Identification and Characterization of a Novel Line of Drosophila Schneider S2 Cells That Respond to Wingless Signaling*

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Wingless (Wg) treatment of Drosophila wing disc clone 8 cells leads to Armadillo (Arm) protein elevation, and this effect has been used as the basis of in vitro assays for Wg protein. Previously analyzed stocks of Drosophila Schneider S2 cells could not respond to added Wg, because they lack the Wg receptor, Dfrizzled-2. However, we found that a line of S2 cells obtained from another source express Dfrizzled-2 and Dfrizzled-1. Thus, we designated this cell line as S2R+ (S2 receptor plus). S2R+ cells respond to addition of extracellular Wg by elevating Arm and DE-cadherin protein levels and by hyperphosphorylating Dsh, just as clone 8 cells do. Moreover, overexpression of Wg in S2R+, but not in S2 cells, induced the same changes in Dsh, Arm, and DE-cadherin proteins as induced in clone 8 cells, indicating that these events are common effects of Wg signaling, which occurs in cells expressing functional Wg receptors. In addition, unphosphorylated Dsh protein in S2 cells was phosphorylated as a consequence of expression of Dfrizzled-2 or mouse Frizzled-6, suggesting that basal structures common to various frizzled family proteins trigger this phosphorylation of Dsh. S2R+ cells are as sensitive to Wg as are clone 8 cells but can grow in simpler medium. Therefore, the S2R+ cell line is likely to prove highly useful for in vitro analyses of Wg signaling.

The Drosophila segment polarity gene wingless (wg, see Refs. 1–4) is a member of the Wnt gene family, which encodes a secreted glycoprotein involved in cell-cell signaling in a number of basic developmental processes in a wide range of animal phyla (5–8). Genetic screening of Drosophila for mutations in segmental patterning has revealed several genes acting sequentially in the Wg signal transduction pathway (9). These genes are dishevelled (dsh, see Refs. 10 and 11), zeste-white 3 (zw-3, see Refs. 12 and 13), and armadillo (arm, see Refs. 14–16), and the order in which they act in the pathway has been defined by genetic and molecular studies (17, 18). wg and dsh negatively regulate zw-3, which is a down-regulator of arm. Thus, the net effect of Wg signaling is the accumulation of a cytoplasmic pool of Arm protein.

The in vitro assay for Wg (19) using soluble Wg protein and the Drosophila imaginal disc cell line, clone 8 (20), has greatly contributed to biochemical analysis of the Wg signaling pathway (21–24). In particular, this system led to the identification of Drosophila frizzled 2 (Dfz2) as a receptor for Wg (24). For identification of Dfz2, Bhanot et al. (24) took advantage of the fact that Schneider S2 cells (25) lack Dfz2 protein and thus do not respond to soluble Wg and that transfection of this new member of the frizzled family (26) makes S2 cells sensitive to Wg. However, we found another line of S2 cells that does respond to Wg by elevating the Arm protein levels.

In this study, we characterized this novel S2 cell line biochemically, and we found that our cell line expresses Dfz2 and Dfz1 proteins, which function as Wg receptors. Therefore, we designated this line as S2R+ (S2 receptor plus). To identify the biochemical changes commonly induced by Wg signaling, we compared the Wg-induced changes in S2R+ and clone 8 cells, both of which express Dfz2 and Dfz1, with those in S2 cells, which express neither. These analyses revealed that Wg signaling induces a common set of biochemical changes as follows: hyperphosphorylation of Dsh, accumulation of Arm, and elevation of DE-cadherin mRNA and protein levels, in S2R+ and clone 8 cells, which express functional Wg receptors, but not in S2 cells, which lack them. We propose S2R+ as a novel cell line for Wg assays in vitro.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Culture—The Drosophila wing imaginal disc cell line clone 8 (20) and Drosophila Schneider S2 cells (25) were obtained from Dr. R. Nusse at Stanford University and cultured as described (19, 21). Another line of Drosophila Schneider S2 cells (later named as S2R+, which referred to S2 cells with Wg receptors) were obtained from Dr. Tadashi Miake at Mitsubishi-kagaku Institute for Life Sciences and cultured in the same manner as the S2 cells in our laboratory. According to Dr. Miake, the S2R+ cells were obtained directly from Dr. Schneider and stored frozen in his laboratory. To use a homogeneous cell population, a clone of S2R+ cell was isolated from the original S2R+ cells by limited dilution and used throughout these experiments. Wg treatment of clone 8, S2, and S2R+ cells was performed by incubating cells with soluble Wg protein secreted in conditioned medium of S2-HS-wg cells (S2 cells expressing Wg under the control of heat-shock promoter, see Ref. 27), details of which were described previously (19).

Expression Constructs and Transfection—Expression plasmids constructed with the pmK33 vector (28), which contains a hygromycin-resistant gene, were transfected into clone 8, S2, and S2R+ cells using the calcium phosphate method as described (19). pMK-Dfz2 (24), a Dfz2 expression plasmid constructed with pmK33, was obtained from Dr. J. Nathans (Johns Hopkins University). A plasmid expressing Wg (named pMK-Wg) was constructed by isolating a 1.9-kb BamHI-ClaI fragment containing the entire coding region of Wg from Wg cDNA (1) and inserting it into the EcoRV site of pmK33. A plasmid-expressing mouse frizzled 6 (Mfz6) protein, named pMK-Mfz6, was made by synthesizing a 2.7-kb double-stranded cDNA fragment containing the entire coding region of Mfz6 (26) by reverse transcriptase-mediated polymerase chain reaction (RT-PCR) and inserting it into the EcoRV site of pmK33. The expression of Dfz2, Mfz6, and wg mRNA in each transfectant was confirmed by Northern blotting with probes specific for each gene (data not shown). The pMK-Wg and the pMK-Mfz6 transfectants used in this study were mixtures of stable clones selected with hygromycin (200 µM). On the other hand, two independent stable clones of pMK-Dfz2 trans-
fectants were used. Wg overexpression was induced in pMK-Wg transfec-
tants by adding 0.5 mM CuSO₄, as described (19).

**Immunoblot Analysis and Antibodies**—Clone 8, S2R+, and S2 cells grown to 80% confluence were lysed in lysis buffer and used to prepare samples for SDS-polyacrylamide gel electrophoresis, as described (22). The samples were loaded on 7.2% SDS-polyacrylamide gels, and the gels were then Western-blotted (21). The antibodies used for Western analysis included the mouse monoclonal anti-Arm antibody N2–7A1 (29), the rat polyclonal anti-Dsh region I antibody (21), affinity purified rabbit anti-Wg IgG (30), the rat monoclonal anti-DE-cadherin antibody DCA-D2 (31), the rat monoclonal anti-Drosophila-a-catenin antibody DCA-A (13), and peroxidase-conjugated secondary antibodies against mouse IgG (Bio-Rad), rat IgG (Jackson ImmunoResearch, West Grove, PA) and rabbit IgG (Cappel, Durham, NC), and the antibodies were diluted as described (22). Blots were visualized with enhanced chemo-
luminescence reagent (ECL, Amersham, UK). The x-ray films were scanned using a densitometer (Shimadzu Corp. Kyoto, Japan).

**RNA Blots**—Total RNAs were extracted by the guanidinium/acid phenol method from an overnight collection of Drosophila embryos, clone 8, S2, and S2R+ cells, and stable transfectants of those cell lines made with pMK-33 or pMK-Wg. Total RNA (20 μg) was separated on 1% formaldehyde-agarose gels and Northern blotted as described (33). The probe for Dfz2 was the 2.6-kb BamHI fragment from pMK-Dfz2. The Dfz1 (34) probe was the 1.0-kb cDNA prepared by RT-PCR, the details of which are described below. The probes for DE-cadherin, Arm, and v-a-catenin were described previously (22).

**Polymerase Chain Reaction**—Poly(A)⁺ RNAs were selected from total RNAs by using Oligotex™-dT30 beads (Daichikagaku, Tokyo, Japan). Single-stranded cDNAs were synthesized using the SuperScript™ Pre-
amplification System for First Strand cDNA Synthesis (Life Technolo-
gies, Inc.). Dfz1 and Dfz2 cDNA fragments were amplified by polymer-
ase chain reaction using the single-stranded cDNAs thus synthesized and the following sets of primers: Dfz1 (an amino acids 1–326): 5’ primer, 5’-ATGGTGCGCTAAATGCTGATT-3’; 3’ primer, 5’-TGGGACAT-
CATCGAAGGCCGCCGAG-3’. Dfz2 (an amino acids 2–290): 5’ primer, 5’-AGCAACATGCTGAAGGTTGCT-3’; 3’ primer, 5’-ATCGAGGT-
TCATCTACAGTG-3’. A 2.1-kb cDNA fragment containing the whole coding region of Mfz6 was amplified by RT-PCR with the single-
stranded cDNA synthesized from mouse embryonic poly(A)⁺ RNA and the following set of primers: 5’ primer, 5’-TCAACTAGTCTGGCAA-
GATGGAAGA-3’; 3’ primer, 5’-TACACTAGTCTCCAGCCTGAAGT-
GCT-3’. The fidelity of the polymerase chain reactions was confirmed by DNA sequencing.

**Immunohistochemistry**—S2R+ cells transfected with pMK-Wg or pMK33 were seeded in 4-well chamber slides and treated with CuSO₄ for 24 h. They were fixed, permeabilized, and blocked (22). The samples were processed for indirect immunostaining with the mouse monoclonal anti-Arm antibody N2–7A1 followed by fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin (Dako, Denmark), with the rat monoclonal anti-DE-cadherin antibody DCA-D2 followed by fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin (Cappel, Durham, NC), or with the affinity purified rabbit anti-Wg IgG (30) followed by fluorescein isothiocyanate-conjugated goat anti-rabbit immu-

**RESULTS**

Identification of a Novel S2 Cell Line That Responds to the Wg Signal—During our experiments to establish S2 cells that express various mouse frizzled proteins, we found that a Schneider S2 cell line (later designated S2R+) obtained from Dr. T. Makie responds to soluble Wg protein produced by S2-

**Identification of a Novel S2 Cell Line That Responds to the Wg Signal**—During our experiments to establish S2 cells that express various mouse frizzled proteins, we found that a Schneider S2 cell line (later designated S2R+) obtained from Dr. T. Makie responds to soluble Wg protein produced by S2-HS-wg cells. Therefore, we obtained the S2 cell line that had been used for identification of Wg receptors (Dfz2) from Dr. R. Nusslein-Volhard, and compared this to two Schneider S2 cell lines biologically. In addition, we established S2 cells expressing Dfz2 protein (named Dfz2/S2) and mouse Fz6 protein (named Mfz6/ S2) by transfecting S2 cells with the pMK-Dfz2 and pMK-Mfz6 plasmids, respectively. We then compared the effects of soluble Wg treatment on Arm accumulation in clone 8, S2R+, S2, Dfz2/S2, and Mfz6/S2 cells (Fig. 1A). We thereby reproduced the published results (24) that clone 8 and Dfz2/S2, but not S2 cells, respond to Wg. In contrast to S2 cells, however, S2R+
cells did elevate Arm (especially the hypophosphorylated form) protein levels in response to soluble Wg protein. In addition, in agreement with the report that Mfz6 does not bind Wg (24), Mfz6/S2 cells did not respond to soluble Wg (Fig. 1A, right panel). Comparison of the Arm protein induction kinetics in clone 8 and S2R+ cells demonstrated that S2R+ cells showed slower onset of Arm accumulation (Fig. 1B). However, both cell lines exhibited marked elevation of Arm protein levels (Fig. 1B) which plateaued between 150 and 180 min (data not shown) after Wg treatment. In addition, comparison of the Wg dose-response curves of Arm protein accumulation in clone 8 and S2R+ cells. Cells were treated in the same way as in A except that conditioned medium (CM) from S2-HS-wg cells serially diluted (shown as X1, X9, X27, and X81) with conditioned medium from control S2 cells was used, in addition to the undiluted S2-HS-wg (shown as X1) or control S2 (shown as S2) conditioned medium.

**FIG. 1.** Comparison of the effects of Wg on Arm protein levels in several Drosophila cell lines. A, Wg induced accumulation of Arm protein in several Drosophila cell lines. Lysates of cells treated for 180 min with conditioned medium from S2-HS-wg (Wg treatment +) or control S2 (Wg treatment −) cells were subjected to Western blotting with anti-Arm antibody (upper panel). The Dfz2/S2 cell line is a clone of S2 cells transfected with pMK-Dfz2. Mfz6/S2 is a mixture of stable clones of S2 cells transfected with pMK-Mfz6. Because basal levels of Dfz2 expression are sufficient to make S2 cells Wg-responsive, and further induction of Dfz2 expression by CuSO₄ leads to a lower response to Wg, Dfz2/S2 cells not treated with CuSO₄ were used. Although CuSO₄ markedly induced Mfz6 mRNA expression, neither induced nor undiluted Mfz6/S2 responded to Wg. Thus, results from undiluted Mfz6/S2 cells are shown. The lower panel shows a β-actin antibody blot as a loading control. B, comparison of Arm protein induction kinetics in clone 8 and S2R+ cells. The time course of the change in the level of Arm protein in clone 8 and S2R+ cells was determined by immunoblotting. Proteins in lysates of cells harvested at various times after Wg treatment were resolved by SDS-polyacrylamide gel electrophoresis, and Arm protein levels were determined. The right-most lanes in both panels show the negative controls (clone 8 or S2R+ cells were treated for 180 min with the conditioned medium (CM) from S2 cells). C, comparison of the Wg dose-response curves of Arm protein accumulation in clone 8 and S2R+ cells. Cells were treated in the same way as in A except that conditioned medium (CM) from S2-HS-wg cells serially diluted (shown as X1, X9, X27, and X81) with conditioned medium from control S2 cells was used, in addition to the undiluted S2-HS-wg (shown as X1) or control S2 (shown as S2) conditioned medium.
lysates from S2R+ visualized by staining with Coomassie Brilliant Blue. SDS-polyacrylamide gel electrophoresis, and the protein bands were containing the same amount of protein (30 µg) were separated on a 7.2% SDS-polyacrylamide gel electrophoresis, and the protein bands were visualized by staining with Coomassie Brilliant Blue.

In contrast, clone 8 cells cannot be maintained in Schneider’s medium supplemented with 10% fetal calf serum (data not shown). Protein electrophoretic profiles of the lysates from S2R+, S2, and clone 8 cells. Aliquots of cell lysate (30 µl) containing the same amount of protein (30 µg) were separated on a 7.2% SDS-polyacrylamide gel electrophoresis, and the protein bands were visualized by staining with Coomassie Brilliant Blue.

Expression of Dfz1 and Dfz2 mRNAs in S2R+ Cells—Studies using Dfz2/S2 cells demonstrated that expression of Dfz2 protein could confer Wg responsiveness on the S2 cell line (see Ref. 24 and Fig. 1A). Furthermore, Dfz1 protein functioned as a Wg receptor in S2 cells (S2 cells expressing Dfz1 protein respond to soluble Wg protein and elevated Arm, see Ref. 24). These results suggested that the Wg responsiveness of the S2R+ cell line could be explained by the expression of Dfz2 or Dfz1 genes. Therefore, expression of Dfz2 and Dfz1 mRNA was examined in S2R+ cells. Northern blot hybridization with probes for Dfz1 and Dfz2 (Fig. 3, upper panels) and RT-PCR experiments with specific sets of primers for Dfz1 and Dfz2 (Fig. 3, lower panels) showed that both Dfz1 and Dfz2 mRNAs were expressed in S2R+ and clone 8 cells but not in S2 cells. Densitometric analyses of Northern blots showed that expression levels of Dfz2 and Dfz1 mRNAs in S2R+ cells were about 1/3 and 1/4 of those in clone 8 cells, respectively.

FIG. 2. Differences in morphology and protein compositions of S2R+, S2, and clone 8 cells. A, morphology of S2R+, S2, and clone 8 cells. Pictures of the subconfluent (upper panels) and fully confluent (lower panels) cultures are shown. B, protein electrophoretic profiles of lysates from S2R+, S2, and clone 8 cells. Aliquots of cell lysate (30 µl) containing the same amount of protein (30 µg) were separated on a 7.2% SDS-polyacrylamide gel electrophoresis, and the protein bands were visualized by staining with Coomassie Brilliant Blue.

Characterization of Dsh Protein in S2R+, S2, and Clone 8 Cells Expressing Dfz2 or Mfz6 Proteins—We have reported that stimulation of clone 8 cells with Wg-conditioned medium led to the increased phosphorylation of Dsh on serine/threonine residues (these hyperphosphorylated Dsh species migrate more slowly than unphosphorylated Dsh) and that this hyperphosphorylation of Dsh correlated with Arm accumulation (21). These results led to the hypothesis that this hyperphosphorylated form of Dsh is the active form. By using the two Wg-responsive cell lines, S2R+ and Dfz2/S2, we examined whether or not the Wg signaling induces hyperphosphorylation of Dsh and concomitant Arm elevation. Clone 8, S2R+, S2, Dfz2/S2, and Mfz6/S2 cells were treated with the conditioned media from either S2-RS-wg or S2 cells for 3 h, and each cell lysate was subjected to Western blotting with anti-Dsh antibody (Fig. 4). We confirmed that naive clone 8 cells display several bands of phosphorylated and unphosphorylated Dsh and that Wg treatment induces hyperphosphorylation of Dsh (increase of lower mobility bands with concomitant decrease of the highest mobility band) in clone 8 cells. The Dsh image from the longer exposure film (Fig. 4, middle panel) showed that a small amount of modified Dsh was also present in naive clone 8+ cells. In contrast, naive S2 cells and pMK-33-transfected S2 cells (data not shown) display a single band of unphosphorylated Dsh protein. Consistent with published results (23), expression of Dfz2 in S2 cells (even the basal level of Dfz2 expression in the absence of CuSO4) led to phosphorylation of Dsh, and further induction of Dfz2 expression by CuSO4 led to predominant modification of Dsh with an additional mobility shift, but this Dfz2 overexpression could not induce Arm accumulation by itself (data not shown). In addition, we found the following changes in Dsh for the first time in this study. First, expression of Mfz6 in S2 cells, like Dfz2, led to phosphorylation of Dsh, suggesting that basal structures common to all frizzled family proteins (such as the seven transmembrane segments) trigger basal phosphorylation of Dsh in some way. Second, Wg treatment had no effect on phosphorylation of Dsh in S2 cells. This is in agreement with the fact that S2 cells can neither receive the Wg signal nor induce Arm accumulation due to the lack of Wg receptors. In contrast, Wg treatment induced hyperphosphorylation of Dsh (Fig. 4, upper and middle panels) and concomitant Arm elevation (Fig. 1A) in S2R+ cells, indicating that the presence of...
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**Fig. 4.** Wg treatment induced modification of Dsh protein in clone 8, S2R+, and Dfz2/S2 cells but not in S2 or Mfz6/S2 cells. Lysates of cells treated for 180 min with conditioned medium from S2-HS-wg (Wg treatment +) or control S2 (Wg treatment −) cells were subjected to Western blotting with anti-Dsh antibody. Upper and middle panels show short and long exposures of the same immunoblot, respectively. As a control for loading, the blot was stripped and incubated with antibody against v-α-catenin (lower panel). Results from Dfz2/S2 or Mfz6/S2 cells not induced with CuSO4 are shown in this figure.

functional Wg receptors and the Wg-induced hyperphosphorylation of Dsh are strongly correlated. Third, similar to clone 8 cells, Wg treatment resulted in an increase in the levels of hyperphosphorylated forms of Dsh (the Wg-induced hyperphosphorylation of Dsh) in Dfz2/S2 cells. Therefore, Dfz2/S2 and clone 8 cells showed very similar Dsh phosphorylation profiles before and after Wg treatment (Fig. 4, upper panel). However, Wg treatment did not result in the Wg-induced hyperphosphorylation of Dsh (Fig. 4, upper panel) or Arm elevation (Fig. 1A) in Mfz6/S2 cells. This may be related to the reported inability of Mfz6 protein to bind to Wg (24). Altogether, expression of Dfz2 protein in S2 cells appeared to first promote basal phosphorylation of Dsh, and next, binding of Wg to Dfz2 further induced hyperphosphorylation of Dsh. The results from clone 8, S2R+, and Dfz2/S2 cells indicate that Wg-induced Dsh hyperphosphorylation is a common biochemical readout in cells expressing functional Wg receptors.

**Wg Overexpression Induces Arm and DE-cadherin Accumulation in S2R+ and Clone 8 Cells, but Not in S2 Cells—**Overexpression of the Wg protein in cells is another way to transmit the Wg signal into cells (19). In this case, the Wg proteins synthesized in a cell bind to the Dfz2 or Dfz1 proteins of that cell itself or neighboring cells and thereby stimulate the Wg pathway in an autocrine or paracrine way, respectively. Therefore, the effects of Wg overexpression on protein levels and modification status of Dsh, Arm, DE-cadherin, and v-α-catenin were examined in clone 8, S2R+, and S2 cells (Fig. 5A). Three cell lines were transfected with the Wg expression plasmid, pMK-Wg, or the negative control vector, pMK-33, and pools of stable transfectants were used. In addition, similar analyses were performed in S2R+ cells treated with soluble Wg for 24 h (Fig. 5A, right panels). In pMK-Wg-transfected clone 8 cells, addition of CuSO4 induced high levels of Wg expression, whereas very little Wg protein expression was detected in the absence of CuSO4. In pMK-Wg-transfected S2 and S2R+ cells, however, cells not induced with CuSO4 already expressed significant levels of Wg protein, and CuSO4 further elevated Wg expression. In clone 8 and S2R+ cells, which express Wg receptors, pMK-Wg transfectants showed elevated levels of Arm and DE-cadherin proteins compared with their pMK-33-transfected counterparts. In contrast, Wg overexpression had no effect on Arm and DE-cadherin protein levels in S2 cells, which lack Wg receptors (Fig. 5A). Despite their poor Wg expression, the pMK-Wg-transfected clone 8 cells not induced with CuSO4 also produced increased levels of Arm and DE-cadherin proteins. This suggests that a very small amount of Wg protein is sufficient to stimulate the Wg pathway in this system. Similar to the results presented in Fig. 4, Wg overexpression led to modification of Dsh protein in S2R+ and clone 8 cells but not in S2 cells. v-α-Catenin protein levels were not affected in any cell type except for the pMK-Wg-transfected S2R+ cells. Probably, in pMK-Wg-transfected S2R+ cells, very high levels of DE-cadherin protein stimulated cadherin adhesion complex formation, and v-α-catenin proteins were stabilized and thus accumulated in these cadherin adhesion complexes. Treatment of S2R+ cells with soluble Wg also elevated both Arm and DE-cadherin protein levels (Fig. 5A, right panels).

Next we examined the amount of Wg protein secreted in the culture medium of the S2 and S2R+ cells transfected with pMK-Wg (Fig. 5B). Induction with CuSO4 markedly increased the levels of secreted Wg protein in the culture medium of S2 and S2R+ transfectants. However, comparison of the Wg protein levels in cell lysate and in the conditioned medium indicated that less than 10% of the total Wg protein produced was secreted in the induced S2 transfectants. These results suggested that the paracrine mode of activation of the Wg pathway also occurred in S2R+ cells expressing Wg and probably in the clone 8 cell counterparts as well. On the other hand, Wg protein present either inside or outside of the S2 cells cannot transmit Wg signals into the S2 transfectants, because the Wg receptor is not present in these cells.

We have reported that Wg signaling caused elevated DE-cadherin mRNA levels in clone 8 cells (22). Therefore, the effects of Wg overexpression on DE-cadherin, Arm, and v-α-catenin mRNA levels were examined in S2R+ cells. Fig. 5C shows that, as in clone 8 cells, Wg overexpression in S2R+ cells resulted in elevated levels of DE-cadherin mRNA but not Arm and v-α-catenin mRNAs, further supporting the idea that DE-cadherin is a target for gene expression regulation by the Wg signaling in a variety of cells (22), whereas Arm and v-α-catenin protein elevations are caused by post-transcriptional regulation.

**Immunofluorescence Analysis of Wg, DE-cadherin, and Arm Protein Distribution in S2R+ Cells Expressing Wg—**To analyze the subcellular localizations of Wg, DE-cadherin, and Arm proteins, S2R+ cells transfected with pMK-Wg or pMK33 were stained with antibody against each protein. The speckled staining observed over the entire cell except for the nucleus (Fig. 6A) indicated that the Wg protein is located in structures related to the secretory pathway, such as the endoplasmic reticulum, Golgi, and secretory vesicles. In agreement with the Western blotting results presented in Fig. 5A, Wg expression resulted in markedly elevated DE-cadherin staining at the cell-cell junctions (Fig. 6C) and cytoplasmic Arm staining (Fig. 6E) in S2R+ cells. This cytoplasmic localization of Arm is consistent with the notion that Wg/Wnt signaling up-regulates the cytoplasmic Arm/β-catenin pools (16, 19, 21). These results showed that S2R+ cells exhibit the same biochemical changes, elevation of the cytoplasmic Arm pools, and DE-cadherin accumulation upon Wg overexpression as do clone 8 cells upon Wg overexpression (22).

**DISCUSSION**

In this study, we identified a novel S2 cell line that expresses Dfz2 and Dfz1 proteins and responds to added Wg protein by elevating the levels of Arm protein. Judging from cell morphology, pattern of growth, and nutritional requirements, this cell line is of S2 type, and was thus designated S2R+. Because the origin of S2R+ cells is poorly documented, we do not know why these two different S2 cell lines appeared. However, the results of this study give some indication of the variation in the behavior of different S2 cell lines. Therefore, if experimental results using S2 cells differ among laboratories, these differences...
should be evaluated with regard to whether the S2 cells used in different laboratories have different characteristics. By using two novel Wg-responsive cell lines, S2R^1 and Dfz2/S2, we tried to evaluate whether or not the effects of Wg signaling that we previously discovered with clone 8 cells, i.e., hyperphosphorylation of Dsh (21) and DE-cadherin induction (22), are generally associated with Wg signaling. As described above, we have shown that these biochemical changes were induced by Wg overexpression or extracellular soluble Wg in S2 cells that lack Wg receptors.

Fig. 3 shows that Dfz2 and Dfz1 mRNAs were expressed in S2R^+ and clone 8 cells but not in S2 cells. Following the procedures described by Bhanot et al. (24), we tried to show that Wg protein binds to the surface of S2R^+ and clone 8 cells but not to that of S2 cells. Whereas the Dfz2/S2 cells showed strong Wg binding, none of these cells showed any significant Wg binding (data not shown), suggesting that relatively little Dfz2 and Dfz1 protein is sufficient to transmit the extracellular Wg signal into cells.

We have shown that expression of Dfz2 or Mfz6 induces phosphorylation of Dsh in S2 cells and that a small proportion of Dsh protein is phosphorylated in S2R^+ and clone 8 cells. These results suggest that expression of frizzled family proteins induces the basal phosphorylation of Dsh. In this regard, Willert et al. (23) have reported that casein kinase 2 (CK2), which binds to the PDZ domain of Dsh, is the major kinase responsible for phosphorylation of Dsh upon Dfz2 overexpression in S2 cells. Therefore, CK2 may take part in the basal phosphorylation of Dsh in Dfz2/S2, Mfz6/S2, clone 8 and S2R^+ cells not stimulated with soluble Wg (Fig. 4). In addition, Axelrod et al. (35) have shown that Dfz1 overexpression led to translocation of Dsh from cytoplasm to plasma membrane. Moreover, Yang-Snyder et al. (36) have reported that overexpression of rat frizzled-1 results in recruitment of Xwnt-8 and XDsh to the plasma membrane in Xenopus embryos. Thus, it is possible that Dfz2 or Mfz6 expression induces translocation of at least a part of Dsh to the plasma membrane in S2 cells and that this Dsh translocation in some way stimulates Dsh phosphorylation by CK2. However, it is not clear whether CK2 also participates in Wg-induced hyperphosphorylation of Dsh or whether other kinase(s) were activated by the binding of Wg to Dfz2 in clone 8, S2R^+, and Dfz2/S2 cells and induced the hyperphosphorylation. In view of the reports indicating association (probably indirect) between frizzled family proteins and...
Dsh and the binding of Dsh to CK2, it is attractive to speculate that Wg binding induces aggregation of Dfz2 receptors, which, in turn, brings the Dsh-CK2 or other kinase complexes close together, and this aggregation stimulates the Dsh phosphorylation by CK2 or other kinases in these Dsh-kinase complexes. This could explain how Wg induces Dsh hyperphosphorylation in clone 8, S2R<sub>1</sub>, and Dfz2/S2 cells. However, it is noteworthy that Dfz2 overexpression led to marked phosphorylation of Dsh, but not to elevation of Arm, in S2 cells (23), indicating that phosphorylation of Dsh, at least by Dfz2 overexpression, cannot activate the Wg signaling pathway by itself. Clearly, further detailed experiments are necessary to evaluate the function of Dsh phosphorylation in Wg signaling.

In vitro analyses of Wg signaling with clone 8 cells (19) have made great contributions to the biochemical analysis of the Wg pathway. However, clone 8 cells absolutely require fly extract and insulin for growth (20). Unfortunately, preparation of fly extract is not easy for biochemists who are not working with flies. In contrast, the S2R<sub>1</sub> cells grow well in Schneider's medium supplemented only with 10% fetal calf serum and are as sensitive as clone 8 cells to the added Wg (Fig. 1). Therefore, we recommend S2R<sub>1</sub> as a new cell line for analyses of Wg signaling in vitro.

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REFERENCES
1. Rijsewijk, F., Schuermann, M., Wagenaar, E., Parren, P., Weigel, D., and Nusse, R. (1987) Cell 50, 649–657
2. Klingensmith, J., and Nusse, R. (1994) Dev. Biol. 166, 396–414
3. Peifer, M., and Bejoovec, A. (1992) Trends Genet. 8, 243–249
4. Perrimon, N. (1994) Cell 76, 781–784
5. Nusse, R., and Varmus, H. E. (1992) Cell 69, 1073–1087
6. Cadigan, K., and Nusse, R. (1997) Genes Dev. 11, 3268–3305
7. Parr, B. A., and McMahon, A. P. (1994) Curr. Opin. Genet. & Dev. 4, 523–528
8. Moon, R. T. (1995) BioEssays 15, 91–97
9. Siegfried, E., and Perrimon, N. (1994) BioEssays 16, 395–404
10. Klingensmith, J., Nusse, R., and Perrimon, N. (1994) Genes Dev. 8, 118–130
11. Thiesen, H., Purrell, J., Bennett, M., Kansagara, D., Syed, A., and Marsh, J. L. (1994) Development 120, 347–360
12. Siegfried, E., Perkins, L., Capaci, T., and Perrimon, N. (1990) Nature 345, 825–829
13. Siegfried, E., Chou, T. B., and Perrimon, N. (1992) Cell 71, 1167–1179
14. Riggelman, B., Wieschaus, E., and Schedl, P. (1989) Genes Dev. 3, 96–113
15. Riggelman, B., Schedl, P., and Wieschaus, E. (1990) Cell 63, 549–560
16. Peifer, M., Sweeton, D., Case, M., and Wieschaus, E. (1994) Development 120, 369–380
17. Noordermeer, J., Klingensmith, J., Perrimon, N., and Nusse, R. (1994) Nature 367, 80–83
18. Siegfried, E., Wilder, E., and Perrimon, N. (1994) Nature 367, 76–80
19. van Leeuwen, F., Harryman Samos, C., and Nusse, R. (1994) Nature 368, 342–344
20. Peel, D. J. and Milner, M. J. (1992) Dev. Biol. 151, 120–123
21. Yanagawa, S., Lee, J.-S., Haruna, T., Oda, H., Uemura, T., Takeichi, M., and Ishimoto, A. (1997) J. Biol. Chem. 272, 25243–25251

FIG. 6. Indirect immunofluorescence analyses showing the distribution of Wg, DE-cadherin, and Arm proteins in S2R+ cells transfected with pMK-Wg. Confocal microscopic images of the pMK-Wg (A, C, and E) and pMK33 (B, D, and F) transfectants induced with CuSO<sub>4</sub> for 24 h are shown. The induced pMK-Wg or pMK33 transfectants were stained with anti-Wg polyclonal antibody (A and B), anti-DE-cadherin monoclonal antibody (C and D), or anti-Arm monoclonal antibody (E and F), respectively. Bars in the panels indicate 10 μm.
23. Willert, K., Brink, M., Wodarz, A., Varma, H. E., and Nusse, R. (1997) *EMBO J.* **16**, 3089–3096
24. Bhanot, P., Brink, M., Harryman Samos, C., Hsieh, J.-C., Wang, Y., Macke, J. P., Andrew, D., Nathans, J., and Nusse, R. (1996) *Nature* **382**, 225–230
25. Schneider, I. (1972) *J. Embryol. Exp. Morphol.* **27**, 353–365
26. Wang, Y., Macke, J. P., Abella, B. S., Andreasson, K., Worley, P., Gilbert, D. J., Copeland, N. G., Jenkins, N. A., and Nathans, J. (1996) *J. Biol. Chem.* **271**, 4468–4476
27. Cumberledge, S., and Krasnow, M. A. (1993) *Nature* **363**, 549–552
28. Koelle, M. R., Talbot, W. S., Segraves, W. A., Bender, M. T., Cherbas, P., and Hogness, D. S. (1991) *Cell* **67**, 59–77
29. Peifer, M. (1993) *J. Cell Sci.* **105**, 993–1000
30. van den Heuvel, M., Nusse, R., Johnston, P., and Lawrence, P. A. (1989) *Cell* **59**, 739–749
31. Oda, H., Uemura, T., Harada, Y., Iwai, Y., and Takeichi, M. (1994) *Dev. Biol.* **165**, 716–726
32. Oda, H., Uemura, T., Shioi, K., Nakajima, A., Tsukita, S., and Takeichi, M. (1993) *J. Cell Biol.* **121**, 1133–1140
33. Yanagawa, S., Kakinuma, K., Tanaka, H., Murakami, A., Nakagawa, Y., Kuo, Y., Yamada, Y., Hashi, H., Kurihara, K., Masuda, T., and Ishimoto, A. (1993) *J. Virol.* **67**, 112–118
34. Vinson, C. R., Conover, S., and Adler, P. (1989) *Nature* **338**, 263–264
35. Axelrod, J. D., Miller, J. R., Shulman, J. M., Moon, R. T., Perrimon, N. (1998) *Genes Dev.* **12**, 2610–2622
36. Yang-Snyder, J., Miller, J. R., Brown, J. D., Lai, C.-J., and Moon, R. T. (1996) *Curr. Biol.* **6**, 1302–1306