A Nuclear Function for Armadillo/β-Catenin

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The Wnt signaling pathway provides key information during development of vertebrates and invertebrates, and mutations in this pathway lead to various forms of cancer. Wnt binding to its receptor causes the stabilization and nuclear localization of β-catenin. Nuclear β-catenin then functions to activate transcription in conjunction with the transcription factor TCF. A recent report has challenged this basic precept of the Wnt signaling field, arguing that the nuclear localization of β-catenin may be unrelated to its function and that β-catenin functions at the plasma membrane to activate this signaling pathway. Here we present evidence that the pathway in fact does depend on the nuclear localization of β-catenin. We reexamine the functionality of various truncations of β-catenin and find that only the most severe truncations are true signaling-null mutations. Further, we define a signaling-null condition and use it to show that membrane-tethered β-catenin is insufficient to activate transcription. We also define two novel loss-of-function mutations that are not truncations, but are missense point mutations that retain protein stability. These alleles allow us to show that the membrane-bound form of activated β-catenin does indeed depend on the endogenous protein. Further, this activity is dependent on the presence of the C-terminus-specific negative regulator Chibby. Our data clearly show that nuclear localization of β-catenin is in fact necessary for Wnt pathway activation.

Introduction

The Wnt signal transduction pathway has been studied extensively in both vertebrate and invertebrate systems. The Drosophila ortholog wingless (wg) is a segment polarity gene that defines posterior cell fates in each of the larval segments (for a review of the various functions of Wg, see Wodarz and Nusse 1998). The pathway is activated when the extracellular ligand Wg binds to the transmembrane receptors Frizzled and Arrow. These in turn activate Disheveled (Dsh), which inactivates a complex composed of Axin, adenomatous polyposis coli (APC), and Zeste-white 3 (Zw3) (the Drosophila homolog of glycogen synthase kinase [GSK3β]). This complex is responsible for the retention of Armadillo (Arm) in the cytoplasm, for its phosphorylation, and thus for its targeting for ubiquitination and destruction. When the complex is activated by Dsh, the intracellular levels of Arm increase, and Arm enters the nucleus, where in combination with the transcription factor TCF/Pangolin, it activates the transcription of genes such as cyclin D and c-myc (Wodarz and Nusse 1998).

We have argued that Axin plays a key role in the Wnt signaling process, functioning both as an anchor for Arm and a scaffold for the degradation complex. Wnt signaling results in a visible reduction in Axin levels, and mutations in Axin cause a relocalization of Arm to the nucleus (Tolwinski and Wieschaus 2001; Tolwinski et al. 2003). The nuclear import and export of Arm are not clearly understood (for a review, see Henderson and Fagotto 2002), but Arm can cross the nuclear membrane by interacting with the nuclear pore complex directly. Once in the nucleus, Arm interacts with a variety of nuclear factors, in particular the transcription factor TCF/LEF (Behrens et al. 1996; Molenaar et al. 1996; Brunner et al. 1997; van de Wetering et al. 1997). The β-catenin–TCF complex releases repression and activates transcription (Cavallo et al. 1998).

A recent study has challenged this view and has questioned the importance of nuclear localization of Arm protein (Chan and Struhl 2002). These authors’ conclusions were based primarily on the observation that a membrane-tethered, stabilized form of Arm (ArmΔArm) causes activation of the Wnt pathway without entering the nucleus. However, this is not the first time that the controversy about the location of Arm/β-catenin function has arisen. Previously, a group working with amphibian embryos had found that membrane-tethered plakoglobin, a close relative of β-catenin, can activate Wnt signaling (Merriam et al. 1997). Another group showed, however, that expression of membrane-tethered forms of β-catenin leads to the nuclear localization of endogenous β-catenin (Miller and Moon 1997). When the endogenous Arm/β-catenin gene was mutated, the activity of membrane-tethered forms was lost (Cox et al. 1999b). These experiments illustrate the importance of following the activity of the endogenous allele in evaluating the activity of membrane-tethered forms. Previously, we had expressed the same membrane-tethered form used by Chan and Struhl (2002) in embryos with various endogenous arm mutations and had concluded that it functions by titrating Axin to the membrane, releasing the endogenous Arm protein and allowing it to move freely into the nucleus (Tolwinski and Wieschaus 2001). These experiments are difficult, because none of the cell-viable alleles are absolute genetic nulls, as Arm plays essential roles in both Wnt signaling and cell adhesion.

In this study, we reexamine Arm function using three classes of previously described arm alleles. We find that by manipulating their levels and localizations, many alleles...
believed to be signaling nulls can still activate transcription. When the cell-adhesive defects of the most severe class of alleles are rescued, however, the mutant protein still fails to signal, allowing us to assay the activity of membrane-tethered Arm in a true signaling-null background. We find that nuclear localization is necessary for pathway activation and that exclusively membrane-bound forms of Arm are insufficient for this. We use two novel missense mutations in arm to assess the nuclear activity of Arm and confirm that negative regulation by the transcriptional regulator Chibby (Cby) is required for patterning.

**Results**

**Membrane-Tethered Arm Is Dependent upon the Endogenous arm Allele**

The original mutants in the arm gene were classified into three groups based upon their phenotypes and the position of stop codons that result in truncated proteins. The “weak” class has the smallest truncations and is represented by armXM19. In germline clones (where maternal and zygotic contribution of protein is removed; Chou and Perrimon 1992), its phenotype is identical to loss-of-function wg mutations (Figure 1B; Peifer and Wieschaus 1990). The “medium” class, represented here by armO43A01, shows defects in adhesion as well as transcription. Here germline clones give embryos that fail to differentiate an intact cuticle (Figure 1C; Tolwinski and Wieschaus 2001). The “strong” class (armXK22) does not allow proper progression through oogenesis and germline clones do not make eggs (Figure 1D; Peifer et al. 1993). Cox et al. (1999b) showed that the junctional defects of the “medium” alleles can be circumvented by coexpression of a membrane-tethered full-length form of Arm (ArmS18) (Figure 2). We have confirmed their findings and extended them to the “strong” allele during oogenesis. We show that uniform expression of ArmS18 allows germ cells to produce normal eggs and rescues the adhesive defects of both armXK22 and armO43A01 embryos.

Expression of ArmS18 does not, however, rescue the signaling defects associated with either of these alleles and the embryos show typical wg phenotypes (see Figure 1G and 1H).

Expression of ArmS18 has no effect on the cuticle of wild-type embryos (compare Figure 1E to 1A), but it does rescue the signaling defects of arm alleles, like armXM19, that have only short C-terminal truncations (Cox et al. 1999b; see Figure 1F). These alleles normally show very low levels of protein (Peifer and Wieschaus 1990), and Cox et al. (1999b) postulated that expression of a membrane-tethered Arm might “free up” the endogenous mutant protein, allowing the “weak” allele to signal. The low levels of armXM19 may reflect degradation of nonsense mRNAs triggered by the premature stop codon in this mutant (reviewed in Wagner and Lykke-Andersen 2002). To eliminate this degradation, we expressed a cDNA version of the armXM19 allele under GAL4/UAS control (Brand and Perrimon 1993) in embryos mutant for armXM19 (Figure 3B). To avoid the possibility of overexpression artifacts, we also expressed a smaller C-terminal deletion from the endogenous promoter (armS8; Orsulic and Peifer 1996). In both experiments, the truncated protein from the transgene accumulated to levels approaching those observed in wild-type (Figure 3G and 3H) and in the characteristic striped pattern indicative of response to the Wg signal (Peifer and Wieschaus 1990). The truncated protein rescued the armXM19 phenotype to a wild-type cuticle pattern and allowed hatching (Figure 3C). When combined with a

**Figure 1. ArmS18 Requires Endogenous Arm**

Endogenous allele indicated at top; ectopically expressed transgenes indicated at left.
(A) The wild-type cuticle of a Drosophila embryo.
(B) The armXM19 “weak” allele phenotype, similar to wg mutations in which the entire cuticle is covered with denticles.
(C) The armO43A01 “medium” allele phenotype shows disintegrated embryos in which cells delaminate owing to an inability to form adherens junctions.
(D) armXK22 “strong” allele does not produce embryos, owing to an oogenesis defect.
(E) A wild-type embryo expressing ArmS18 shows a wild-type cuticle.
(F) armXM19 mutant expressing ArmS18 is rescued to a wild-type cuticle.
(G) armO43A01 mutant expressing ArmS18 shows rescued adhesion, but a wg mutant signaling phenotype.

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K) Coexpression of Arm

vented by coexpression of a membrane-tethered full-length tethered, unstabilized form of Arm (ArmS18) leads to pathway activation only in cells that receive Wg signal, because this form of Arm is still armXK22

(H) armXK22 mutant expressing ArmS18 also shows rescued adhesion, as well as a wg mutant signaling phenotype.

(l) Coexpression of ArmArm and ArmS18 in wild-type embryos leads to naked cuticle or the uniform Wg active phenotype.

(K) Coexpression of Arm Arm and ArmS18 leads to naked cuticle or the uniform Wg active phenotype in an armXM19 mutant background.

(L) However, coexpression of ArmArm and ArmS18 in “strong” mutant armXK22 background shifts embryos back to the wg mutant phenotype. Expression of the membrane-tethered, stabilized form of Arm (ArmArm) leads to uniform activation of signaling in all cells. This effect is independent of whether the cell is exposed to Wg signal or not, because ArmArm functions independently of Wg ligand. The membrane-tethered, unstabilized form of Arm (ArmS18) leads to pathway activation only in cells that receive Wg signal, because this form of Arm is still subject to Wg-dependent phosphorylation and phosphorylation-dependent degradation.

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mutation in the kinase zw3. ArmS8 causes the cuticles of these embryos to appear uniformly naked (compare Figure 3E to 3D), as would be expected since the Arm S8 protein is expressed to high uniform levels throughout the epidermis when Zw3 is removed (Figure 3I). These experiments argue that the C-terminus is not essential for signaling or transcriptional activation of Wnt targets required for cuticle patterning. However, as we do not obtain adult flies containing exclusively the truncated alleles, it is very likely that the C-terminus is not entirely expendable and must have important functions later in development.

Null Allele Background Proves That ArmS8 Cannot Signal on Its Own

The fact that armXM19 is able to signal when expressed at normal levels invalidates its use in tests for a direct activity of membrane-tethered Arm in Wnt signaling (Chan and Struhl 2002). Therefore, expression of ArmS8 in a "weak" allele background cannot address whether membrane-tethered Arm activates transcription without ever entering the nucleus, since a membrane-un tethered, signaling-competent form of Arm is also present. To directly address whether the ArmS8 transgene can transmit Wg signal on its own, we turned to the "strong" and "medium" alleles. Although ArmS18 is not sufficient to restore signaling to these alleles, it...
raises the possibility that stronger expression of stabilized, membrane-tethered Arm (Arm\textsuperscript{DArm}) might reveal some signaling capacity of those alleles as well. Experiments of this kind have been difficult with Arm\textsuperscript{DArm}, given that it lacks the \(\alpha\)-catenin-binding site and fails to rescue the junctional defect in “medium” and “strong” endogenous arm allele backgrounds. We have found that by expressing both Arm\textsuperscript{DArm} and Arm\textsuperscript{S18}, we can recover intact embryos in all backgrounds tested. We find that “medium” and “weak” alleles can be induced to activate transcription, but the “strong” arm allele cannot (see Figure 1J–1L), consistent with the position of the “medium” alleles in the hypomorphic allelic series. These findings demonstrate that Arm\textsuperscript{DArm} is dependent upon the endogenous form of arm, as it cannot activate transcription in the “strong” allele background.

Loss-of-Function Missense Mutations

When Arm\textsuperscript{DArm} is expressed in a wild-type embryo, it strongly activates Wg signaling (Figure 4C; Chan and Struhl 2002). Chan and Struhl (2002) suggest that this is because this membrane-tethered form of Arm can signal on its own. The results presented above argue, on the other hand, that it does so by stabilizing the endogenous protein. To further test this, we asked whether expression of Arm\textsuperscript{DArm} can induce Wg signaling when endogenous Arm is replaced by signaling-deficient Arm. We turned to two novel missense mutations where the rest of the arm coding region remains intact. Because these alleles do not produce truncations through stop codons, they are immune to nonsense mRNA degradation (Wagner and Lykke-Andersen 2002). Both mutations result in amino acid substitutions close to repeat seven, a key hinge region postulated to be important in binding of TCF (Huber et al. 1997; Graham et al. 2000). Both mutants retain the phosphorylation sites required for degradation and therefore accumulate in stripes in response to Wg signal (Figure 5I and 5J). They supply apparent wild-type junctional
activity and accumulate to high levels in all cells when the kinase responsible for the degradation signal (Zw3) is removed (Figure 5K and 5L). The primary phenotype of these alleles is a loss or reduction of Wnt transcriptional responses (Figure 5A and 5B). The armF1a allele produced a partial loss-of-function phenotype, and germline clone embryos show some residual naked cuticle. armLM134 produces a stronger phenotype comparable to a loss of wg function, although it may not be a signaling null (see below).

We asked whether these signaling-deficient alleles could block the cell fate transformation and Wnt target activation observed when ArmΔArm is expressed in wild-type epidermis. If ArmΔArm functions independently of the endogenous protein, then all cells should assume the naked cell fate. However, this does not occur (Figure 5C and 5D). Instead, both point mutants produce a cuticle pattern with periodic denticle belts and regions of intervening naked cuticle. This periodicity may reflect the fact that armF1a and armLM134 can still be controlled by Wg even when ArmΔArm is expressed. This periodicity is, in fact, abolished when Zw3 activity is removed from such embryos (i.e., in triply mutant armΔArm embryos). Under these conditions, all cells in the cuticle take on the naked cell fate (Figure 5E and 5F). Since ArmΔArm lacks the N-terminal sites that respond to Zw3, the sensitivity of the double-mutant phenotype confirms that the pattern of the double mutant is dependent on the endogenous Arm protein.

The behavior of membrane-tethered ArmΔArm contrasts with that of other stabilized forms of Arm that would be predicted to move more freely between the cytoplasm and the nucleus. ArmS10, for example, contains a small N-terminal deletion that blocks Zw3 phosphorylation, but preserves binding sites for various nuclear proteins (see Figure 2; Pai et al. 1997). ArmS10 is not membrane-tethered, but the cell fate transformations it produces are identical to those produced by ArmΔArm (compare Figure 4C and 4D). They do not, however, depend on the endogenous allele and are still observed in an armF1a or armLM134 germline clone background (Figure 5G and 5H).

ArmΔArm Causes Nuclear Localization and Mobility Shift of Endogenous Arm

All of our experiments argue that ArmΔArm produces its effect on transcription by activating the endogenous alleles. To investigate the mechanism that underlies this effect, we looked at the in situ localization of the endogenous Arm protein and its migration pattern on Western blots. Expression of ArmΔArm is sufficient to drive both wild-type and the point mutant forms of Arm into nuclei (see Figure 4A and 4B; Miller and Moon 1997; Tolwinski and Wieschaus 2001).

Generally, the most obvious feature observed upon removal of any of the negative factors of the Wg pathway is the rapid accumulation of Arm in cells. However, another feature is the phosphorylation state of the Arm protein. Peifer et al. (1994a) found that a fast-migrating band of Arm corresponds with active Wg signaling and that a slower-migrating band corresponds with Wg’s being off. Therefore, it is the unphosphorylated band that corresponds with signaling. Here we show that, on Western blots, endogenous Arm protein responds to ArmΔArm expression in much the same way that it does to the removal of negative components of the pathway such as Axin and APC1 and APC2 (see Figure 4E).

We see a downshift of the protein, which is directly opposite to what is seen when a positive component of the pathway is removed (Dsh or Wg; see Figure 4E). Wild-type embryos show the expected intermediate phenotype, as they have both active and inactive forms of Arm protein (see Figure 4E). The observed shift is most likely the result of phosphorylation (Peifer et al. 1994a), though we do not address this directly in this study.

The C-Terminus of Arm Is Necessary for Cby-Mediated Repression

Although the missense mutations we have used in our studies produce (on average) weaker phenotypes, they are more effective at blocking the cell-fate transformation induced by ArmΔArm than the “medium” C-terminal truncation mutants (compare Figure 1K with Figure 5C and 5D). The comparison is somewhat indirect, owing to the necessity of expressing ArmS18 in the “medium” arm allele background in order to get intact embryos. However, we find that expression of ArmS18 in an armF1a background has no visible effect on the cuticle (data not shown). Therefore, the activity of C-terminally truncated arm alleles in response to ΔArm expression suggests that, under certain conditions, removal of the C-terminus may actually enhance the transcriptional activity of Arm. One possibility is suggested by the recent discovery of Cby (Takemaru et al. 2003), a nuclear negative regulator of the Wg pathway that binds to the C-terminus of Arm. To test whether nuclear Cby affected the transcription produced by ArmΔArm, we used RNA interference (RNAi) to reduce Cby levels in armF1a embryos with and without ArmΔArm. In the absence of ArmΔArm, i.e., in embryos where most ArmF1a protein is cytoplasmic, Cby RNAi has no effect (Figure 6D). However, when ArmΔArm is present, lowering Cby levels leads to increased naked cuticle characteristic of Wnt pathway activation (compare Figure 6B to 6C). We propose that Cby’s effect on armF1a protein is dependent on ArmΔArm relocalizing Arm to the nucleus.

Discussion

In this study we offer genetic proof that the nuclear localization of Arm is important for the activation of the pathway. The dissenting view (Chan and Struhl 2002) relied on C-terminal truncations that we have shown retain their ability to signal if their levels are increased. These alleles also appear to bypass the normal nuclear regulation by Cby. We show that full-length loss-of-function forms of Arm provide a novel way of assessing the activity of the pathway. Finally, we show that in an approximate signaling-null condition, ArmΔArm cannot activate transcription on its own. Based on these findings, we propose that membrane-tethered Arm, whether wild-type or activated, cannot activate transcription on its own. It does, however, have a profound effect on the endogenous form, forcing both “weak” and “medium” alleles to translocate to the nucleus and activate transcription. Our findings extend and build upon the original nuclear localization of Arm model (Miller and Moon 1997; Cox et al. 1999b). Further support for the nuclear localization of Arm model has recently been provided by the publication of a study that uses tissue culture experiments to show that nuclear localization of Arm is required (Cong et al. 2003).

Our results also point to an unexpected feature of Arm,
namely that the C-terminus, although it has been shown to supply transcriptional activation (Hsu et al. 1998), does not appear to be required for Wnt activation. Cox et al. (1999a) studied this aspect of Arm function and found that a C-terminally truncated form of Arm can significantly rescue the signaling defects of arm mutants, but is not as good as the wild-type form at transcriptional activation. Further, given that arm mutant flies expressing the transgene that lacks the C-terminus do not survive to adulthood, the C-terminus may not be entirely expendable. This may point to the requirement for Cby-based repression or Teashirt-mediated activation at a later stage of development, as both these proteins function by binding the C-terminus of Arm (Gallet et al. 1999; Takemaru et al. 2003). However, taken together with the finding that an N-terminally truncated Arm sent to the nucleus fails to activate transcription (Chan and Struhl 2002), it appears that it is the N-terminus that is most important for the nuclear transactivation and chromatin remodeling functions ascribed to β-catenin (Hsu et al. 1998; Hecht and Kemler 2000; Takemaru and Moon 2000; Barker et al. 2001; Tutter et al. 2001; Bienz and Clevers 2003).

We have previously shown that the “medium” arm mutant (armO43A01), which creates a stop codon eliminating repeats 10 through 12 and the entire C-terminus) does not signal in the presence of uniform ArmΔArm (Tolwinski and Wieschaus 2001). Chan and Struhl (2002) found that armO43A01 embryos expressing high levels of ArmΔArm from the paired GAL4 driver were able to activate Wnt targets. But since neither ArmΔArm nor armO43A01 can provide junctional Arm activity, the abnormalities of these embryos make these experiments difficult to interpret. As an alternative, we used a membrane-tethered but otherwise wild-type form of Arm (ArmS18), which we expressed in armO43A01 mutant embryos (see Figure 1G). The ArmS18 allele rescues the junctional defects, but does not allow signaling. Similar results have been obtained with another “medium” allele, armRPS7 (Cox et al. 1999b). However, when combined with ArmΔArm and ArmS18, armO43A01 can now be clearly seen to activate naked cell fates. It thus appears that even the “medium” alleles of arm actually do retain some ability to function when ArmΔArm is present. This is not observed in the larger truncations (“strong” alleles), consistent with the “medium” alleles retaining the TCF-binding region (Graham et al. 2000).

The question now becomes what is ArmΔArm doing at the membrane that causes such drastic change in the signaling kinetics of the pathway. We have previously argued that ArmΔArm may function by titrating the cytoplasmic anchoring activity of Axin and by therefore allowing rapid enrichment of Arm in the nucleus. We have in fact observed such an enrichment and have shown that it is counteracted by increasing the level of Axin (Tolwinski and Wieschaus 2001). Further work has pointed to the importance of controlling Axin stability in pathway activation (Salic et al. 2000; Mao et al. 2001; Lee et al. 2003; Tolwinski et al. 2003). Expression of large quantities of a stabilized, membrane-tethered form of Arm might also remove additional cytoplasmic inhibitory factors, preventing them from interacting with non-tethered Arm. In turn, even lower-level or lower-activity alleles will now be able to activate transcription, simply owing to the complete lack of inhibiting factors.

The missense mutations described here provide a glimpse of the in vivo activity of Arm protein. Structural studies of β-catenin found that although the central repeat region forms a uniformly repeating super helix, one α-helix was missing from repeat seven. The missing helix might allow a local flexibility in the structure and led the authors to define this region as a potential hinge (Huber et al. 1997). Further
crystallographic analysis concluded that this region was important for TCF binding (Graham et al. 2000). Both our point mutations cluster around this repeat and would probably lead to structural consequences for this hinge. The apparent specificity of these alleles for the transcriptional response to Wnt signaling provides in vivo evidence that the postulated hinge may be very important for that aspect of Arm protein function.

Note

As the final version of this paper was being prepared, the paper by Chan and Struhl (2002) was retracted.

Materials and Methods

Fly Strains

The wild-type strain used was Oregon R. See Flybase (http://flybase.bio.indiana.edu) for details on mutants used. Hypomorphic mutants of arm are as follows: armLM134 TCC to TTC at nucleotide 2776, armLM134 CGC to CAC at nucleotide 2990, armLM134 stop codon at nucleotide 3466, armLM134 stop codon at nucleotide 3850, armLM134 stop codon at nucleotide 3404, armLM134 stop codon at nucleotide 3401, Other alleles used were armS044230 zw3M11–1, dsh276, apc2690 and apcG222.

Crosses and Expression of UAS Constructs

arm mutants. As Arm and many other Drosophila proteins are contributed maternally, to fully evaluate the function of a mutant protein, one needs to make embryos maternally and zygotically mutant. Therefore, maternally mutant eggs were generated by the dominant female sterile technique (Chou and Perrimon 1992). For all expression experiments, the Arm–GAL4 driver was used. All X-chromosome mutants use FRT 101. The arm mutants used were as follows: armLM134 zw3M11–1 (zygotic) armLM134 zw3M11–1 (maternal)/Y (zygotic) armLM134 (maternal)/Y (zygotic) armLM134 (maternal)/Y (zygotic) armLM134 (maternal)/Y (zygotic); Arm–GAL4/UAS–Arm (zygotic) armLM134 zw3M11–1 (zygotic); Arm–GAL4/UAS–Arm (zygotic) armLM134 (maternal)/Y (zygotic); Arm–GAL4/UAS–Arm (zygotic)

Supporting Information

Accession Numbers

The GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) accession numbers for the genes and alleles discussed in this paper are apc1Q8 (U77947), apc2690 (AF901430), arm (X54468), armS044230 (AF086811), dsh276 (U02491), apcG222 (NM 104746), and zw3M11–1 (X54005).

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Conflicts of interest

The authors have declared that no conflicts of interest exist.

Author contributions

NST and EW conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, and wrote the paper.

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