

# Expression of *Wnt-1* in PC12 Cells Results in Modulation of Plakoglobin and E-Cadherin and Increased Cellular Adhesion

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**Abstract.** The *Wnt-1* gene plays an essential role in fetal brain development and encodes a secreted protein whose signaling mechanism is presently unknown. In this report we have investigated intracellular mechanisms by which the *Wnt-1* gene induces morphological changes in PC12 pheochromocytoma cells. PC12 cells expressing *Wnt-1* show increased steady-state levels of the adhesive junction protein plakoglobin, and an altered distribution of this protein within the cell. This effect appears similar to a modulation of the plako-

globin homolog, Armadillo, that occurs in *Drosophila* embryos in response to the *Wnt-1* homolog, *wingless* (Riggleman, B., P. Schedl, and E. Wieschaus. 1990. *Cell*. 63:549–560). In addition, PC12/*Wnt-1* cells show elevated expression of E-cadherin and increased calcium-dependent cell–cell adhesion. These results imply evolutionary conservation of cellular responses to *Wnt-1/wingless* and indicate that in certain cell types *Wnt-1* may act to modulate cell adhesion mechanisms.

THE *Wnt* gene family constitutes a large group of developmentally regulated genes involved in cell–cell signaling in a wide range of animal phyla (for reviews see references 38, 47). Members of the *Wnt* family have been implicated in diverse biological processes, including specification of the vertebrate body axis, development of specific compartments of the central nervous system, axial specification in limb development, mouse mammary gland development, and pattern formation in *Drosophila* (16, 38, 44, 47, 73). The best studied members of this family are mouse *Wnt-1* and its *Drosophila* ortholog, the segment polarity gene *wingless* (46, 64).

*Wnt-1* is expressed in a restricted pattern within the developing neural tube of several vertebrates (43, 67, 82, 83) and its functional significance is evident from the severe mid-brain and cerebellar defects observed in mice homozygous for mutant *Wnt-1* alleles (39, 75, 76). Although the structural defects in such mice are limited to the brain, *Wnt-1* is also expressed along the dorsal mid-line of the neural tube in the spinal cord region where its function may be redundant with other members of the *Wnt* gene family (66, 82, 83). Ectopic expression of *Wnt-1* can have dramatic effects in other tissues. Although not normally expressed in the mammary gland, the gene was first identified as a proto-oncogene activated by retroviral insertion in mouse mammary tumors and its expression in the mammary glands of transgenic mice causes hyperplasia and an increased incidence of carcinomas (46, 48, 77). In addition, injection of *Wnt-1* RNA into early *Xeno-*

*pus* embryos induces dorsal mesoderm with resulting duplication of the embryonic axis (40, 71).

The protein products of *Wnt-1* are cysteine-rich secreted glycoproteins of 41–44 kD which have been found associated with extracellular matrix and cell surfaces (3, 5, 52–54). The secretory nature of these products, together with experimental evidence that the gene can act via a paracrine mechanism in cell culture (28), strongly suggests that *Wnt-1* protein functions as an extracellular signaling factor. Specific receptors for the protein have not yet been identified, however, and it is unclear what intracellular changes are elicited by *Wnt-1* in the different responsive tissues. One approach towards investigating such responses is to characterize the phenotypic changes elicited by expression of the gene in cultured cell lines. *Wnt-1* causes morphological transformation and deregulated growth of certain mammary epithelial cells (4, 65) and also induces distinct morphological changes in PC12 pheochromocytoma cells, a neural crest-derived tumor cell line with both neuronal and epithelial characteristics (19, 23, 68).

In seeking to understand the changes in cell phenotype induced by *Wnt-1* in these cells, we have followed clues derived from studies of the *Drosophila Wnt-1* homolog, *wingless*, which is 54% identical to mouse *Wnt-1* at the amino acid level and also encodes a secreted protein (8, 21, 22, 64, 79). *Wingless* plays a vital role in pattern formation within the embryonic body segments and imaginal discs, and also functions in development of the nervous system and malpighian tubules (1, 11, 49, 70, 72). Through the extensive genetic analysis of segmental patterning, several genes have been identified whose mutant phenotypes are similar to that of *wingless* and whose gene products are thought to act in the

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same developmental pathway (26, 57). One such gene is *armadillo* (62, 81). *Armadillo* mRNA is expressed uniformly in the embryonic epidermis, but in each segment an elevated level of Armadillo protein is found in a broad stripe of cells including those which express *wingless* (60, 63). This localized increase in Armadillo protein is dependent on *wingless* function and so appears to be a specific cellular response to the *wingless* signal (58, 60, 63).

Armadillo protein is closely related to two mammalian proteins, plakoglobin and  $\beta$ -catenin, both of which are components of intercellular adhesive junctions (7, 13, 36, 45, 51, 56). Plakoglobin and  $\beta$ -catenin can be found at the cytoplasmic face of the plasma membrane in protein complexes physically associated with the intracellular domain of E-cadherin or N-cadherin (30, 36, 51, 59). These complexes are thought to play an important role in stabilizing the adhesion function of cadherins and/or in mediating connections to the subcortical cytoskeleton (reviewed in references 12, 32).

In this report we have investigated the phenotypic changes induced by expression of *Wnt-1* in PC12 cells. In view of the *wingless*-dependent modulation of Armadillo protein in *Drosophila* embryos (63), we examined the possibility that its mammalian homologs and associated adhesion molecules might be regulated in response to *Wnt-1*. Our results show modulations in the expression level and subcellular location of plakoglobin which show similarities to those described for Armadillo in response to *wingless*. Concomitant with these changes were elevated levels of E-cadherin at lateral cell boundaries and an increase in calcium-dependent cell-cell adhesion. Our data imply evolutionary conservation of cellular responses to *wingless/Wnt-1* signals, and suggest that the phenotypic effect of *Wnt-1* in PC12 cells may be due in part to regulation of cadherin-based cell adhesion mechanisms.

## Materials and Methods

### Cell Lines

PC12 rat pheochromocytoma cells (23) were obtained both from M. Chao and from J. Wagner (Cornell University Medical College) and were maintained in DME supplemented with 10% fetal calf serum, 5% horse serum, and 1% penicillin and streptomycin. To express *Wnt-1* efficiently in PC12, cells were infected with helper-free stocks of the murine sarcoma virus (MSV)<sup>1</sup>-based retrovirus vector *MVWnt-1* (28) which contains mouse *Wnt-1* cDNA together with a bacterial neomycin phosphotransferase resistance gene (*neo*). In parallel, cells were infected with MVFs, a retroviral vector identical to *MVWnt-1* except for a frameshift mutation at codon 77 of the *Wnt-1* reading frame which renders the protein non-functional (4). Retroviral infection was performed as described by Brown and Scott (6) and 4 d later infected PC12 cells were selected in the presence of 600  $\mu$ g/ml G418 (GIBCO-BRL, Gaithersburg, MD). To avoid spurious effects due to clonal variation among PC12 cells, 50-100 resistant colonies were pooled and grown as mixed populations; in addition, infections were repeated with PC12 cells obtained from two other laboratories. The PC12-F cell line, a spontaneously flat and adhesive variant of PC12, was isolated by repeatedly rinsing PC12 cultures with PBS to remove non-adherent cells. Madin-Darby Canine Kidney (MDCK) epithelial cells were obtained from E. Rodriguez-Boulton (Cornell University Medical College).

### Immunoblotting and Cell Fractionation

Cell lysates for immunoblot analysis of plakoglobin and E-cadherin were prepared by scraping cell cultures in RIPA buffer (25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1%

$\beta$ -mercaptoethanol, 1% aprotinin, and 1 mM PMSF) at 80°C. For cell fractionations (29), cells were rinsed three times in 10 mM Tris-HCl (pH 7.4), 140 mM NaCl, 5 mM EDTA, 2 mM DTT, 1 mM PMSF, and then scraped at 4°C into the same buffer containing 0.1% Triton X-100, 0.1 mg/ml DNase I, and 0.1 mg/ml RNase A. Lysates were homogenized with 30 strokes of a Dounce homogenizer (Kontes Glass Co., Vineland, NJ) and centrifuged at 2,000 g for 10 min to remove large particles. The supernatant was then centrifuged at 100,000 g for 2 h at 4°C. The resulting supernatant was saved as the soluble (cytosolic) fraction. The pellet, representing insoluble cytoskeletal, desmosomal, and membranous material, was solubilized in 1 $\times$  Laemmli buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 50 mM DTT, 5% glycerol) by boiling for 5 min. Total protein content of cell lysates or fractions was determined by Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA) and equal quantities were loaded onto 10% SDS-polyacrylamide gels for electrophoresis and subsequent transfer to nitrocellulose. Filters were pre-incubated with a 5% solution of nonfat dry milk, and then incubated with primary antibody for 2 h at room temperature. Mouse monoclonal antibody against plakoglobin (ascites fluid; 13), and rabbit anti-uvomorulin antiserum (80) were each used at a dilution of 1:2,000. Protein bands were visualized by using alkaline phosphatase-conjugated secondary antibodies and NBT/BCIP substrates (Promega Biotec, Madison, WI).

### Immunofluorescence

Cells grown on glass coverslips until confluent were washed twice in PBS containing 1 mM CaCl<sub>2</sub> (PBSC), fixed in 2% paraformaldehyde at room temperature for 1 h, and permeabilized with 0.075% saponin. Autofluorescence was quenched by treatment with 50 mM NH<sub>4</sub>Cl. Coverslips were then rinsed in PBSC containing 0.2% BSA and incubated for 1 h at 37°C in plakoglobin antibody (1:50 dilution of ascites fluid) or E-cadherin antiserum (1:100 dilution). Texas red-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were applied at 1:100 dilution and incubated for 30 min at room temperature. Cells were viewed under epifluorescence and photographed using T-Max 400 film.

### Northern Blotting

RNA for Northern analysis was prepared by the single-step thiocyanate-phenol-chloroform extraction method of Chomczynski and Sacchi (9) and poly A<sup>+</sup> RNA was selected by binding to oligo-dT cellulose. 5- $\mu$ g samples were analyzed on a 1% formaldehyde-agarose gel and transferred to nitrocellulose. The hybridization probe for plakoglobin mRNA was a 60-bp EcoRI-PstI fragment derived from the 3' end of bovine plakoglobin cDNA (13). A rat cyclophilin cDNA probe, provided by G. Ciment (Oregon Health Sciences University, Portland, OR) was used to control for RNA content of the lanes (31). Gel-purified DNA fragments were labeled with <sup>32</sup>P by random hexamer priming (Boehringer-Mannheim Biochemicals, Indianapolis, IN).

### Cell Aggregation Assays

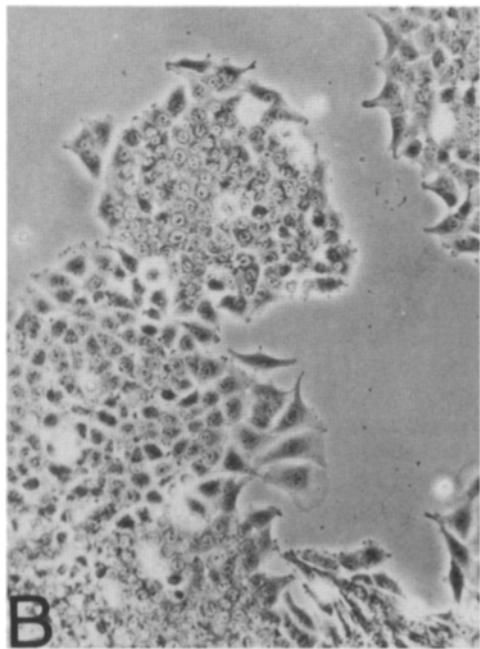
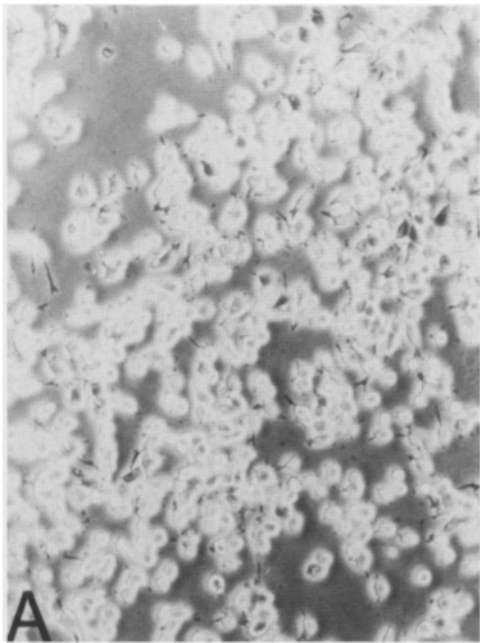
Aggregation assays were performed largely as described by Urushihara et al. (78). Cultures were dissociated into single cell suspensions by treatment with 0.01% trypsin in PBSC. Cells were pelleted by centrifugation at 1,000 g and washed twice in PBSC containing 0.01% soybean trypsin inhibitor (Sigma Immunochemicals, St. Louis, MO). After further washing in PBSC, the cells were resuspended in either PBSC or PBS, each containing 1% BSA and 0.1 mg/ml DNase I (Sigma Immunochemicals). Aliquots of 2  $\times$  10<sup>5</sup> cells in a volume of 0.5 ml were added to the wells of a 24-well tissue culture plate and incubated at 37°C on a gyratory shaker at 80 rpm. At various time points, the total number of particles in suspension was counted using a hemocytometer. The extent of cell aggregation was calculated as the ratio of the number of particles at each time point (N<sub>t</sub>) to the initial number of particles plated (N<sub>0</sub>). Data shown represent the mean values of three similar experiments.

## Results

### Morphological Effects of *Wnt-1* in PC12 Cells

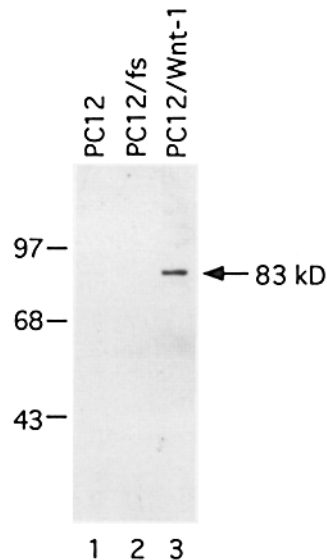
When growing on a plastic substratum, PC12 pheochromocytoma cells are normally rounded and refractile in appearance, make very few cell-cell contacts, and appear poorly adherent. As originally observed by Shackelford et al. (68),

1. Abbreviations used in this paper: MSV, murine sarcoma virus; PBSC, PBS containing 1 mM CaCl<sub>2</sub>.



**Figure 1.** Morphological effect of *Wnt-1* in PC12 cells. Phase contrast photographs of PC12/fs cells, expressing a frameshift mutant *Wnt-1* allele (A), and PC12/*Wnt-1* cells expressing wildtype *Wnt-1* (B).

expression of *Wnt-1* in PC12 cells causes striking phenotypic changes. In the present study we used retrovirus vectors to infect PC12 cells and express either wild-type *Wnt-1* or a functionally defective frameshift mutant allele. Cells expressing the mutant allele (PC12/fs cells) were similar in morphology to uninfected PC12 cells (Fig. 1 A), while those expressing wild-type *Wnt-1* (PC12/*Wnt-1* cells) were morphologically flat and showed extensive cell-cell contacts (Fig. 1 B). Unlike the control populations, PC12/*Wnt-1* cells grew as discrete colonies of adherent cells that were epithelioid in appearance and went on to form a confluent monolayer. Immunoblot analysis of these cells confirmed that they



**Figure 2.** Plakoglobin protein levels are elevated in PC12/*Wnt-1* cells. Immunoblot analysis of uninfected PC12 (lane 1), PC12/fs cells (lane 2), and PC12/*Wnt-1* cells (lane 3). Whole-cell lysates normalized for total protein content were analyzed by Western blotting using anti-plakoglobin antibody and secondary antibody conjugated to alkaline phosphatase. Positions of molecular weight markers, indicated in kD, are shown at left. The 83-kD plakoglobin protein is considerably more abundant in the cells expressing *Wnt-1* (lane 3, arrow).

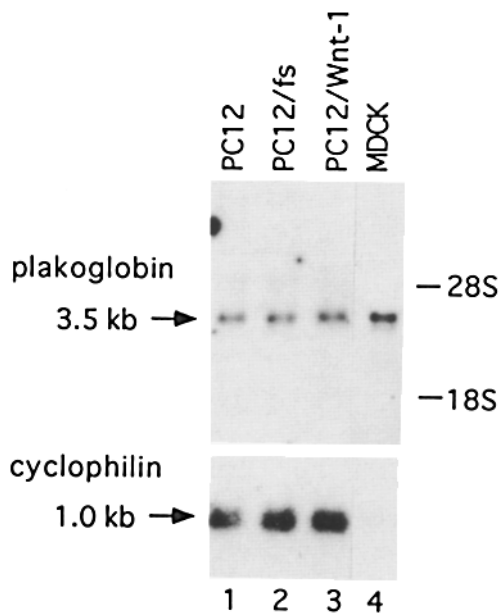
express *Wnt-1* protein, some of which was associated with extracellular matrix as has been shown in other cell lines (data not shown; 3, 28).

#### ***PC12/Wnt-1* Cells Express Elevated Levels of Plakoglobin Protein**

In view of the modulation of Armadillo protein levels caused by *wingless* signaling in *Drosophila* embryos, we sought to determine whether expression of *Wnt-1* in responsive mammalian cells might lead to analogous changes in either plakoglobin or  $\beta$ -catenin, the two mammalian homologs of Armadillo. Accordingly, we examined the steady-state levels of these proteins in PC12/*Wnt-1*, PC12/fs, and uninfected cells by immunoblotting. While analysis of  $\beta$ -catenin using anti-Armadillo antibody (59) showed no obvious differences between the cell populations (data not shown), significant differences were observed in plakoglobin levels. Using a monoclonal antibody specific for plakoglobin, a faint plakoglobin protein band of 83 kD was detected in total cell lysates of PC12 and PC12/fs cells (Fig. 2, lanes 1 and 2). In PC12/*Wnt-1* cell lysates, however, the protein was approximately fivefold more abundant (lane 3). Since pooled populations of infected cells were used in these experiments, this effect was specifically attributable to *Wnt-1* expression and not simply to clonal variation among PC12 cells. Moreover, this analysis was repeated by expressing *Wnt-1* in PC12 populations obtained from two other laboratories and comparable changes in plakoglobin levels were obtained in each case (data not shown).

#### ***Plakoglobin* Levels in PC12 Are Regulated at a Posttranscriptional Level**

To investigate whether the elevated levels of plakoglobin protein in PC12/*Wnt-1* cells result from modulation at the transcriptional or posttranscriptional level, we performed Northern analysis using bovine plakoglobin cDNA as a probe. A single mRNA species of  $\sim 3.5$  kb was detected in all of the PC12 derivatives (Fig. 3) as well as in MDCK cells, which are known to be enriched for plakoglobin (13, 61). The size of this RNA corresponds to that of the plakoglobin tran-



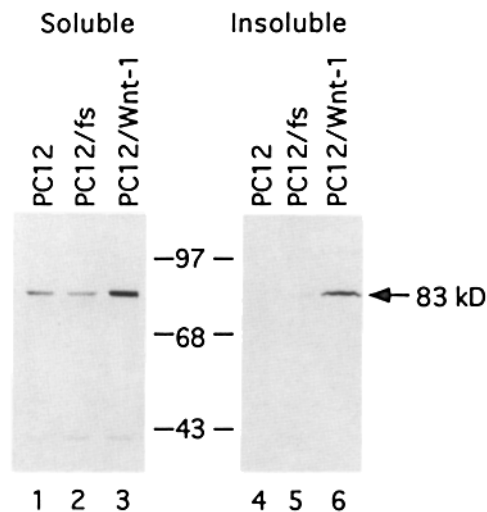
**Figure 3.** Plakoglobin mRNA levels are unaltered by *Wnt-1* expression. PolyA<sup>+</sup> RNA from PC12 derivatives was analyzed by Northern blotting and probed with a <sup>32</sup>P-labeled plakoglobin cDNA fragment (*top*). The blot was re-hybridized with rat cyclophilin probe to control for RNA loading (*bottom*). Lane 1, uninfected PC12 cells; lane 2, PC12/fs; lane 3, PC12/*Wnt-1*; lane 4, MDCK cells. A single plakoglobin mRNA species of approximately 3.5 kb is expressed at similar levels in all three PC12 cell derivatives.

script previously detected in rodent and other mammalian cells (13). Despite the elevated plakoglobin protein levels in PC12/*Wnt-1* cells, the relative abundance of the 3.5-kb transcript in these cells was not significantly different from that in PC12/fs or uninfected PC12 cells (Fig. 3). This indicates that the increase in plakoglobin protein levels associated with *Wnt-1* expression in PC12 cells is the result of posttranscriptional regulation.

#### Subcellular Localization of Plakoglobin Is Altered in PC12/*Wnt-1* Cells

Since plakoglobin has been described in both cytosolic and membrane-associated fractions of other cells, we next examined the subcellular distribution of the protein by biochemical fractionation of infected PC12 populations. Cells were lysed with 0.1% Triton X-100 and the membrane-associated and cytoskeletal components were separated from soluble proteins by centrifugation. In uninfected PC12 and PC12/fs cells, at least 90% of the plakoglobin was found in the detergent-soluble fraction (Fig. 4, lanes 1, 2, 4, and 5) while in PC12/*Wnt-1* cells ~50% of the cellular plakoglobin was detergent insoluble (lanes 3 and 6). Thus, in PC12 cells expressing *Wnt-1* there is both an increase in overall levels of plakoglobin and an altered partitioning of the protein upon detergent extraction.

To localize plakoglobin more precisely, we studied its subcellular distribution in confluent cell cultures by indirect immunofluorescence. In PC12/fs cells we observed diffuse cytoplasmic staining (Fig. 5 B), consistent with the detergent-soluble plakoglobin detected by immunoblotting. Similar staining was also seen in uninfected PC12 cells (data not



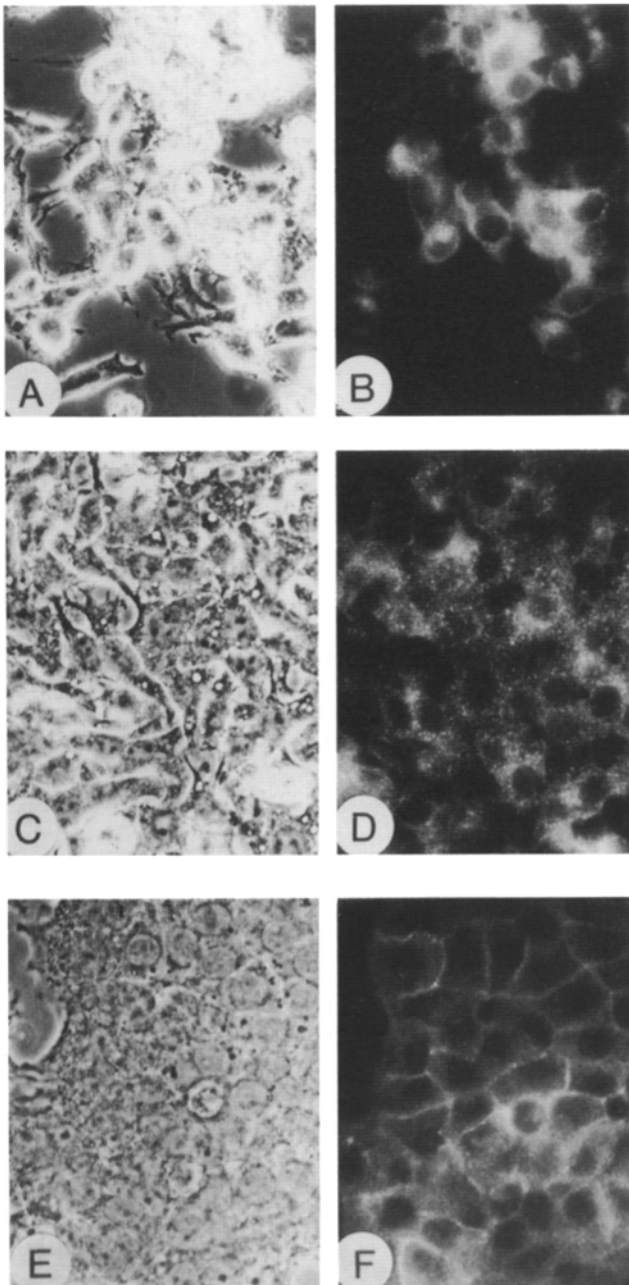
**Figure 4.** Immunoblot analysis of cell lysates fractionated into detergent-soluble and -insoluble components. Lysates prepared in 0.1% Triton X-100 were centrifuged at 100,000 *g* to separate soluble (lanes 1–3) and insoluble fractions (lanes 3–6) and were analyzed by Western blotting with anti-plakoglobin antibody. Lanes 1 and 4, uninfected PC12 cells; lanes 2 and 5, PC12/fs cells; lanes 3 and 6, PC12/*Wnt-1* cells. The 83-kD plakoglobin protein band is indicated. In PC12 and PC12/fs, the majority of plakoglobin is detergent-soluble while in PC12/*Wnt-1* cells a significant proportion is found in the insoluble fraction (lane 3).

shown). In contrast, PC12/*Wnt-1* cells exhibited both cytoplasmic staining and peripheral staining that appeared uniform at the cell membrane wherever there was contact between neighboring cells (Fig. 5 F). This presumably corresponds to or includes the detergent-insoluble fraction of plakoglobin.

We also studied the subcellular distribution of plakoglobin in a spontaneously adherent variant cell line designated PC12-F, which was derived from uninfected PC12. PC12-F cells exhibit a flat adherent morphology and show extensive cell–cell contacts somewhat similar to PC12/*Wnt-1* cells. Immunostaining of PC12-F cells showed only cytoplasmic fluorescence, however, with no evidence of membrane-associated plakoglobin (Fig. 5 D). These results suggest that localization of plakoglobin to the peripheral membrane is not an obligatory feature of adherent PC12 derivatives and that in PC12/*Wnt-1* cells the altered distribution of the protein is a consequence of *Wnt-1* expression rather than the altered morphology per se.

#### PC12/*Wnt-1* Cells Express Elevated Levels of E-Cadherin

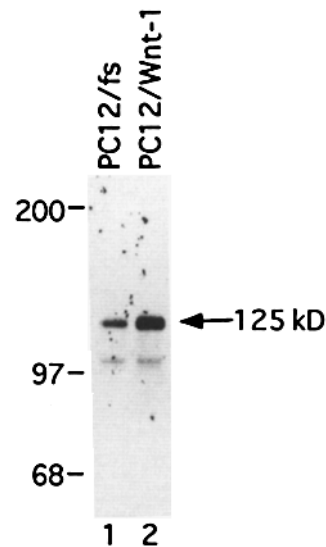
In other cells in which plakoglobin is located at the plasma membrane, the protein has been found physically associated with either E-cadherin or N-cadherin (7, 30, 36, 61). To investigate whether either of these proteins is coregulated with plakoglobin in PC12/*Wnt-1* cells, we examined their expression by immunoblotting. While N-cadherin levels did not differ significantly between the *Wnt-1* positive and *Wnt-1* negative cell lines (data not shown), a striking change was observed in E-cadherin expression. The 125-kD form of E-cadherin was detected in both cell populations but was ap-



**Figure 5.** Plakoglobin is located at the cell membrane of PC12/*Wnt-1* cells. Indirect immunofluorescence analysis of PC12/*fs* cells (A and B), a spontaneously adherent PC12 derivative PC12-F (C and D), and PC12/*Wnt-1* cells (E and F). A, C, and E, phase-contrast views; B, D, and F, epifluorescence. In addition to diffuse cytoplasmic staining, PC12/*Wnt-1* cells show distinct membrane-associated staining of plakoglobin at the cell margins (F).

proximately 5–10 times more abundant in PC12/*Wnt-1* cells than in PC12/*fs* (Fig. 6). As with plakoglobin, Northern blot analysis showed similar levels of E-cadherin mRNA in both cell lines, again implying regulation at the posttranscriptional level (data not shown).

We next examined the sub-cellular distribution of E-cadherin by immunofluorescence of permeabilized cells. In the majority of PC12/*fs* cells, E-cadherin staining was weak and diffuse (Fig. 7 A), as was also observed in the adherent PC12



**Figure 6.** Elevated E-cadherin expression accompanies the increased plakoglobin levels in PC12/*Wnt-1* cells. Whole-cell lysates of PC12/*fs* cells (lane 1) and PC12/*Wnt-1* cells (lane 2) were analyzed by immunoblotting with anti-E-cadherin antibody. The 125-kD E-cadherin protein band is indicated. E-cadherin is substantially much more abundant in the cells expressing *Wnt-1*.

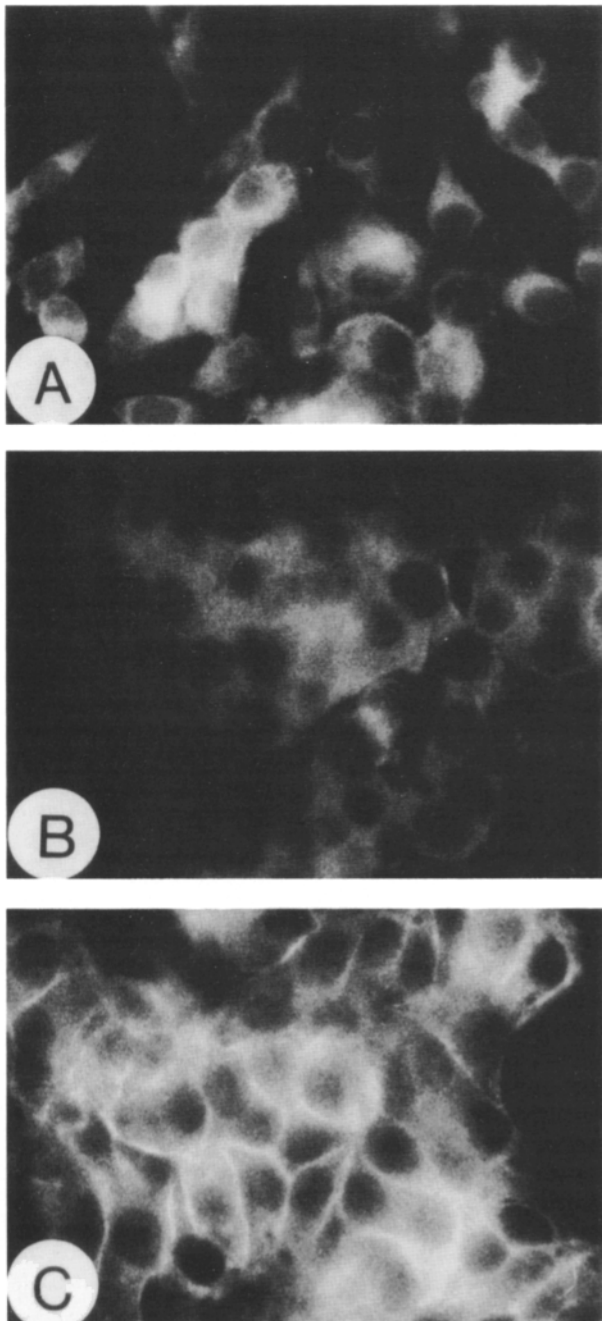
sub-line PC12-F (Fig. 7 B). In PC12/*Wnt-1* cells, however, superimposed upon the diffuse staining pattern we observed consistent staining concentrated at the borders between neighboring cells (Fig. 7 C). These data raise the possibility that a proportion of the E-cadherin expressed in PC12/*Wnt-1* cells may be involved in adhesive interactions between adjacent cells.

#### **PC12/*Wnt-1* Cells Show Increased Calcium-dependent Adhesion**

To investigate whether the elevated plakoglobin and E-cadherin levels in PC12/*Wnt-1* cells were accompanied by increased cellular adhesion, we tested the ability of the cells to aggregate after dissociation into single-cell suspensions. As shown in Fig. 8, dissociated PC12/*fs* cells and uninfected PC12 failed to aggregate in these assays and after 2.5 h 95% of them remained as single cells. In contrast, PC12/*Wnt-1* cells aggregated readily, and the number of particles in suspension was reduced by ~35% after only 1 h. As expected for cadherin-based adhesion, the aggregation of PC12/*Wnt-1* cells required the presence of calcium in the buffer (Fig. 8). Thus the expression of *Wnt-1* in PC12 cells resulted in a significant increase in calcium-dependent intercellular adhesion.

#### **Discussion**

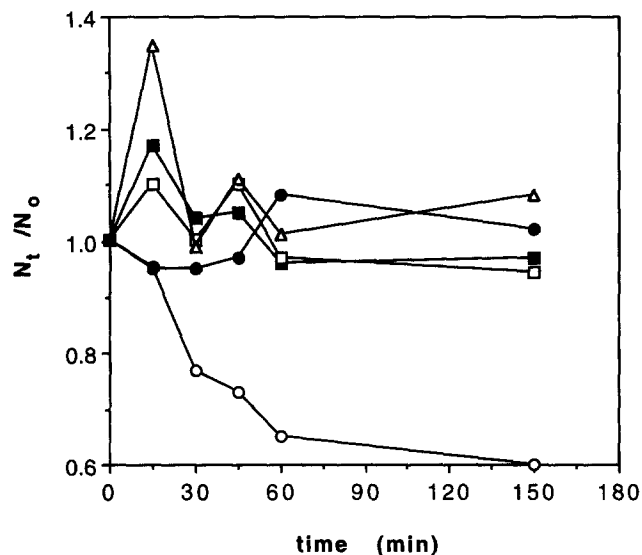
The *Wnt-1* gene is known to play an essential role in fetal brain development, but the mechanisms by which it elicits intracellular changes in target cells have not been clearly elucidated. Secreted Wnt-1 protein is thought to act in an autocrine or paracrine manner (3, 28, 47, 53), but specific receptors for Wnt-1 have not yet been identified and detailed analysis has been hampered by difficulties in isolating significant quantities of Wnt-1 protein in soluble form. However, considerable information about signaling pathways utilized by *wingless*, the *Drosophila* homolog of *Wnt-1*, has emerged from the extensive genetic analysis of segment polarity performed in that organism (for reviews see references 26, 57). One of the gene products whose expression is regulated by *wingless* in *Drosophila*, and which is implicated as an important downstream target of the *wingless* sig-



**Figure 7.** E-cadherin is associated with cell margins in PC12/*Wnt-1* cells. Indirect immunofluorescence analysis of PC12/fs (A), PC12-F (B), and PC12/*Wnt-1* cells (C), using E-cadherin antibody. Staining at the cell margins is evident in PC12/*Wnt-1* cells and is not seen in the control cell populations.

nal in segmental patterning, is Armadillo (60, 63). This protein shows 63–69% sequence identity with the vertebrate adhesive junction proteins plakoglobin and  $\beta$ -catenin (7, 20, 36, 56, 62), and Armadillo itself has recently been shown to be part of a multi-protein complex in *Drosophila* which resembles the adherens junctions of vertebrate cells (55).

In this report we have investigated the mechanisms by which vertebrate *Wnt-1* induces phenotypic changes in the neural cell line PC12. In response to expression of *Wnt-1*,



**Figure 8.** PC12/*Wnt-1* cells display calcium-dependent cell–cell adhesion in aggregation assays. Cells were dissociated by mild trypsin treatment in the presence of calcium and their ability to aggregate was measured over a 2.5-h period. Aggregation is expressed as the ratio of the number of particles in suspension at specific time points ( $N_t$ ) to the number at time zero ( $N_0$ ). In the presence of calcium, PC12 (■), PC12/fs (□), and PC12-F cells (△) showed little or no aggregation, while PC12/*Wnt-1* cell suspensions showed a 40% reduction in particle number (○). PC12/*Wnt-1* cells showed no significant aggregation in the absence of calcium (●).

PC12 cells adopt a flat morphology reminiscent of epithelial cells, form extensive cell–cell contacts, and become refractory to neuronal differentiation induced by nerve growth factor (Fig. 1; 68). We have shown that these changes are associated with modulation of plakoglobin, one of the two vertebrate homologs of Armadillo. In addition, *Wnt-1* causes increased accumulation of E-cadherin at the borders between adjacent cells and an accompanying change in calcium-dependent cell adhesion.

Plakoglobin is expressed at low levels in control PC12 cells, and is found mostly in the Triton-soluble fraction which in other cells corresponds to the cytosolic dimeric form of plakoglobin (13, 29). Following the expression of *Wnt-1* cDNA in any of three different sub-lines of PC12, the steady state level of whole-cell plakoglobin increased significantly, apparently as a result of regulation at the post-transcriptional level. This increased abundance of plakoglobin was accompanied by a relative change in the intercellular distribution of the protein. In PC12/*Wnt-1* cells a substantial fraction of the cellular plakoglobin became Triton-insoluble and immunolocalization studies showed plakoglobin at the plasma membrane wherever neighboring cells were in contact.

Plakoglobin is a component of both adherens junctions and desmosomes, and has been shown to be physically associated with the cytoplasmic domains of E-cadherin, N-cadherin, or desmoglein (12, 18, 30, 36, 51, 59). In concert with the modulation of plakoglobin in PC12/*Wnt-1* cells, we observed similar changes in the abundance and distribution of E-cadherin, which became concentrated at regions of intercellular

contact. Our data therefore suggest that plakoglobin and E-cadherin are co-localized at the membrane of PC12/*Wnt-1* cells, a notion further supported by the detection of plakoglobin in protein complexes immunoprecipitated from these cells with E-cadherin antibodies (R. Bradley, unpublished data).

In other cell systems, the presence of these proteins at regions of cell-cell contact is indicative of cadherin-based adhesive interactions. In MDCK cells, for example, following the induction of calcium-dependent adhesion, plakoglobin and E-cadherin undergo a coordinated redistribution and accumulate at adherens junctions and cell boundaries (61). The membrane localization of these proteins in PC12/*Wnt-1* cells therefore implies that they are functional in intercellular adhesion, and this prediction is borne out by the calcium-dependent re-aggregation behavior exhibited by PC12/*Wnt-1* cells in suspension (Fig. 8). Collectively these data suggest that the modulation of E-cadherin and plakoglobin accounts, at least in part, for the increased adhesion observed in PC12/*Wnt-1* cells, and this may in turn bring about the altered cell morphology. The molecular mechanisms by which *Wnt-1* elicits these changes are presently unclear and may be difficult to elucidate until the putative cell surface receptors for *Wnt-1* protein are identified. However, given the likelihood that adhesive junctions can be regulated by phosphorylation (10, 12, 35), one might speculate that *Wnt-1* receptors, or their associated signal transduction machinery, might modify the phosphorylation state of one or more components of the cadherin-catenin-plakoglobin complex, leading to enhanced stability of such complexes at the plasma membrane.

The mammary epithelial cell lines that can be transformed by *Wnt-1* undergo a distinctly different morphological transition from that described here for PC12 cells. Although we have not characterized the effect in detail, the appearance of the mammary cells suggests a possible reduction in cell-cell adhesion in response to *Wnt-1*. One explanation of these contrasting effects might be that *Wnt-1* protein acts via different *Wnt* receptors in the different cell lines, or that the receptors are coupled to distinct signaling pathways. To elucidate this further, we are currently investigating the role of specific adhesion molecules in *Wnt-1*-mediated transformation of mammary cell lines.

It remains to be determined whether regulation of cell adhesion is a common cellular response to *Wnt-1*, or is limited to specific cell types. Given the important roles of differential cadherin expression during development (74), regulation of cadherin function could potentially be a common mechanism by which *Wnt* genes influence morphogenesis and pattern formation. Although the specific role of cell adhesion in the formation of fetal brain compartments mediated by *Wnt-1* is not yet known, an effect of *Wnt-1* on regulating intercellular junctions in vivo has been demonstrated in *Xenopus* embryos. Olson et al. (50) have shown that injection of *Wnt-1* RNA into fertilized *Xenopus* eggs caused increased gap junctional communication between ventral blastomeres at the 32-cell stage, before the duplication of the embryonic axis which ensues. Since cadherin-mediated cell adhesion appears to be a prerequisite for the formation of gap junctions in several cell types (24, 27) and the abundance of gap junctions in the adjoining membranes of cohesive cells can be regulated by cadherin function (42), it is possible that the effect of *Wnt-1* on gap junctions in vivo might result from

modulation of cell-cell adhesion. Further evidence linking *Wnt-1* to adhesive changes in *Xenopus* comes from the recent observation of McCrea et al. that injection of antibodies to  $\beta$ -catenin can produce an axis duplication phenotype similar to that obtained with *Wnt-1* (37). This reinforces the possibility that catenins and associated cadherins might be downstream effectors of the signal provided by *Wnt-1* in *Xenopus* embryo injection experiments.

The modulation of plakoglobin by *Wnt-1* that we have described in PC12 cells appears strikingly similar to the effects on Armadillo protein mediated by *wingless* in *Drosophila* embryos. The steady-state levels of both plakoglobin and Armadillo are increased in cells responding to *Wnt-1/wingless* signals, and in each case the cytosolic fraction of the protein becomes more abundant (60, 63). In PC12 cells this is accompanied by a conspicuous increase in membrane-associated plakoglobin, although in *Drosophila* the effects on this fraction appear more subtle (60). The potentially analogous interactions between these pairs of homologous gene products suggest that elements of the signal transduction and/or cellular response pathways for *Wnt-1/wingless* in certain cells may have been conserved between *Diptera* and mammals. Further evidence of such conservation has emerged from studies of the homeobox gene *engrailed*, another downstream target of the *wingless* signal (26, 57). For a defined period during establishment of segment polarity in the *Drosophila* embryo, expression of *engrailed* is dependent on the *wingless* signal in adjacent cells (17, 25, 34). In the mouse embryo a homolog of *engrailed* is expressed in a pattern partially overlapping that of *Wnt-1* in the brain, but the expression is lost in *Wnt-1*-deficient mice (14, 15, 41). In addition, mouse midbrain tissue expressing *Wnt-1* can cause ectopic induction of chicken *engrailed* when grafted into chick embryonic forebrain, consistent with the notion that *Wnt-1* may regulate *engrailed* in vertebrate species as well as in *Drosophila* (2, 33). It will be interesting to determine whether homologs of other components of the *wingless* signaling pathway, such as glycogen synthase kinase 3, the homolog of *zeste-white-3/shaggy* (69), are involved in cellular responses to *Wnt-1* in vertebrates.

In view of the apparent conservation in the response of plakoglobin and Armadillo to *Wnt-1/wingless* signals, our results in PC12 cells have potential implications for understanding the cellular mechanisms underlying segment polarity in *Drosophila* and suggest that the modulation of Armadillo by *wingless* may be accompanied by localized changes in the degree of cell-cell adhesion within the segment. Such differential adhesion within a broad stripe of cells in each segment might in turn affect the distribution of gap junctions, and so help establish gradients of small intracellular signaling molecules. Alternatively, a non-uniform distribution of adhesive junctions within the segment might itself provide patterning cues by causing local asymmetries in organization of the cytoskeletal network. The combination of *Drosophila* genetics and vertebrate cell biology now offers powerful tools with which to address these issues.

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