Direct interaction of Frizzled-1, -2, -4, and -7 with PDZ domains of PSD-95

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Received 4 April 2002; revised 26 April 2002; accepted 30 April 2002

First published online 24 May 2002

Edited by Gianni Cesareni

Abstract In *Drosophila*, the *frizzled* gene plays a critical role in the establishment of tissue polarity, but the function of the Frizzled family of proteins in mammals is largely unknown. Recent evidence suggested that Frizzleds are receptors for the Wnt family of secreted glycoproteins which are involved in cell fate determination. However, it is unclear how Frizzled receptors transduce Wnt signals to intracellular signaling components. Here we show that the mouse Frizzled-1, -2, -4 and -7 can bind to proteins of the PSD-95 family, which are implicated in the assembly and localization of multiprotein signaling complexes in the brain. Moreover, PSD-95 can form a ternary complex with Frizzled-2 and the adenomatous polyposis coli protein, a negative regulator of Wnt signaling, suggesting that members of the PSD-95 family may serve to recruit intracellular signaling molecules of the Wnt/Frizzled pathway into the vicinity of the receptor.

Key words: Frizzled; Wnt; Adenomatous polyposis coli; PSD-95; PDZ interaction

1. Introduction

The Frizzled family of proteins is involved in the control of cell and tissue polarity (reviewed in [1]). The Frizzled pathway is best characterized for the control of hair polarity on the *Drosophila* wing, where it regulates the subcellular localization of its signaling components, and directs the polymerization of the actin cytoskeleton at particular positions on the cell cortex [2–4]. Frizzleds are seven-transmembrane receptors that can recognize Wnt ligands [5,6]. The Wnt signaling pathway directs cell fates during embryogenesis and regulates cell proliferation in adult tissues (reviewed in [7]). In addition, several components of the Wnt signal cascade are implicated in a number of human cancers (reviewed in [8,9]). Central to the Wnt pathway is a multiprotein complex including β-catenin/armadillo, the glycogen synthase kinase-3β (GSK-3β), Axin and adenomatous polyposis coli protein (APC). However, it is still unsolved how this signaling complex is localized to specific subcellular sites, and how it is linked to Frizzled receptors.

Recently, proteins of the PSD-95 family have been proposed to function as scaffolds that assemble signaling complexes at specific sites on the plasma membrane (reviewed in [10,11]). This family includes the PSD-95/SAP90, SAP97/hDlg and Chapsyn-110/PSD-93. They are characterized by the presence of three PDZ domains in the N-terminal half, followed by a SH3 and a guanylate kinase (GK) domain. The PDZ domains bind to a specific C-terminal sequence in interacting proteins. For the first two PDZ domains (PDZ1+2) of PSD-95 family proteins the consensus motif E-S/T-X-V has been identified. For instance, the Kv1.4 Shaker type K+ channel (C-terminal sequence E-T-D-V) and the *N*-methyl-D-aspartic acid receptor NR2 subunits (E-S-D-V) bind to PSD-95 [12–14]. PSD-95 also binds to other transmembrane receptors, signaling proteins and cytoskeletal components, supporting the idea that PSD-95 family proteins function as a scaffold for the assembly of signaling complexes.

In mammals, more than eight Frizzled homologs have been identified [15–17]. They differ in their spatial and temporal expression pattern and in their affinity for different Wnt ligands, indicating diverse functions for the receptors during development and in the adult organism [6,18]. Interestingly, several of the Frizzled proteins share the E-S/T-X-V sequence motif in their C-terminal cytoplasmic tail, suggesting that they can bind to PDZ proteins. Here we show that Frizzled-1, -2, -4, and -7 bind to PSD-95 family proteins via a classical C-terminus/PDZ interaction. Moreover, we demonstrate that Frizzled, PSD-95 and APC can form a ternary complex, providing evidence that PSD-95 may serve to assemble components of the Wnt/Frizzled signaling pathway.

2. Materials and methods

2.1. DNA constructs

For the Frizzled C-terminal fragments used in the yeast two-hybrid assay, the C-terminal sequence of Frizzled was either amplified by PCR (for mouse Frizzled-3 and -8) or complementary oligonucleotides comprising the C-terminal sequence (for mouse Frizzled-1, -2, -4, and -7) were annealed and subcloned into the *Eco*RI sites of pGAD10. The yeast two-hybrid constructs of PSD-95, Chapsyn-110, SAP97, hDlg, and Kv1.4 were described elsewhere [12]. For the construction of Frizzled expression vectors used in the coclustering and coimmunoprecipitation experiments, the cDNA of mouse Frizzled-7 was subcloned into the *Eco*RI and *Bcl*I site of the expression vector GW1-CMV (British Biotechnology). An *Asc*I restriction site at D187 was introduced by site-directed mutagenesis (transformer site-directed mutagenesis kit, Clontech), and a myc-tag was inserted into this site. The mFz7V/A mutant was generated by PCR amplification using mFz7 as template and the following primers: sense 5′-ggactagtccg-tccttgacacgaagc-3′, antisense 5′-ggaattctcatgccgcagtttccccttgctgc-3′. The PCR product was subcloned into the *Eco*RI site of GW1-CMV. Hemagglutinin (HA)-rFz2 was obtained by inserting a HA-tag into the unique *Asc*I site (G193) of rat Frizzled-2 in pCS2+.

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0014-5793/02/$22.00 © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
PII: S0014-5793(02)02831-4

Human APC cDNA was ligated into the *BclI* site of GW1-CMV. The PSD-95 expression construct has been described in [19].

2.2. Yeast two-hybrid assay

Protein interaction assays in the two-hybrid system have been described [12,20]. Positive interactions between two proteins (one fused to the lexA DNA binding domain, the other to the GAL4 activation domain) result in activation of the reporter genes β-galactosidase and HIS3.

2.3. Transfection and immunocytochemistry

COS-7 cells grown to ~80% confluency were transfected using Lipofectamine (Gibco BRL). Two days later, the cells were fixed in 4% formaldehyde in 20 mM phosphate buffer pH 7.4 for 15 min at room temperature. Immunocytochemistry was performed according to standard protocols using phosphate buffered saline pH 7.4 containing 0.1% Triton X-100 and 0.1% bovine serum albumin for all incubation steps. The following antibodies were used according to the manufacturer's recommendation: anti-myc (clone 9E10, Santa Cruz), anti-α-tubulin (B-5-1-2, Sigma), anti-HA (Y11, Santa Cruz), anti-PSD-95 (guinea pig antiserum HM319, described in [12]), anti-APC (Ab-7, Oncogene), and FITC-, Cy3- or Cy5-conjugated anti-mouse, -rabbit or -guinea pig antibody (Jackson Immunoresearch).

2.4. Immunoprecipitation

Two days after transfection, COS cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris pH 7.4, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 1 mM EDTA) containing 2 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin and 1 μg/ml leupeptin for 1 h at 4°C. After centrifugation at 16 000×*g* for 30 min the supernatant was incubated with 20 μl of agarose-coupled myc antibody (clone 9E10, Santa Cruz) for 2 h at 4°C. Immunoprecipitates were washed in RIPA buffer and analyzed by immunoblotting with the following antibodies: anti-myc (9E10, Santa Cruz) and anti-PSD-95 ('CSK') [12,21].

3. Results

3.1. A subset of Frizzled receptors binds to PSD-95 via their C-terminus

We tested in the yeast two-hybrid system whether Frizzled receptors can bind to members of the PSD-95 family via a C-terminus/PDZ interaction. The highly homologous mouse Frizzled-1 and -2 both terminate with the putative PDZ binding sequence E-T-T-V at their cytoplasmic C-terminal end. Similarly, the C-terminal sequences for mouse Frizzled-4 and -7 (E-T-V-V and E-T-A-V, respectively) are also putative binding motifs for PSD-95 and related proteins. The PDZ2 domain of PSD-95 bound strongly and the PDZ1 domain bound weakly to the C-termini of Frizzled-1, -2, -4, and -7, as assessed semi-quantitatively by yeast two-hybrid assay (Fig. 1). The combined PDZ1 and PDZ2 domains (PDZ1+2) bound more strongly than either repeat alone. The strength of this interaction in the yeast two-hybrid system was similar to the binding of the Shaker-type K^+ channel Kv1.4 to the PDZ1+2 domains of PSD-95. No interaction was observed between PSD-95 and the C-termini of Frizzled-3 and -8, which terminate in G-S-T-A and L-S-Q-V, respectively. None of the tested Frizzled C-termini interacted with PDZ3 of PSD-95. The PDZ domains of Chapsyn-110/PSD-93 showed a similar pattern of binding to Frizzled-1, -4, and -7. The same Frizzled receptors also bound with high affinity to the PDZ1+2 of SAP97 and its *Drosophila* homolog Dlg. In contrast, no interaction of any of the tested Frizzled receptors was observed with the PDZ domains of Lin-7 and Veli-2, indicating specificity of the PDZ interaction.

3.2. Coclustering of Frizzled and PSD-95

To demonstrate that an interaction between Frizzled and PSD-95 can also occur with the full length proteins in mammalian cells, we transiently transfected myc-tagged mouse Frizzled-7 (myc-mFz7) and PSD-95 in COS-7 cells. From COS-7 cells coexpressing myc-mFz7 and PSD-95, myc-antibodies immunoprecipitated myc-mFz7 and PSD-95 (Fig. 2A), indicating association of these proteins. PSD-95 itself was not immunoprecipitated by the myc-antibody in the absences of myc-mFz7. To show that the binding of myc-mFz7 to PSD-95 was dependent on the C-terminal sequence of the receptor, we generated a Frizzled-7 mutant (myc-mFzV/A) in which the C-terminal valine had been substituted by alanine (E-T-A-A). PSD-95 failed to be coimmunoprecipitated with myc-mFzV/A, indicating that the C-terminus of Frizzled is essential for the interaction with PSD-95.

PSD-95 can form coclusters with interacting membrane proteins when coexpressed in heterologous cells [12]. To test if Frizzled receptors also cocluster with PSD-95, we cotransfected COS cells with myc-mFz7 and PSD-95 and examined the distribution of these proteins by immunostaining. When expressed individually, Frizzled accumulated in an intracellular reticular pattern suggestive of accumulation in the endoplasmic reticulum, whereas PSD-95 expressed alone was

pGAD10 \ pBHA		Frizzled-1 E-T-T-V	Frizzled-2 E-T-T-V	Frizzled-3 G-S-T-A	Frizzled-4 E-T-V-V	Frizzled-7 E-T-A-V	Frizzled-8 L-S-Q-V	Kv1.4 E-T-D-V
		ß-Gal	ß-Gal	ß-Gal	ß-Gal	ß-Gal	ß-Gal	ß-Gal
PSD-95	PDZ 1	+	+	-	+	+	-	n.d.
	PDZ 2	++	++	-	++	++	-	n.d.
	PDZ 3	-	-	-	-	-	-	n.d.
	PDZ 1+2	+++	+++	-	+++	+++	-	+++
Chapsyn 110	PDZ 1	+	n.d.	n.d.	+	+	n.d.	n.d.
	PDZ 2	++	n.d.	n.d.	++	++	n.d.	n.d.
	PDZ 3	-	n.d.	n.d.	-	-	n.d.	n.d.
	PDZ 1+2	+++	n.d.	n.d.	+++	+++	n.d.	n.d.
SAP97	PDZ 1+2	+++	n.d.	n.d.	+++	+++	n.d.	n.d.
Dlg	PDZ 1+2	+++	n.d.	n.d.	+++	+++	n.d.	n.d.
Veli-2		-	-	-	-	-	-	n.d.
Lin-7		-	-	-	-	-	-	n.d.
pGAD10		-	-	-	-	-	-	-

Fig. 1. Interaction of members of the Frizzled family with members of the PSD-95 family. Strength of interaction was tested for Frizzled-1, -2, -3, -4, -7, and -8 in the yeast two-hybrid system and measured as the degree of activation of the reporter gene β-Gal. β-Gal activity was measured by time taken for colonies to turn blue in X-gal filter lift assays [20]: +++ (< 25 min), ++ (25–50 min), + (50–100 min), – (no significant β-Gal activity). n.d., not determined; β-Gal, β-galactosidase.

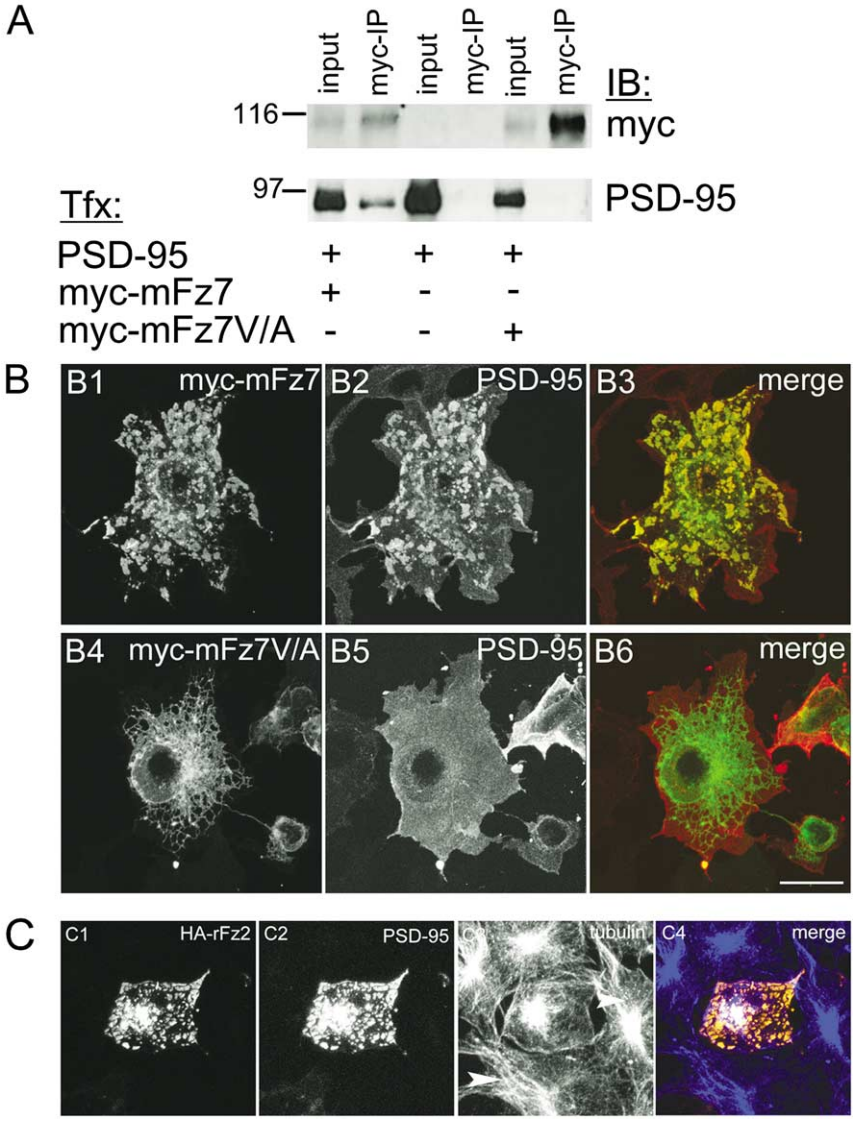

Fig. 2. Coimmunoprecipitation and coclustering of Frizzled and PSD-95. A: COS-7 cells were transfected with PSD-95 and myc-mFz7 or the C-terminal mutant myc-mFz7V/A. Immunoprecipitation with anti-myc antibodies (myc-IP) from COS-7 cell lysate was assayed by immunoblotting (IB) with antibodies against myc and PSD-95. Input lanes are loaded with 10% of the extract used for the immunoprecipitation. B: COS-7 cells were transfected with PSD-95 and myc-mFz7 or the C-terminal mutant myc-mFz7V/A and immunostained with antibodies against myc and PSD-95. Myc-antibodies were detected with FITC-conjugated (green), and PSD-95 antibodies with Cy3-conjugated (red) secondary antibodies. B1–B3 represent the same cell. B1 and B2 show the staining pattern for either one of the two cotransfected proteins, PSD-95 and myc-mFz7. In B3 the two images are superimposed (colocalization appears in yellow). B4 and B5 show the staining pattern for either one of the two cotransfected proteins, PSD-95 and myc-mFz7V/A, which are superimposed in B6. C: COS-7 cells were transfected with PSD-95 and HA-rFz2 and immunolabeled with antibodies against HA, PSD-95 and α-tubulin. C1 and C2 show the staining pattern for either one of the two cotransfected proteins, PSD-95 and HA-rFz2, and C3 for endogenous α-tubulin. Arrowheads in C3 are pointing to tubulin staining in untransfected cells. In C4 the three images are superimposed. HA-antibodies were detected with FITC-conjugated (green), PSD-95 antibodies with Cy3-conjugated (red), and α-tubulin antibodies with Cy5-conjugated (blue) secondary antibodies.

found diffusely distributed throughout the cell (data not shown). Coexpression of myc-mFz7 and PSD-95 in the same cell resulted in the redistribution of both proteins into irregularly shaped clusters (Fig. 2B1–3). The formation of clusters was observed in more than 90% of all cells that were cotransfected with myc-mFz7 and PSD-95. Coclustering of PSD-95 and myc-mFz7 was dependent on the C-terminal sequence of the receptor, because coexpression of PSD-95 with the mutant myc-mFz7V/A failed to induce the formation of clusters. Instead, myc-mFz7V/A showed the reticular distribution characteristic of Frizzled in the absence of PSD-95, and PSD-95 appeared diffuse in the cytoplasm (Fig. 2B4–6). Similar results were obtained for the coexpression of myc-mFz7 with SAP97 and Chapsyn-110 (data not shown). Coclustering of Frizzled receptors and PSD-95 family members did not change the gross morphology or viability of the cells as demonstrated by staining of microtubules in transfected COS cells with an antibody against α-tubulin (Fig. 2C). In untransfected cells microtubule bundles emerge from a site near the nucleus and traverse the cell to the periphery (Fig. 2C4, arrowheads). A similar staining pattern for tubulin was observed in cells cotransfected with HA-tagged rFz2 and PSD-95 (Fig. 2C1–4), indicating that the formation of clusters did not affect the overall structure of the cell.

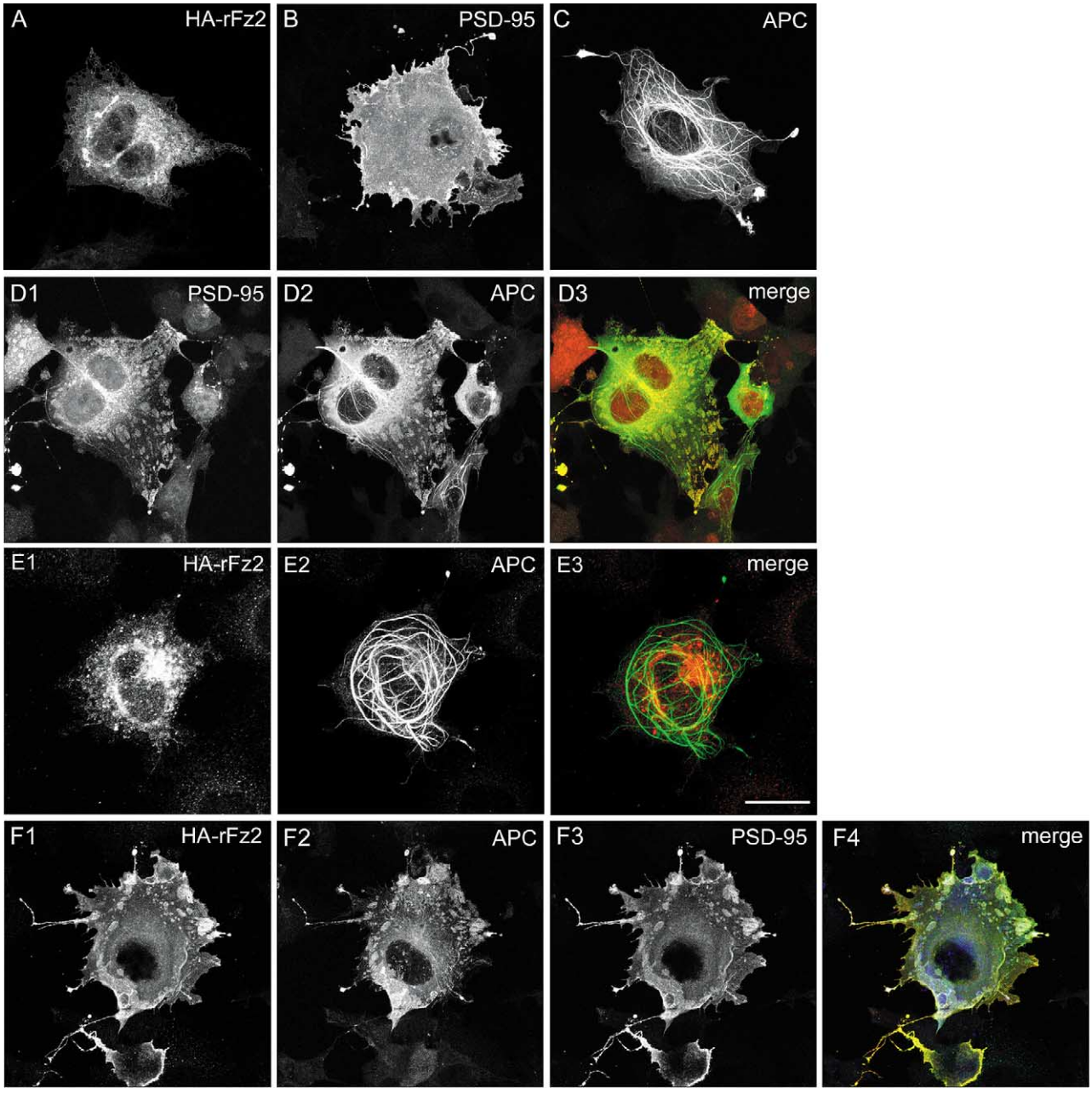

Fig. 3. Coclustering of Frizzled, PSD-95 and APC. COS-7 cells were transfected with HA-rFz2, PSD-95 and APC and immunostained with antibodies against HA, PSD-95 and APC. Primary antibodies were detected with either FITC-conjugated (green), Cy3-conjugated (red) or Cy5-conjugated (blue) secondary antibodies. A–C: Localization of HA-rFz2 (A), PSD-95 (B) and APC (C) in cells transfected with each of the constructs alone. D1–3: Localization of cotransfected PSD-95 (D1, green) and APC (D2, red); D3 is the superimposed image of D1 and D2 (colocalization appears in yellow). E1–3: Localization of cotransfected HA-rFz2 (E1, red) and APC (E2, green), superimposed in E3. F1–4: Localization of triple-transfected HA-rFz2 (F1, green), APC (F2, blue) and PSD-95 (F3, red), superimposed in F4 (colocalization of the three proteins appears white). Scale bar, 40 μm.

3.3. Frizzled, PSD-95 and APC can form a ternary complex

The APC protein has been shown to bind PSD-95 family proteins [22,23]. To test if PSD-95 can support a ternary protein complex containing Frizzled and APC, we triple-transfected COS-7 cells with APC, PSD-95 and HA-tagged rat Frizzled-2 (HA-rFz2). Fig. 3A–C shows the distribution of the individually transfected proteins. HA-rFz2 accumulated in an intracellular reticular pattern (Fig. 3A), whereas PSD-95 was diffusely distributed throughout the cytoplasm (Fig. 3B). APC was found mainly in a filamentous pattern reminiscent of microtubules (Fig. 3C), consistent with its ability to bind and rearrange the microtubule cytoskeleton [24,25]. When doubly transfected, PSD-95 and APC redistributed into coclusters (Fig. 3D1–3), similar to those formed between PSD-95 and myc-mFz7 (Fig. 2B) or HA-rFz2 (Fig. 2C). APC and HA-rFz2 showed no coclustering when expressed together in the absence of PSD-95 (Fig. 3E1–3). In contrast, when coexpressed with PSD-95, APC and HA-rFz2 colocalized in clusters, suggesting that they can assemble into the same complex dependent on PSD-95 (Fig. 3F1–4). Clusters containing all three proteins were observed in more than 90% of the cells that were triple-transfected with PSD-95, APC, and HA-rFz2.

Taken together, this suggests that PSD-95 can link together Frizzled receptors and a downstream component of the Wnt signaling pathway.

4. Discussion

Mechanisms through which Frizzled receptors transduce extracellular signals to intracellular pathways are still unclear. Given the existence of many Frizzled homologs, their differential expression in time and space, and the diversity of cellular processes controlled by Wnt signaling, it is likely that the specificity of Frizzled signaling is determined by the assembly of specific signaling complexes associated with the receptor. Thus, a multitude of intracellular binding partners for Frizzled receptors may exist in vivo. To date only two proteins, Kermit and GOPC, have been identified that directly interact with the cytoplasmic tail of Frizzled receptors [26,27]. In both proteins the binding site of Frizzled was localized to a PDZ domain, a common protein binding module in many scaffolding proteins. In neurons the PDZ-containing proteins of the PSD-95 family are implicated in the synaptic clustering of neurotransmitter receptors and their coupling to cytoplasmic signaling molecules in the postsynaptic density. PDZ1 and PDZ2 of PSD-95 family proteins recognize the C-terminal consensus sequence E-S/T-X-V, which is found in a subset of Frizzled receptors. In this study we demonstrate that certain Frizzled receptors can interact with members of the PSD-95 family and form a ternary complex together with APC, a negative regulator of Wnt signaling. Several different Frizzled receptors are expressed in the developing and adult brain, consistent with the putative interaction with members of the PSD-95 family. However, we were unable to show association between Frizzled receptors and PSD-95 in the brain by coimmunoprecipitation because of the lack of good antibodies recognizing mammalian Frizzled proteins. Other components of the Wnt/Frizzled signaling pathways (e.g. APC and β-catenin/armadillo) are present at synapses [22,23,28], and synaptic differentiation in the cerebellum appears to be regulated by Wnt7a signaling [29,30], supporting the idea that a Frizzled signaling complex might exist at synapses in the central nervous system.

During the course of our experiments and consistent with other reports [26,27], we observed that when expressed alone Frizzled accumulates in an intracellular compartment, most likely the endoplasmic reticulum. This could be due to the high expression level of the protein in the cell which might overload the transport machinery and consequently lead to a failure in the proper targeting of the receptor to the plasma membrane. Alternatively, Frizzled might fail to be transported to the plasma membrane in the absence of binding partners which are essential for the membrane localization of the receptor. In fact, a role in the translocation of Frizzled from an intracellular compartment to the cell membrane has been demonstrated for the two so far known C-terminal binding partners of Frizzled, GOPC and Kermit [26,27]. In neurons binding of Frizzled to PSD-95 might help to target and stabilize the receptor in the synaptic membrane. The correct subcellular localization of Frizzled seems to be essential for its proper function as demonstrated for the Frizzled-dependent establishment of planar cell polarity in hair cells of the *Drosophila* wing [3,4]. Frizzled signaling in hair cells ultimately affects the actin cytoskeleton underlying the cuticular hair structure [31]. Frizzled signaling might also play a role in the control of the postsynaptic actin cytoskeleton, which interfaces with the PSD-95 signaling complex localized at synapses.

Acknowledgements: We thank Dr. Randall Moon for providing the mouse Frizzled-7 clone and Dr. Jeremy Nathans for the rat Frizzled-2 clone. M.S. is Associate Investigator of the Howard Hughes Medical Institute. This work was supported by a postdoctoral fellowship of the Deutscher Akademischer Austauschdienst for H.H.

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