Biochemical characterization of the *Drosophila* Axin protein

Shin-ichi Yanagawa*, Jong-Seo Lee, Yukihiro Matsuda, Akinori Ishimoto

Department of Viral Oncology, Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto 606-8507, Japan

Received 9 February 2000; received in revised form 5 May 2000

Edited by Veli-Pekka Lehto

Abstract In the Wnt/Wingless pathway, accumulation of β-catenin/Armadillo protein is a key regulatory step. Vertebrate Axin is a negative regulator of Wnt signaling, promoting glycogen synthase kinase-3β-mediated phosphorylation of β-catenin and thereby destabilizing β-catenin. Using *Drosophila* cell culture systems, we demonstrated that a *Drosophila* homolog of Axin (Daxin) inhibits Wingless-induced Armadillo accumulation and *Drosophila* T-cell factor-dependent transcription induced by Wingless, Dishevelled, and Armadillo. The carboxy-terminal portion of Daxin is not essential for the inhibitory activity, but a mutant only consisting of this portion behaves as a dominant-negative protein. Moreover, interactions between Daxin and Zeste-white 3, Armadillo, Dishevelled, protein phosphatase 2A and Daxin itself were shown, providing direct evidence that Daxin is a scaffold protein in the Wingless pathway.

Key words: Axin; Wingless; Armadillo; Zeste-white 3; Dishevelled; *Drosophila*

1. Introduction

The Wnt family of secretory protein plays pivotal roles in a number of basic developmental processes. Components of the Wnt/Wingless (Wg) signaling pathway are structurally and functionally conserved in vertebrates and invertebrates [1–3]. The stabilization of β-catenin/Armadillo (Arm) is a key regulatory step in this pathway [3–5] and its stability is regulated by glycogen synthase kinase-3β (GSK-3β)/Zeste-white3 (Zw3)-mediated phosphorylation, which targets β-catenin/Arm to the ubiquitin/proteasome pathway [6]. Wnt/Wg inhibits GSK-3β/Zw3 function through the Dishevelled (Dsh) family proteins, thereby upregulating β-catenin/Arm levels [7,8]. Accumulated β-catenin/Arm then forms a complex with the T-cell factor (TCF)/*Drosophila* TCF (D-TCF) family of transcription factors, and this complex translocates into the nucleus and regulates expression of Wnt/Wg target genes [9]. Axin and its homolog Conductin/Axil regulate Wnt signaling negatively [10–28]. Axin stimulates GSK-3β-mediated phosphorylation of β-catenin by binding and bridging these proteins and thus destabilizing β-catenin [11–15,19,27,28]. Moreover, Axin acts as a scaffold protein which binds other components of the Wnt signaling pathway, such as the adenomatous polyposis coli gene product (APC), [12,17,19], mouse Dsh, Dvls [22–24], protein phosphatase 2A catalytic subunit (PP2Ac), [25] and Axin itself [23,25,26,28]. The APC–Axin complex downregulates β-catenin [15,18], but its mechanism of action remains elusive [19,29]. Recently, genetic studies have revealed that an Axin homolog in *Drosophila*, Daxin, interferes with Wg and DWnt-2 signaling [30,31]. These in vivo studies include analyses of the ventral cuticle pattern in mutant embryo [30], and mosaic analyses of *Daxin* in wing, leg, and eyes [30,31], and indicate that loss of function or overexpression of *Daxin* and *wg* produce opposite phenotypes.

Here we describe the biochemical characterization of the Daxin protein using a *Drosophila* cell culture system. We demonstrated that Daxin inhibits Wg-induced Arm accumulation and D-TCF-dependent transcription induced by Wg, Dsh, and Arm. Using a series of Daxin mutants, we mapped domains essential for this negative regulation. In addition, interaction of Daxin with Zw3 (both wild-type and kinase-dead forms), Arm, Dsh, PP2Ac and Daxin itself was shown by pull-down assays with glutathione *S*-transferase (GST)–Daxin fusion proteins and co-immunoprecipitation experiments. These results are direct evidence that Axin family proteins mediate a set of conserved biochemical processes which suppress Wnt/Wg signaling.

2. Materials and methods

2.1. Expression constructs

The Flag epitope was added to the amino-terminus of the full-length Daxin proteins as follows: the entire coding sequence of Daxin was amplified by polymerase chain reaction (PCR) using a pBluescript IISK plasmid containing the A1 form of Daxin cDNA ([31], gift from R. Nusse and K. Willert, Stanford University) as template, and a sense primer (5′-AGTAAGCTTAGTGGCCATCCATCGGGA-3′) containing a *Hin*dIII site and an antisense primer (5′-AAAATCGATATATTAATCGGATGGCTT-3′) containing the termination codon of Daxin and a *Cla*I site. The Daxin PCR products were double-digested with *Hin*dIII and *Cla*I and subcloned into the *Hin*dIII-*Cla*I-cleaved pFlagCMV2 (Eastman Kodak). The resulting plasmid was named pFlagCMV2-Daxin. Then a series of deletion mutants (see Fig. 1) were generated as follows: for mutant #2, pFlagCMV2-Daxin was double-digested with *Eco*52I and *Eco*RV, blunted with T4 polymerase and recircularized by ligation with T4 ligase; likewise, for mutant #3, #5, and #6, pFlagCMV2-Daxin was double-digested with *Nru*I and *Eco*RV, *Hin*dIII and *Nru*I, and *Hin*dIII and *Eco*52I, respectively, blunted, and recircularized; for mutant #4, pFlagCMV2-Daxin was digested with *Bst*PI, partially filled in with T4 polymerase in the presence of GTP and TTP, blunted with mung bean nuclease, digested again with *Hin*dIII, blunted with T4 polymerase and recircularized; for mutants #7 and #8, mutants #5 and #4, respectively, were double-digested with *Eco*52I and *Eco*RV, blunted and recircularized. To generate expression plasmids in *Drosophila* cell lines, the sequences encoding Flag-tagged Daxin were amplified by PCR by using the pFlagCMV2-based Daxin constructs as templates and a sense primer, named 5′ Flag (5′-GACGCAAATGGGCGGTAGGCGTGTACGG-TG-3′, a sequence that starts 82 bp upstream of the initiator methionine codon for the Flag epitope in pFlagCMV2), and an antisense primer, named 3′ Flag (5′-GACAAGGCTGGTGGGCACTGGAG-TGGCAAC-3′, which is present 46 bp downstream of the polylinker site of pFlagCMV2). In addition, the sequence encoding Flag-tagged Daxin lacking the DIX domain (mutant #1 in Fig. 1) was amplified

*Corresponding author. Fax: (81)-75-751 3995.
E-mail: syanagaw@virus.kyoto-u.ac.jp

Abbreviations: PAGE, polyacrylamide gel electrophoresis

PII: S0014-5793(00)01601-X

using the 5′ Flag primer, an antisense primer (5′-TCCTATGGCTGAGTACCGGGTATTTTTA-3′, corresponding to amino acids 675–682 and a termination codon), and the pFlagCMV2-Daxin plasmid. These PCR products were blunted and cloned into the *Eco*RV site of pMK33 [32]. The resulting plasmid expressing Flag-tagged full-length Daxin was named pMKFlag-Daxin. A plasmid expressing C-terminally Myc-tagged full-length Daxin under the control of heat shock promoter ([31], pMKHS-Daxin-mycD5′) was obtained from R. Nusse. The fidelity of the PCR reactions with Pfu Turbo DNA polymerase (Stratagene) was confirmed by DNA sequencing. pMKDsh-Myc, pMKZw3-HA (which expresses wild or kinase-dead type of Zw3), pMK-Wg, pMKArm-Myc (expressing Myc-tagged full-length Arm), and pMK-del-Arm-Myc (expressing Myc-tagged Arm with an amino-terminal deletion) have been described [33–35].

2.2. Cell cultures and transfections

The *Drosophila* S2R+ cells responding to Wg and SW480 cells were cultured as described in [13,35], respectively. Expression plasmids were introduced into S2R+ cells using calcium phosphate-mediated transfection as described [33]. The pMKFlag-Daxin, pMKDsh-Myc, pMKZw3HA, and pMK33 transfectants used in this study were mixtures of stable S2R+ cell clones selected with hygromycin (200 μM). The transfectants generated with pMK33-based vectors were induced to overexpress the transfected genes by adding 0.5 mM $CuSO_4$ as described [33].

2.3. Wg treatment of S2R+ cells expressing Daxin proteins

S2R+ cells transfected with pMK33 or pMK33-based Daxin expression plasmids, either exposed or not to $CuSO_4$ for 24 h, were grown until fully confluent in T24 flasks. For Wg treatment, co-cultivation with S2-HS-wg cells (S2 cells expressing Wg under the control of heat shock promoter) was performed [36]. A total of 2 ml of S2-HS-wg or control S2 cell suspension in Schneider medium containing 10% fetal calf serum (2×10^6 cells/ml) was added to the S2R+ transfectants for 3 h (for cells induced with $CuSO_4$ for 24 h, co-culture was performed with $CuSO_4$). Then the S2-HS-wg or S2 cells overlaid on the S2R+ culture were removed by three cycles of washing with 5 ml of phosphate-buffered saline (PBS). Next, cell lysates were prepared from the S2R+ cells remaining in the flask and subjected to SDS–PAGE.

2.4. Luciferase assay

Subconfluent cultures of S2R+ cells in T25 flasks were transfected with 1 μg of one of the luciferase reporters TOPFLASH or FOPFLASH [37], 1 μg of pAclacZ, a plasmid expressing β-galactosidase under the control of the *Drosophila* actin 5C promoter, and 4 μg of either the pMK33, pMK-Wg, pMKDsh-Myc, pMKArm-Myc or pMK-del-Arm-Myc plasmid, in combination with 4 μg of the pMK33 or pMKFlag-Daxin plasmid, so that the total amount of plasmids transfected per flask was 10 μg. Twenty-four hours after transfection, cells were washed with PBS and cultured for a further 24 h in the presence of 0.5 mM $CuSO_4$, then luciferase and β-galactosidase activities in the cell lysates were determined using kits from Promega. Relative light units were measured with a luminometer. β-Galactosidase activities were used to normalize transfection efficiency. The assays were performed three times as duplicate transfections.

2.5. Immunoblot analyses, and antibodies

The cell lysates whose protein concentration was normalized were subjected to Western blot analysis as described [33]. The rabbit antibody against *Drosophila* PP2Ac was obtained from Dr. Uemura [38]. The mouse monoclonal anti-Arm antibody N2-7A1, the rat polyclonal anti-Dsh region I antibody, the rat monoclonal anti-*Drosophila* α-catenin antibody DCAT-1, and the mouse monoclonal antibodies against β-catenin, GSK-3β, HA epitope (12CA5), Flag epitope (M2), human c-Myc epitope (9E10) were used as described [33–35]. In addition, peroxidase-conjugated secondary antibodies against mouse IgG, rat IgG, and rabbit IgG were used as described [33–35]. The blots were visualized with the Enhanced Chemiluminescence Reagent (Amersham Pharmacia Biotech).

2.6. Immunoprecipitations, and pull-down assay with GST–Daxin fusion proteins

The cell lysates for immunoprecipitation and pull-down assay were prepared with lysis buffer containing protease inhibitors and phosphatase inhibitors as described [33]. 10 μg of anti-Flag antibody (M2) was added to the cell lysate and the mixture was incubated for 4 h at 4°C, then 30 μl of goat anti-mouse IgG (H+L)-Sepharose 4B (Zymed) was added and the mixture incubated for a further 1 h at 4°C. The immune complexes were washed four times with lysis buffer, resuspended in SDS–PAGE sample buffer and resolved by SDS–PAGE, and immunoblotted. Three pGEX-Daxin plasmids were constructed as follows: the 1.3 kb *Bst*PI–*Xho*I fragment and the 0.3 kb *Nru*I–*Xho*I fragment of the Daxin cDNA encoding amino acids 152–567 and 462–567 of the Daxin protein, respectively, were cloned into the *Bam*HI site and the *Sal*I site, respectively, of pGEX5X-3 by blunt end ligation. The 0.3 kb *Eco*RI–*Nru*I fragment encoding amino acids 358–461, whose *Nru*I site was filled in with T4 polymerase, was cloned into the *Eco*RI and *Sma*I sites of pGEX5X-3. The GST–Daxin fusion proteins were expressed in *Escherichia coli* XL1Blue and purified with glutathione-Sepharose CL4B beads as described by the manufacturer (Amersham Pharmacia). 500 μl of cell lysate from S2R+ cells stably transfected with pMK-Zw3HA or pMK33 or SW480 cells was incubated with 30 μl of glutathione-Sepharose CL4B beads carrying GST or the GST–Daxin fusion proteins for 1 h at 4°C. The beads were washed three times with lysis buffer, and resuspended in SDS–PAGE sample buffer. Bound proteins were subjected to Western blotting.

3. Results and discussion

3.1. Effect of overexpression of full-length or mutant forms of Daxin on Arm levels in S2R+ cells

In vivo studies [31,32] have shown that Daxin is a negative regulator of Wg signaling. To characterize the functional domains in Daxin, we generated a series of Flag-tagged Daxin mutants (Fig. 1). We first examined the effect of overexpression of full-length or a series of mutant Daxin on Arm levels in S2R+ cells. As shown in Fig. 2, induction of full-length, #1, #2, and #3 Daxin led to slight decreases of Arm protein levels in S2R+ cells. In contrast, overexpression of #4 (lacking the N-terminal portion including the RGS domain) and #5 (con-

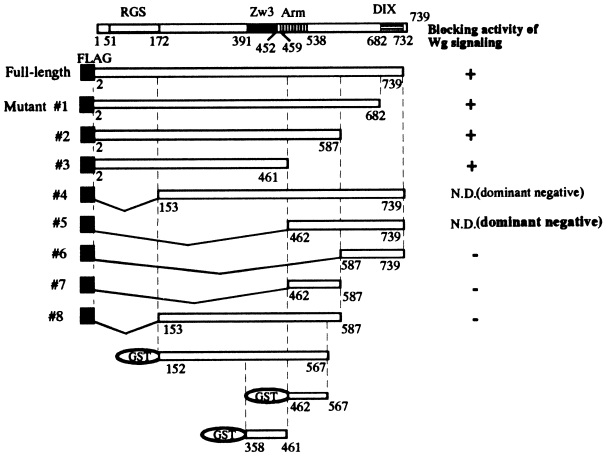

Fig. 1. Schematic representation of Flag-tagged Daxin, its deletion mutants and GST–Daxin fusion proteins. The diagram at the top indicates Daxin structure. The most highly conserved domains between mouse Axin and Daxin, the RGS and DIX domains, are indicated by an open and a horizontally hatched box, respectively. The Arm and putative Zw3 binding domains are presented as a filled and a vertically hatched box, respectively. Numbers under the diagrams indicate amino acid positions of the Daxin protein. The Flag epitopes are labeled and indicated by thick filled boxes. GST proteins are presented as ovals. A summary of the Wg blocking activity of the Daxin transgenes is shown on the right of the diagrams. The Wg blocking activity of the Daxin transgenes is referred to effect of overexpression of each transgene on Wg-induced Arm accumulation. N.D., not determined. 'Dominant negative' in bold letters indicates that this Daxin mutant is markedly dominant-negative.

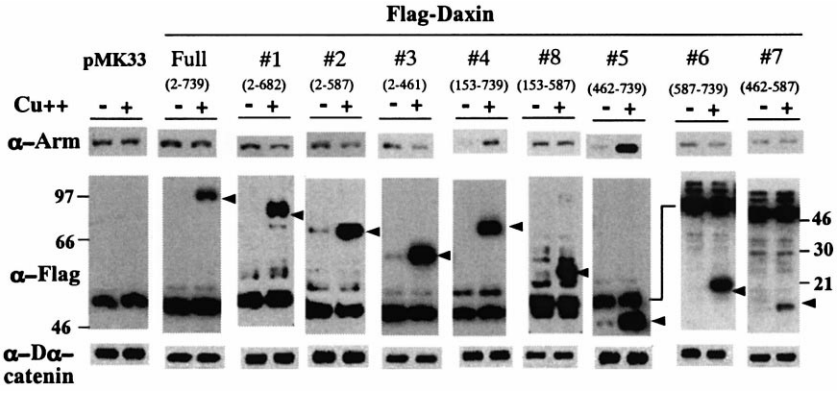

Fig. 2. Effect of overexpression of full-length or mutant forms of Daxin on Arm levels in S2R+ cells. The stable S2R+ transfectants expressing full-length or mutant forms of Daxin were induced (+) or not (−) with $CuSO_4$ for 24 h. S2R+ cells transfected with pMK33 were used as negative control. Cell lysates were subjected to Western blot analysis with anti-Arm (top panel), anti-Flag (middle panel), or anti-Da-catenin (bottom panel) antibodies. The Daxin proteins are indicated by arrowheads and the positions of molecular weight markers are indicated on the sides of the panels. Blots with anti-Da-catenin were used as loading controls.

sisting of the region from the Arm binding domain to the carboxy-terminus, which includes binding domains for Dsh and PP2Ac and for oligomerization, see below) slightly and markedly elevated Arm levels, respectively, suggesting that these two proteins are dominant-negative mutants of Daxin. Mutants #6 and #7, which correspond to the N- and the C-terminal half, respectively, of #5, as well as #8, had little effect on Arm levels. The result for mutant #4 suggests that the RGS domain of Daxin, which can interact with D-APC protein [30], participates in downregulation of Arm. This observation is consistent with the report that mouse Axin mutants lacking the RGS [10,14,19] or a N-terminal portion including the RGS domain (which is similar to our mutant #4 [19]) have dominant-negative functions in *Xenopus*. However, Daxin with a deletion of the RGS domain behaves the same as wild-type Daxin, blocking Wg signaling in vivo [31]. The additional N-terminal 51 amino acid deletion only present in our #4 mutant could explain this apparent discrepancy.

3.2. Negative regulation of Wg signaling by Daxin

To demonstrate that Wg signaling is negatively regulated by Daxin, we examined effects of overexpression of several Daxin proteins on Wg-induced accumulation of Arm in vitro (Fig. 3). Compared with the pMK33 transfectant used as control, Wg-induced elevation of Arm was blocked by expression of full-length Daxin, and mutants #2 and #3, but not mutants #6, #7, and #8 (data for #7 and #8 not shown). These findings suggest that the N-terminal portion of Daxin (N-terminus to amino acid 461) can block Wg signaling, and that the region from the N-terminus to the RGS domain is essential for this blocking activity. These results correspond well to the observations on Axin mutants in *Xenopus* embryos [19], showing that the RGS and GSK-3β binding site are required, whereas the carboxy-terminal sequences, including the PP2Ac binding site and DIX domain, are not essential for inhibition of dorsal axis formation and for β-catenin downregulation.

3.3. Interaction of Daxin with components of Wg/Wnt signaling pathway

We next examined physical interactions between Daxin and certain components of the Wg and Wnt pathway using two methods: pull-down assays with GST–Daxin fusion proteins and co-immunoprecipitation with anti-Flag antibody. GST

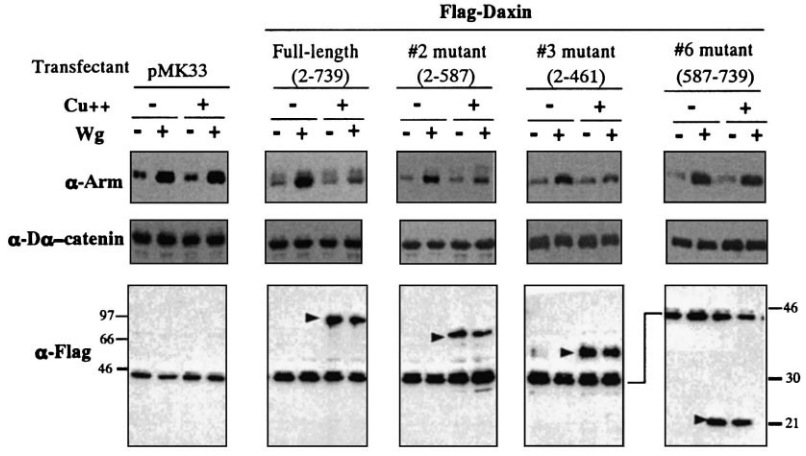

Fig. 3. Negative regulation of Wg signaling by Daxin. The S2R+ cells expressing full-length or mutant forms of Daxin were induced (+) or not (−) with $CuSO_4$ for 24 h. These cultures were overlaid with a suspension of either S2 (−) or S2-HS-wg (+) cells for 3 h. Total cell lysates were prepared from the co-cultivated S2R+ cells and subjected to Western blot analysis with anti-Arm (top panel), anti-Da-catenin (middle panel), or anti-Flag (bottom panel) antibodies. The Flag-tagged Daxin proteins induced are indicated by arrowheads.

fusion proteins expressing three distinct regions of Daxin were generated (see Fig. 1). The experiments with lysates from S2R+ cells, S2R+ cells expressing HA-tagged Zw3, and SW480 cells revealed that endogenous Arm or β-catenin, and Zw3 or GSK-3β, bind to amino acids 462–567 (Fig. 4A, panel a) and 358–461 (Fig. 4A, panel b), respectively, of the Daxin protein. None of these GST–Daxin fusion proteins could pull down Dsh or PP2Ac from lysate from S2R+ cells (data not shown). These results are consistent with the report

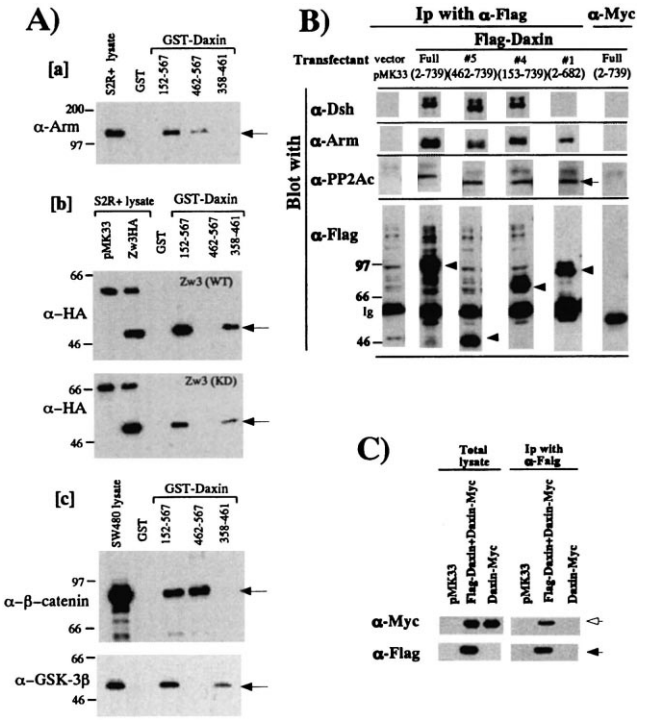

Fig. 4. Physical interaction of Daxin with components of Wg and Wnt signaling pathway. A: Pull-down assays to demonstrate the interaction of Daxin with Arm, wild-type and the kinase-dead form of Zw-3, β-catenin, and GSK-3β. GST and three types of GST–Daxin fusion proteins whose Daxin portions are indicated above the panels were immobilized to glutathione-Sepharose. These beads were incubated with lysates from S2R+ cells (a), S2R+ cells expressing HA-tagged wild-type (upper panel) or kinase-dead form (lower panel) of Zw-3 (b), or SW480 cells (c), and total cell lysates and the proteins bound to each bead were subjected to Western analysis with anti-Arm (a), anti-HA (b), anti-β-catenin (c, upper panel) or anti-GSK-3β (c, lower panel) antibodies. The protein bands of interest are indicated by arrows on the right, and the positions of molecular weight markers shown on the left side of each panel. B: Co-immunoprecipitation showing association of Daxin with Dsh, Arm, and PP2Ac. Total cell lysates prepared from S2R+ cells stably expressing Flag-tagged Daxin proteins or negative control cells (transfected with pMK33), were subjected to immunoprecipitation with anti-Flag or anti-Myc antibodies as described in Section 2. An arrow indicates the position of PP2Ac and the arrowheads in the bottom panels indicate the position of the Daxin proteins. C: Daxin oligomerization. S2R+ cells expressing Flag-tagged and Myc-tagged Daxin proteins were established by cotransfection of pMKFlag-Daxin and pMKHS-Daxin-mycD5′. S2R+ cells transfected with pMK33 or pMKHS-Daxin-mycD5′ were used as negative controls. The three cell lines were induced with $CuSO_4$ for 24 h, followed by a heat shock for 30 min at 37°C. Total cell lysates were subjected to immunoprecipitation with anti-Flag antibody. The total lysates and immune complexes were Western blotted with mouse monoclonal anti-Myc (upper panels) or mouse monoclonal anti-Flag (lower panel) antibodies. Myc-tagged Daxin and Flag-tagged Daxin are shown by the open and solid arrows, respectively.

by Willert et al. [31] demonstrating that Zw3 and Arm co-immunoprecipitate with Daxin but they differ from the report by Hamada et al. [30] showing that Arm but not Zw3 interacts with Daxin. Our results with a heterologous system also indicate that the Arm/β-catenin and Zw3/GSK-3β binding motifs of Axin family proteins are stretches of amino acids conserved between mouse and fly Axin proteins (amino acids 446–458 in the putative Zw3 binding site, and amino acids 494–510 in Arm binding sites of Daxin). In addition, binding of both wild-type and kinase-dead (a lysine residue in the ATP binding region was changed to arginine) forms of Zw3 to Daxin (and to mouse Axin, S. Yanagawa, an unpublished result) was demonstrated (Fig. 4A, panel b). This demonstration of binding of the kinase-dead form of Zw3 (and probably GSK-3β) to Axin proteins provides an attractive model to explain why a kinase-dead form of Zw3/GSK-3β is dominant-negative and thus functions as a very potent inducer of Arm/β-catenin [4,5,33]: the kinase-dead form of Zw3/GSK-3β complexes with Daxin/Axin and Arm/β-catenin but cannot phosphorylate Arm/β-catenin, thereby inducing formation of a non-functional Daxin/Axin complex which no longer facilitates Arm/β-catenin degradation. Also, kinase-dead Zw3/GSK-3β will not phosphorylate Daxin/Axin [9]. Since phosphorylated Axin binds β-catenin better than unphosphorylated Axin [27], β-catenin will no longer be recruited to the Axin/GSK-3β complex, thus preventing β-catenin phosphorylation plus targeting to the proteasome. In contrast to our result, however, Ikeda et al. have reported that wild-type but not the kinase-dead form of GSK-3β binds to rat Axin [11].

Next, we demonstrated with co-immunoprecipitation experiments that Arm and PP2Ac interact with the C-terminal portion (amino acids 462–682) of Daxin (Fig. 4B). Dsh co-immunoprecipitated with full-length Daxin, mutants #4 and #5 but not with mutant #1 (Fig. 4B). In addition, the DIX domain alone is not sufficient for association of Dsh (data not shown). These results indicated that the C-terminal portion (amino acid 462 to the C-terminus including the DIX domain) of Daxin is required for Dsh binding. Finally, oligomerization of Daxin was examined. Fig. 4C shows that Myc-tagged Daxin co-immunoprecipitated with Flag-tagged Daxin, when cell lysates from S2R+ transfectants expressing both Flag-tagged and Myc-tagged Daxin were subjected to immunoprecipitation with anti-Flag antibody.

3.4. Effect of Daxin on D-TCF-dependent transcription

Axin has been shown to inhibit TCF-dependent transcription induced by Wnt-1 [20,31], Wnt-3A [21] or Dvl-2 [22] in mammalian cells. In *Drosophila* cells, however, it has not been elucidated whether Wg, Dsh, and Arm indeed affect D-TCF-dependent transcription. Therefore, we performed a reporter assay with the standard reporter plasmids for TCF-dependent transcription in vertebrate, TOPFLASH and FOPFLASH [37], to analyze the effect of Daxin overexpression on D-TCF-dependent transcription induced by Wg, Dsh or Arm in S2R+ cells (Fig. 5). Co-transfection of pMK-Wg markedly induced transcription from TOPFLASH but not from FOPFLASH in a dose-dependent manner (4 and 8 μg of pMK-Wg plasmid induced 8- and 13-fold increases in luciferase activity, respectively, compared to pMK33). In addition, expression of Dsh, full-length Arm, and N-terminally deleted Arm induced 4-, 1.8- and 6-fold increases in luciferase activity from TOP-

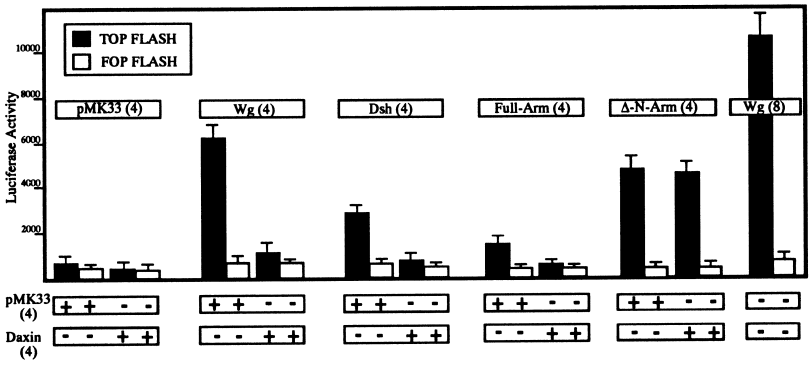

Fig. 5. Effect of Daxin on D-TCF-dependent transcription induced by Wg, Dsh, and full-length or N-terminal deleted Arm in S2R+ cells. The combination and the amount (numbers in parentheses indicate μg of the plasmid used) of plasmids transfected are indicated. The luciferase activities from TOPFLASH and FOPFLASH reporter plasmid are shown as filled and open bars, respectively. The right-most column shows the result with 8 μg of pMK-Wg plasmid.

FLASH, respectively, but that from FOPFLASH was not affected significantly. Note that the N-terminally deleted Arm caused a much higher increase in D-TCF-dependent transcription compared with full-length Arm, as expected. Interestingly, D-TCF-dependent transcription induced by Wg, Dsh, and full-length Arm was inhibited by co-expression of Daxin, while that induced by N-terminally deleted Arm was not. This finding supports a current model for Daxin function in Wg signaling: overexpression of Wg or Dsh leads to an elevation of Arm in S2R+ cells [35]. Daxin, on the other hand, enhances Zw3-mediated Arm phosphorylation at a specific motif in the N-terminus and thereby facilitates degradation of Arm. Wild-type Arm induced directly from its expression construct or indirectly by transfection of Wg or Dsh expression plasmids was downregulated by Daxin. Therefore, the Wg-, Dsh- and full-length Arm-induced transcription from TOPFLASH is inhibited by Daxin. In contrast, the N-terminally deleted Arm, which is no longer phosphorylated by Zw3 and thus escapes Daxin-mediated degradation, can still bind to D-TCF through its Arm repeats and functions as a coactivator for D-TCF. These findings are consistent with the reports that Axin [19] and Conductin [16] downregulate wild-type β-catenin but not mutant β-catenin lacking phosphorylation sites for GSK-3β.

3.5. Concluding discussion

In this paper, we analyzed structural and functional relationships of the Daxin protein in Wg signaling using a *Drosophila* cell culture system. Although sequence homology between fly and vertebrate Axin is mainly restricted to the RGS and DIX domains, our results substantiated functional conservation of Axin family proteins: we demonstrated that Daxin blocks Wg-induced accumulation of Arm as well as Wg-, Dsh-, and Arm-induced D-TCF-dependent transcription in vitro; we also showed an association of Arm, Zw3, Dsh, and PP2Ac with the C-terminal half of Daxin and oligomerization of Daxin.

Acknowledgements: We thank Drs. Karl Willert and Roel Nusse for Daxin cDNA and pMKHS-Daxin-mycD5. We also thank Dr. H. Clevers (University of Utrecht) for TOPFLASH and FOPFLASH plasmids, and Dr. S. Hayashi (National Institutes of Genetics, Mishima, Japan) for pAclacZ plasmid.

References

[1] Cadigan, K. and Nusse, R. (1997) Genes Dev. 11, 3286–3305.
[2] Wodarz, A. and Nusse, R. (1998) Annu. Rev. Cell Biol. 14, 59–88.
[3] Polakis, P. (1999) Curr. Opin. Genet. Dev. 9, 15–21.
[4] Yost, C., Torres, M., Miller, J., Huang, E., Kimelman, D. and Moon, R. (1996) Genes Dev. 10, 1443–1454.
[5] Peifer, M., Sweeton, D., Casey, M. and Wieschaus, E. (1994) Development 120, 369–380.
[6] Maniatis, T. (1999) Genes Dev. 13, 505–510.
[7] Cook, D., Fry, M.J., Hughes, K., Sumathipala, R., Woodgett, J.R. and Dale, T.C. (1996) EMBO J. 15, 4526–4536.
[8] Ruel, L., Stambolic, V., Ali, A., Manoukian, A.S. and Woodgett, J.R. (1999) J. Biol. Chem. 274, 21790–21796.
[9] Bienz, M. (1998) Curr. Opin. Cell Biol. 10, 366–372.
[10] Zeng, L., Fagotto, F., Zhang, T., Hsu, W., Vasicek, T.J., Perry III, W.L., Lee, J.J., Tilghman, S.M., Gumbiner, B.M. and Costantini, F. (1997) Cell 90, 181–192.
[11] Ikeda, S., Kishida, S., Yamamoto, H., Murai, H., Koyama, S. and Kikuchi, A. (1998) EMBO J. 17, 1371–1384.
[12] Nakamura, T., Hamada, F., Ishidate, T., Anai, K., Kawahara, K., Toyoshima, K. and Akiyama, T. (1998) Genes Cells 3, 395–403.
[13] Sakanaka, C., Weiss, J.B. and Williams, L.T. (1998) Proc. Natl. Acad. Sci. USA 95, 3020–3023.
[14] Itoh, K., Krupnik, V.E. and Sokol, S.Y. (1998) Curr. Biol. 8, 591–594.
[15] Hart, M.J., de los Santos, R., Albert, I.N., Rubinfeld, B. and Polakis, P. (1998) Curr. Biol. 8, 573–581.
[16] Behrens, J., Jerchow, B.A., Wurtele, M., Grimm, J., Asbrand, C., Wirtz, R., Kuhl, M., Wedlich, D. and Birchmeier, W. (1998) Science 280, 596–599.
[17] Yamamoto, H., Kishida, S., Uochi, T., Ikeda, S., Koyama, S., Asashima, M. and Kikuchi, A. (1998) Mol. Cell. Biol. 18, 2867–2875.
[18] Kishida, S., Yamamoto, H., Ikeda, S., Kishida, M., Sakamoto, I., Koyama, S. and Kikuchi, A. (1998) J. Biol. Chem. 273, 10823–10826.
[19] Fagotto, F., Jho, E.-h., Zeng, L., Kurth, T., Joos, T., Kaufmann, C. and Costantini, F. (1999) J. Cell Biol. 145, 741–756.
[20] Yamamoto, H., Kishida, S., Kishida, M., Ikeda, S., Takada, S. and Kikuchi, A. (1999) J. Biol. Chem. 274, 10681–10684.
[21] Kishida, M., Koyama, S., Kishida, S., Mastubara, K., Nakashima, S., Higano, K., Takada, R., Takada, S. and Kikuchi, A. (1999) Oncogene 18, 979–985.
[22] Smalley, M.J., Sara, E., Paterson, H., Naylor, S., Cook, D., Jayatilake, H., Fryer, L.G., Hutchinson, L., Fry, M.J. and Dale, T.C. (1999) EMBO J. 18, 2823–2835.
[23] Kishida, S., Yamamoto, H., Hino, S.-I., Ikeda, S., Kishida, M. and Kikuchi, A. (1999) Mol. Cell. Biol. 19, 4414–4422.

[24] Lin, L., Yuan, H., Weaver, C.D., Mao, J., Farr III, G.H., Susman, D.J., Jonkers, J., Kimelman, D. and Wu, D. (1999) EMBO J. 18, 4233–4240.
[25] Hsu, W., Zeng, L. and Costantini, F. (1999) J. Biol. Chem. 274, 3439–3445.
[26] Sakanaka, C. and Williams, L.T. (1999) J. Biol. Chem. 274, 14090–14093.
[27] Willert, K., Shibamoto, S. and Nusse, R. (1999) Genes Dev. 13, 1768–1773.
[28] Hedgepeth, C.M., Deardorff, M.A., Rankin, K. and Klein, P.S. (1999) Mol. Cell. Biol. 19, 7147–7157.
[29] Seeling, J.M., Miller, J.R., Gil, R., Moon, R.T., White, R. and Virshup, D.M. (1999) Science 283, 2089–2091.
[30] Hamada, F., Tomoyasu, Y., Takatsu, Y., Nakamura, M., Nagai, S-i., Suzuki, A., Fujita, F., Shibuya, H., Toyoshima, K., Ueno, N. and Akiyama, T. (1999) Science 283, 1739–1742.
[31] Willert, K., Logan, C.Y., Arora, A., Fish, M. and Nusse, R. (1999) Development 126, 4165–4173.
[32] Koelle, M.R., Talbot, W.S., Segraves, W.A., Bender, M.T., Cherbas, P. and Hogness, D.S. (1991) Cell 67, 59–77.
[33] Yanagawa, S., van Leeuwen, F., Wodarz, A., Klingensmith, J. and Nusse, R. (1995) Genes Dev. 9, 1087–1097.
[34] Yanagawa, S., Lee, J.-S., Haruna, T., Oda, H., Uemura, T., Takeichi, M. and Ishimoto, A. (1997) J. Biol. Chem. 272, 25243–25251.
[35] Yanagawa, S., Lee, J.-S. and Ishimoto, A. (1998) J. Biol. Chem. 273, 32353–32359.
[36] van Leeuwen, F., Harryman Samos, C. and Nusse, R. (1994) Nature 368, 342–348.
[37] Korinek, V., Barker, N., Morin, P.J., van Wichen, D., de Weger, R., Kinzler, K.W., Vogelstein, B. and Clevers, H. (1997) Science 275, 1784–1787.
[38] Shiomi, K., Takeichi, M., Nishida, Y., Nishi, Y. and Uemura, T. (1994) Development 120, 1591–1599.