Roles of the C Terminus of Armadillo in Wingless Signaling in Drosophila

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ABSTRACT

Drosophila melanogaster Armadillo and its vertebrate homolog β-catenin play multiple roles during development. Both are components of cell-cell adherens junctions and both transduce Wingless (Wg)/Wnt intercellular signals. The current model for Wingless signaling proposes that Armadillo binds the DNA-binding protein dTCF, forming a bipartite transcription factor that activates Wingless-responsive genes. In this model, Armadillo's C-terminal domain is proposed to serve an essential role as a transcriptional activation domain. In Xenopus, however, overexpression of C-terminally truncated β-catenin activates Wnt signaling, suggesting that the C-terminal domain might not be essential. We reexamined the function of Armadillo's C terminus in Wingless signaling. We found that C-terminally truncated mutant Armadillo has a deficit in Wg-signaling activity, even when corrected for reduced protein levels. However, we also found that Armadillo proteins lacking all or part of the C terminus retain some signaling ability if overexpressed, and that mutants lacking different portions of the C-terminal domain differ in their level of signaling ability. Finally, we found that the C terminus plays a role in Armadillo protein stability in response to Wingless signal and that the C-terminal domain can physically interact with the Arm repeat region. These data suggest that the C-terminal domain plays a complex role in Wingless signaling and that Armadillo recruits the transcriptional machinery via multiple contact sites, which act in an additive fashion.

CELL-CELL signals and their attendant signal transduction pathways shape the fates of virtually all cells within the body, both during normal embryonic development and as a part of the physiology of the adult animal. Further, the inappropriate activation of these pathways is a contributing cause of many human cancers. It is thus important to understand in detail the mechanisms by which signals are transduced. In addition, many of these signaling pathways already existed in the common ancestor of most if not all multicellular animals. Identification of both the conserved features of these pathways and also the ways in which their deployment differs in different animals can help us understand the forces that shape evolutionary change.

We focus on the Wingless (Wg)-/Wnt-signaling pathway (reviewed in Cadigan and Nusse 1997). Members of the Wg/Wnt family of ligands direct a wide variety of cell fate decisions in all animals examined. This pathway mediates some of the earliest cell fate choices in both the vertebrate Xenopus and the nematode *Caenorhabditis elegans*, as well as directing fine-scale patterning in the embryonic ectoderm of Drosophila, the mammalian CNS, and fly and mammalian limbs. In many of these cases, the signal is transduced by a common set of components that have been identified by genetic screens in Drosophila, *C. elegans*, and mammals, as well as by biochemical approaches.

Drosophila Armadillo (Arm) and its vertebrate homolog β-catenin (βcat) are key effectors of Wg signal (reviewed in Cadigan and Nusse 1997). They also play additional roles in the cell; for example, they act as key components of cell-cell adherens junctions. These different roles are reflected in Arm/βcat's subcellular distribution. Arm/βcat accumulates in adherens junctions of most cells; in cells that do not receive Wg/Wnt signals, nonjunctional Arm/βcat is unstable and rapidly degraded by a multiprotein complex that includes Zeste white3 kinase (Zw3; vertebrate homolog is GSK-3β), APC, and Axin. Wg/Wnt signals act through a family of receptors related to the fruit fly Frizzled protein. Certain Frizzled family receptors activate the protein Dishevelled, leading to stabilization and thus accumulation of nonjunctional Arm/βcat via inactivation of the destruction machinery.

The data concerning the subsequent steps in the pathway have been interpreted in several ways. The data in Drosophila support a model in which Arm is stabilized by Wg signaling and thus enters the nucleus and binds to the HMG-class DNA-binding protein dTCF (also called Pangolin; Brunner *et al.* 1997; van de Wetering *et al.* 1997). This complex is thought to act as a bipartite transcription factor that activates Wg-responsive genes, with dTCF contributing the DNA-binding domain and

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the proximal C terminus of Arm acting as a transcriptional activation domain. Several studies support this model: (1) mutations truncating the C terminus of Arm disrupt its ability to transduce Wg signaling *in vivo* (Klingensmith *et al.* 1989; Peifer *et al.* 1994a; Orsulic and Peifer 1996; White *et al.* 1998), (2) Arm's C terminus acts as a transcription-activation domain in a simplified mammalian gene expression assay (van de Wetering *et al.* 1997), and fusion of the Arm C terminus to TCF makes it an Arm independent activator (Roose *et al.* 1998; Vleminckx *et al.* 1999), and (3) mammalian βcat, the C terminus of which diverges from that of Arm, has reduced signaling ability in Drosophila (White *et al.* 1998). However, these results contrast with data obtained in Xenopus. Overexpression of a βcat mutant encoding only the Arm repeat region (and thus lacking the C-terminal domain) activates Wnt signaling (Funayama *et al.* 1995), suggesting that in Xenopus βcat's C terminus is not essential for signaling. Wnt signaling is also activated by the Arm repeat region of plakoglobin, a βcat paralog (Karnovsky and Klymkowsky 1995; Rubenstein *et al.* 1997).

These latter data can be interpreted several ways. C-terminally truncated βcat should bind to components of the destruction machinery, such as APC or Axin. By doing so, it could shield the endogenous full-length β-catenin in frog embryos from destruction, allowing it to accumulate and thus to signal. This interpretation is consistent with the current model. Alternately, the current model may be wrong. We now realize that in the absence of Arm/βcat, TCF/LEF represses Wg/Wnt-responsive genes (Brannon *et al.* 1997; Riese *et al.* 1997; Cavallo *et al.* 1998; Roose *et al.* 1998). Further, genetic data from *C. elegans* suggest that the TCF relative Pop-1 antagonizes Wnt signaling during early embryogenesis (Rocheleau *et al.* 1997; Thorpe *et al.* 1997). This led to the suggestion that the only role of Arm/βcat might be to bind to and thus inhibit the repressor activity of TCF/LEFs, allowing the activation of Wg/Wnt-responsive genes (Merriam *et al.* 1997). C-terminally truncated Arm retains the dTCF-binding site (van de Wetering *et al.* 1997) and thus could potentially inhibit TCFs.

The two models make different predictions about the signaling ability of C-terminally truncated Arm/βcat in the absence of endogenous wild-type Arm/βcat. If C-terminally truncated Arm/βcat acts only by stabilizing endogenous Arm/βcat, there should be no signaling in the absence of wild-type protein. In contrast, if Arm/βcat's C terminus is dispensable for signaling, the absence of endogenous protein should have no effect. In Drosophila we can remove the endogenous protein by mutation or replace it with mutated versions of the protein, retaining only certain functions. We thus carried out detailed tests of several C-terminally truncated versions of Arm, both in the presence and absence of wild-type endogenous Arm.

MATERIALS AND METHODS

Mutant constructs: The mutant genes created for use in this study encode different Arm domains. Arm-R (Arm repeats R1–13, encoding amino acids 127–719) and Arm-NR (the N-terminal domain plus Arm repeats 1–13, encoding amino acids 1–719) were subcloned from pBSArmR1–13 and pBSArmNR (Pai *et al.* 1996), respectively, either into pUAST (Brand and Perrimon 1993; Arm-R), or into pUAST-myc (Arm-Rmyc and Arm-NR), derived from pUAST by adding an initiator methionine and a single c-myc epitope immediately after the promoter. We also cloned full-length Xenopus β-*cat* into pUAST.

Biochemical analyses: Levels of protein expression and phosphorylation isoforms were analyzed by collecting embryos from crosses of individual transformant lines to *e22c*-GAL4/*CyO*, making and analyzing embryo extracts by SDS-PAGE and immunoblotting with anti-myc, followed by enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL) detection (for total levels) or detection with alkaline phosphatase-coupled secondary Ab, NBT, and BCIP (Promega, Madison, WI; for isoforms). Since ECL detection is not strictly linear, we examined several exposures of each blot and repeated individual experiments to confirm differences. For immunoblotting we used monoclonal anti-c-myc 9E10 culture supernatant directly. Other antibodies were diluted as follows: monoclonal anti-Arm 7A1 (Peifer *et al.* 1994a; 1:500), monoclonal anti-BicD (Suter and Steward 1991; 1:30). Membrane fractionation was as in Peifer (1993).

Immunofluorescence and antibody staining: Embryos were collected and dechorionated as in Cox *et al.* (1996), and antibody incubations and washes are described in Peifer *et al.* (1993). For monoclonal Armadillo antibody 7A1 (1:200), embryos were fixed for 5 min in 37% formaldehyde, incubated in primary antibody for 1 hr at 25° or overnight at 4° in antibody wash (1% BSA in disc wash), and washed in antibody wash three times for 5 min each time. Secondary antibody incubation was as in Cox *et al.* (1996).

Genetics: GAL4 stocks were provided by the Bloomington Drosophila Stock Center. The zygotic and maternal phenotypes of arm^{YD35}, $arm^{H8.6}$, and arm^{XM19} are described in Peifer *et al.* (1993, 1994a) and Peifer and Wieschaus (1990). All crosses were done at 25° with two or more independent lines of each mutant (mutant constructs, *UAS-arm-X*). For F_1 progeny, hatch rates were determined and cuticles of hatched larvae and unhatched embryos were prepared as in Wieschaus and Nüsslein-Volhard (1986). We crossed *e22c*-GAL4/*CyO* females with *UAS-arm-X* homozygous males to look for dominant effects. We tested for rescue of animals with a maternal and zygotic contribution composed entirely of arm^{XM19}-mutant protein by generating females who were heterozygous for *e22c*-GAL4 and were carrying arm^{XM19} germline clones as in Peifer *et al.* (1993). These females were mated to *UAS-arm-X* homozygous males.

Two-hybrid analysis: Two-hybrid experiments were carried out as described in Pai *et al.* (1996). Yeast cells were transformed with plasmids encoding portions of the Arm repeat region fused to the LexA DNA-binding domain, along with the GAL4 activation domain plasmid containing either the C-terminal region of Arm from isoleucine 451 in Arm repeat 10 to the C terminus or a control plasmid (pCK4) expressing only the Gal4p transcriptional activation domain. Values shown are averages from duplicate β-galactosidase assays performed on at least six independent transformants.

RESULTS

C-terminally truncated Arm signals less effectively than wild-type Arm: Arm protein can be divided into

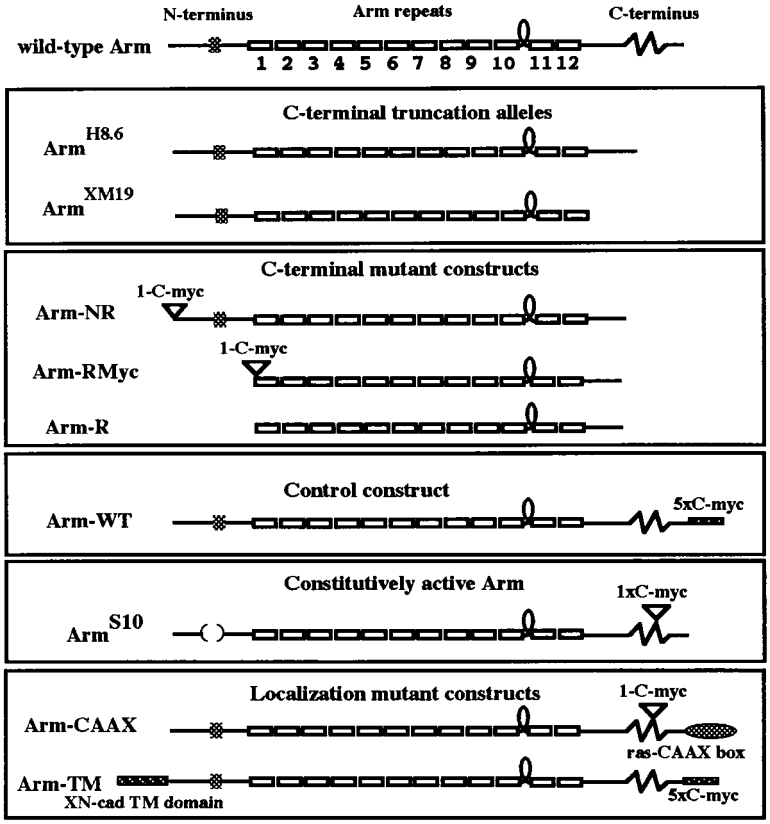


Figure 1.—*arm* mutants used in this study.

three "domains" (Figure 1), based on its sequence and on the crystal structure of the central portion of βcat (Huber *et al.* 1997). The central two-thirds of Arm is composed of a series of Arm repeats, a structural motif present in Arm/βcat as well as numerous otherwise unrelated proteins. There are 125–150 amino acid regions both N-terminal and C-terminal to the Arm repeats. While we originally defined the boundary between the Arm repeats and the C-terminal domain at amino acid 707, we now suspect from the crystal structure that the C-terminal domain begins at amino acid 678, thus including what we originally viewed as Arm repeat 13. This repositioning of the boundary between the Arm repeats and the C-terminal domain means that the most proximal part of the C-terminal domain is quite well conserved between Arm and βcat, while the distal C terminus is less well conserved.

While Wg signaling determines the fate of cells in numerous tissues, we most often focus on its effect on anterior-posterior cell fate choices in the larval epidermis. The ventral epidermis is secreted by a dozen rows of cells along the anterior-posterior axis per embryonic segment; the anterior rows of cells secrete small hairs known as denticles, while posterior cells secrete naked cuticle (Figure 2A). Wg is secreted by the tenth row of cells and is required for proper cell fate choices. In *wg* mutants, all cells choose anterior fates and secrete denticles; in contrast, if one removes the negative regulator *zw3*, all cells choose posterior fates and secrete naked cuticle. The original mutagen-induced *arm* mutants form a natural truncation series, sequentially removing more and more protein from the C terminus (Peifer and Wieschaus 1990). Some remove part (*arm*$^{H8.6}$; truncated after amino acid 725) or all (*arm*^{XM19}; truncated after amino acid 680) of the C-terminal domain (Figure 1), while others delete further, removing Arm repeats. *arm* mutations that reduce or remove the proximal C-terminal domain retain function in adherens junctions, but have severely reduced signaling function. At 25°, such embryos resemble *wg* null mutants both in cuticle phenotype (*e.g.*, Figure 2B is an *arm*^{XM19} maternal and zygotic mutant) and in the expression of Wg-responsive genes (Klingensmith *et al.* 1989; Peifer *et al.* 1991, 1994a; Orsulic and Peifer 1996). Mutations that delete further, removing portions of the Arm repeat region, also abolish function in adherens junctions (Cox *et al.* 1996), resulting in disruption of epithelial structures.

Our previous tests of the function of C-terminally truncated Arm proteins were done in the absence of endogenous wild-type Arm and thus suggested that the C terminus played an essential role in signaling. However, all of the truncated *arm* mutants produce protein at levels somewhat lower than that of wild-type (Peifer and Wieschaus 1990), perhaps due to mRNA instability triggered by a premature stop codon (reviewed in Ruiz-Echevarria *et al.* 1996). Arm^{XM19} and Arm$^{H8.6}$ proteins accumulate to about 10 and 30% of the level of wild-

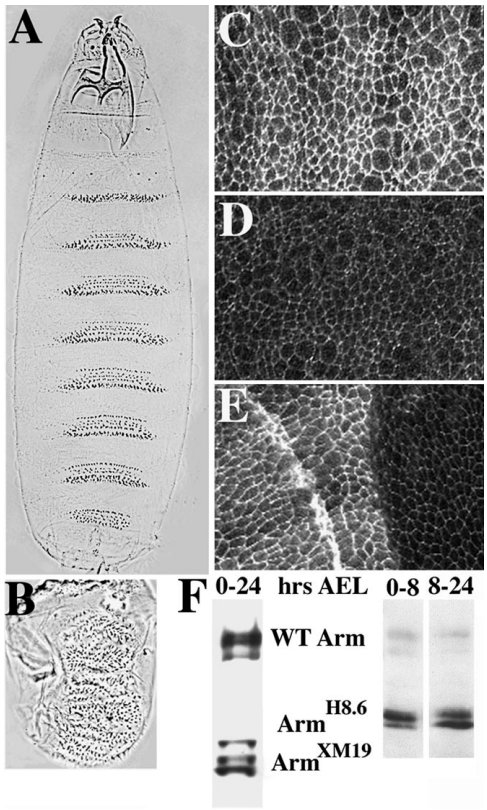


Figure 2.—C-terminal truncation of Arm dramatically reduces activity in Wg signaling. (A) Ventral view of the cuticle of a wild-type embryo at hatching. The body is divided into segments; on the ventral side anterior cells of each thoracic and abdominal segment secrete denticles and posterior cells secrete naked cuticle. (B) Cuticle of an arm^{XM19} maternal and zygotic mutant (a *wg* null mutant or an $arm^{H8.6}$ maternal and zygotic mutant would be essentially indistinguishable from this). Body size is dramatically reduced and all surviving ventral cells secrete denticles. (C–E) Embryos stained with anti-Arm antibody. (C) Zygotically wild-type sibling. Note stripes of cells accumulating cytoplasmic/nuclear Arm. (D) Embryo maternally and zygotically arm^{XM19} mutant. All cells accumulate Arm exclusively at the plasma membrane. (E) Side-by-side comparison of an embryo maternally and zygotically arm^{XM19} mutant (right) and a zygotically wild-type sibling (left), to show the difference in the intensity of Arm immunofluorescence. (F) Accumulation levels of C-terminally truncated proteins. Total cell extract was made from embryonic progeny of a cross of females with germlines homozygous for arm^{XM19} (left) or $arm^{H8.6}$ (right) to arm^{+}/Y males and immunoblotted with anti-Arm antibody. Age in hours after egg-laying (AEL) is indicated. (Left) Arm^{XM19} protein in arm^{XM19} maternally and zygotically mutant embryos accumulates at levels similar to that of wild-type Arm in their maternally mutant but zygotically wild-type siblings. (Right) $Arm^{H8.6}$ protein in $arm^{H8.6}$ maternally and zygotically mutant embryos accumulates at higher levels than wild-type protein in their maternally mutant but zygotically wild-type siblings.

type Arm protein in a wild-type embryo (Peifer and Wieschaus 1990). This raised the possibility that the phenotype of Arm^{XM19} and $Arm^{H8.6}$ reflected their reduced level of accumulation and not a defect in signaling function.

To address this, we designed a strategy by which we could equalize the levels of wild-type and mutant proteins and then assess function. We did so by reducing the level of wild-type Arm by removing the wild-type maternal contribution. We crossed females with germlines homozygous for either $arm^{H8.6}$ or arm^{XM19} to wild-type males. The resulting progeny all have a maternal contribution composed exclusively of mutant protein. Because *arm* is on the X chromosome, half the progeny get a paternal Y chromosome and make only mutant protein zygotically; the other half of the embryos receive a paternal wild-type *arm* gene and thus produce wild-type Arm protein zygotically. We compared the level of wild-type protein in paternally rescued embryos to the level of mutant protein in maternally and zygotically mutant embryos (we measured levels of wild-type and mutant proteins in a mixed population of embryos, all of whom were maternally *arm* mutant and half of whom had received a paternal wild-type *arm* gene). Paternally rescued embryos have substantially reduced levels of wild-type protein compared to embryos with a wild-type maternal contribution (data not shown). The level of wild-type protein in maternally mutant but zygotically wild-type embryos is roughly equal to the level of Arm^{XM19} protein in arm^{XM19} maternally and zygotically mutant embryos (Figure 2F) and is only about 25% of the level of $Arm^{H8.6}$ protein in $arm^{H8.6}$ maternally and zygotically mutant embryos (Figure 2F; note that the nonlinearity of ECL detection may mean that the absolute differences may vary somewhat from our quantitated levels). Levels remain equivalent from early embryogenesis (0–8 hr), when the maternally contributed mutant proteins might be predominant, through later stages of embryogenesis (8–24 hr) when zygotic wild-type and mutant genes have come on to their full levels (Figure 2F).

This allows us to further interpret our previous phenotypic studies. Maternally and zygotically $arm^{H8.6}$- or arm^{XM19}-mutant embryos are null for Wg signaling as measured either by cuticle phenotype or by gene expression (Klingensmith *et al.* 1989; Peifer *et al.* 1994a; when assayed at 25°—$arm^{H8.6}$ has a slightly weaker phenotype at 18°). In contrast, maternally mutant embryos that receive a paternal wild-type *arm* gene are wild-type in phenotype and survive to adulthood. Thus wild-type Arm can transduce Wg signal even when its levels are reduced to levels equal to or lower than those of the C-terminally truncated mutants. This allows us to conclude that C-terminal truncation of Arm substantially impairs its ability to signal; this is not due solely to reduced levels of mutant protein.

These results do not, however, rule out the possibility that the reduced level of accumulation of mutant protein may influence its ability to signal. We previously observed that the signaling ability of Arm can be influenced by its subcellular localization. Thus, for example, when levels of wild-type Arm are low, DE-cadherin can serve as a sink, such that all remaining Arm in the cell

is sequestered in the cadherin-bound pool (Cox *et al.* 1996), leaving none available for signaling. We thus reexamined the subcellular distribution of Arm^{XM19} protein and compared it to the subcellular distribution of wild-type protein in the zygotically rescued siblings (we previously explored this in Peifer *et al.* 1994a). As we previously noted, in embryos in which all maternal and zygotic protein was Arm^{XM19}, virtually all of the mutant Arm was found at the cell surface, even in cells receiving Wg signal (Figure 2, C *vs.* D).

Taken alone, these data suggest that Arm^{XM19} is synthesized at such low levels that all is sequestered in the cadherin-associated pool, leaving none available for signaling. By this model, however, there should be a similar sequestration of wild-type Arm at the junctions of zygotically rescued siblings, since wild-type Arm and Arm^{XM19} accumulate at equivalent levels (Figure 2F). This is not the case: in zygotically rescued embryos Arm accumulated both in the cadherin-associated pool at the plasma membrane and in the cytoplasm of cells that receive Wg signal (Figure 2C). Thus Wg signal can stabilize wild-type Arm but cannot stabilize Arm^{XM19}. These data are further reinforced by our previous observations on the ability of mutations in *zw3*, a component of the destruction machinery, to alter Arm accumulation. Mutation of *zw3* dramatically elevates the levels of wild-type Arm while *zw3* mutations have no effect on levels of Arm^{XM19} protein (Peifer *et al.* 1994a,b). Together, these data suggest that C-terminally truncated Arm cannot be stabilized by Wg signal and that its destruction is independent of Zw3 activity.

We should note an apparent discrepancy between the levels of Arm^{XM19} as measured by immunoblotting, where Arm^{XM19} protein accumulated to levels similar to or higher than that of wild type, and the levels of Arm^{XM19} as measured by immunofluorescence, where levels of Arm^{XM19} protein appear significantly lower than those of wild-type protein (Figure 2E). Our antibody, which is directed against the N-terminal domain, may access wild-type and mutant protein equally on an immunoblot, where both proteins have been denatured, but not *in situ*, perhaps because the conformations of wild-type and mutant proteins differ in a way that alters accessibility to the antibody epitope.

Design and expression of different C-terminally truncated Arm mutants: To complement these experiments, we set out to examine the effect of elevating the level of C-terminally truncated Arm to fully wild-type levels, to see if this might partially rescue the reduction in signaling function observed in $arm^{H8.6}$ or arm^{XM19}. These experiments were designed to match more closely the experiments in Xenopus, where C-terminally truncated βcat was expressed at levels that met or exceeded those of the wild-type endogenous βcat. We designed a series of mutants that altered the termini of Arm protein, focusing on the role of the C terminus (Figure 1). In Arm-N terminus plus repeats (Arm-NR) the C terminus beyond amino acid 719 was removed, and an N-terminal myc-epitope tag was added; this truncation was thus intermediate in extent between $arm^{H8.6}$ or arm^{XM19}. However, as this mutation does not generate an mRNA with coding sequence flanking a premature stop codon, we expected the mRNA to be stable and thus encode normal levels of protein (we show in Figure 6 below that this is the case). The second set of mutants, Arm-Repeats (Arm-R) and Arm-Repeats plus myc (Arm-Rmyc), contain only the Arm repeat region and thus lack both N and C termini; Arm-Rmyc also carries an N-terminal myc-tag. Since Arm-R and Arm-Rmyc were identical in their phenotypic effects, we refer to both as Arm-R unless otherwise noted. Arm-R mimics the "repeat only" mutants of βcat that activate Wnt signaling when injected into wild-type Xenopus embryos (Funayama *et al.* 1995). We also generated a construct encoding full-length Xenopus β-*catenin*; while Arm and βcat are 71% identical in protein sequence overall, the divergence in the C-terminal region is much more substantial. All of these proteins were expressed under the control of the GAL4-UAS system (Brand and Perrimon 1993), in which the gene of interest is cloned downstream of a minimal promoter and a series of GAL4-binding sites. The gene of interest can be introduced in a silent state into flies lacking GAL4 and activated by crossing these flies to flies expressing GAL4 in the desired temporal and spatial pattern. We used the *e22c*-GAL4 driver, which is expressed essentially ubiquitously in the ectoderm beginning late in embryonic stage 9 (Cox *et al.* 1999).

The Arm repeats alone activate Wg signaling in a wild-type background: To look for dominant effects, we first expressed our mutant proteins in a wild-type background. Fly embryos are not sensitive to slight elevation in the level of expression of wild-type Arm. Increasing Arm levels using a chromosomal duplication, a wild-type transgene (Orsulic and Peifer 1996), or by overexpression of Arm-WT using *e22c*-GAL4 (Pai *et al.* 1997; data not shown) has no effect on the embryonic pattern. Likewise, when we expressed Arm-NR in wild-type embryos, it also did not have any dominant effects; in fact, animals expressing Arm-NR survived to adulthood (data not shown; Arm-NR was expressed at wild-type levels, unlike the C-terminally truncated mutants; see Figure 6 below). Expression of Xenopus βcat also had no dominant effects (data not shown).

In contrast, when we used *e22c*-GAL4 to express Arm-R, containing only the Arm repeat region, in the presence of wild-type endogenous Arm, we saw activation of Wg signaling (Figure 3, B and C). Expression of Arm-R led to a partial conversion of anterior epidermal cells to a posterior naked cuticle fate, suggesting uniform activation of Wg signaling. We previously observed a similar dominant phenotype when, using the same GAL4 driver, we expressed Arm^{S10} (Figure 3D), a mutant Arm protein retaining the C terminus but also

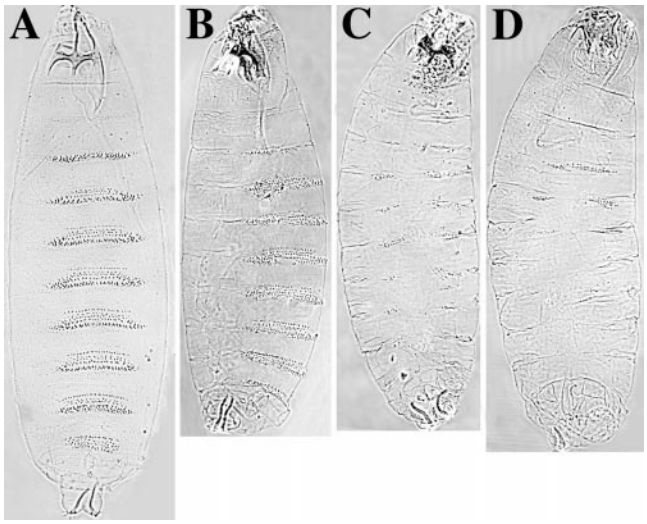


Figure 3.—Arm-R expression in a wild-type background activates Wg signaling. (A) Wild-type embryo. (B and C) Different lines expressing Arm-Rmyc (B and C) in an otherwise wild-type background, showing the range of dominant effects from a nearly wild-type pattern (B) to a strong conversion of denticles to naked cuticle (C). (D) For comparison, the very strong phenotype resulting from expressing Arm^{S10} (Pai *et al.* 1997), which retains the Arm repeats and C terminus but has a deletion in the N-terminal region, in an otherwise wild-type background.

missing the N-terminal region that downregulates protein stability (Pai *et al.* 1997). The dominant phenotypes induced by Arm-R were, on average, less severe than those induced by Arm^{S10}, suggesting that the C-terminal domain is required for full signaling activity. The difference between Arm-R and Arm-NR in their effect on the wild-type pattern likely reflects the fact that, like Arm^{S10}, Arm-R lacks the N-terminal stability-regulating domain, which is retained by Arm-NR.

The dominant phenotype of Arm-R in the wild-type background could be due to signaling by Arm-R itself. Alternatively, Arm-R might displace wild-type Arm from adherens junctions and also block the destruction machinery, allowing endogenous Arm to accumulate outside junctions and signal. To determine whether Arm-R exerts its dominant effects by stabilizing wild-type endogenous Arm, we examined the levels and localization of wild-type Arm in embryos expressing either Arm-R or Arm-NR (Figure 4). To do so, we used our N-terminal anti-Arm monoclonal, which recognizes wild-type Arm and Arm-NR but not Arm-R. Consistent with the failure of Arm-NR to affect normal development, the localization of Arm-NR plus wild-type Arm resembled that of wild-type Arm in a nontransgenic embryo (Figure 4, A and E *vs.* C). In contrast, Arm-R dramatically altered the accumulation of wild-type Arm (Figure 4, B and F). These differences were first seen at stage 11, when the levels of Arm-R driven by *e22c*-GAL4 begin to rise (see below). While levels of wild-type endogenous Arm at the plasma membrane remained stable or dropped, levels of wild-type Arm inside cells rose dramatically, so that all cells resembled cells receiving Wg signal (Figure 4, B *vs.* C). This stabilization of cytoplasmic wild-type Arm is very similar to that seen in a *zw3* mutant (Peifer *et al.* 1994a) and thus could easily account for the dominant effects of Arm-R. The elevation in the level of intracellular Arm by Arm-R continues throughout the rest of embryonic development (Figure 4F). Despite the significant increase in intracellular Arm caused by Arm-R expression, the total levels of endogenous wild-type Arm were not increased, as assayed either by immunofluorescence or immunoblotting (Figures 4D and 8B).

C-terminally truncated Arm retains significant signaling activity when expressed at wild-type levels: To assess

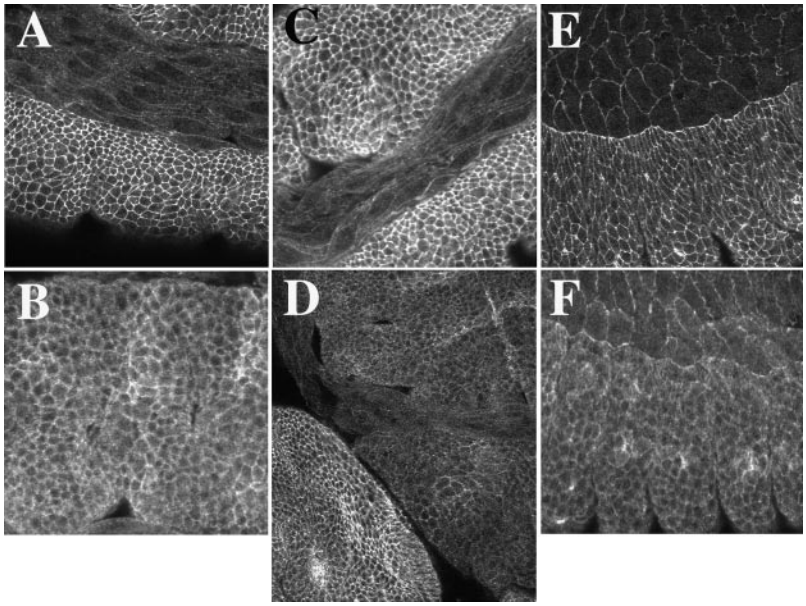


Figure 4.—Arm-R expression elevates the level of endogenous wild-type Arm in the cytoplasm and depresses its accumulation at the membrane. All embryos were stained with anti-Arm antibody, which recognizes endogenous Arm and Arm-NR, but not Arm-R. (A–D) Stage 11 embryos. (A) Arm-NR expression does not alter the level or localization of endogenous Arm, and its own localization resembles that of the endogenous protein. (B) In contrast, Arm-R expression results in the accumulation of elevated levels of endogenous wild-type Arm in the cytoplasm relative to the plasma membrane. (C) Nontransgenic sibling for comparison. (D) The total level of endogenous Arm in a Arm-R-expressing embryo (top) is not elevated relative to a nontransgenic sibling; in fact the levels seem lower. (E) Stage 13 embryo expressing Arm-NR. The pattern of Arm accumulation is unaltered from wild-type. (F) Stage 12 embryo expressing Arm-R, with elevated levels of intracellular Arm.

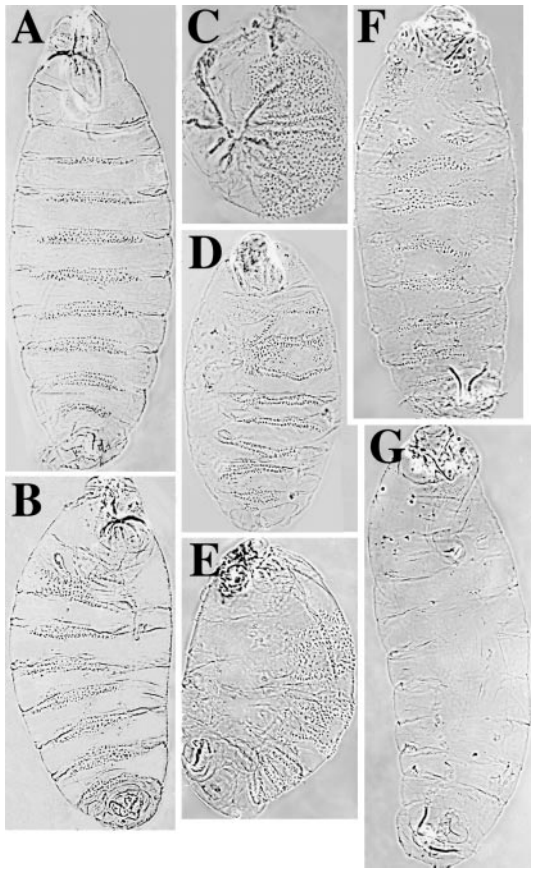


Figure 5.—Expression of wild-type levels of C-terminally truncated Arm can partially rescue signaling. (A and B) Two lines expressing Arm-WT in an arm^{XM19} background. The segment polarity phenotype is either completely (A) or substantially (B) rescued, with no gain of function effects on pattern. (C) Embryo maternally and zygotically mutant for arm^{XM19}. (D) Embryo expressing Arm-NR in an arm^{XM19} background. The segment polarity phenotype is partially rescued, but to a lesser extent than by Arm-WT. (E and F) Different lines expressing either Arm-Rmyc (E) or Arm-R (F) in an arm^{XM19} background. The segment polarity phenotype is rescued to varying degrees; in the embryos with the strongest rescue, vestiges of the dominant phenotype are observed as ablation of denticles along the ventral midline. Rescue by Arm-R is noticeably less than that by Arm-WT. (G) Embryo expressing Arm^{S10} in an arm^{XM19} background. Note the completely penetrant dominant phenotype.

the signaling ability of C-terminally truncated Arm when expressed at wild-type levels, we expressed Arm-R and Arm-NR in an *arm*-mutant background in the absence of endogenous wild-type Arm. As discussed above, $arm^{H8.6}$ and arm^{XM19} encode proteins partially or completely lacking the C-terminal domain, respectively. Embryos maternally and zygotically mutant for either $arm^{H8.6}$ or arm^{XM19} are null for Wg signaling at 25° (Klingensmith *et al.* 1989; Peifer *et al.* 1991, 1994a). $arm^{H8.6}$ retains intrinsic signaling activity, however, as revealed by the weaker phenotypes of $arm^{H8.6}$ at 18° (Klingensmith *et al.* 1989) and of $arm^{H8.6}zw3$ double mutants, in which levels of $Arm^{H8.6}$ protein are elevated by reducing its degradation (Peifer *et al.* 1994a,b). In contrast, neither reduction in temperature nor simultaneous mutation of *zw3* leads to any rescue of arm^{XM19}. We thus used arm^{XM19} as a background we expected to be null for Wg signaling.

To create embryos expressing C-terminally truncated protein at wild-type levels, we expressed Arm-NR, which lacks the distal C terminus (it is six amino acids shorter than $Arm^{H8.6}$; Figure 1), in embryos maternally and zygotically arm^{XM19} mutant. We expected that as Arm-NR lacks most of the C-terminal domain, it would not rescue signaling. Instead, we found that Arm-NR substantially rescued the Wg-signaling defects of arm^{XM19} mutants (embryos were restored to a more normal size and the segment polarity phenotype was substantially but not completely rescued, as fusions of denticle belts were often observed; Figure 5D). Arm-NR was diminished in signaling ability, however, as it rescued the phenotype less completely than did Arm-WT (Figure 5, A and B). Expression of Xenopus βcat also led to substantial but not complete rescue of signaling, to an extent essentially identical to that of Arm-NR (data not shown). Since Arm and βcat diverge substantially in sequence in the C-terminal region, the βcat C terminus may not function effectively in flies. Together these data suggest that removing Arm's C terminus diminishes, but does not eliminate its signaling function. White *et al.* (1998) recently reported similar experiments with βcat and C-terminally truncated Arm; in their experiments, however, the signaling ability of C-terminally truncated Arm was less than what we observed, perhaps due to differences in the constructs and the means through which they were expressed.

Expression of Arm-R, containing the Arm repeats alone, at wild-type levels also substantially rescued the Wg-signaling defect of arm^{XM19} mutants (Figure 5, E and F). The extent of rescue was on average greater than that conferred by Arm-NR (Figure 5D), but less than that conferred by Arm-WT (Figure 5, A and B). Arm-R expression in an arm^{XM19} mutant also sometimes resulted in a dominant activated Wg-signaling phenotype similar to, but weaker than, that induced by Arm-R in a wild-type background (Figure 5F). The dominant activated phenotype predominated along the ventral midline, leading to partial ablation of the denticle belt. However, extra denticles were observed at the lateral margins, suggesting failure to completely rescue Wg signaling. The dominant effects of Arm-R in the arm^{XM19}-mutant background were substantially weaker than those of Arm^{S10} (Figure 5G), which also lacks the critical regulatory region in the N terminus but which retains the C terminus. Together, these data suggest that Arm proteins lacking the distal C-terminal domain retain reduced but still significant intrinsic signaling ability.

Arm-R accumulates to higher levels than wild-type Arm, partially explaining its activated phenotype: Arm-R was significantly more potent than Arm-NR: Arm-R was bet-

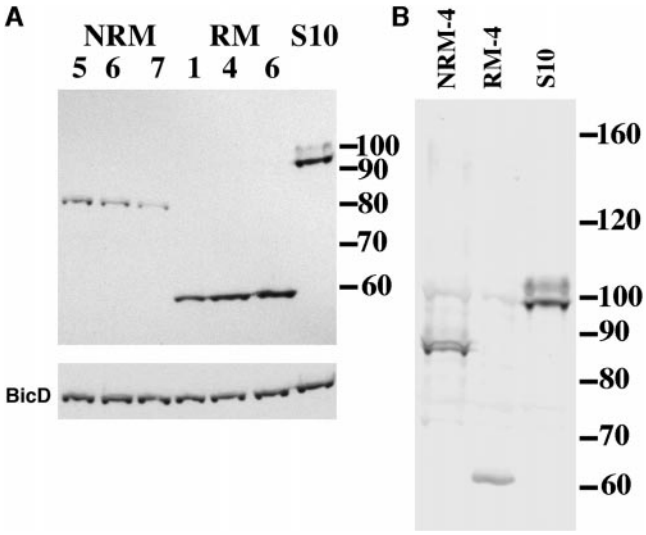


Figure 6.—Accumulation levels of Arm mutant proteins. Flies carrying UAS-Arm constructs as indicated were crossed to flies carrying the GAL4 driver *e22c*-GAL4. Embryo extracts were immunoblotted with anti-myc epitope antibody. MW markers are indicated at right. (A) Levels of expression of different Arm-NR (NRM) and Arm-Rmyc (RM) transgene insertion lines (three lines of each are presented, indicated by the line number below the label). They are compared to Arm^{S10} (S10), which accumulates to higher levels than wild-type (Pai *et al.* 1997). Arm-Rmyc lines accumulate high levels of protein, while Arm-NR lines accumulate levels of protein more similar to wild-type. The samples represent 0 to 19-hr-old embryos. (B) Phosphorylation of mutant Arm proteins. Arm^{S10} (S10) shows a set of phosphorylated isoforms similar to those of wild-type Arm. In contrast, only two isoforms of Arm-NR (line NRM-4) and one or two isoforms of Arm-Rmyc (line RM-4) are detectable.

ter at rescuing the signaling defect of arm^{XM19}, and Arm-R expression caused a dominant activated phenotype. We previously found that removal of part of the N-terminal domain of Arm (creating the mutant Arm^{S10}) stabilizes the mutant protein and thus renders it constitutively active in Wg signaling (Pai *et al.* 1997). This region is absent in Arm-R but present in Arm-NR, and thus the difference in their biological activities might be accounted for in part by differences in their level of accumulation. Neither Arm-R nor Arm-Rmyc is recognized by anti-Arm antibody, as they lack its N-terminal epitope. Arm-Rmyc and Arm-NR were both myc tagged, however, and thus we compared their level of expression with each other and with other myc-tagged Arm proteins. Arm-Rmyc accumulated to high levels (Figure 6A); the elevated levels of Arm-Rmyc, lacking the entire N and C termini, were similar to those attained by Arm^{S10} (Figure 6A), which lacks the key regulatory region in the N terminus (Pai *et al.* 1997). In contrast, Arm-NR, which retains both the N terminus and the repeats, accumulated at levels more similar to wild-type Arm (Figure 6A; data not shown). In the course of examining protein levels, we also looked at phosphorylation. Arm is phosphorylated on both Ser/Thr and Tyr residues (Peifer *et al.* 1994b). Ser/Thr phosphorylation alters Arm's mobility on SDS-PAGE; we can use this to roughly estimate whether different mutant proteins are normally phosphorylated. Neither Arm-NR nor Arm-Rmyc has a full set of phosphorylation isoforms. Arm-NR, which lacks the C terminus, has only two visible isoforms (Figure 6B); this resembles the phosphorylation state of Arm^{XM19} (Peifer *et al.* 1994b). Arm-Rmyc showed only a single isoform (Figure 6B). Thus at least some of the phosphorylation sites that alter Arm mobility are likely to be in the N and C termini; these proteins may remain phosphorylated on sites that do not alter the mobility of the protein, however.

Arm^{XM19} retains residual signaling ability: The data above suggest that Arm-R and $Arm^{H8.6}$, both of which lack the distal C-terminal region but retain the proximal conserved domain, retain some ability to signal, albeit less than wild-type Arm. Arm^{XM19} lacks the entire C-terminal domain, retaining only three amino acids past the end of the Arm repeats. In all of our previous experiments, Arm^{XM19} behaved as if it were null for signaling. However, in the course of other experiments (Cox *et al.* 1999), we identified circumstances that revealed that even Arm^{XM19} retains signaling activity. This activity was revealed by use of a membrane-tethered form of Arm, Arm-CAAX, which is tethered to the membrane using the lipid modification signal of mammalian K-ras. Arm-CAAX cannot signal on its own—when it is expressed in an animal lacking essentially all endogenous Arm (an embryo maternally and zygotically mutant for the near-null allele arm^{XP33}), Arm-CAAX rescues Arm's function in adherens junctions but has no signaling activity (Cox *et al.* 1999). However, when Arm-CAAX was expressed in embryos maternally and zygotically mutant for arm^{XM19}, these embryos were rescued to a nearly wild-type pattern (Figure 7, C and D). As Arm-CAAX cannot signal on its own (Cox *et al.* 1999), this suggested that Arm^{XM19} retains residual signaling activity, which is somehow promoted by Arm-CAAX coexpression.

We were surprised that Arm-CAAX had such a strong effect. One possible explanation is that Arm-CAAX binds to and blocks the destruction machinery, allowing Arm^{XM19} protein to accumulate to higher levels. We tested this. Arm-CAAX coexpression does not detectably increase the total level of Arm^{XM19} (Figure 8A, left) or the level of wild-type Arm in sibling embryos; in fact, levels of wild-type Arm slightly decrease (Figure 8A; likewise, Arm-WT, Arm-TM, and Arm-R coexpression does not elevate total levels of Arm^{XM19}; Figure 8B).

Arm-CAAX localizes to the plasma membrane in complex with DE-cadherin and α-catenin and retains full function in adherens junctions as tested genetically (Cox *et al.* 1999). By binding to DE-cadherin, Arm-CAAX could displace Arm^{XM19} from adherens junctions (most Arm in embryos is found in the junctional pool; Peifer 1993). Even without elevating the total pool of

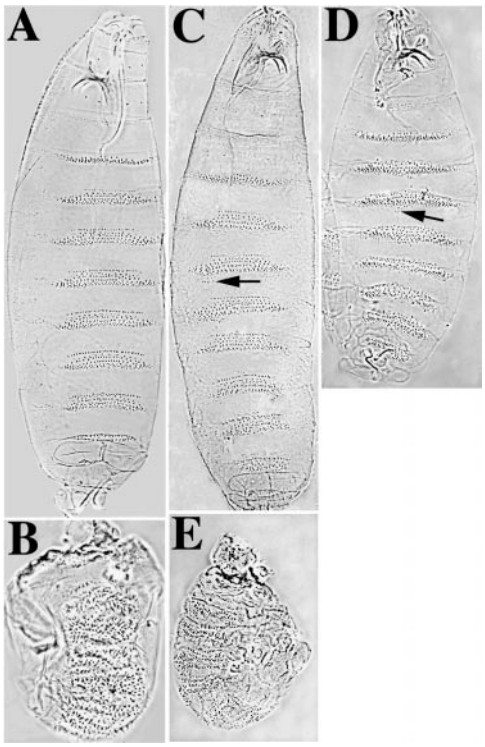


Figure 7.—Arm-CAAX substantially rescues the signaling defect of *arm*^{XM19}. (A) Wild-type embryo. (B) Embryo maternally and zygotically mutant for *arm*^{XM19}. (C and D) Unhatched and hatched embryos maternally and zygotically mutant for *arm*^{XM19} and also expressing Arm-CAAX. The segment polarity phenotype is almost totally rescued, leaving only a few denticles in the naked cuticle region. (E) Embryo maternally and zygotically mutant for *arm*^{XM19} and also expressing Arm-TM. Arm-TM has little rescuing ability.

Arm^{XM19}, Arm-CAAX could thus substantially elevate the pool of Arm^{XM19} available for signaling. To address this possibility, we fractionated cells derived from embryos maternally mutant for *arm*^{XM19} and isolated a membrane fraction. We found that expression of Arm-CAAX reduced the levels of both wild-type Arm and of Arm^{XM19} protein in the membrane fraction (Figure 8A, right). Quantitation of these blots followed by normalization for loading suggested that wild-type Arm in the membrane fraction was reduced more than threefold while levels of Arm^{XM19} protein in the membrane fraction were reduced more than fourfold (note that potential nonlinearity of ECL signal may affect the absolute levels, but should not alter conclusions about relative levels). Since total levels of Arm^{XM19} protein remained unaffected by Arm-CAAX coexpression (Figure 8A, left), this suggests that Arm-CAAX coexpression elevates levels of Arm^{XM19} protein in the cytoplasm (attempts to directly measure the levels of wild-type and mutant Arm in the soluble pool were prevented by limited quantities of material and the low level of Arm that is normally found in the soluble fraction). Further support for this model is provided by our previous observation that a different membrane-tethered form of Arm, Arm-TM, which carries the transmembrane domain of N-cadherin, did not significantly rescue embryos maternally and zygotically mutant for *arm*^{XM19} (Cox *et al.* 1999; Figure 7E). Arm-TM, unlike Arm-CAAX, does not localize to adherens junctions but instead appears to be trapped in the ER/Golgi (Cox *et al.* 1999) and thus may not be able to displace Arm^{XM19} into the signaling pool.

The C-terminal region of Arm can interact with the Arm repeat region: The crystal structure of the Arm repeat region of β-catenin revealed that the Arm repeats form a folded domain (Huber *et al.* 1997). The structure of the N- and C-terminal regions remains unknown, as these were absent in the crystal; however, protease resistance studies (Huber *et al.* 1997) and the ability of small fragments of the N-terminal domain to interact with α-catenin (Aberle *et al.* 1996; Pai *et al.* 1996) suggest that these regions may not form independently folded domains and could exist as extended peptides or individual α-helices. If this model is correct, such peptides might be able to fold back into the groove formed by the Arm repeats of Arm/βcat. In the course of a two-hybrid screen for novel Arm interactors using most of the Arm repeat region as bait, we found that one interactor was a piece of Arm itself. This fragment of Arm extends from isoleucine 451 in Arm repeat 10 to the C terminus. We further mapped the interacting region using the two-hybrid system (Figure 9). The minimal piece of Arm with which the C-terminal fragment can strongly interact is Arm repeats 3–8; this is the same binding site occupied by DE-cadherin (Pai *et al.* 1996), dTCF (van de Wetering *et al.* 1997), and dAPC (C. Kirkpatrick and M. Peifer, unpublished data). Very weak interactions were also detected with other more amino terminal fragments (Arm repeats 1–7, 1–6, and 1–4) but these βgal levels were much closer to the background levels in the absence of the C-terminal fragment.

DISCUSSION

Redefining the C-terminal domain: Arm protein has a modular structure. Its most prominent feature is the Arm repeats that make up the central two-thirds of the protein and serve as docking sites for a number of protein partners, including DE-cadherin, dAPC, and dTCF. On the basis of sequence alignment, we originally proposed that there were 13 Arm repeats, ending at amino acid 707, and thus the C-terminal domain began at this point. The Arm repeats came into sharper focus recently with the solution of the X-ray crystal structure of the Arm repeat region of βcat (Huber *et al.* 1997). This allowed us to revise our model of Arm structure (Figure 10A); it is likely that there are only 12 Arm repeats, suggesting that the C-terminal domain begins at amino acid 678. This fits well with the biology, which suggests that the C terminus, as defined by its action in transcriptional activation, begins at this position.

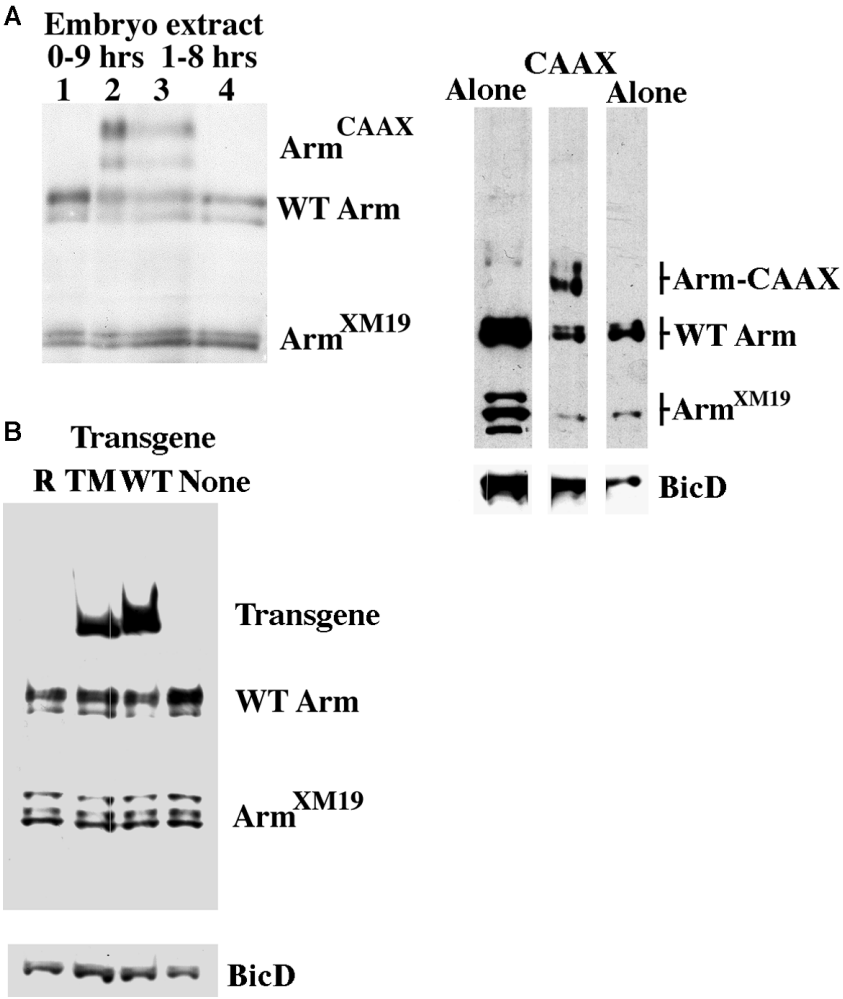


Figure 8.—Arm-CAAX does not raise total levels of Arm^{XM19} protein, but does lower its levels in the membrane-bound fraction. (A) Left: Coexpression of Arm-CAAX does not detectably elevate levels of total Arm^{XM19} protein in *arm^{XM19}*-mutant embryos. Females with germlines homozygous for *arm^{XM19}* were crossed to *arm^{+} / Y* males, expressing (lanes 2 and 3) or not expressing (lanes 1 and 4) Arm-CAAX. Total cell extracts were made from embryonic progeny of indicated ages after fertilization and immunoblotted with anti-Arm. Right: Coexpression of Arm-CAAX reduces levels of Arm^{XM19} protein in the membrane fraction. Females with germlines homozygous for *arm^{XM19}* were crossed to *arm^{+} / Y* males, expressing (middle lane) or not expressing (outside lanes) Arm-CAAX. Cell extracts were made from embryonic progeny and fractionated into membrane (P100) and soluble fractions. The membrane fraction was immunoblotted with anti-Arm. Due to the extremely limited number of embryos available, the entire sample was loaded in each case, and as a result, loading levels are not equal. BicD served as a loading standard. While the right lane has substantially less total protein than the central lane, the levels of Arm^{XM19} protein are equal. The left lane has only slightly more total protein than the central lane, but has substantially more Arm^{XM19} protein. (B) Coexpression of Arm-R (R), Arm-TM (TM), and Arm-WT (WT) does not elevate total levels of Arm^{XM19} protein and appears to decrease levels of endogenous wild-type Arm (WT Arm). Females with germlines homozygous for *arm^{XM19}* were crossed to *arm^{+} / Y* males expressing the indicated transgene. Total cell extracts were made and immunoblotted with anti-Arm. The blot was reprobed with BicD to control for loading differences.

Armadillo's C terminus can be divided into several regions that together play a complex role in Wg signaling: Our previous work led us to suggest that the proximal C terminus of Arm plays an essential role in Wg signal transduction by acting as a transcriptional activation domain. Inconsistent with this model, however, βcat mutants lacking the C terminus activate Wnt signaling when misexpressed in Xenopus (Funayama *et al.* 1995). To address this issue, we further characterized mutant Arm proteins lacking the C terminus. This analysis confirmed that the Arm C terminus potentiates signaling. We equalized the expression levels of wild-type and C-terminally truncated Arm by reducing wild-type Arm levels. Wild-type Arm remains fully functional in signaling when expressed at this reduced level. In contrast, Arm^{XM19}, lacking the entire C terminus, is null for Wg signaling at these levels of expression (Peifer *et al.* 1991, 1994a; Figure 2).

However, our analysis also revealed that C-terminally truncated Arm retained significant signaling function and reinforced the idea that different C-terminally truncated mutants differ in their signaling ability (Figure 10B). Arm$^{H8.6}$ and Arm-NR, which have 40–50 amino acids of the C-terminal domain intact, retain substantially more signaling ability than Arm^{XM19}, which lacks the entire C-terminal domain. The contrast is most striking when comparing Arm$^{H8.6}$ and Arm^{XM19}. Arm$^{H8.6}$ retains clear residual signaling activity, as revealed by its weaker phenotype at lower temperatures and by the weaker phenotype seen when Arm degradation was decreased in an *arm$^{H8.6}$zw3* double mutant (Klingensmith *et al.* 1989; Peifer *et al.* 1994a). In contrast, there is no rescue of the signaling ability of Arm^{XM19} either by reduction of the temperature or by simultaneous mutation of *zw3* (Peifer *et al.* 1994a). Full-length vertebrate βcat, when expressed in Drosophila, signals about as well as Arm-NR. Consistent with this, while βcat is highly related to Arm in the N terminus, the Arm repeats, and the proximal C terminus (retained in Arm-NR), βcat diverges extensively in the distal C terminus (missing

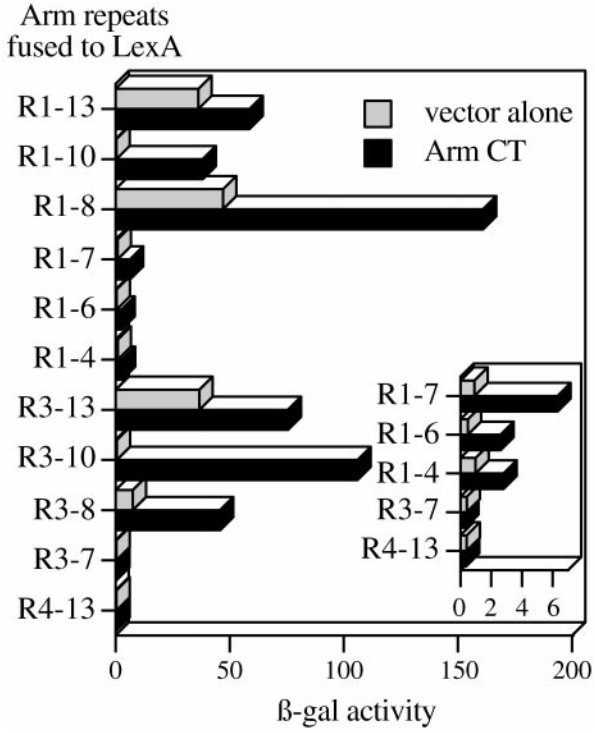


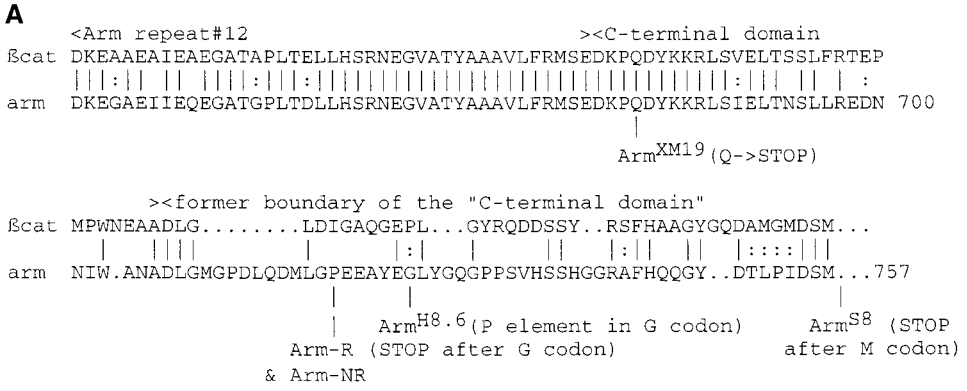
Figure 9.—The Arm C-terminal region can physically interact with Arm's central Arm repeats. A fusion protein containing a C-terminal portion of Arm fused to the GAL4 activation domain, originally isolated in a two-hybrid screen using Arm repeats 1–10 of Arm as bait, was tested for interaction with different portions of the Arm repeat region of Arm fused to the LexA DNA-binding domain (constructs as in Pai *et al.* 1996). β-Galactosidase levels were determined as in materials and methods; cells carrying the activation domain vector alone (pCK4) show the background levels of β-galactosidase activity from the different Arm repeat fragments. The inset shows the data for weakly interacting or noninteracting fragments at higher resolution.

in Arm-NR). White *et al.* (1998) previously demonstrated the reduced signaling ability of βcat in Drosophila; in their experiments it had even less signaling function.

Together, our current experiments and previous data suggest that the C terminus consists of at least three functional regions, two of which contribute to activation of Wg-responsive genes in an additive fashion (Figure 10). The C terminus distal to amino acid 757 is dispensable for Wg signaling, as demonstrated by the wild-type phenotype of the truncation mutant Arm^{S8}, which lacks this region (Orsulic and Peifer 1996). The region between amino acids 710 and 757 stimulates the ability of Arm to signal but is not essential, as demonstrated by the partial signaling ability of mutants such as Arm-NR or $Arm^{H8.6}$, which lack this region. Finally, the region of the C terminus from amino acids 678–710 plays a critical role in full signaling ability, as revealed by the further reduction in signaling function of Arm^{XM19}, which lacks this region. This latter region of the C terminus (amino acids 678–710) was recently revealed to contain the binding site for Drosophila Teashirt protein (Gallet *et al.* 1999), a zinc-finger transcription factor that binds Arm and helps mediate the late response to Wingless signaling (Gallet *et al.* 1998). Thus the difference in signaling ability between Arm^{XM19} and Arm-NR or $Arm^{H8.6}$ may reflect the fact that only the latter two are likely to bind Teashirt. These differences in signaling assayed *in vivo* are also reflected in the *in vitro* gene activation assay, which assesses the ability of Arm and dTCF to activate expression of a reporter construct with several dTCF-binding sites upstream. In this assay, Arm^{S8} behaves indistinguishably from wild type, $Arm^{H8.6}$ retains strong ability to coactivate the reporter, and the coactivation ability of Arm^{XM19} is sharply reduced but not completely eliminated (van de Wetering *et al.* 1997; M. van de Wetering and H. Clevers, personal communication).

The C terminus also appears to play a second function: regulating Arm stability. Nonjunctional Arm is normally unstable, but is stabilized by Wg signal. We found that while embryos maternally and zygotically arm^{XM19} mutant have levels of total Arm similar to those of their wild-type paternally rescued siblings, wild-type Arm accumulates in the cytoplasm / nuclei of cells receiving Wg signal, while Arm^{XM19} protein does not (Peifer *et al.* 1994a; Figure 2). Further, we previously found that mutations in *zw3* greatly stabilize wild-type Arm but do not substantially stabilize Arm^{XM19} and only slightly stabilize $Arm^{H8.6}$ (Peifer *et al.* 1994b). These latter data may help explain the fact that mutations in *zw3* have no effect on the phenotype of an arm^{XM19} mutant, while they slightly suppress the phenotype of an $arm^{H8.6}$ mutant (Peifer *et al.* 1994a). Together these data suggest that C-terminally truncated Arm cannot be stabilized by Wg signaling. This idea was independently suggested by White *et al.* (1998), who misexpressed mammalian plakoglobin and βcat in flies. βcat was stabilized in cells receiving Wg signal, while plakoglobin was not. They suggested that this might result from the divergence in C-terminal domains of Arm and plakoglobin and, thus, that the C terminus might regulate stability. The mechanism by which this occurs remains unknown.

In addition to these roles in transcriptional activation and in regulating stability, our data suggest a possible additional function for the C-terminal domain: it can bind, at least in two-hybrid experiments, to the central-most Arm repeats of Arm itself. Such an interaction could be either intermolecular, via Arm proteins forming homodimers, or intramolecular, with the tail folding back upon the Arm repeats of the same protein. Previous data failed to provide any support for the presence of Arm dimers (Orsulic and Peifer 1996; Pai *et al.* 1997), so if this interaction occurs *in vivo* it seems more likely to be intramolecular. Such an interaction would provide interesting possibilities for regulation. The C terminus could provide a competitor for other Arm partners such as cadherin or dTCF, allowing regulation



B

Arm repeats / C-terminus		Wg signaling "on own"	Stabilized in *zw3* mutant	Residual signaling activity revealed by
1 1 1 2	WT	++	++	NA
	Arm^{S8}	++	ND	NA
	$Arm^{H8.6}$	+/-	+/-	*zw3* mutation or reduced temp.
	Arm-NR	+	ND	Expression at wildtype levels
	Arm^{XM19}	--	--	Expression of Arm-CAAX

C

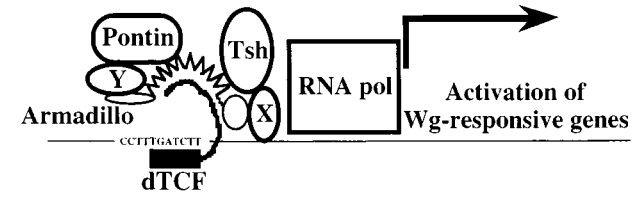


Figure 10.—Signaling and the C terminus. (A) Comparison of the proximal C-terminal domains of Arm and βcat, displaying the old and new boundaries of the C-terminal domain and the positions of the truncations in different Arm mutants. (B) Summary of the signaling activities of different C-terminally truncated mutant Arm proteins. NA, not applicable; ND, not determined. (C) Model in which Arm contacts the transcriptional machinery via multiple additive contacts.

of their binding by modulation of the affinity of the interaction of the C terminus *vs.* that of other heterologous partners. Such competition might even help explain the effect of the C terminus on stability discussed above—by competing for Arm interaction with cadherins and/or APC, the C terminus could regulate Arm stability, and its removal might thus alter the stability of Arm.

Arm protein lacking the entire C-terminal domain retains residual signaling ability: While previous work suggested that $Arm^{H8.6}$ might retain some signaling function, we were quite surprised to learn that Arm^{XM19}, lacking the entire C-terminal domain, retains detectable activity in Wg signaling. This was revealed by expression of membrane-tethered Arm-CAAX in an *arm*^{XM19} background. Arm-CAAX retains no signaling ability of its own (Cox *et al.* 1999), and thus the ability of Arm-CAAX to strongly stimulate signaling by Arm^{XM19} demonstrates that Arm^{XM19} retains residual activity in Wg signaling.

This raises the question of how membrane-tethered Arm promotes signaling by Arm^{XM19}. We first examined whether Arm-CAAX blocks the machinery that normally targets free Arm for destruction (as does tethered βcat in Xenopus; Miller and Moon 1997). Arm-CAAX coexpression did not noticeably stabilize total Arm^{XM19}. Instead, we suspect that the major effect of Arm-CAAX is mediated by its ability to displace Arm^{XM19} from adherens junctions. Arm-CAAX retains function in adherens junctions and binds both DE-cadherin and α-catenin (Cox *et al.* 1999); as it is tethered to the membrane it may outcompete Arm^{XM19} for the available cadherin binding sites. Consistent with this, Arm-CAAX expression reduced the levels of Arm^{XM19} in the membrane-bound pool. Thus, Arm-CAAX may displace Arm^{XM19} into the soluble pool, where it would be available for signaling. Consistent with this model for the effect of Arm-CAAX, a different form of membrane-tethered Arm, Arm-TM, which does not localize to adherens junctions, does not significantly augment signaling by Arm^{XM19}.

Our data reinforce the idea that small changes in total Arm levels can produce large changes in signaling. A twofold increase in maternal *arm* (Wieschaus and

Noell 1986) or slight changes in cadherin dosage, which alter free Arm levels (Cox *et al.* 1996; Sanson *et al.* 1996), significantly stimulate signaling. In Xenopus, twofold or less differences in βcat levels on the dorsal *vs.* ventral sides trigger dorsal axis formation (Larabell *et al.* 1997). Several factors are likely to contribute to this threshold effect. Most important, the multiplicity of Arm partners and their differences in affinity for Arm mean that one partner can serve as a sink for active Arm. In a normal cell, most Arm is bound to cadherin; signaling-active dTCF-bound Arm is likely only a small fraction of the total Arm present. Once cadherins are saturated with Arm, small increases in total Arm lead to large increases in Arm available to interact with dTCF. Arm^{XM19} protein, present at lower levels than normal wild-type protein, may be particularly susceptible to such sequestration.

Theoretically, C-terminally truncated Arm, which retains the ability to bind dTCF (van de Wetering *et al.* 1997), could also promote signaling by titrating dTCF, preventing it from acting as a repressor (Cavallo *et al.* 1998). A comparison of the phenotypes of different mutants, however, suggests that this is not the case. *wg* null mutants have a very strong segment polarity phenotype, reflecting the lack of activation of Wg-responsive genes by Arm/dTCF complexes, but they retain repression mediated by dTCF/Groucho complexes (Cavallo *et al.* 1998). In contrast, embryos that are null for zygotic dTCF have substantial reductions in both activator and repressor functions of dTCF and thus have a milder phenotype, intermediate between that of a wild-type embryo and a *wg* mutant, retaining residual expression of Wg-responsive genes (Cavallo *et al.* 1998). If C-terminally truncated Arm, by binding dTCF, abolished its ability to repress Wg-responsive genes, the phenotype of such a mutant should be like that of *dTCF*. Instead, embryos carrying only C-terminally truncated Arm resemble *wg* null mutants, consistent with a failure to effectively antagonize repression.

Armadillo is likely to contact the transcriptional machinery through several protein interfaces: Since Arm protein lacking the C terminus retains detectable function in signaling, other regions of Arm may also interact with the basal transcriptional machinery or with other transcription factors, supplementing the role of the C terminus (Figure 10C). One accessory signaling interface is likely to reside in the C-terminal half of the Arm repeat region, as assayed by genetic and functional experiments in both Drosophila (Peifer and Wieschaus 1990; Orsulic and Peifer 1996) and in Xenopus (Behrens *et al.* 1996; Fagatto *et al.* 1996; Rubenstein *et al.* 1997). A second contact site resides in the N-terminal Arm repeats (Arm repeats 2–5), where βcat has been shown to bind to Pontin52, which in turn binds TATA-box binding protein; βcat and Pontin52 can form a tripartite complex with LEF/TCF proteins (Bauer *et al.* 1998). Finally, the N-terminal domain, while not essential for signaling *in vivo* (Orsulic and Peifer 1996; Pai *et al.* 1997), does contain sequences that can act as a transcriptional activation domain in cultured cells (Hsu *et al.* 1998). Together with the one or more binding sites for the transcriptional machinery present in the C-terminal domain, these multiple protein contacts may contribute in an additive or synergistic fashion to Arm transcriptional activation *in vivo*.

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