Critical Scaffolding Regions of the Tumor Suppressor Axin1 Are Natively Unfolded

Maria Noutsou1, Afonso M. S. Duarte2, Zeinab Anvarian1, Tatiana Didenko2, David P. Minde2, Ineke Kuper1, Isabel de Ridder1, Christina Oikonomou1, Assaf Friedler3, Rolf Boelens4, Stefan G. D. Rüdiger2* and Madelon M. Maurice1*

1Department of Cell Biology, University Medical Center Utrecht, Utrecht, The Netherlands
2Cellular Protein Chemistry, Bijvoet Center for Biomolecular Research, Utrecht University, Utrecht, The Netherlands
3Department of Organic Chemistry, The Hebrew University of Jerusalem, Jerusalem, Israel
4Biomolecular NMR Spectroscopy, Bijvoet Center for Biomolecular Research, Utrecht University, Utrecht, The Netherlands

Received 30 June 2010; received in revised form 5 November 2010; accepted 5 November 2010
Available online 16 November 2010

Edited by P. Wright

Keywords:
Wnt pathway;
natively unfolded;
scaffold;
Axin;
β-catenin degradation

The Wnt pathway tumor-suppressor protein Axin coordinates the formation of a critical multiprotein destruction complex that serves to downregulate β-catenin protein levels, thereby preventing target gene activation. Given the lack of structural information on some of the major functional parts of Axin, it remains unresolved how the recruitment and positioning of Wnt pathway kinases, such as glycogen synthase kinase 3β, are coordinated to bring about β-catenin phosphorylation. Using various biochemical and biophysical methods, we demonstrate here that the central region of Axin that is implicated in binding glycogen synthase kinase 3β and β-catenin is natively unfolded. Our results support a model in which the unfolded nature of these critical scaffolding regions in Axin facilitates dynamic interactions with a kinase and its substrate, which in turn act upon each other.

© 2010 Elsevier Ltd. All rights reserved.

Introduction

The Wnt/β-catenin signaling pathway controls essential cellular decisions on growth and differen-

*Corresponding authors. E-mail addresses:
s.g.d.rüdiger@uu.nl; M.M.Maurice@umcutrecht.nl.

Abbreviations used: APC, adenomatous polyposis coli; CK1, casein kinase 1; GSK3β, glycogen synthase kinase 3β; AxinCR, central region of Axin; GST, glutathione S-transferase; siRNA, small interfering RNA; WCM, Wnt3a-conditioned medium; TFE, 2,2,2-trifluoroethanol; HSQC, heteronuclear single-quantum coherence; JNK, c-Jun N-terminal kinase; TEV, tobacco etch virus; TCEP, tris(2-carboxyethyl)phosphine; CV, column volumes; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate.
Wnt binding to its receptors Frizzled and Lrp5/6 at the cell surface results in the recruitment of the Axin–GSK3β complex, which now turns its activity to phosphorylate the cytoplasmic tail of Lrp6. Together, these events lead to an accumulation of dephosphorylated β-catenin and its migration to the nucleus to induce target gene transcription.

Axin possesses multiple functional domains through which it orchestrates β-catenin phosphorylation (Fig. 1). The N-terminal RGS (regulator of G-protein signaling) domain mediates binding to the large scaffolding protein APC. APC itself carries three short Axin binding motifs and multiple binding sites for β-catenin.23 The C-terminal DIX domain of Axin mediates self-association and binding to the DIX domain of Dishevelled, which is believed to modulate its signaling activity.24–26 The central part of Axin contains binding sites for GSK3β, β-catenin, and CK1.5,27 Scaffolding of APC, GSK3β, and β-catenin by Axin dramatically enhances GSK3β-mediated phosphorylation of β-catenin.28,29 At the same time, GSK3β-mediated phosphorylation of Axin enhances its stability.30

While crystal structures are available for both the RGS domain and the DIX domain of Axin,26,31 experimental structural information on the central region of Axin (AxinCR) is limited to small helical Axin peptides that were coocrystallized with GSK3β and β-catenin.32,33 Insight into the structural properties of this region is crucial to understanding how Axin coordinates protein–protein interactions in the β-catenin destruction complex to mediate its tumor-suppressor activity. The functional importance of the Axin central region is illustrated by an increasing number of reported missense mutations in AxinCR reported in a variety of human tumors.18–22

Using various biophysical and biochemical methods, we demonstrate here that the central domain of Axin is intrinsically disordered. Our findings support a model in which crucial scaffolding functions of Axin are exerted by a large highly flexible region.

Results

Recombinant AxinCRG295-A500 is functional in vitro and in vivo

Functional understanding of AxinCR, which connects the folded N-terminal RGS domain and the C-terminal DIX domain (Fig. 1), is limited due to a lack of experimental structural information. To determine the structural properties of AxinCR in an experimental approach, we generated and purified a recombinant fragment of AxinCR spanning Gly295 to Ala500 and including the GSK3β-binding domain and the β-catenin binding domain. First, we set out to determine if AxinCRG295-A500, lacking the N-terminal RGS domain and the C-terminal DIX domain of Axin, is functional in isolation. We recombinantly produced His-NusA-tagged GSK3β and His-tagged β-catenin, both His-NusA-tagged GSK3β and His-tagged β-catenin, but not control His-NusA protein, were able to bind AxinCRG295-A500 in a pull-down approach (Fig. 2a–c). To verify that this interaction also takes place in vivo, we expressed glutathione S-transferase (GST)-tagged AxinCRG295-A500 in HEK293T cells and confirmed its binding to endogenous GSK3β and β-catenin (Fig. 2d). These results are consistent with current models, which propose that AxinCR is able to perform its scaffolding function by bringing together kinase and substrate. In addition, ectopic expression of the GST-tagged AxinCRG295-A500 fragment in mammalian cells strongly induced β-catenin-mediated transcription in a luciferase reporter assay (TOPflash), whereas the GST-tagged full-length Axin protein fully suppresses Wnt-induced activation (Fig. 2e). These findings suggest that the AxinCRG295-A500 fragment competes with endogenous Axin (estimated ratio, 50:1) for binding to GSK3β and β-catenin, and dominantly

Fig. 1. Domain organization of Axin. Well-defined Axin domains that are important for Wnt pathway regulation are shown. The N-terminal RGS domain binds APC, and the C-terminal DIX domain is responsible for homodimerization and heterodimerization. The central region (indicated as AxinCR), connecting the RGS and DIX domains, binds to GSK3β and β-catenin. The experimental fragment AxinCRG295-A500 is indicated. Asterisks indicate the locations of missense mutations in AxinCR reported in a variety of human tumors.
interferes with the formation of a functional endogenous destruction complex. As a consequence, β-catenin accumulates and translocates to the nucleus to initiate the transcription of the luciferase reporter gene. In concordance, TOPflash reporter activity is induced by AxinCR\textsubscript{G295-A500} in a Wnt-independent manner (Fig. 2e).

To verify that the TOPflash activity enhanced by AxinCR\textsubscript{G295-A500} expression in cells depends on the activation of β-catenin, we performed the TOPflash
assay in the presence of small interfering RNA (siRNA) oligonucleotides that silence β-catenin expression (Fig. 2e). siRNA treatment for 48 h reduces endogenous β-catenin expression levels to 30% (Fig. 2f and g) and abrogates TOPflash activity in the presence or in the absence of AxinCRG295-A500, as well as upon addition of Wnt3a-conditioned medium (WCM). These results argue that AxinCRG295-A500 retains its GSK3β and β-catenin binding functions independent of the rest of the protein in vivo and in vitro.

**AxinCRG295-A500 does not undergo thermal denaturation and displays an extended conformation**

To determine whether AxinCRG295-A500 carries a three-dimensionally folded hydrophobic core, we performed thermal denaturation studies by monitoring intrinsic protein (Trp-mediated) fluorescence as a measure of tertiary structural changes. Two Trp residues are present in AxinCRG295-A500 (W297 and W444). We monitored the fluorescence spectrum of AxinCRG295-A500 while raising the temperature from 20 °C to 80 °C (Fig. 3a and b). The fluorescence intensity of AxinCRG295-A500 peaked at similar wavelengths at minimal and maximal temperatures (Fig. 3a). In concordance, the thermal denaturation curve of AxinCRG295-A500 did not reveal a transition in fluorescence intensity (Fig. 3c). In contrast, a recombinantly produced Axin RGS domain, which is known to exist in a tightly folded conformation, significantly shifts in peak fluorescence intensity when comparing minimal and maximal temperatures (Fig. 3b). Moreover, the RGS domain yielded a clear transition in fluorescence intensity during thermal denaturation, reflecting an unfolding of the hydrophobic core (Fig. 3d). These findings indicate that AxinCRG295-A500, in contrast to the Axin RGS domain, lacks folded domains with a hydrophobic core, or that the two Trp residues in the AxinCRG295-A500 sequence are not inside any folded region. To assess whether AxinCRG295-A500 lacks a hydrophobic core and consequently adopts an extended shape, we performed analytical size-exclusion chromatography (Fig. 4a). AxinCRG295-A500 eluted from the column with an elution volume of 9.92 ml. According to the calibration curve of standard markers, this volume corresponds to a molecular mass of 70 kDa, a value
that is significantly higher than the calculated mass of 23 kDa based on the primary amino acid sequence. The large apparent size of AxinCRG295-A500 during size-exclusion chromatography could be attributed to either an unfolded extended shape or oligomerization. To exclude the latter possibility, we analyzed whether His-tagged AxinCRG295-A500 could bind the untagged form by utilizing a pull-down assay. We could not detect any interaction between the two AxinCRG295-A500 forms (Fig. 4b). These findings rule out the possibility that AxinCRG295-A500 forms oligomers and support the assumption that AxinCRG295-A500 bears an extended conformation lacking a hydrophobic core.

AxinCRG295-A500 exhibits a random-coil secondary structure with residual helical elements

Next, we applied circular dichroism (CD) to gain insight into the presence of secondary structural elements in AxinCRG295-A500. The far-UV CD spectrum yielded a negative peak of ellipticity at 201 nm, a typical feature of random-coil structures.35 To uncover whether residual helical elements in the AxinCRG295-A500 fragment could be induced, we titrated increasing amounts of 2,2,2-trifluoroethanol (TFE), a helix-stabilizing agent that only acts on proteins that carry residual unstable helical structures by strengthening local H-bond interactions.36 Addition of TFE shifted the negative peak to 208 nm and induced another negative peak at 222 nm and a positive peak at 190 nm, yielding a spectrum of a protein with significant α-helical content (Fig. 5). With the use of CD deconvolution algorithms (DichroWeb37), the α-helical content of Axin is estimated to be 21% under physiological conditions and increases to 58% under conditions of 20% TFE. These results indicate that AxinCRG295-A500 has the tendency to form α-helical elements that are stabilized by conditions favoring intramolecular interactions.

AxinCR is predicted to lack order

To gain insight into the localization of helical elements and the overall structural organization of AxinCRG295-A500 (Fig. 6a), we utilized bioinformatic tools to predict secondary structure binding sites and disorder tendency. We submitted the AxinCRG295-A500 protein sequence to eight publicly available servers that predict secondary structure based on the primary sequence and to seven servers that predict protein disorder, implementing 12 algorithms (Fig. 6c and d). The chosen algorithms for disorder prediction were based on different criteria (reviewed by Ferron et al.38).

Individual prediction programs for secondary structure consistently predict three helical regions, two of which overlap with α-helical peptides previously cocrySTALLized in complex with GSK3β and β-catenin, respectively (Fig. 6c and d).32,33 In contrast, the predicted extent of disordered regions is inconsistent, differing significantly between algorithms (Fig. 6d). Noticeably, the amino acid composition of AxinCRG295-A500 reveals 52.8% disordered...
promoting residues (D, M, K, R, S, Q, P, and E) and 33.7% order-promoting residues (C, W, Y, I, F, V, L, H, T, and N); in comparison, for the folded RGS domain, the respective fractions compose 44.4% and 45.2%. Together with our experimental data, these predictions argue that AxinCR is unfolded while containing three short helices, which might only be stabilized by cofactors in the cell. As two of these helices perform protein–protein interactions and do not contain hydrophobic patches on their surface, we consider it unlikely for the three helical modules to form a hydrophobic core.

1H–15N heteronuclear single-quantum coherence NMR spectrum of AxinCRG295-A500 reveals an unstructured conformation

To gain insight into the tertiary structure of AxinCR, we performed two-dimensional 1H–15N HSQC NMR analysis (Fig. 7). The resulting HSQC spectrum showed strongly clustered resonances in a narrow range of 7.6–8.6 ppm, which is characteristic of unfolded proteins.40 To compare the properties of AxinCRG295-A500 in physiological buffer with an entirely unfolded AxinCRG295-A500, we repeated the two-dimensional 1H–15N HSQC NMR experiment in the presence of the denaturing agent urea. Both spectra reveal only minor differences, which most likely are caused by an increase in buffer viscosity upon addition of 5 M urea.41 These results confirm that AxinCRG295-A500 is intrinsically disordered under physiological conditions.

AxinCRG295-A500 strongly enhances the GSK3β-mediated phosphorylation of β-catenin

To address the scaffolding role of AxinCRG295-A500, we incubated GSK3β with a fixed amount of β-catenin substrate and titrated purified AxinCRG295-A500 in this reaction. Titration of AxinCRG295-A500 strongly enhanced the efficiency of GSK3β in phosphorylating β-catenin in vitro (Fig. 8a), whereas no effect was observed on AxinCR variants lacking either the β-catenin binding site (AxinCRΔβcat) or the GSK3β binding site (AxinCRΔGSK3β), suggesting that Axin binding of both GSK3β and β-catenin is required for its scaffolding function (Fig. 8b and c). Phosphorylation of the AxinCRG295-A500 and AxinCRΔβcat fragments occurs equally well (Fig. 8a and b), suggesting that both variants adequately bind GSK3β. In contrast, AxinCRΔGSK3β remains largely unphosphorylated (Fig. 8c), confirming a lack of GSK3β binding. Quantification of β-catenin phosphorylation levels in these experiments demonstrates that the enhancement of β-catenin phosphorylation reaches a plateau and drops upon further titration of AxinCRG295-A500 (Fig. 8d). These findings suggest the need for the formation of a trimolecular complex to most efficiently achieve β-catenin phosphorylation. AxinCRG295-A500 excess likely titrates GSK3β away from β-catenin, thereby diminishing the efficiency of phosphorylation (Fig. 8e). We conclude that the highly flexible AxinCRG295-A500 fragment scaffolds and targets GSK3β kinase activity towards its substrate, β-catenin.

Discussion

The tumor-suppressor protein Axin plays a crucial role in the control of Wnt signaling by coordinating the formation of a multiprotein complex that mediates the phosphorylation of the transcriptional regulator β-catenin.4 The N-terminal RGS domain and the C-terminal DIX domain assist in the assembly of the complex by binding the scaffolding protein APC and by mediating Axin oligomerization, respectively, whereas AxinCR brings together the kinases CK1 and GSK3β and their substrate, β-catenin.3 The structures of the RGS and DIX domains were solved previously,26,31 but as of yet, the tertiary structure of the connecting and functionally important middle part of Axin has just been predicted to be disordered42 and has not adequately been addressed.
Here, we have used a variety of biophysical and biochemical techniques to analyze the conformation of AxinCR. Our results convincingly show that the large AxinCR is intrinsically disordered under physiological conditions. Despite the clear lack of rigid tertiary structure in AxinCR, residual helical elements may be present, as shown by CD analysis. The unstable interconverting helical elements are stabilized by conditions that favor intramolecular hydrogen-bond formation (Fig. 5). In accordance with these findings, two independent AxinCR peptides that were cocryrstallized with GSK3β and β-catenin showed α-helical conformation. Together, these results argue that residual helical regions in unbound AxinCR may be stabilized with their interaction partners during complex formation. Indeed, disorder-to-order transitions that accompany the molecular recognition of disordered proteins are reported in an increasing number of cases. How does the large intrinsically disordered region facilitate the multiple roles of Axin in different and crucial developmental signaling pathways? Besides mediating β-catenin phosphorylation, the middle part of Axin accommodates multiple additional protein–protein interactions, the majority of which act in important signaling pathways [e.g., transforming growth factor β, c-Jun N-terminal kinase (JNK), and p53] (Fig. 9). Several of the binding sites of these interaction partners overlap. It remains unresolved how Axin, typified as the limiting component of the β-catenin destruction complex, may serve various functions in the cell. To meet this requirement, the intrinsically disordered propensity of AxinCR would provide a highly flexible and extended surface for interactions of low affinity and high specificity (Fig. 9). As a consequence, implicated protein interactions are fast and transient. For specificity to be secured, selective induction of (partial) folding or secondary structure formation by interacting proteins may serve to shape the conformation of the disordered region to facilitate the multiple roles of Axin in different and crucial developmental signaling pathways.
Mutations in Axin are strongly linked to the development of cancer. Many missense mutations, as reported in colorectal carcinoma, medulloblastoma, and hepatocellular carcinoma, hit AxinCR and AxinRGS (Fig. 1). Inhibition or downregulation of the tumor-suppressor protein Axin is believed to contribute to tumor formation. Regarding the lack of structure of AxinCR, missense mutations are unlikely to affect protein function and stability by misfolding, but rather would interfere with the binding of partner proteins to linear motifs, or may disturb interaction due to conformational selection. Remarkably, however, many of these mutations affect residues outside the linear binding motifs for β-catenin and GSK3β. How these mutations deregulate GSK3β activity towards β-catenin remains the subject of further studies.

In summary, we demonstrate that the functionally important AxinCR is natively unfolded under physiological conditions. These findings bear importance for our current study of the mechanism by which Axin performs its tumor-suppressor function.

Materials and Methods

Construct generation and protein expression

The fragments encompassing the central regions of human Axin1, AxinCR, AxinRGS, and AxinΔNcat (G295-G430), and AxinΔGSK3β (G433-V600), as well as the RGS domains of Axin (N2-V220), human β-catenin (full length and N138-L781), and human GSK3β, were amplified by PCR. All AxinCR fragments were cloned in the bacterial expression vector pET-24a(+) (Novagen). All proteins were expressed in Escherichia coli Rosetta2 cells (Merck4Biosciences). Cells expressing Axin fragments were grown in Luria–Bertani medium. M9 minimal medium enriched with 15NH4Cl (Cambridge Isotope Laboratories, Inc.) was used for NMR purposes. At the time point where the optical density (OD600) had reached 0.6, protein expression was induced by addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (Roche). Bacterial cultures expressing all AxinCR fragments and β-cateninN138-L781 were incubated at 20 °C for 4 h, and cultures expressing AxinRGS were incubated at 18 °C for 16 h.

Cells transformed with the β-catenin and GSK3β constructs were grown in Terrific broth autoinduction medium at 30 °C. At the time point where the OD600 had reached 3, the temperature was decreased to 15 °C, and the cells grew until the OD600 had reached 15.

Protein purification

Bacterial Rosetta cells expressing Axin fragments and β-cateninN138-L781 were pelleted, resuspended in buffer A

| AxinCRG295-A500 is predominantly unfolded. The 1H–15N HSQC spectrum of AxinCRG295-A500 in 10 mM phosphate buffer (pH 7.2) with anionic-strength 300 mM NaCl, 0.5 mM TCEP, 10% 2H2O, and 2.5% DSS is represented in red. The fingerprint resonance from amide protons of peptide bonds is not well dispersed and ranges from 7.6 ppm to 8.5 ppm, reflecting an unstructured conformation. The 1H–15N HSQC spectrum of AxinCRG295-A500 in 10 mM phosphate buffer (pH 7.2) with 300 mM NaCl, 0.5 mM TCEP, 10% 2H2O, 2.5% DSS, and 5 M urea is represented in blue. The two spectra show significant overlap, indicating a lack of structure of AxinCR under physiological conditions. |
[65 mM Tris–HCl (pH 7.5), 300 mM NaCl, 20 mM imidazole, 0.5 mM tris(2-carboxyethyl)phosphine (TCEP), and Complete Protease Inhibitor Cocktail EDTA-Free Tablets (Roche)], and lysed by sonication (Sonifier B12; Branson Ultrasonic). A subsequent ultracentrifugation step (30,000 rpm for 45 min at 4 °C) separated the soluble proteins from the insoluble proteins. The soluble fraction was filtered and applied to a POROS 20MC column (metal-chelate affinity chromatography; Applied Biosystems) connected to an Äkta Purifier chromatography system (GE Healthcare). The column was equilibrated with 10 column volumes (CV) of buffer A, and a linear gradient between buffer A and buffer B [50 mM Tris–HCl (pH 7.5), 300 mM NaCl, 1 M imidazole, and 0.5 mM TCEP] was applied to elute His-tagged proteins. Fractions were collected and analyzed by SDS-PAGE. Appropriate fractions containing His-tagged proteins were applied to a HiTrap desalting column (GE Healthcare) to exchange buffer B with 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 0.5 mM TCEP. Recombinant His-tagged TEV protease, which was modified to enhance stability (L56V/S135G; the expression construct was a kind gift of Cabrita et al.58), was added at a His-tagged protein/TEV ratio of 8:1. Cleavage took place at 37 °C for 3 h in the presence of 1 mM dithiothreitol (DTT). Next, the cleaved AxinCR was separated by the TEV protease using a second affinity purification. Bacterial cells expressing GSK3β and β-catenin were pelleted, resuspended in buffer C [50 mM Tris–HCl (pH 7.5), 500 mM NaCl, 1 mM TCEP, 10% glycerol, and Complete Protease Inhibitor Cocktail EDTA-Free Tablets (Roche)], and lysed mechanically by two passages through a cell cracker (EmulsiFlex-C5; Avestin). The lysate was cleared from aggregates at 45,000g for 50 min, and the supernatant was filtered and loaded on a POROS 20MC column. The column was equilibrated with 10 CV of

Fig. 8. AxinCR facilitates Axin fragment phosphorylation. In vitro phosphorylation of β-catenin by GSK3β in the absence and in the presence of a titration series of (a) AxinCR, (b) AxinCRΔβ, and (c) AxinCRΔGSK3β. The phosphorylation of Axin fragments is also shown. (d) Quantification of the incorporation of β-catenin for all reactions (a–c). The intensities were normalized against the value of the phosphorylated β-catenin in the absence of the Axin fragment. (e) Model depicting the effects of low (left), moderate (middle), and high (right) abundances of AxinCR on GSK3β-mediated kinase activity towards β-catenin. Under optimal stoichiometric conditions (middle), the majority of molecules are involved in trimolecular complex formation, providing the maximal phosphorylation of β-catenin. AxinCR excess (right) inhibits the formation of complexes composed of all three proteins, leading to a decrease in phosphorylation efficiency.
buffer C, and a linear gradient between buffer C and buffer D [50 mM Tris–HCl (pH 7.5), 500 mM NaCl, 1 mM TCEP, 10% glycerol, and 1 M imidazole] was applied to elute His-tagged proteins.

Interaction assays

Purified AxinCRG295-A500 was pulled down via affinity chromatography using purified His-NusA-GSK3β or His-β-catenin. His-NusA was used as negative control to verify the specificity of the interaction. His-AxinCRG295-A500 was used in order to examine the possibility of AxinCRG295-A500 self-interaction. In all assays, AxinCRG295-A500 was incubated with each His tagged protein for 2 h at 4 °C. Samples were loaded on gravity flow columns (Bio-Rad) with Ni²⁺ beads (Qiagen) equilibrated in buffer F [50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 20 mM imidazole, and 1 mM TCEP] and incubated for 1 h at 4 °C. Through gravity flow, unbound proteins were removed, and the beads were washed with 10 CV of buffer F. Bound protein complexes were eluted with 4 CV of buffer G [50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 500 mM imidazole, and 1 mM TCEP]. Samples were taken from all steps and analyzed on SDS-PAGE.

HEK293T cells were transfected with pEBG-2T (a mammalian GST expression vector) with or without AxinCRG295-A500 as fused protein. Cells were lysed in lysis buffer [50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 50 mM NaF, 1% Triton, 1 mM DTT, 1 mM Na3VO4, and protease inhibitors] at 4 °C. The soluble proteins were separated from cell debris via centrifugation and incubated with preequilibrated Glutathione Sepharose™ 4B bead (GS-4B) slurry (GE Healthcare) for 2 h at 4 °C. The beads were extensively washed with lysis buffer to eliminate aspecific binding. GST fusion protein complexes were eluted from the GS-4B beads with SDS sample buffer and denaturation (105 °C for 5 min). Samples were taken from all steps and analyzed by Western blot analysis.

Antibodies and siRNA

This study used mouse anti-GST antibody (Abcam), mouse anti-β-catenin antibody (BD Transduction Laboratories), rabbit anti-GSK3β antibody (Cell Signaling Technology), goat anti-Axin antibody (R&D Systems), mouse-anti-tubulin antibody (Sigma), and control siRNA and siRNA against β-catenin (Ambion).

Silencing of β-catenin

Cells were transfected with 64 nM control siRNA or siRNA against β-catenin. Transfection was performed using Lipofectamine 2000 in accordance with the manufacturer’s instructions. Forty eight hours after transfection, cells were lysed and β-catenin levels were analyzed by Western blot analysis.

Mammalian expression constructs and reporter assay

Human AxinCRG295-A500 was subcloned into a GST mammalian expression vector (pEBG-2T), and full-length...
Highly Flexible Scaffolding by Axin1

783

GST-tagged Axin was subcloned into pCS2. As mock, we used pEBG-2T without any fused protein. HEK293T cells were seeded in 24-well plates and cotransfected in duplicate with 100 ng of the indicated Axin constructs and 30 ng of the luciferase reporter construct TOPflash or FOPflash. Transfection efficiency in luciferase reporter assays was controlled and normalized by including a constant amount of TK Renilla reporter plasmid in all transfections. At 24 h after transfection, cells were treated with WCM or control L-cell medium for 16 h. Cells were lysed in passive lysis buffer (Promega), and luciferase activities were measured with the dual-luciferase reporter assay system (Promega) in accordance with the manufacturer’s instructions.

Fluorometric analysis

Purified AxinCRG295-A500 (10 μM) or AxinRGS (10 μM) in 10 mM sodium phosphate (pH 7.2), 150 mM NaCl, and 1 mM DTT was measured on a spectrophotometer (Perkin-Elmer LS55) with a 1.5-mm cuvette (Hellma) with a magnetic stirrer using the software Fluo_pe (D. Veprintsev; LMB Cambridge, UK). Fluorometric analysis was performed at increasing temperatures ranging from 20.1 °C to 80.1 °C for AxinCRG295-A500 and from 21.4 °C to 72.7 °C for AxinCR, with a 1 °C step increase. Fluorescence was measured with excitation at 280 nm and with emission at a range from 300 nm to 400 nm.

Analytical gel filtration

Analytical gel filtration of purified AxinCRG295-A500 at a concentration of 500 μM was performed on an Äkta Purifier (GE Healthcare) using a Bio-Silect SEC250 analytical column (300 mm × 7.8 mm) with a guard column (50 mm × 7.8 mm; Bio-Rad) equilibrated with 25 mM Tris–HCl (pH 7.2), 150 mM NaCl, and 1 mM TCEP. AxinCR was eluted at a flow rate of 1 ml/min, averaged over 10 acquisitions. Spectra were corrected for buffer contributions. Different concentrations of TFE were used: 10%, 20%, 30%, and 40% (vol/vol).

Disorder and secondary structure predictions

The primary protein sequence of AxinCR from Gly295 to Ala500 (GenBank accession number AAH44648.1) was submitted to seven publicly available servers implementing 12 different algorithms for protein disorder prediction. Eight publicly available algorithms were used for secondary structure predictions. In all cases, we used the default parameters. The servers used are as follows: SCRATCH/DisPro, DisEMB, GlobPlot, RONN, DripPrep, IUPred, PONDOR, Porter, TNIN, SORMA, GOR4, PSIPRED, APSSP2, and PROF.

NMR spectroscopy

15N–1H HSQC experiment was performed using 0.6 mM purified 15N-labeled AxinCRG295-A500 in buffer E [10 mM phosphate buffer (pH 7.2), 300 mM NaCl, 0.5 mM TCEP, 10% D2O, and 2.5% 2,2-dimethyl-2-silapentane-5-sulfonate (DSS)] and in buffer E plus 5 M urea. Spectra were measured at 25 °C on a Bruker DRX600 instrument equipped with a TCI cryoprobe. Spectra were processed with Topspin (Bruker) and analyzed with SPARKY software (T. D. Goddard and D. G. Keller, SPARKY 3, University of California, San Francisco).

In vitro phosphorylation

GSK3 (7 nM; BioVision) was added to phosphorylation buffer [50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 10 mM MgCl2, 1 mM DTT, and 10 μCi of [γ–32P]ATP (3000 Ci/μmol)] to phosphorylate 1 μM recombinant β-catenin in a total volume of 30 μl. Samples were incubated at 30 °C for 45 min in the absence or in the presence of purified AxinCRG295-A500 or AxinCRΔfcat (G295-G430), and AxinCR-ΔGSK3 (G433-V600) (0.1 μM, 0.2 μM, 0.4 μM, 0.8 μM, 1.6 μM, 3.2 μM, 6.4 μM, 12.8 μM, and 25.6 μM). Samples were mixed with SDS sample buffer and loaded on gels. The gels were dried, and a phospho imager screen (Molecular Dynamics) was used to measure radioactivity with the Storm scanner (Amer sham Biosciences). The intensities were quantified by ImageQuant software (Molecular Dynamics).

Acknowledgements

This work was supported by the Dutch Cancer Society (UU 2006–3508), the European Research Council (European Research Council starting grant 242958 to M.M.M.), the Utrecht University (High Potential Grants to M.M.M. and S.G.D.R.), the European Union (Marie-Curie-Excellence Grant to S.G.D.R.), and the Netherlands Organization for Scientific Research NWO (VIDI career development grant to S.G.D.R.). A.F. was supported by European Research Council starting grant 203413. We thank Jacques P. F. Doux and Tania Rutters-Meijneke (Biochemistry of Membranes, Bijvoet Center) for help with CD experiments.

Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2010.11.013
References


