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Wnt Family Proteins Are Secreted and Associated with the Cell Surface

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Members of the *Wnt* gene family are proposed to function in both normal development and differentiation as well as in mammary tumorigenesis. To understand the function of *Wnt* proteins in these two processes, we present here a biochemical characterization of seven *Wnt* family members. For these studies, AtT-20 cells, a neuroendocrine cell line previously shown to efficiently process and secrete *Wnt*-1, was transfected with expression vectors encoding *Wnt* family members. All of the newly characterized *Wnt* proteins are glycosylated, secreted proteins that are tightly associated with the cell surface or extracellular matrix. We have also identified native *Wnt* proteins in retinoic acid-treated P19 embryonal carcinoma cells, and they exhibit the same biochemical characteristics as the recombinant proteins. These data suggest that *Wnt* family members function in cell to cell signaling in a fashion similar to *Wnt*-1.

INTRODUCTION

The *Wnt* gene family is representative of a growing class of multifunctional factors that play a role in both tumorigenesis and patterning events required for normal differentiation and development (Nusse and Varmus, 1992). The best-studied member of the *Wnt* family is *Wnt*-1, which is transcriptionally activated upon mouse mammary tumor virus (MMTV) integration and subsequently contributes to mammary tumorigenesis (Nusse and Varmus, 1982; Brown *et al.*, 1986; Rijsewijk *et al.*, 1987b; Tsukamoto *et al.*, 1988). Although *Wnt*-1 can function in mammary transformation, it is not normally expressed in the mammary gland but is restricted to male germ cells in the adult mouse and the developing neural tube of midgestation mouse embryos (Shackleford and Varmus, 1987; Wilkinson *et al.*, 1987). Analysis of mice lacking a functional *Wnt*-1 gene has demonstrated that *Wnt*-1 plays an instrumental role in neural development (McMahon and Bradley, 1990; Thomas and Capecchi, 1990; Thomas *et al.*, 1991).

Wnt-1 is one member of a gene family whose additional members were isolated either as a target for MMTV insertion (*Wnt*-3, *Wnt*-3A was subsequently isolated by homology to *Wnt*-3), fortuitously from a chromosomal walk directed around the cystic fibrosis gene (*Wnt*-2), or from mouse embryo RNA using the polymerase chain reaction (*Wnt*-4, -5A, -5B, -6, -7A, and -7B) (Wainwright *et al.*, 1988; Gavin *et al.*, 1990; Roelink *et al.*, 1990). There are now at least 10 known members of the *Wnt* family in the mouse; all of which are expressed during development, many in the developing nervous system with some expressed in adult brain as well (Gavin *et al.*, 1990). In addition, five members of the Wnt family are expressed in the normal mammary gland in the mouse and are differentially regulated during pregnancy and lactation (Gavin and McMahon, 1992). This family has been remarkably well conserved throughout evolution, with homologues present in both invertebrates and vertebrates (McMahon, 1992; Sidow, 1992). In addition to the predicted amino acid sequence similarities among family members, a role in cell signaling has also been documented for several *Wnt* family members. For example, *wingless*, the *Drosophila* homologue of *Wnt*-1, is necessary for proper segmental patterning of the embryo and is proposed to function locally via cell-cell interactions (Rijsewijk *et al.*, 1987a; Gonzalez *et al.*, 1991). Ectopic expression of members of the *Wnt* family in *Xenopus* leads to duplication of dorsal axial structures (*Xenopus* *Wnt*-3A and -8 and mouse *Wnt*-1) and can also affect

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gap junctional communication during development (*XWnt*-8 and *Wnt*-1) (Christian *et al.*, 1991; Olson *et al.*, 1991; Wolda *et al.*, 1993). In addition, injection of mouse *Wnt*-1 and *Xenopus Wnt*-8 mRNA into *Xenopus* embryos can rescue axis-deficient, UV-irradiated embryos (Smith and Harland, 1991; Sokol *et al.*, 1991).

In spite of similarities in sequence, overlap in expression patterns, and a putative role in signaling, there are apparent differences in function between *Wnt* family members that might indicate biochemical differences. For example, not all *Wnt* proteins are able to elicit partial transformation of mammary epithelial cells (Wong *et al.*, unpublished data; Olson and Papkoff, unpublished data), or cause axis duplication or increased gap junctional communication in *Xenopus* (Olson *et al.*, 1991; Wolda *et al.*, 1993). Thus a complete understanding of the roles these genes play in growth control and development will be facilitated by analysis of the biochemical characteristics of the encoded proteins. The predicted amino acid sequences of the *Wnt* genes suggest that they are cysteine-rich proteins, all containing a putative signal sequence and N-linked glycosylation sites (Gavin *et al.*, 1990). Antibodies necessary for a biochemical analysis of *Wnt* proteins have previously only been available for *Wnt*-1 and -2. Analysis of native *Wnt*-1 protein has proven to be difficult because very few cell lines express this protein and those that do express very little, although native *Wnt*-1 has been characterized recently in P19 cells (Papkoff, 1993). However, studies of recombinant *Wnt*-1 and -2 proteins, carried out in cell lines transfected with high level expression vectors, indicates that these proteins are glycosylated, secreted, and associated with the cell surface and/or extracellular matrix (Bradley and Brown, 1990; Papkoff and Schryver, 1990; Blasband *et al.*, 1992), possibly through association with a proteoglycan (Hinck *et al.*, unpublished data). It is presumed that *Wnt* proteins function through interaction with an extracellular receptor, perhaps on an adjacent cell, although no receptor has been identified at this point.

As a first step toward investigating the function of the newly identified *Wnt* proteins in the mouse and to compare them to the previously characterized *Wnt*-1 and -2 proteins, we have analyzed the biochemical properties of seven new *Wnt* family members in transfected cells. We find that, similar to *Wnt*-1 and -2, the other family members are also glycosylated, secreted proteins that interact with the cell surface. In addition we have characterized some of the native *Wnt* proteins that we find exhibit properties similar to the recombinant species.

MATERIALS AND METHODS

Antibodies

Antibodies were generated from peptides chosen from nonoverlapping regions of the *Wnt* sequences as indicated: *Wnt*-3A, residues 281–296, CEPNPETGSFGTRDRT (this sequence overlaps with *Wnt*-3); *Wnt*-4, residues 258–271, RNAQFKPHTDEDLVC; *Wnt*-5A, residues 341–354, RGYDQFKTVQTERC; *Wnt*-5B, residues 332–346, CGRGYDRFKSVQVER; *Wnt*-6, residues 253–267, CFHGASRVMGTNDGKA; *Wnt*-7A, residues 133–148, CDKEKQGQYHWDEGWK; *Wnt*-7B, residues 255–268, KQLRSYQKPMETDLC. Peptides were obtained from either Multiple Peptide Systems (San Diego, CA) or Applied Biosystems (Foster City, CA, kindly provided by P. Hoeprich), coupled to Keyhole limpet hemocyanin via a terminal cysteine residue, and injected into rabbits. Positive sera were identified by testing for immunoreactivity to the immunizing peptide in an enzyme-linked immunoabsorption assay.

Vectors

Full-length *Wnt* cDNAs were cloned into Bluescript vectors (Stratagene, La Jolla, CA) for in vitro transcription and translation (some were generous gifts from T. Dale, ICR, Sutton, England). The *Wnt* expression vectors used to transfect the AtT-20 cell line were constructed by a modification of pRSVint-1 (Papkoff and Schryver, 1990), in which *int*-1 (currently called *Wnt*-1) cDNA sequences located downstream of the promoter contained within the RSV long terminal repeat were replaced with the various *Wnt* cDNA sequences (Gavin *et al.*, 1990). pRSVneo, supplied by B. Schryver (Syntex Research, Palo Alto, CA), contains the neomycin coding sequences from pSV2neo (Southern and Berg, 1982) cloned into pRSVint-1 replacing the *int*-1 sequences.

Cell Lines

AtT-20 cells (provided by R. Kelly, University of California, San Francisco) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Cells were cotransfected with pRSV-*Wnt* and pRSVneo vectors using lipofection as previously described (Felgner *et al.*, 1987). Cells were initially selected using growth media containing 750 μg/ml geneticin (G418; GIBCO, Grand Island, NY) until no cells remained on the control dishes. Transfectants were then switched to medium containing 125 μg/ml G418, and individual clones were screened for *Wnt* expression by immunoprecipitation with anti-peptide antibodies. For each *Wnt* family member, an individual clone was selected for the experiments shown.

P19 cells (P19S18) were cultured as described in Rudnicki and McBurney (1987). Cells were differentiated as monolayers in 5×10^{-7} M retinoic acid (RA). Cells were cultured for 2 d in RA followed by 1 d without RA before harvesting.

In Vitro Transcription and Translation

Bluescript vectors containing *Wnt* cDNAs were linearized with the appropriate restriction enzymes and transcribed according to the supplier's specifications (Promega, Madison, WI). The resulting in vitro transcribed RNA was translated in a reticulocyte lysate (Promega) in the presence of ^{35}S-cysteine. Aliquots of each reaction were either analyzed directly on a sodium dodecyl sulfate (SDS)-12.5% polyacrylamide gel (1 μl of the reaction) or immunoprecipitated with the appropriate anti-peptide antibody (5 μl of the reaction) followed by SDS polyacrylamide gel electrophoresis.

Metabolic Labeling, Immunoprecipitation and Gel Electrophoresis

For the continuous metabolic labeling experiments shown in Figure 2 and for the pulse-chase analysis shown in Figure 4, duplicate 6-cm-diameter dishes of *Wnt*-expressing cells were incubated in 2 ml DMEM lacking cysteine and methionine (label medium) for 20 min at 37°C. For a continuous label, the cells were then incubated in label medium (1.5 ml) containing 0.15 mCi/ml ^{35}S-cysteine for 3 h in the presence or absence of 1 mM suramin (CB Chemicals, Oxford, CT) or for 10 min in the absence of suramin. The cells were extracted with 1 ml RIPA buffer (0.01 M tris(hydroxymethyl) aminomethane pH 7.0,

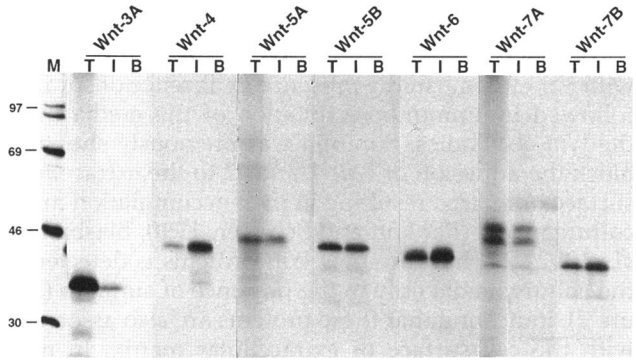

Figure 1. In vitro translation and immunoprecipitation of *Wnt* proteins. cDNAs for the indicated *Wnt* proteins were transcribed and translated in vitro, and aliquots of the reaction were analyzed on an SDS-12.5% polyacrylamide gel. Shown in lane T is the translation product (1 μl of the reaction) before immunoprecipitation. Lane I is an immunoprecipitate of the translation product (5 μl of the reaction) with the appropriate anti-peptide antibody. Lane B is an immunoprecipitate (5 μl of the reaction) blocked by incubation with the cognate peptide. Lane M shows the ^{14}C-labeled rainbow markers (Amersham) that were used to determine the molecular weight of each protein.

0.15 M NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS) on ice for 30 min. Both cell extracts and media were centrifuged for 15 min at 4°C. For the pulse-chase experiments, cells were incubated in label medium containing 0.2 mCi/ml ^{35}S-cysteine for 30 min (pulse). After the pulse label, cells were washed once with growth medium containing additional cysteine and methionine at 0.3 mM (chase medium) and then incubated in 1.5 ml chase medium containing 1 mM suramin. Both cells and media were harvested as above at 0, 0.5, 2.5, 6, or 18 h of chase time. To label cells in the presence of tunicamycin, 6-cm-diameter dishes were first incubated in growth medium containing 60 μg/ml tunicamycin (CalBiochem, San Diego, CA) for 30 min at 37°C. Dishes were then switched to label medium containing 0.15 mCi/ml ^{35}S-cysteine with 34 μg/ml tunicamycin for 2 h. Cells were harvested as described above. For all experiments one-third of each sample was immunoprecipitated using 3–5 μl of anti-peptide antibody directed against *Wnt* protein. Samples were immunoprecipitated by mixing cell extract or media, 3–5 μl antibody, and 50 μl of a 20% slurry of protein-A sepharose (Pharmacia, Piscataway, NJ) and rotating for 3–4 h at 4°C. For the blocked immunoprecipitates the antibody was preincubated with 2.5 μg of immunizing peptide. The antibody-protein A complexes were pelleted and washed three times with 0.5 ml RIPA buffer. The washed pellets were resuspended in 40 μl 1× Laemmli sample buffer, boiled 5 min, and analyzed on a SDS-12.5% polyacrylamide gel that was subsequently fluorographed with Amplify (Amersham, Arlington Heights, IL) according to the supplier's instructions.

To detect native *Wnt* proteins, 10-cm dishes of RA-treated P19 cells were labeled with 0.25 mCi/ml ^{35}S-cysteine (4 ml total) for 5 h in the presence of suramin. Eight hundred microliters of media was immunoprecipitated and analyzed as described above.

RESULTS

Characterization of Antibodies and In Vitro Translation Products

To generate anti-peptide antibodies for identification and characterization of new *Wnt* family members, peptides were chosen from the predicted amino acid sequences of *Wnt*-3/3A, -4, -5A, -5B, -6, -7A, and -7B that were unique, thus providing antibodies specific for each *Wnt* protein that would not cross-react with the others. To test the antibodies and to obtain a first approximation of the size of the *Wnt* proteins, we obtained full-length cDNA clones to each of the *Wnt* genes (except *Wnt*-3A that is lacking the first 50 amino acids), translated them in vitro, and immunoprecipitated the translation products using the specific antibodies. As shown in Figure 1, each antibody immunoprecipitated the appropriate in vitro translation product, and the immunoprecipitation was blocked by preincubation of the antibody with the cognate peptide. In addition, the sizes of the translation products are close to the predicted molecular weights on the basis of the deduced amino acid sequence (from Gavin *et al.*, 1990) (Table 1).

Identification of Wnt Proteins in Transfected Cells

On the basis of the previous characterizations of *Wnt*-1 and -2 proteins (Papkoff and Schyver, 1990; Blasband *et al.*, 1992), the most productive approach to initially identify and characterize *Wnt* proteins is to generate cell lines that express high levels of the recombinant protein. We transfected a murine neuroendocrine cell line, AtT-

Table 1. Sizes of Wnt proteins in AtT-20 cells

Wnt	Predicted size (kDa)[a]	IVT[b]	In vivo	Tunicamycin	Secreted
3A	39.3	33	60.5, 44.5, 41.5	55, 39	60.5*, 44.5*
4	39	41	46, 43	40	46*, 43'
5A	42.1	43	49, 47	41	49*, 47*
5B	41.6	41.5	49, 46.5	40	49*
6	39.5	42	46, 43.5, 40.5	40.5	46*
7A	38.9	42	47.5, 46	40.5	47.5*
7B	39.3	40.5	65, 46, 43.5, 41	39	46*

[a] Gavin *et al.*, 1990.

[b] In vitro translation.

[c] Secreted protein is slightly smaller than species detected in cell extracts.

20, that has previously been shown to efficiently process and secrete *Wnt*-1 protein (Papkoff and Schyver, 1990). Cells were cotransfected with pRSVneo and pRSV-*Wnt* expression vectors by lipofection. Individual clones were selected in G418 and screened for *Wnt* protein expression by immunoprecipitation with the appropriate anti-peptide antibody. A single positive clone for each *Wnt* protein was selected for further analysis. To identify *Wnt* proteins in the cells, each clone was metabolically labeled with ^{35}S-cysteine, and cell extracts were immunoprecipitated with the specific *Wnt* antibody. As seen in Figure 2, multiple protein species were detected in extracts of each cell line (lane I) that were blocked by preincubation of antibody with the cognate peptide (lane B). In most cases all of the bands detected in cell extracts were larger than the in vitro translation products (Table 1), presumably because of intracellular processing (see below). Although most of the proteins are 40–50 kDa, within the predicted size range of *Wnt* proteins, two cell lines expressing *Wnt*-3A and -7B contain in addition higher molecular weight species (60.5 and 65 kDa, respectively) whose immunoprecipitation is blocked by peptide. These proteins are likely to be cross-reacting cellular proteins and not *Wnt*-associated proteins, because they were present in multiple transfected clones generated with each of these two *Wnt* expression vectors and are immunoprecipitated even under highly denaturing conditions (Smolich and Papkoff, unpublished data). The nature of these protein species is currently under further investigation.

The prediction of a signal sequence for all of these new *Wnt* proteins (Gavin *et al.*, 1990) as well as the homology to *Wnt*-1 and -2, which have been characterized previously, suggests the possibility that the new *Wnt* proteins may be secreted. To determine this, *Wnt*-expressing AtT-20 cell lines were metabolically labeled with ^{35}S-cysteine in the presence or absence of suramin followed by immunoprecipitation of the media using the *Wnt* antibodies. Suramin was previously shown to block the adhesion of *Wnt*-1 and -2 to the extracellular surface or matrix, resulting in their accumulation in the culture media (Papkoff and Schyver, 1990; Blasband *et al.*, 1992). Each of the new *Wnt* proteins is detected in the culture media only in the presence of suramin (Figure 2), indicating that these proteins are also associated with the cell surface or extracellular matrix. In most cases only the largest form of the protein is detected in the media, suggesting that this is the fully processed mature form. In addition we noticed a slight size shift between the intracellular and secreted forms, possibly because of carbohydrate processing in the secretory pathway similar to that observed for *Wnt*-1 (Papkoff and Schryver, 1990). Interestingly, the 60.5-kDa protein recognized by the *Wnt*-3A antibody in extracts of the *Wnt*-3A-expressing cell line is also detected in medium only in the presence of suramin suggesting that it is also secreted. The 65-kDa protein immunoprecipitated by the *Wnt*-7B antibody, however, does not appear to be secreted, because it is not released into the medium with suramin treatment.

Wnt Proteins Are Glycosylated

The predicted amino acid sequences of each of the *Wnt* proteins indicate possible sites for N-linked glycosylation. To determine if the multiple bands present in the labeled immunoprecipitates are glycosylation products, *Wnt*-expressing cell lines were labeled for 3 h in the

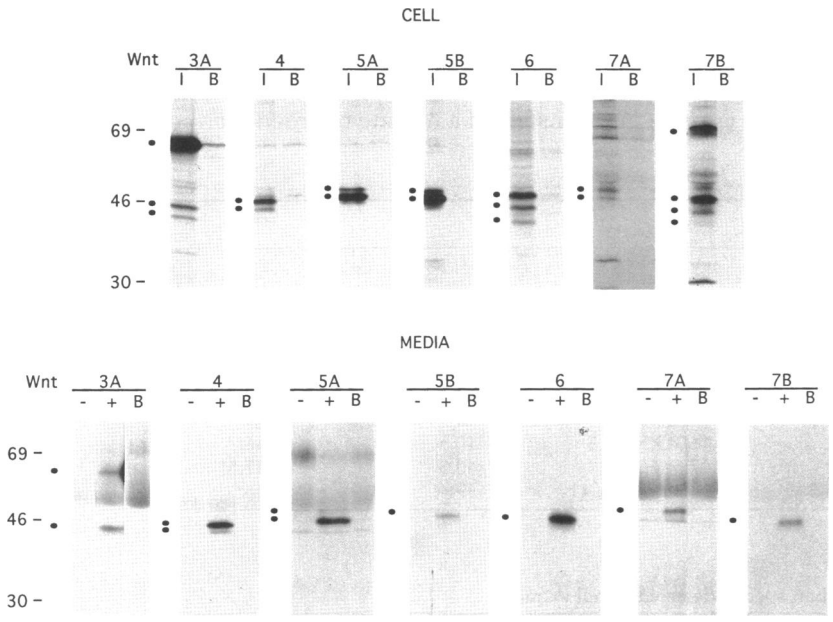

Figure 2. Identification of *Wnt* proteins in transfected AtT-20 cells. Cell lines transfected with expression vectors for the indicated *Wnt* proteins were metabolically labeled with ^{35}S-cysteine for 3 h, either in the presence or absence of suramin, and one-third of the total cell extract and media was immunoprecipitated with the appropriate anti-peptide antibody. For the cell extracts, cells labeled without suramin were analyzed. The immunoprecipitates were analyzed on an SDS-12.5% polyacrylamide gel. Lane I shows the immunoprecipitate, and lane B is an immunoprecipitate blocked with cognate peptide. The large dots indicate each protein species reproducibly immunoprecipitated and blocked with cognate peptide. For the media samples, lanes are: –, immunoprecipitate from media of cells labeled in the absence of suramin; +, immunoprecipitate from media of cells labeled in the presence of suramin; B, blocked immunoprecipitate from media of cells labeled in the presence of suramin. The large dots indicate the secreted proteins. The sizes of the ^{14}C-labeled rainbow markers are indicated.

presence of tunicamycin, which inhibits N-linked glycosylation. For comparison cells were labeled without tunicamycin for either 3 h (Figure 3, lane L; this is the label time used in Figure 2) or for 10 min (Figure 3, lane S) to detect early events in *Wnt* processing. As can be seen in Figure 3, all of the *Wnt* proteins appear to be glycosylated, because treatment with tunicamycin results in the appearance of a single smaller molecular weight protein (lane T, marked with an *). For each *Wnt*-expressing cell line the protein detected upon tunicamycin treatment is slightly smaller than the in vitro translation product (see Table 1 for size comparisons), presumably because of cotranslational cleavage of the signal peptide sequence in the transfected cell lines. The 60.5-kDa protein present in the *Wnt*-3A-expressing cell line is also glycosylated because it is reduced in size by tunicamycin treatment. The effect of tunicamycin treatment on the 65-kDa protein present in the *Wnt*-7B-expressing cell line is less clear; however, after analyzing the data from several experiments the 65-kDa protein appears to be unaffected by tunicamycin treatment and therefore is unglycosylated.

Treatment of the *Wnt*-expressing cell lines with tunicamycin revealed differences in the kinetics of glycosylation within the protein family. In the case of *Wnt*-6, the size of the tunicamycin product appears to correspond to the smallest protein present in either the 3-h or 10-min labeled cell extracts, confirming that this is likely to represent the protein backbone lacking the signal sequence. For each of the other *Wnt* proteins examined, the tunicamycin product is smaller than any protein specifically detected in untreated extracts after any label time. This suggests that whereas the tunicamycin product likely represents the unmodified protein backbone, the glycosylation of these proteins occurs very quickly and efficiently, such that after only a 10-min labeling period the initial modifications have already taken place, and the protein backbone is no longer detectable.

Wnt Proteins Are Efficiently Processed and Secreted

To examine the kinetics of processing and secretion of the *Wnt* proteins, pulse-chase experiments were performed with the *Wnt*-expressing cell lines. Cells were pulse-labeled for 30 min with ^{35}S-cysteine followed by a chase in the absence of label for 0.5, 2.5, 6, or 18 h with suramin included in the chase media. At each time point, both cell extracts and media were immunoprecipitated with the appropriate *Wnt* antibody as shown in Figure 4. As indicated above, the pattern of *Wnt* bands immunoprecipitated from cell extracts after a 30-min pulse label is the same as that seen after just a 10-min label. Thus at the 0 time point (harvested immediately after the 30-min pulse), with the exception of *Wnt*-6, the fully processed forms are already apparent, and the lowest molecular weight precursor, corresponding to the peptide backbone, cannot be detected. By 2.5–6 h all of the *Wnt* proteins are efficiently converted to the most mature form, similar to what has previously been observed for *Wnt*-1 in AtT-20 cells (Papkoff and Schryver, 1990).

In immunoprecipitates made from the labeled media, no *Wnt* proteins are detected until 2.5–6 h into the chase period (Figure 4), and only the higher molecular weight forms are detected, as seen previously in the media from a continuous label in the presence of suramin (Figure 2). The *Wnt* proteins continue to accumulate in the chase medium for ≥18 h, similar to what has been observed for *Wnt*-1 (Papkoff and Schryver, 1990).

Identification of Native Wnt Proteins

Very few cell lines have been identified that endogenously express *Wnt* proteins, and those that do express them at such a low level that characterization of these proteins is technically difficult. However, given the information gathered from the preceeding characterization of the recombinant proteins, we attempted to identify native *Wnt* proteins in P19 cells, where native *Wnt*-1 protein has been identified and characterized (St. Ar-

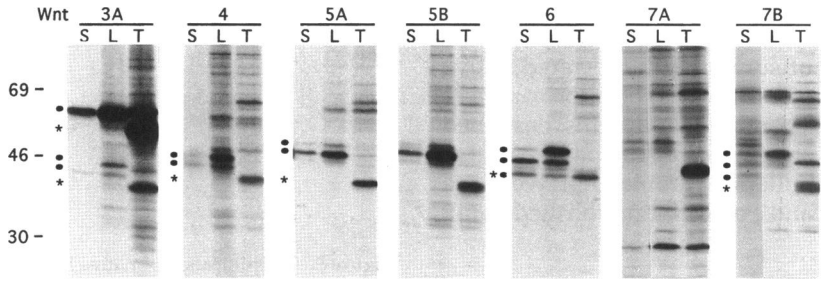

Figure 3. Immunoprecipitation of *Wnt* proteins from tunicamycin-treated cells. Replicate dishes of cells expressing the indicated *Wnt* proteins were metabolically labeled with ^{35}S-cysteine for a short time period, (10 min without tunicamycin, lane S), a longer period (3 h without tunicamycin, lane L), or 3 h with tunicamycin (lane T). Two-thirds of each cell extract was immunoprecipitated for the 10-min label, and one-third for the 3-h label extracts. Immunoprecipitations were analyzed on an SDS-12.5% polyacrylamide gel. The large dots mark the *Wnt* protein species detected in the untreated extracts, and the (*) indicates the tunicamycin product. The positions of the ^{14}C-labeled rainbow markers are indicated.

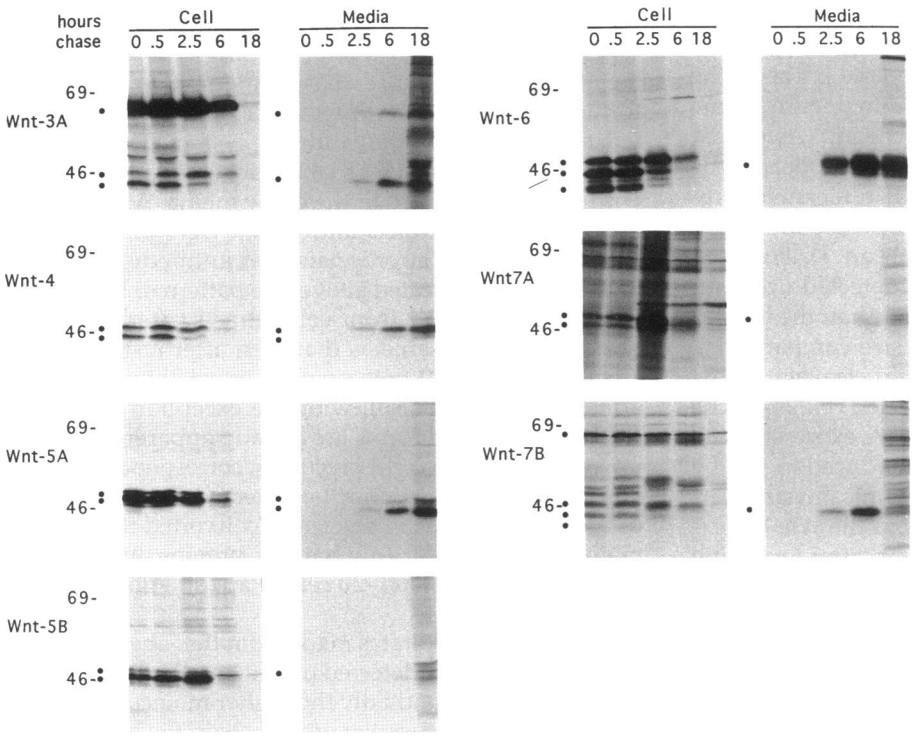

Figure 4. Pulse-chase analysis of *Wnt* proteins. Replicate dishes of *Wnt*-expressing cells were pulse-labeled for 30 min with ^{35}S-cysteine, followed by incubation in the absence of label (chase) for 0, 0.5, 2.5, 6, or 18 h. Suramin was present during the entire chase period. Both cell extracts and media were harvested at the indicated times, and one-third of each sample was immunoprecipitated with the appropriate anti-peptide antibody and analyzed on an SDS-12.5% polyacrylamide gel. The positions of the *Wnt* proteins are denoted by dots. The positions of the ^{14}C-labeled rainbow markers are indicated.

naud *et al.*, 1989; Papkoff, 1993). The P19 cell line is a murine embryonal carcinoma cell line that will differentiate along a neuroectodermal lineage in response to RA. Several *Wnt* genes are transcriptionally regulated upon RA treatment (Smolich and Papkoff, unpublished data). To detect native *Wnt* proteins in these cells, cultures were treated with RA for 3 d, sufficient time for induction of *Wnt* family mRNA accumulation (Smolich and Papkoff, unpublished data). Cultures were then metabolically labeled with ^{35}S-cysteine for 5 h in the presence of suramin, and media samples were immunoprecipitated using each of the *Wnt* antibodies. *Wnt* proteins were detected using antibodies against *Wnt*-3A, -4, -5A, -6, and -7B (Fig. 5); no proteins were detected in immunoprecipitates that were blocked with cognate peptide. Similar to the results seen with the recombinant proteins, nothing was detectable in cultures labeled in the absence of suramin. The secreted native *Wnt* proteins from P19 cells appear to be approximately the same size as the recombinant proteins described above. In addition, the protein expression patterns agree with the mRNA accumulation profile that we have characterized (Smolich and Papkoff, unpublished data).

DISCUSSION

This report describes the first biochemical characterization of seven newly identified *Wnt* family members. Because there are so few cell lines that endogenously express *Wnt* proteins and because those that do generally express very low levels of the proteins (i.e., P19 cells, St. Arnaud *et al.*, 1989; Papkoff, 1993), we began our characterization of *Wnt* proteins in transfected cells that have been engineered to express high levels of recombinant protein. We have chosen the murine neuroendocrine cell line AtT-20 because this has previously been shown to efficiently process and secrete *Wnt*-1 protein (Papkoff and Schryver, 1990), unlike several other cell lines that have been examined in which *Wnt*-1 appears to accumulate inside the cell (Brown *et al.*, 1987; Papkoff *et al.*, 1987; Papkoff, 1989). The data show that each of the new *Wnt* proteins are secreted glycoproteins that

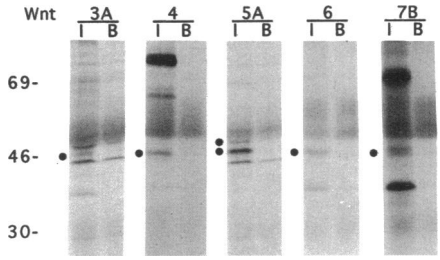

Figure 5. Identification of native *Wnt* proteins. RA-treated P19 cells were metabolically labeled for 5 h with ^{35}S-cysteine in the presence of suramin. The media was harvested and immunoprecipitated with the appropriate anti-peptide antibody followed by electrophoresis on an SDS-12.5% polyacrylamide gel. Lane I shows an immunoprecipitate. Lane B is an immunoprecipitate blocked with cognate peptide. The large dots mark the positions of the *Wnt* proteins. The positions of the ^{14}C-labeled rainbow markers are indicated.

associate with the cell surface or extracellular matrix. *Wnt* proteins are detected in the culture media only in the presence of suramin, which blocks association with the cell surface and allows the proteins to accumulate in the culture medium. Suramin treatment may bypass the internalization and subsequent degradation of *Wnt* proteins that presumably follows their delivery to the cell surface in untreated cells.

The demonstration that Wnt family proteins are secreted has important implications for Wnt function, because it has been suggested that secretion of *Wnt*-1 is necessary for transformation. Fibroblasts expressing *Wnt*-1, although not transformed themselves, can elicit partial transformation of mammary epithelial cells in coculture via a paracrine mechanism (Jue *et al.*, 1992). In addition, a *Wnt*-1 mutant that lacks the signal sequence is not capable of transforming mammary epithelial cells (Mason *et al.*, 1992). Because other members of the *Wnt* family are also active in this transformation assay (Blasband *et al.*, 1992; Wong *et al.*, unpublished data), the demonstration that these proteins are surface-associated suggests that they function via a similar mechanism of action.

Similar to *Wnt*-1, AtT-20 cells efficiently process and secrete the other *Wnt* proteins. The initial processing of these proteins, with the exception of *Wnt*-6, is extremely rapid. After a pulse-label of 10 min, the protein backbone, probably lacking the signal peptide, has already been modified by the addition of carbohydrate moieties such that the unmodified form is not detectable. *Wnt*-6, the one exception, is more similar to *Wnt*-1 in that the primary translation product, lacking signal peptide, is still detectable after a 30-min pulse label (Papkoff and Schryver, 1990). Whereas the kinetics of the initial processing events appear to vary, the overall half-life of mature forms is the same for all *Wnt* proteins examined with the majority turned over from the cell by 2.5–6 h of chase. The functional basis, if any, for the differences observed in the initial processing events is unclear at the present time. It is possible that differences inherent in the various *Wnt* proteins, which are not obvious on the basis of the biochemical properties described here, are responsible for the processing variation.

Aside from the expected protein species predicted from the estimated molecular weights and putative glycosylation sites, we have detected larger molecular weight proteins in the *Wnt*-3A and -7B expressing cell lines. Particularly intriguing is a large 60.5-kDa protein recognized in cells transfected with a *Wnt*-3A cDNA, which appears to be a secreted glycoprotein with properties very similar to other *Wnt* proteins. This protein does not appear to be a *Wnt*-3A-associated protein, because it is immunoprecipitated from lysates prepared even under highly denaturing conditions and is recognized on a Western blot (Smolich and Papkoff, unpublished data). There is at least one other Wnt gene, a member of the *Drosophila Wnt* family, that also encodes a larger protein (Eisenberg *et al.*, 1992; Russell *et al.*, 1992). We are currently investigating the possibility that the large protein found in Wnt-3A expressing cells represents a novel cross-reacting *Wnt* protein.

On the basis of our characterization of recombinant *Wnt* proteins and preliminary northern blot analysis in P19 cells (Smolich and Papkoff, unpublished data), we were able to identify some *Wnt* proteins native to P19 cells. Because native *Wnt* proteins are expressed at such low levels, their identification was greatly facilitated both by the prior analysis of the recombinant proteins and by the Northern blot analysis of *Wnt* mRNA accumulation during RA-induced differentiation. We have identified native *Wnt* proteins in the culture medium of RA-induced P19 cells treated with suramin, thus suggesting that the native proteins are also secreted and surface-associated. The expression of the proteins also correlates well with the mRNA expression patterns (Smolich and Papkoff, unpublished data). The P19 cell line, because it provides access to native *Wnt* proteins, could serve as a useful system in which to investigate the signaling pathways that involve the *Wnt* family proteins.

Although the secretion, cell surface association, and turnover of *Wnt* proteins appears to be fairly uniform, functional differences have been demonstrated within the family. *Wnt*-1 and some of the other *Wnt* family members are able to elicit partial transformation of mammary epithelial cell lines, whereas other *Wnt* proteins, such as *Wnt*-4 and -5A, have no effect when expressed in the same cells (Wong *et al.*, unpublished data; Olson and Papkoff, unpublished data). Differences are also apparent in other species such as *Xenopus*, where ectopic expression of mouse *Wnt*-1 and *Xenopus Wnt*-1, -3A, and -8, but not *Xenopus Wnt*-5A, causes duplication of the dorsal axis and affects gap junctional communication (McMahon and Moon, 1989; Christian *et al.*, 1991; Olson *et al.*, 1991; Wolda *et al.*, 1993). The phenotypic variations caused by overexpression of different *Wnt* family members suggests that the *Wnt* genes can be divided into at least two functionally distinct classes. An initial explanation for these functional differences, before the experiments reported here, might have been based on differences in biochemical properties. However our results demonstrate, at least in terms of glycosylation and secretion patterns, that all *Wnt* proteins examined behave similarly, probably utilizing a common mechanism of action from secreted factor to receptor binding and activation.

The biochemical similarities reported here further suggest that the basis for the functional differences that are apparent within the *Wnt* family may reside in the responding cell. It is possible that different subsets of family members interact with distinct nonoverlapping sets of receptors that exhibit restricted expression patterns or ligand specificity. Furthermore, these different *Wnt* receptors could activate distinct signal transduction

pathways. In addition, the normally restricted and often nonoverlapping expression of each *Wnt* protein may contribute to different biological effects. The identification of receptors that interact with *Wnt* proteins will greatly aid in understanding the molecular nature of the functional differences apparent in the *Wnt* family.

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