Accumulation of Armadillo Induced by Wingless, Dishevelled, and Dominant-negative Zeste-white 3 Leads to Elevated DE-cadherin in Drosophila Clone 8 Wing Disc Cells*

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Drosophila genetic studies suggest that in the Wingless (Wg) signaling pathway, the segment polarity gene products, Dishevelled (Dsh), Zeste-white 3 (ZW-3), and Armadillo (Arm), work sequentially; wg and dsh negatively regulate zw-3, which in turn down-regulates arm. To biochemically analyze interactions between the Wg pathway and Drosophila E-cadherin (DE-cadherin) which bind to Arm, we overexpressed Dsh, ZW-3, and Arm, in the Drosophila wing disc cell line, clone 8, which responds to Wg signal. Dsh overexpression led to accumulation of Arm primarily in the cytosol and elevation of DE-cadherin at cell junctions. Overexpression of wild-type and dominant-negative forms of ZW-3 decreased and increased Arm levels, respectively, indicating that modulation in zw-3 activity negatively regulates Arm levels. Overexpression of an Arm mutant with an amino-terminal deletion elevated DE-cadherin levels, suggesting that Dsh-induced DE-cadherin elevation is caused by the Arm accumulation induced by Dsh. Moreover, the Dom-, dominant-negative ZW-3-, and truncated Arm-induced accumulation of DE-cadherin protein was accompanied by a marked increase in the steady-state levels of DE-cadherin mRNA, suggesting that transcription of DE-cadherin is activated by Wg signaling. In addition, overexpression of DE-cadherin elevated Arm levels by stabilizing Arm at cell-cell junctions.

The Wnt gene family encodes a secreted glycoprotein of 41–44 kDa involved in the cell-cell signaling of a number of basic developmental processes in a wide range of animal phyla (1). The best studied members of this family are mouse Wnt-1 (2, 3) and its Drosophila orthologue, the segment polarity gene wingless (4–6). Drosophila geneticists, ectopic expression of Wnt in Xenopus embryo, several Wnt gene knock-out mice, and cell biological analyses of mammalian and Drosophila cells have implicated Wnt genes in a wide range of biological processes, such as Drosophila pattern formation (4–6), specification of the body axis in Xenopus (7), axial specification of limb and wing in Drosophila and vertebrate (8–11), development of the specific compartment of the central nervous system (12–14), somite formation (15), mammary gland development (16, 17), nephrogenesis in the kidney (18), induction of mitogenesis and transduction in mammalian epithelial cells (19), and modulation of calcium-dependent cell adhesion (20–22).

Through Drosophila genetic screens for mutations in segmental patterning, genes acting in the Wg signal transduction pathway have been identified (23). These genes are dishevelled, zeste-white 3, and armadillo, and their order of action in the pathway has been defined by genetic and molecular studies (24–26). The dsh gene encodes a 65-kDa cytoplasmic protein of unknown function which has several vertebrate homologues (27–30). The zw-3 gene encodes serine-threonine kinases homologous to vertebrate glycogen synthase kinase 3 (GSK3, Refs. 31–34), and the arm gene encodes for a β-catenin homologue (35–37). Genetic and cell biological studies suggest that Wg activates Dsh through a recently characterized Wg receptor, Drosophila frizzled 2 (38). Dsh then negatively regulates the Ser/Thr protein kinase ZW-3, which normally stimulates the instability of Arm protein in the cytoplasm and nucleus. Consequently, the Wg signal stabilizes intracellular Arm, which is thought to alter the expression of the target genes of Wg such as engrailed. The vertebrate counterparts of these components of the Wg signaling cascade in Drosophila function similarly in the Wnt signaling pathway (39) because the Xenopus homologue of Dsh (30, 40), GSK3 (41–43), and β-catenin (44) also participate in the Wnt axis induction pathway in Xenopus embryos (7). Furthermore, β-catenin binding to the tumor suppressor gene product APC (adenomatous polyposis coli gene (45, 46)) and GSK3β binding to the APC-β-catenin complex have been identified (47), leading to the proposal that APC can be placed between GSK3β and β-catenin in the Wnt pathway. Arm was originally discovered as the product of a segment polarity gene (48), but this protein is closely related to the mammalian protein β-catenin (49, 50), a component of intercellular adhesion junctions that binds to the intracellular domain of cadherin (51, 52). Recently, Drosophila a-catenin (Du-catenin, 53) and DE-cadherin (54) have been cloned, and a cadherin-catenin cell adhesion system has been proven in Drosophila. Therefore, Arm/β-catenin is considered to have dual functions as an element of the adhesion apparatus and a transducer of the Wg/Wnt signal.

In some mammalian cells, however, Wnt-1 expression up-regulates steady-state levels of plakoglobin and E-cadherin (20) or stabilizes β-catenin and N-cadherin (21), leading to an increase in calcium-dependent cell-cell adhesion. These findings suggest that Wnt modulates cell-cell interaction during

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１The abbreviations used are: GSK3, glycogen synthase kinase 3; bp, base pair; PAGE, polyacrylamide gel electrophoresis; kb, kilobase pairs; HA, hemagglutinin; APC, adenomatous polyposis coli; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)methyl]glycine; mAb, monoclonal antibody; HMG, high mobility group.
morphogenesis by regulating the properties of cell-cell adherens junctions. In this regard, several studies of Drosophila genetics and the Wg in vitro assay using the Drosophila imaginal disc cell line, clone 8 (55), have shown that activation of the Wg pathway results in Arm accumulation (25, 26, 35, 37, 55, 56), indicating that DE-cadherin can be regulated by the Wg signaling cascade.

In this study, we analyzed the effects of modulating components of the Wg pathway on DE-cadherin expression levels in clone 8 cells. We demonstrated that activation of the Wg pathway in clone 8 cells either directly by Wg or by the overexpression of Dsh or dominant-negative ZW-3 led to Arm accumulation and elevation of DE-cadherin protein levels. Moreover, overexpression of amino-terminal deleted Arm resulted in DE-cadherin elevation. In addition, the Dsh- dominant-negative ZW-3, and truncated Arm-induced accumulation of DE-cadherin protein was preceded by a marked increase in the steady-state levels of DE-cadherin mRNA, suggesting that DE-cadherin induction by the Wg signaling is at least in part, regulated at the transcriptional level.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—The Drosophila imaginal disc cell line clone 8 (57) and Drosophila Schneider S2 cells were cultured as described (55, 56). Expression plasmids constructed with the pMK33 vector (56), which contains a hygromycin-resistant gene, were transfected into clone 8 and S2 cells using the calcium phosphate method as described (55). The transfected cells were a mixture of stable clones selected with hygromycin (200 μg/ml) Dsh, ZW-3, DE-cadherin, and Arm overexpression were induced in transfected cells by adding 0.5 μM CuSO4, as described by Yanagawa et al. (56). Clone 8 cells overexpress full-length and mutant (lacking the discs large homology region, Ref. 60), details of which were described (56). Wingless treatment of clone 8 cells were performed by treating the transformed S2 cell line clone 8 (55), have shown that activation of the Wg pathway results in Arm accumulation (25, 26, 35, 37, 55, 56), indicating that DE-cadherin can be regulated by the Wg signaling cascade.

To construct a DE-cadherin expression plasmid, a 5.4-kb BamHI fragment, which covers the whole coding region of Arm from Arm cDNA (48) and inserting it into the BamHI site of PMK33. Furthermore, we constructed plasmids expressing two types of Myc-tagged Arm as follows. A 36-bp double-stranded oligonucleotide 5’-GATCCACTACGTTACGATGAGCGGT-3’, consisting of a BamHI site, was used to generate the HA epitope- tagged Arm cDNA clones as templates. A 30-base sense primer 5’-GATCCACTACGTTACGATGAGCGGT-3’, consisting of a BamHI site, was used to generate the HA epitope- tagged Arm cDNA clones as templates. A 30-base sense primer 5’-GATCCACTACGTTACGATGAGCGGT-3’, consisting of a BamHI site, was used to generate the HA epitope- tagged Arm cDNA clones as templates. A 30-base sense primer 5’-GATCCACTACGTTACGATGAGCGGT-3’, consisting of a BamHI site, was used to generate the HA epitope- tagged Arm cDNA clones as templates. A 30-base sense primer 5’-GATCCACTACGTTACGATGAGCGGT-3’, consisting of a BamHI site, was used to generate the HA epitope- tagged Arm cDNA clones as templates.
and induced (with CuSO$_4$) action of the method described by Hinck et al. (21). Briefly, noninduced and induced (with Cu$^{2+}$ for 16 h as described) Dsh or DE-cadherin transfectants were plated at very low density (10$^7$ cells/250-ml T flask) and allowed to grow for 16 h. The cultures were rinsed with phosphate-buffered saline containing 5 mM EDTA and plated onto a dish coated with 2% agarose containing M3 medium supplemented with 5 mM EDTA.

RESULTS

Dsh Overexpression Induces Arm and DE-cadherin Proteins—Clone 8 cells expressing Dsh under the control of inducible metallothionein promoter were induced with CuSO$_4$, and the effects of Dsh overexpression on Arm, DE-cadherin, ZW-3, and Ds-catenin protein levels were examined. The blots against anti-Dsh and anti-Myc antibodies in Fig. 1A showed that the metallothionein promoter can induce higher levels of Dsh expression than endogenous Dsh. The Dsh induction kinetics shown in Fig. 1A also demonstrate that CuSO$_4$ causes rapid and marked accumulation of Dsh protein and that slower migrating forms of Dsh (due to Ser/Thr phosphorylation as described by Yanagawa et al. (56)) appear as Dsh protein accumulates. Concomitantly, Arm protein levels were quickly and markedly elevated. On the other hand, DE-cadherin accumulation was first detected 12 h after induction (5-fold), and at 24 h, there was a 15-fold accumulation of DE-cadherin. In contrast, the levels of Ds-catenin and ZW-3 remained unchanged upon Dsh overexpression. Overexpression of a Dsh mutant (56), which lacks 51 amino acids (amino acids 286–336) in the GLGF/DHR motif (59), did not induce either Arm or DE-cadherin. To determine if this also occurs in other cells, the effect of Dsh overexpression on Arm and DE-cadherin protein levels were analyzed in the Drosophila cell line, Schneider S2. Dsh overexpression elevated Arm and DE-cadherin protein levels in S2 cells (Fig. 1B) similarly to that in clone 8 cells.

Wg-induced Accumulation of DE-cadherin—Since Wg protein caused Arm to accumulate in clone 8 cells (55) and Wnt-1 overexpression data obtained from the embryo Xenopus (33) demonstrated that negative regulation of $wz$-3 gene function leads to Arm protein accumulation in Drosophila embryos. Moreover, the ectopic expression of $\beta$-catenin (44) and of a dominant-negative form of glycogen synthase kinase 3 (vertebrate homologue of Wnt-3, Refs. 41–43) in the Xenopus embryo both induce the same phenotype as the ectopic expression of some Wnts, namely duplication of the body axis. However, in vitro findings showing that Arm protein levels are negatively regulated by WZ-3 have not been demonstrated. Therefore, the effects of overexpression of wild-type and dominant-negative
accumulation of Arm protein and the increase in DE-cadherin (shown in Fig. 1) suggest that there is a correlation between genetic epistasis. This experiment and Dsh overexpression model for the Wg signal transduction pathway deduced from were received. These protein levels regardless of whether signals from Dsh or Wg were loaded. For the dominant-negative form of ZW-3, data from cells established by two independent transfections are shown.

(kinase dead) forms of ZW-3 on Arm protein levels were analyzed in the clone 8 cell culture system. Both forms of ZW-3 were HA-tagged and cloned into PMK-33 vector, and stable clone 8 cell lines expressing HA-tagged ZW-3 were established. Following the method of Sutherland et al. (63), their kinase activities were checked by an in vitro kinase assay (data not shown) using the immunoprecipitates obtained with anti-HA antibody from cell lysates, labeled ATP, and a substrate peptide for ZW-3 kinase (GS peptide 2, Ref. 63). Upon induction with CuSO4, there were few-fold increases in ZW-3 protein levels in wild-type and dominant-negative transfectants (Fig. 3, ZW-3 panel). The overexpression of wild-type and dominant-negative forms of ZW-3 decreased and increased Arm protein levels, respectively. In particular, cells from the second transfection with a dominant-negative ZW-3 construct showed much higher levels of Arm accumulation compared with the cells from the first transfection (Fig. 3, Arm panel). Corresponding to these Arm levels, slight and marked increases in DE-cadherin levels were observed in cells from the first and the second transfections, respectively. However, Dsh and D-catenin levels remained constant. These results showed that the modulation of zw-3 activity alone induces opposite changes in Arm protein levels regardless of whether signals from Dsh or Wg were received. These in vitro findings further supported the model for the Wg signal transduction pathway deduced from genetic epistasis. This experiment and Dsh overexpression (shown in Fig. 1) suggest that there is a correlation between accumulation of Arm protein and the increase in DE-cadherin protein levels.

**Armadillo-induced Accumulation of DE-cadherin**—To determine whether or not Arm protein accumulation induced by Wg signaling leads to elevated DE-cadherin protein in clone 8 cells, we studied the consequences of Arm overexpression. An Arm expression plasmid (named PMK-Arm) was constructed with the PMK vector and transfected into clone 8 cells to establish stable cell lines. Despite the enormous induction of Arm mRNA, the Arm and DE-cadherin protein levels were only slightly increased (Fig. 4A), thus indicating the very tight post-transcriptional regulation of Arm protein. However, because of this marginal induction of Arm protein, we could not precisely evaluate the correlation between Arm accumulation and DE-cadherin protein elevation. Extensive analyses of mammalian

![image](https://example.com/image.png)

**FIG. 3.** Overexpression of wild-type and dominant-negative forms of ZW-3 decreases and increases Arm protein levels, respectively. Western blots showing the DE-cadherin, Arm, ZW-3, D-catenin, and Dsh protein levels in lysates from noninduced (−) and induced (+) clone 8 cells transfected with the construct expressing either wild-type (wild-type) or dominant-negative (K→R) forms of ZW-3. Cells were induced with CuSO4 for 16 h. In each lane, the same amount of protein (assayed with Protein Assay Kit, Bio-Rad) was loaded. For the dominant-negative form of ZW-3, data from cells established by two independent transfections are shown.

![image](https://example.com/image.png)

**FIG. 4.** Arm protein elevation leads to the accumulation of DE-cadherin. A, protein and total RNA samples were prepared from induced (+) or noninduced (−) clone 8 cells stably transfected with an Arm expression construct (PMK-Arm). Protein levels of Arm or DE-cadherin and mRNA levels of Arm were determined by Western and Northern blotting, respectively. B, transient expression of Myc-tagged Arm in clone 8 cells. PMK33 vector (lane 1), a construct with full-length Arm (lane 2) and a construct with amino-terminal deleted Arm (lane 3) were introduced into clone 8 cells with calcium phosphate. The transfected cells were cultured in normal medium for 24 h, induced with CuSO4 for a further 16 h, and cell lysates were Western blotted against anti-Myc antibody. C, mutant Arm overexpression resulted in DE-cadherin accumulation. The stable transfectants established with either full-length (shown as Full) or amino-terminal deleted (shown as Deletion) Arm constructs were induced (+) or not (−) with CuSO4 for 16 h. Protein samples were prepared from the cells and Western blotted against anti-DE-cadherin, anti-Myc, or anti-D-catenin antibodies. Total RNA samples were prepared from the same cells and Northern blotted with Arm cDNA probe (bottom panel). The positions of molecular weight markers and the expected migration positions for full-length (arrowhead) and mutant (arrow) Arms are indicated on the right side of the panels for Myc-tagged Arm in B and C.

E-cadherin (reviewed in Ref. 64) have demonstrated that the so-called Arm repeat region of β-catenin is responsible for the binding of both E-cadherin and the high mobility group (HMG) box transcription factor lef/TCF (65, 66). Moreover, Rubinfeld et al. (47) showed that a mutant β-catenin lacking the amino-terminal 89 amino acids but not the full-length protein accumulates at high levels in SW 480 colon cancer cells. Therefore, to induce the marked accumulation of Arm protein with the Arm repeats, a plasmid that expresses a mutant form of Arm lacking the amino-terminal 114 amino acids was constructed with the PMK 33 vector. Since the epitope of the anti-Arm antibody, N2–7A1, is located in this deleted region, full-length and amino-terminal deleted Arm were both Myc-tagged (see “Experimental Procedures”). The transient expression shown in Fig. 4B revealed that the amino-terminal deletion mutant
FIG. 5. DE-cadherin overexpression resulted in the accumulation of Arm protein without affecting its mRNA levels. Cell lysates and total RNAs were prepared from DE-cadherin transfectant either noninduced (−) or induced (+) with CuSO4 for 16 h. Protein levels of DE-cadherin, Arm, Dsh-catenin, and Dsh were determined by Western blotting (shown as protein). Steady-state levels of mRNA of DE-cadherin, Arm, and Dsh-catenin were determined by Northern blotting (shown as RNA) with specific DNA probes. The arrowhead in the Arm protein panel indicates highly modified Arm.

(a) Cu++
(b) D-sh-cat
(c) D-Cat
(d) Dishevelled

PMK-Cadherin

Protein mRNA

Cu++ + + − −
DE-Cadherin
Arm
Catenin

PMK-Dsh

Cu++ +

PMK-del

Cu++ −

-Arm

(A) Anti-Cadherin
(B) Anti-Armadillo
(C) Anti-Cadherin
(D) Anti-Armadillo
(E) Anti-Cadherin
(F) Anti-Armadillo
(G) Anti-Cadherin
(H) Anti-Myc

FIG. 6. Indirect immunofluorescence analyses showing the distribution of Arm and DE-cadherin in transfectants overexpressing Dsh, DE-cadherin, and mutant Arm. Confocal microscopic images of the Dsh transfectant (PMK-Dsh; A–D), the DE-cadherin transfectant (PMK-Cad; E and F), and mutant Arm transfectants (PMK-del-Arm; G and H) are shown. The noninduced (A and B; − Cu++) or induced (C–H; + Cu++) cells were stained with either anti-DE-cadherin mAb (A, C, E, and G), anti-Arm mAb (B, D, and F), or anti-Myc mAb (H). A and B show typical background stainings with anti-DE-cadherin mAb and anti-Arm mAb, respectively, in noninduced cells. Bar in F indicates 10 μm.

Modulation of DE-cadherin Expression by Wingless Signaling

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Overexpression of DE-cadherin Leads to Arm Accumulation—It is well documented that the Wg/Wnt pathway competes with DE-cadherin/cadherin for Arm/β-catenin under conditions where Arm is limiting (67–69). In addition, β-catenin (70, 71) protein levels are post-translationally regulated (apparently at the same level as that of Wg/Wnt-mediated Arm/β-catenin regulation) by cadherin protein levels in certain mammalian cells. This means that sensitivity of cells to Wg/Wnt signaling can be influenced by cadherin protein expression levels in cells. Therefore, to understand what kind of DE-cadherin-mediated regulation of Arm is operative in clone 8 cells, the effect of DE-cadherin overexpression on Arm, Dsh-catenin, and Dsh was examined. Incubating the transfectant with CuSO4 markedly induced DE-cadherin mRNA with DE-cadherin protein increasing 20-fold. This DE-cadherin protein accumulation induced a marked increase in the Arm protein levels without affecting the steady-state levels of Arm mRNA (Fig. 5). This finding indicated that Arm protein levels are post-transcriptionally controlled. The blots of Arm protein showed that the hyperphosphorylated form(s) of Arm (an arrowhead in the Arm protein panel) was dominantly accumulated in DE-cadherin-induced cells. When Arm accumulation was induced by Wg or by overexpression of Dsh, it was a hypophosphorylated form (Refs. 55, 56, and 72 and Fig. 1A). This contrast suggests that the Arm accumulation mechanism functioning in DE-cadherin overexpression differs from those involved in induction by Wg or Dsh overexpression. DE-cadherin overexpression induced a slight increase in Dsh-catenin levels but had no effect on Dsh. Higher expression levels of DE-cadherin provide a large amount of Arm-binding sites, and the association of DE-cadherin with Arm may retard the turnover of Arm, thus inducing its accumulation. Hinck et al. (73) noted that in Madin-Darby canine kidney cells, E-cadherin forms a complex with β-catenin shortly after synthesis. Regarding co-localization of DE-cadherin and Arm in a DE-cadherin overexpressor (Fig. 6, see below), overexpressed DE-cadherin may stabilize Arm and lead to Arm accumulation.

ImmunoFluorescent Analysis of Arm and DE-cadherin Distribution in Dsh, DE-cadherin, and Mutant Arm Transfectants—To confirm the accumulation of Arm and DE-cadherin and to analyze their subcellular distribution in transfectants overexpressing Dsh and DE-cadherin, the cells were stained with anti-Arm or anti-DE-cadherin monoclonal antibodies. Consistent with the Western blot results presented in Fig. 1A and Fig. 5, Arm and DE-cadherin stainings were marked in induced (Fig. 6, C–F) but not noninduced cells (Fig. 6, A and B). As expected, DE-cadherin localized at cell-cell junctions in both cell types, and the DE-cadherin transfectant (Fig. 6E) was more intensely stained for cadherin than the Dsh transfectant (Fig. 6C). Interestingly, Arm localized primarily at cell-cell junctions in DE-cadherin transfectants (Fig. 6F), but in Dsh cells, Arm staining was intense primarily in the cytoplasm (Fig. 6D). This sharp contrast corresponds to the observation that hypo- and hyperphosphorylated forms of Arm accumulated in cells expressing Dsh (Fig. 1A) and DE-cadherin (Fig. 5), respectively. Immunostaining of a mutant Arm expressor with anti-Myc or anti-DE-cadherin antibodies revealed that the mutant form of Arm accumulates primarily in the cytoplasm (Fig. 6H).
and that DE-cadherin staining at cell-cell junctions is increased (Fig. 6G), suggesting that this Arm overexpressor is similar to the Dsh overexpressor in terms of Arm and DE-cadherin distribution.

**Cell Fractionation to Confirm Subcellular Localization of Arm and DE-Cadherin in Dsh and DE-cadherin Transfectants**—To quantify the subcellular distribution of Arm and DE-cadherin protein in Dsh and DE-cadherin cells, we performed cell fractionation analysis. A comparison of noninduced and induced Dsh transfectants showed that Dsh overexpression leads to a marked increase in Arm, especially in the S100 fraction, and to a clear elevation of DE-cadherin and the corresponding accumulation of Arm in the P100 fraction (Fig. 7, left panels). In noninduced Dsh cells, a very small amount of Myc-tagged Dsh protein was found as an apparently unmodified protein only in the S100 fraction. In induced Dsh cells, large amounts of unmodified and modified forms of Dsh protein accumulated in the S100, and several modified but not unmodified forms of Dsh accumulated in the P100 fraction. The most highly modified form of Dsh (arrowhead in Fig. 7, left panel) preferentially accumulated in the P100 fraction of induced cells. In the DE-cadherin transfectant, DE-cadherin overexpression also resulted in the marked accumulation of Arm, but in sharp contrast to Dsh transfectants, most of it was localized in the P100 fraction (Fig. 7, right panels). Furthermore, a large proportion of the Arm that accumulated in the P100 fraction was modified (arrow on the right of the Arm blot). This Arm distribution data revealed by cell fractionation was consistent with the Arm staining profiles shown in Fig. 6, D and F.

**Dsh or DE-cadherin Expressing Cells Show Increased Cadherin-dependent Cell Adhesion**—Fig. 6, C and E, shows significant and marked accumulation of DE-cadherin on the cell surface of Dsh and DE-cadherin-expressing cells, respectively. To investigate if the elevated DE-cadherin levels in these cells were accompanied by increased intercellular adhesion, we assayed the strength of cell-cell adhesion by the method used by Hinck et al. (21), i.e., vigorous trituration of cells following their aggregation in suspension culture. Noninduced and induced Dsh cells form similar size aggregates (about 50 cells per aggregate), but trituration of aggregates from noninduced Dsh cells dissociated 67% of the aggregates into groups of <10 cells. In contrast, a large proportion of aggregates from induced Dsh cells survived this treatment and only 25% of the aggregates dissociated into small size aggregates (Fig. 8A). Similar to the situation in Dsh cells, induced DE-cadherin cells showed marked resistance against trituration compared with their noninduced counterparts (Fig. 8A). Findings from control experiments without calcium indicated that calcium is required for strong cell-cell adhesion in both transfectants (Fig. 8B). These results showed that elevated expression of DE-cadherin in Dsh and DE-cadherin transfectants increases cadherin-mediated cell-cell adhesion.

**Dsh, a Dominant-negative ZW-3 and Amino-terminal Deleted Arm Overexpression Leads to an Increase in the Steady-state Levels of DE-cadherin mRNA**—To investigate the regulatory mechanisms responsible for the increased levels of DE-cadherin and Arm proteins in the Dsh transfectant, we examined changes in steady-state levels of mRNAs of DE-cadherin, Arm, and β-catenin (Fig. 9A). A comparison of induction kinetics of DE-cadherin mRNA (Fig. 9A) and DE-cadherin protein (Fig. 1A) suggested that mRNA induction precedes protein accumulation because a marked increase of mRNA, but not the protein, was detectable 4 h after induction. This Dsh overexpression system is the first to show that DE-cadherin mRNA is upregulated in vitro. Despite the marked accumulation of Arm protein (Fig. 1A), its mRNA levels were constant, further demonstrating that Arm protein levels are tightly controlled by a post-transcriptional mechanism (37) which probably regulates...
We showed that amino-terminal deleted but not full-length Arm protein can be overexpressed in either transient or stable transfection systems (Fig. 4). We also found that Myc-tagged full-length Arm protein accumulates when the transfectants were incubated with Wg (data not shown). These findings suggest that the NH2-terminal sequences in Arm proteins are responsible for their rapid turnover. In this regard, Yost et al. (74) reported that a β-catenin mutant lacking the amino-terminal region is more stable and more active in an ectopic axis induction in Xenopus embryos and that specific phosphorylation of Ser and Thr residues in the amino-terminal domain of the protein by GSK3β facilitates rapid degradation of β-catenin. Zecca et al. (75) noted similar data in Drosophila. Munemitsu et al. (76) also noted deletion of an amino-terminal sequence stabilizes β-catenin in cultured mammalian cells. Moreover, Morin et al. (77) found a special class of mutant β-catenins that eliminate the specific amino acids that become phosphorylated by GSK3β. These mutant β-catenins escape GSK3β-mediated degradation and thus accumulate in high levels and function as oncogenic proteins in colon carcinoma cell lines with normal levels of wild-type APC. The amino-terminal deleted Arm protein used in this experiment may be stabilized by a similar mechanism because these specific amino acid residues of the ZW-3 kinase target are conserved in the amino-terminal domain of Arm.

Compared with clone 8 cells, we did not detect obvious morphological changes in cells transfected with Dsh, a dominant-negative ZW-3 or truncated Arm constructs, even after induction with CuSO4. However, the induced DE-cadherin transfectants formed firm cell aggregates in which individual cells made tight cell-cell junctions. Fig. 8 shows that the accumulation of DE-cadherin in cells overexpressing Dsh and DE-cadherin (Fig. 6, C and E) actually increases cadherin-dependent cell adhesivity.

Fig. 5 shows the DE-cadherin induced modulation of Arm and Dα-catenin. Our findings were in agreement with those of the following studies. Oda et al. (54) showed that overexpression of DE-cadherin results in increases of Arm and Dα-catenin in Schneider S2 cells. Uemura et al. (78) reported Western blot data from the DE-cadherin mutant fly showing that shotguns null mutant embryos lacking zygotic DE-cadherin have markedly reduced total DE-cadherin levels (about 20% of the wild-type level) and a concomitant sharp decrease of the larger isoform of Arm, which are predominantly associated with DE-cadherin. Tepass et al. (79) also showed that Arm and Dα-catenin levels are significantly reduced in the embryonic epidermis of shotguns null mutant embryos.

Oda et al. (54) and Uemura et al. (78) compared lateral views of stage 10 embryos stained for DE-cadherin and Arm. They confirmed that Arm accumulated at high levels in the area of Wg expression. Thus, Arm was stained in a repeated segment manner. However, the uniform staining of the embryos with anti-DE-cadherin suggests that distribution of DE-cadherin is uniform in the embryos and that Arm accumulation cannot induce an increase in DE-cadherin protein at least in the epidermis of the extended germ band stage embryos. This finding seems incompatible with our in vitro data from clone 8 cells. However, it seems that fluctuations in Arm protein levels sharply affect DE-cadherin levels in clone 8 cells, which contain a very limited amount of DE-cadherin, but not in the embryonic epidermis where DE-cadherin is highly expressed. Wg signaling-dependent DE-cadherin regulation (either transcriptional or post-transcriptional) is probably operative in embryonic epidermis, but this regulation mechanism may be overwhelmed by other Wg-independent regulations of DE-cadherin in these cells. In this regard, Hinck et al. (21) noted that Wnt-1

**Fig. 9. Increase in steady-state levels of DE-cadherin mRNA upon induction of Dsh, amino-terminal deleted Arm, and dominant-negative ZW-3 proteins.** A, kinetics of DE-cadherin, Arm, and Dsh-catenin mRNA levels upon Dsh overexpression. Total RNAs were isolated from the dsh transfectants at different periods after CuSO4 treatment, and they were subjected to Northern analysis. Steady-state levels of DE-cadherin, Arm, and Dsh-catenin mRNAs were analyzed with specific DNA probes. B, comparison of induction kinetics of DE-cadherin protein and mRNA in cells overexpressing amino-terminal deleted Arm. Dsh-catenin protein levels are also shown. C, DE-cadherin protein accumulation in dominant-negative ZW-3-expressing cells was accompanied by an increase in its mRNA level. Total RNA from noninduced (0 h) and induced (24 h) cells from the second transfection (Fig. 3) were subjected to Northern analysis with DE-cadherin, Arm, and ZW-3-specific probes.

Arm protein breakdown (55). The protein and mRNA levels of Dα-catenin, on the other hand, were not affected by Dsh overexpression. Similar kinetic analysis on cells expressing amino-terminal deleted Arm also showed that accumulation of DE-cadherin protein was preceded by its mRNA elevation (Fig. 9B). Furthermore, a dominant-negative ZW-3-induced accumulation of DE-cadherin protein was accompanied by an increase in the steady-state levels of DE-cadherin mRNA (Fig. 9C). These findings suggest that the DE-cadherin elevation observed in cells expressing Dsh, a dominant-negative ZW-3 and amino-terminal deleted Arm is caused by increased levels of DE-cadherin protein synthesis. These mRNA accumulations may be due to either stimulation of DE-cadherin transcription or stabilization of its RNA.

**DISCUSSION**

Using a Drosophila cell culture system that responds to the Wg signal, a cDNA clone of DE-cadherin and a monoclonal antibody against DE-cadherin, we analyzed the interaction between Wg signaling and DE-cadherin expression. We overexpressed three components of the Wg signal transduction pathway, Dsh, ZW-3, and Arm, in clone 8 cells and investigated their effects on DE-cadherin levels. The effect of DE-cadherin overexpression on the levels of Arm and Dα-catenin were also analyzed. This study showed that activations of the Wg signaling pathway by overexpression of downstream components of this cascade all increase mRNA and protein levels of DE-cadherin.
expression results in the induced accumulation of β-catenin and N-cadherin in AT at 20 cells and C57MG cells, whereas Wnt-1 has no effect on the modulation of cadherin-catenin levels in Madin-Darby canine kidney cells that express very high levels of cadherin and catenins and thus show tight cell-cell adhesion in the presence of calcium. It is also of interest to see whether DE-cadherin levels in zy-3 mutant embryos are increased because very high levels of Arm accumulate in these mutant embryos compared with the wild type (33).

Hinck et al. (21) and Bradley et al. (20) demonstrated that Wnt-1 expression in some mammalian cultured cells leads to increases in the steady-state levels of β-catenin and plakoglobin (another vertebrate Arm homologue) and elevated E- or N-cadherin, resulting in a concomitant increase in the strength of calcium-dependent cell-cell adhesion. They demonstrated that the elevation of cadherin was primarily caused by stabilization of this protein by complex formation with β-catenin or plakoglobin. Thus, they did not analyze the effect of Wnt-1 expression on cadherin mRNA levels. We have shown in this study that modulation of the more downstream components of Wg pathway, Dsh, ZW-3, and Arm can induce up-regulation of DE-cadherin. We cannot rule out the possibility that Dsh- and dominant-negative ZW-3-induced elevation of cytoplasmic Arm also increases DE-cadherin-Arm complex formation on the plasma membrane, which leads to DE-cadherin stabilization. This partial translocation mechanism may partly contribute to the DE-cadherin accumulation induced by Dsh and dominant-negative ZW-3 overexpression. However, a major new insight of this study is that the DE-cadherin protein accumulation induced by overexpression of Dsh, dominant-negative ZW-3, and deleted Arm is preceded by elevation of steady-state levels of DE-cadherin mRNA that probably leads to increased DE-cadherin protein synthesis. Furthermore, elevation of cytoplasmic pools of amino-terminal deleted Arm alone induce DE-cadherin mRNA levels, supporting the idea that DE-cadherin mRNA accumulation observed in cells overexpressing Dsh and a dominant-negative ZW-3 is caused by Arm elevation induced by overexpression of these proteins. To address whether overexpression of these proteins activates DE-cadherin transcription, chloramphenicol acetyltransferase assays with the DE-cadherin promoter region are now underway. If transcriptional activation occurs, the transcription factor(s) that seems modulated by elevated cytoplasmic pools of Arm may be characterized using gel shift assays with the DE-cadherin promoter region.

Recent studies showing interaction of β-catenin/Arm with HMG box transcriptional factors, Lef-1(65)/Xtcf-3 (66) and NURD, result in a concomitant increase in the strength of calcium-dependent cell-cell adhesion. They demonstrated that the stabilization of cadherin was primarily caused by stabilization of this protein by complex formation with β-catenin or plakoglobin. Thus, they did not analyze the effect of Wnt-1 expression on cadherin mRNA levels. We have shown in this study that modulation of the more downstream components of Wg pathway, Dsh, ZW-3, and Arm can induce up-regulation of DE-cadherin. We cannot rule out the possibility that Dsh- and dominant-negative ZW-3-induced elevation of cytoplasmic Arm also increases DE-cadherin-Arm complex formation on the plasma membrane, which leads to DE-cadherin stabilization. This partial translocation mechanism may partly contribute to the DE-cadherin accumulation induced by Dsh and dominant-negative ZW-3 overexpression. However, a major new insight of this study is that the DE-cadherin protein accumulation induced by overexpression of Dsh, dominant-negative ZW-3, and deleted Arm is preceded by elevation of steady-state levels of DE-cadherin mRNA that probably leads to increased DE-cadherin protein synthesis. Furthermore, elevation of cytoplasmic pools of amino-terminal deleted Arm alone induce DE-cadherin mRNA levels, supporting the idea that DE-cadherin mRNA accumulation observed in cells overexpressing Dsh and a dominant-negative ZW-3 is caused by Arm elevation induced by overexpression of these proteins. To address whether overexpression of these proteins activates DE-cadherin transcription, chloramphenicol acetyltransferase assays with the DE-cadherin promoter region are now underway. If transcriptional activation occurs, the transcription factor(s) that seems modulated by elevated cytoplasmic pools of Arm may be characterized using gel shift assays with the DE-cadherin promoter region.

Recent studies showing interaction of β-catenin/Arm with HMG box transcriptional factors, Lef-1(65)/Xtcf-3 (66) and Pangolin (80, 81), and their translocation to the nucleus sup-
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