\)} antibodies, detection was performed using anti-FLAG, anti-GFP, or anti-G\textsubscript{\(\alpha_{13}\)} antibodies. Specific immunoprecipitation with \(\beta\)-arrestin-2 and Go\textsubscript{\(\alpha_{13}\)} is elevated in the presence of DVL1. b, Western blot analysis (IB) showing levels of expression of the constructs following transfection: GFP-\(\beta\)-arrestin, FLAG-DVL1, Go\textsubscript{\(\alpha_{13}\)}, and \(\beta\)-arrestin. b. HEK-293 cells were transiently transfected with WT Go\textsubscript{\(\alpha_{13}\)}, DVL1, and GFP-\(\beta\)-arrestin. After PAR1 activation, immunoprecipitation using anti-G\textsubscript{\(\alpha_{13}\)} antibodies was performed. Maximal interaction between Go\textsubscript{\(\alpha_{13}\)} and \(\beta\)-arrestin is observed by 15 min and declines after 2 h of PAR1 activation.

**FIGURE 9.** Inhibition of LRP5/6 or antagonizing extracellular Wnt does not affect PAR1-induced \(\beta\)-catenin stabilization. a, siRNA-LRP5/6 inhibits Wnt3A but not PAR1-induced \(\beta\)-catenin stabilization. HEK-293 cells were transiently transfected with Wnt3A, and PAR1-induced \(\beta\)-catenin stabilization. HEK-293 cells were transiently transfected with FLAG-\(\beta\)-catenin. The cells were PAR1-activated (e.g. TFLRNPNDK, 5 h), followed by application of either SFRP2- or SFRP5-containing conditioned medium (CM). No effect was seen on PAR1-induced \(\beta\)-catenin levels. c, activation of PAR1 reduces the association of GSK-3\(\beta\) with axin in a manner similar to Wnt2A. HCT-116 colon cancer cells were transiently transfected with FLAG-axin and activated for PAR1. Immunoprecipitation was carried out using anti-FLAG antibodies, and Western blot detection using anti-GSK-3\(\beta\) antibodies was employed. A marked reduction in the GSK-3\(\beta\) levels co-immunoprecipitated with axin is seen following the application of Wnt3A. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
induces DVL1 membrane translocation, leading to β-catenin stabilization, recent studies in Drosophila revealed that Wnt signaling transduces its signals via Goα. It was shown that the βy subunits of Goα bind and relocalize DVL to the plasma membrane, whereas the α-subunit of Goα interacts with and recruits axin to the plasma membrane, leading to β-catenin stabilization (57, 58). Although binding of DVL to the C-terminal portion of Fz is mediated via the PDZ domain (37), many other DVL functions are mediated via the DIX domain. The highly conserved DIX domain is located within the N-terminal region of DVL and is essential for its signaling activity (59). Interestingly, only two other proteins contain a close relative of the DIX domain: axin, in its C-terminal domain, as well as a less well known protein, Ccd1, which is found exclusively in vertebrates (60, 61). DVL has been shown to have a strong tendency to form self-associating protein assemblies in the cytoplasm that are visible as puncta and are mediated via the DIX domain. Overall, the DIX domain is well known to mediate oligomerization and binding to axin (36, 62, 63). The phosphorylation of DVL is another critical step in the regulation of DVL-mediated downstream signal transduction. The best studied serine/threonine kinases are casein kinase I ε and δ (CKI ε/δ). CKI ε/δ are positive regulators of the Wnt signaling pathway, phosphorylating DVL in response to Wnt signaling at the SXSS motif, located at sites 139 and 142 of DVL, respectively. In fact, this site is present beyond the DIX domain, residing within the linker region between the DIX and PDZ (e.g. the DIX domain resides between 10–94) (64, 65). Whether activation of PAR1 leads to stimulation of the traditional Wnt-elicited kinases resides between 10–94) (64, 65). Whether activation of PAR1 leads to stimulation of the traditional Wnt-elicited kinases and/or others remains to be determined. We demonstrate that PAR1 activation causes DVL phosphorylation (Fig. 6c). It is therefore postulated that the activation of PAR1 stimulates DVL binding (to Goα) and activation (e.g. serine phosphorylation). It was recently demonstrated that β-arrestin binds DVL in the vicinity of the CKI ε/δ phosphorylation sites following Wnt signaling. Indeed, inhibition of Wnt signaling by CKI inhibitors reduced the binding of β-arrestin to DVL and β-catenin stabilization (50). β-Arrestins are known to regulate GPCR desensitization as well as signaling (49). The phosphorylation of PAR1 is critical for its rapid termination, mediated most likely via GRK4 and GRK5 (GPCR kinases) (66, 67), regulated via β-arrestin-1 (68). We demonstrate here that PAR1 activation also promotes the association of β-arrestin-2 with DVL. We suggest that activation of PAR1 leads to receptor desensitization and internalization mediated via β-arrestin-1 (68), whereas β-arrestin-2 is free to act as a signaling protein that associates with DVL to elicit β-catenin stabilization. It is postulated that β-arrestin identifies phosphorylated (primed) DVL and subsequently recruits other kinases for the completion of DVL activation and dynamics. In this regard, activation of PAR1 resembles DVL activation by Wnt3A stimulation. Furthermore, although we demonstrate that Wnt antagonist SFRPs do not impair PAR1-induced β-catenin stabilization, this does not exclude the possibility that there is cross-talk between GPCRs (e.g. Fz and PAR1) mediated by a junctional-labile cytoplasmic protein. It is possible that PAR1-induced β-catenin levels converge with the traditional canonical Wnt pathway downstream of DVL. One should keep in mind that Wnts act through both Fz and LRP receptors to bring them into proximity (14). It is further believed that Wnt stimulates the β-catenin pathway by relocating axin to the plasma membrane, thereby inactivating its function as a tumor suppressor protein. It is still not clear whether Wnt-induced axin membrane translocation is a prerequisite for its dissociation from the β-catenin degradation complex. In addition, although we still have gaps in our understanding of the β-catenin stabilization pathway, it appears that two separate axes exist: the LRP-axin axis and the Fz-DVL phosphorylation axis. These two branches ultimately converge at a later stage, eventually resulting in β-catenin stabilization and nuclear translocation (69). In the Wnt-induced Fz-LRP6 receptor complex, Fz recruits DVL through the PDZ domain, leaving the DVL-DIX domain accessible for further interactions. DVL-DIX then recruits the axin-GSK3β complex via oligomerization and puncta formation (64), thus promoting GSK3β-induced LRP phosphorylation. Axin is therefore required for LRP6 phosphorylation and activation. Supporting evidence for the distinct routes of axin-LRP and Fz-DVL is provided by several studies showing that truncated LRP5/6 activates β-catenin independently of DVL (14, 15, 70, 71). This suggests that it can recruit axin to the plasma membrane independently of DVL. The relative role of the PAR1 Goα-DVL axis in leading to β-catenin stabilization remains to be evaluated. Whether it can be bypassed via the axin-LRP path, shown to provide a route distinct from the Fz-DVL phosphorylation axis, is yet unknown.

We demonstrated here a new pathway for DVL that associates via the DIX domain with Goα, which is recruited to the C-terminal tail of PAR1. Overall, we propose an original Wnt-independent pathway for the PAR1-induced Goα-DVL axis. This pathway provides new cellular targets central in cancer progression.

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