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Mutational Analysis of Mouse *Wnt*-1 Identifies Two Temperature-Sensitive Alleles and Attributes of *Wnt*-1 Protein Essential for Transformation of a Mammary Cell Line

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The proto-oncogene *Wnt*-1 encodes a cysteine-rich, secretory glycoprotein implicated in virus-induced mouse mammary cancer and intercellular signaling during vertebrate neural development. To attempt to correlate structural motifs of *Wnt*-1 protein with its function, 12 mutations were introduced singly and in several combinations into the coding sequence of *Wnt*-1 cDNA by site-directed mutagenesis. Mutant alleles in a retroviral vector were tested for their ability to transform the mouse mammary epithelial cell line C57MG in two ways: by direct infection of C57MG cells and by infection of NIH3T3 cells that serve as donors of *Wnt*-1 protein to adjacent C57MG cells in a secretion-dependent (paracrine) assay. In addition, the synthesis and secretion of mutant proteins were monitored in multiple cell types by immunological assays. Deletion of the signal peptide demonstrated that transformation in both direct and paracrine assays depends upon entry of *Wnt*-1 protein into the endoplasmic reticulum. Changes in potential proteolytic processing sites (two basic dipeptides and a probable signal peptidase cleavage site) did not adversely impair biological activity or protein processing and uncovered a second site for cleavage by signal peptidase. Replacement of each of the four asparagine-linked glycosylation sites did not affect transforming activity at normal temperatures, but one glycosylation site mutant was found to be temperature-sensitive for transformation. An allele encoding a protein that lacks all four glycosylation sites was also transformation competent. In two of four cases, substitution of serine for a cysteine residue impaired transforming activity at the usual temperature, and transformation was temperature sensitive in a third case, implying that at least some of the highly conserved cysteine residues are important for *Wnt*-1 function.

INTRODUCTION

The *Wnt*-1 (originally *int*-1) gene was initially identified as a frequent target for insertional activation by mouse mammary tumor virus (MMTV) proviral DNA (Nusse and Varmus, 1982; Nusse *et al.*, 1984; van Ooyen and Nusse, 1984). Its status as a proto-oncogene has been confirmed by its ability to alter the morphology and growth properties of mammary epithelial cell lines (Brown *et al.*, 1986; Rijsewijk *et al.*, 1987a) and to cause mammary gland hyperplasia and tumors in transgenic mice (Tsukamoto *et al.*, 1988) when linked to viral regulatory sequences. Unlike most other proto-oncogenes, *Wnt*-1 is expressed in very few cell types in normal mice, principally in restricted portions of the central nervous system in midgestational embryos and in round spermatids in adults (Jakobovits *et al.*, 1986; Shackleford and Varmus, 1987; Wilkinson *et al.*, 1987). *Wnt*-1 has been more directly linked to developmental events by the discovery that the homologue of *Wnt*-1 in *Drosophila melanogaster* is the segment polarity gene, *wingless* (Rijsewijk *et al.*, 1987b), and by the disruption of the mouse *Wnt*-1 gene, preventing formation of a normal cerebellum and midbrain (Thomas and Capecchi, 1990;

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McMahon and Bradley, 1990). Further, injection of synthetic *Wnt*-1 RNA into fertilized *Xenopus* eggs produces anterior duplication of the embryonic axis (McMahon and Moon, 1989).

In the past few years, *Wnt*-1 has been recognized as a member of a large gene family, with at least 10 members identified in the mouse (McMahon and McMahon, 1989; Gavin *et al.*, 1990; Roelink *et al.*, 1990; Roelink and Nusse, 1991) and one or more genes characterized in man (van Ooyen *et al.*, 1985; Wainwright *et al.*, 1988), *Xenopus* (Noordermeer *et al.*, 1989; Christian *et al.*, 1991a; Christian *et al.*, 1991b), zebrafish (Molven *et al.*, 1991), *Drosophila* (Rijsewijk *et al.*, 1987b; Russell *et al.*, 1992; A.M.C. Brown, personal communication), *Axolotl* (Busse *et al.*, 1990), *C. elegans* (Shackleford *et al.*, unpublished observations), and several other organisms (Sidow, 1992). Mouse *Wnt*-1 encodes a protein 370 amino acids in length, with many of the hallmarks of a secreted factor. The primary translation product has a hydrophobic signal peptide, four sites for asparagine-linked glycosylation, 23 cysteine residues, and four dibasic peptides that are potential cleavage sites for serine proteases (van Ooyen and Nusse, 1984; Fung *et al.*, 1985). Similar features are found in the predicted protein products of other *Wnt* genes. Amino acid sequence identities among the proteins encoded by *Wnt* gene family members range from 35 to >90%, with regions of strong homology scattered throughout the coding sequence. Strikingly, of the 23 cysteine residues in the *Wnt*-1 protein, 22 are found in most of the *Wnt* proteins whose sequence has been deduced to date.

Current information about *Wnt* proteins is derived mostly from studies with cultured cell lines programmed to express exogenous mouse *Wnt*-1 coding sequences. During synthesis, *Wnt*-1 protein enters the secretory pathway (Brown *et al.*, 1987; Papkoff *et al.*, 1987) where it binds the binding protein BiP (Kitajewski *et al.*, 1992), undergoes removal of its N-terminal signal peptide, and is sequentially glycosylated at up to four sites, producing five protein species that range from 36 kDa (unglycosylated) to 44 kDa (fully glycosylated). After secretion, *Wnt*-1 protein binds tightly to the extracellular matrix or other extracellular components (Papkoff, 1989; Papkoff and Schryver, 1990; Bradley and Brown, 1990). Notably, no biologically active, cell-free *Wnt*-1 protein has yet been prepared from any expression system.

Although most cultured cells appear to be unresponsive to an exogenous *Wnt*-1 gene, expression of *Wnt*-1 in the mouse mammary epithelial cell line C57MG causes pronounced morphological changes and augmented growth potential in monolayers (Brown *et al.*, 1986). The transformed cells are not, however, able to form colonies in soft agar or tumors in nude mice. When expressed in fibroblasts, *Wnt*-1 has no apparent effects; however, when the cells are cocultivated with the responsive C57MG cells, changes characteristic of direct transformation are induced in nearby C57MG cells by a paracrine mechanism (Jue *et al.*, 1992).

To address the significance of various sequence motifs in *Wnt*-1 protein, we made twelve site-directed mutations in the coding sequence of mouse *Wnt*-1 and tested their effects on protein production, secretion, and biological activity, with the use of direct (autocrine) and paracrine transformation assays with the C57MG cell line. The mutant alleles show that *Wnt*-1 proteins must enter the endoplasmic reticulum to transform cells, that glycosylation sites are dispensible for activity, and that at least some cysteine residues are required. In addition, we have identified two alleles that are temperature-sensitive for transformation, and used glycosylation and cleavage site mutants to judge the frequency of modifications at these sites.

MATERIALS AND METHODS

Cell Lines and Viruses

C57MG cells (Vaidya *et al.*, 1978) were supplied by A.M.C. Brown and maintained as described (Brown *et al.*, 1986). Because the parental cell line exhibits spontaneous morphological alterations, we isolated by limited dilution cloning a subline that more stably maintains a flat morphology. This subline, designated C57MG2, was used for all the experiments described in this paper. NIH 3T3 and Ψ2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics. Derivatives of these cell lines, obtained by infection with retroviral vectors expressing neomycin phosphotransferase (*neo*), were maintained in the same medium, with the addition of 400 μg/ml G418 (Geneticin, GIBCO Laboratories, Grand Island, NY). Viruses expressing wild-type and frameshift alleles of *Wnt*-1, MXIN and MXIN fs, are described in Brown *et al.* (1986). Quail QT6 cells (Moscovici *et al.*, 1977) were maintained in Medium 199 containing Hanks' balanced salt solution, supplemented with 2.95 g/l tryptose phosphate broth, 5% FCS, and 1% chicken serum (heat inactivated).

Construction of Mutant Proviral Plasmids

Plasmid pV101, supplied by A. Tsukamoto, contains the full-length *Wnt*-1 cDNA clone 26 (Fung *et al.*, 1985), truncated at the *Mlu* I site in the 3′ untranslated sequence and cloned into the *Eco*RI site of pSP64. An *Eco*RI fragment of pV101, encompassing the entire *Wnt*-1 coding sequence, was introduced into the M13 vectors mp19 and tg130 (Kieny *et al.*, 1983). Mutagenesis was carried out with the use of a site-directed mutagenesis kit (Amersham, Arlington Heights, IL) according to the manufacturer's instructions. The presence of each mutation was confirmed by DNA sequencing with the use of a Sequenase kit (USB). In addition, 99% of the coding region of the mutated cDNA for N359Q was sequenced.

The following mutants were made by site-directed mutagenesis with oligonucleotides whose sequences are given below:

R100Q: AGTTCCAGCGCTGGTTTCGGAA

R157Q: GACTACCGGCAGCGCGGCCC

C121S: CAACCGAGGCAGCCGAGAAA

C143S: GCGCGCTCCAGCTCCGAAG

C151S: CGCAGGTGCTGGACTCGATG

N316Q: CGCGGGAGAACTACTCTGGCAAGCTCG

N359Q: AGCTGCCGCCAGTGCACGCAC

C369W: ACCTCATAGCCACTCGTGC

N346Q: GGAGCGCTGCCAGTGCACCTTCC

A27V, N29T and A27VN29T were made with the mixed oligonucleotide GCCACTACTG(GT)TAGCA(AG)CCAGGGCTGC. After sequencing, clones that contained each individual and both mutations were selected. To delete the 25 amino terminal amino acids of *Wnt*-1 protein with the ΔL mutation, oligonucleotide L26M TGGCAGCCATGGCTGCGGG was used to introduce an *Nco* I site (underlined) at the position of leucine 26. There is also an *Nco* I site at the initiator methionine codon. By cutting at both sites and religating the cohesive ends, the amino terminal sequence is deleted and a new initiator methionine is introduced at the previous position of leucine 26. −3N was constructed with the use of an M13 clone already containing N316Q as a substrate for mutagenesis with both oligonucleotides N346Q and N359Q simultaneously. To construct −4N, *Eco*RI fragments from M13 clones containing −3N and N29T were subcloned into pBluescriptII (Stratagene). A *Cla* I fragment extending from the polylinker to nucleotide 704 of the *Wnt*-1 cDNA with N29T was substituted for the equivalent fragment of the −3N subclone, thus generating −4N. Full details of all plasmid constructions are available on request to the authors.

Each mutant allele was introduced into the *Eco*RI site of the MLV based retroviral vector pMV7 (Kirschmeier *et al.*, 1988). This vector expresses *Wnt*-1 from the retroviral LTR and *neo* from an internal Herpes simplex virus thymidine kinase promoter. After transfection into the ecotropic packaging cell line Ψ2 (Mann *et al.*, 1983) by calcium phosphate co-precipitation, virus-containing supernatants were harvested from pools of stably infected G418 resistant Ψ2 clones and used to infect C57MG (Vaidya *et al.*, 1978) or NIH 3T3 cells, as described by Brown *et al.* (1986). To obtain pools of NIH3T3 cells expressing detectable levels of protein from some of the mutant alleles (i.e., N346Q, N359Q, R100Q, A27V, C143S, and C369W), the proviral constructs were transiently transfected into the amphotropic packaging cell line PA317 (Miller and Buttimore, 1986). After 72 h, the medium was removed and used to infect the ecotropic packaging cell line PE501 (Miller and Rosman, 1989). Stably infected clones were selected in 400 μg/ml G418, and helper free stocks of virus were harvested from confluent plates of pools of >50 colonies. These viral stocks were then used to infect NIH3T3 cells as above.

Assays for Wnt-1 Transforming Activity

C57MG Assay. Approximately 1×10^5 pooled, infected, G418-resistant C57MG cells were plated in one well of a six-well tissue culture plate (Corning) in DMEM supplemented with 10% FCS and 10 μg/ml insulin (Sigma, St. Louis, MO). The morphological changes induced by *Wnt*-1 are more readily observed by placing the cells in serum-free medium 24 h after plating and allowing them to grow to confluence in this medium. After 24 h, the culture medium was replaced with HB-CHO (Hana Biologics, Irvine, CA), a defined medium optimized for fibroblast cell lines. The morphology was assessed and cells photographed at confluence, usually after 3–5 d, with Polaroid film and an Olympus inverted microscope.

Paracrine Assay. In a slight variation of the protocol described by Jue *et al.* (1992), $\sim 4 \times 10^4$ 3T3 cells expressing one of the mutant alleles and an equal number of C57MG cells were cocultivated in one well of a six-well tissue culture plate. Again, the cells were cultured in 10% FCS DMEM with 10 μg/ml insulin for 24 h, which was then replaced with the defined medium, HB-CHO. Cultures were photographed after a total of 3–5 d.

Temperature Shift Experiments

These were carried out as described above for the normal autocrine and paracrine assays, except that plates were set up in duplicate, with sister plates incubated at 32, 35, 37, and 40°C.

Expression in QT6 Cells

Wnt-1 and mutant *Wnt*-1 cDNAs were subcloned into pON expression vectors (Geballe *et al.*, 1986) that utilize the cytomegalovirus immediate early promoter/enhancer to drive expression. Subconfluent 60-mm dishes of QT6 cells were calcium-phosphate transfected with 10 μg of plasmid followed by treatment with 10% DMSO in phosphate-buffered saline (PBS). At 40 h posttransfection, cells were washed with PBS, preincubated for 15 min at 37°C in DMEM lacking cysteine and methionine (labeling media), and then incubated for 4 h at 37°C with 1.5 ml of labeling media supplemented with 0.125 mCi/ml [^{35}S]translabel and 0.125 mCi/ml [^{35}S]cysteine (ICN Radiochemicals, Irvine, CA). After labeling, cells were washed twice with cold PBS, 0.5 ml of TENT buffer (20 mM Tris, pH 8.0, 2 mM EDTA, 150 mM NaCl, 1% Triton-X100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin) was added to each dish, and the dishes were rocked for 20 min at 4°C. The lysates were centrifuged for 10 min at 10 000 × *g* and the supernatants were normalized for protein content. The cell lysates were incubated for 1 h at 4°C with 1 μl ascites fluid containing monoclonal antibodies against *Wnt*-1 specific peptide A (residues 200–212) (Brown *et al.*, 1987). Protein A-Sepharose (Sigma) was used to collect the antigen-antibody complexes. Immunoprecipitates were washed four times with cold TENT buffer and then electrophoresed on a 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel.

Immunoblot Analysis

C57MG and NIH3T3 cell lines were grown to subconfluence in 100-mm dishes. The culture medium was then replaced with DMEM supplemented with 1% FCS and 100 μg/ml of heparin (from porcine intestinal mucosa, Sigma). After 24 h, the medium was collected and cells were harvested. To prepare lysates for intracellular protein analysis, cells were washed twice with cold PBS, and then removed from the dish with a rubber policeman in 5 ml of cold PBS. Cells were pelleted by centrifugation at 1000 × *g* for 10 min at 4°C, and then lysed in 90 μl of TENT buffer (see above) for 20 min at 4°C. The lysate was clarified by centrifugation at 10 000 × *g* for 10 min at 4°C, and protein content was determined. For extracellular protein analysis, conditioned medium was precleared by centrifugation at 1000 × *g* for 10 min, the supernatant was spun at 150 000 × *g* for 1.5 h at 4°C, and the pellet was solubilized in 50 μl of Laemmli buffer. For immunoblot analysis of C57MG cell samples, 160 μg of protein from the cell lysates and a proportional volume of the extracellular sample were run through 10% SDS-polyacrylamide gels and then electroblotted onto nitrocellulose filters. The protein blot was blocked for 1 h at 25°C in *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (Tris)-buffered saline (TBS) (10 mM Tris, pH 8.0, 150 mM NaCl) containing 0.2% Tween-20 and 0.2% bovine serum albumin (blocking buffer A), and then incubated overnight at 4°C with anti-*Wnt*-1 monoclonal antibodies raised against peptide A (residues 200–212) diluted 1:1000 in blocking buffer A. The primary antibody was removed, the blot washed three times for 5 min each in TBST (TBS with 0.5% Tween-20), incubated for 1 h at 4°C with 0.5 μCi/ml [^{125}I]anti-mouse IgG antibodies in blocking buffer A, and then washed as before. The blot was dried and then subjected to autoradiography. For immunoblot analysis of NIH3T3 cell samples, 80 μg of protein from the cell lysates and a proportional volume of the extracellular sample were run through 10% SDS-polyacrylamide gels and then electroblotted onto nitrocellulose filters. The protein blot was blocked for 1 h at 25°C in TBS containing 0.2% Tween-20 and 1% bovine serum albumin (blocking buffer B) and then incubated overnight at 4°C with anti-*Wnt*-1-peptide A monoclonal antibodies diluted 1:2000 in blocking buffer B. The primary antibody was removed, the blot washed three times for 5 min each in TBST, incubated for 1 hr at 4°C in 1:20 000 diluted horseradish peroxidase conjugated anti-mouse IgG antibodies (Amersham) in blocking buffer B, and then washed as before. The blot was processed with the enhanced chemiluminescence (ECL) detection system (Amersham).

RESULTS AND DISCUSSION

Experimental Strategy

Site-Directed Mutagenesis. We have made 12 site-directed mutations in potentially significant sequences of

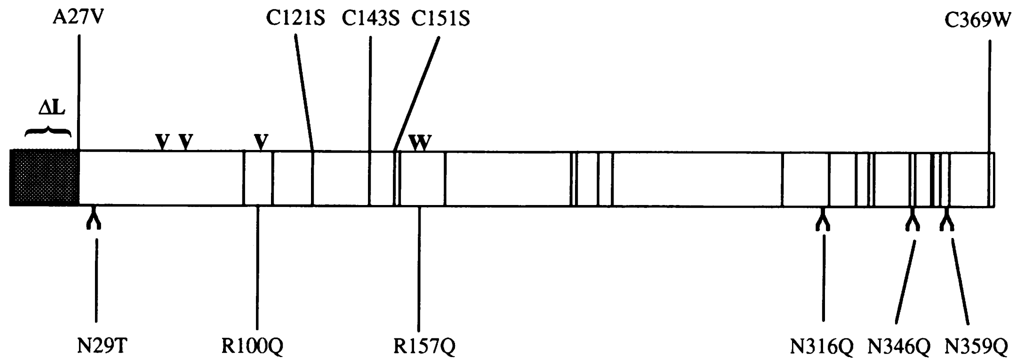

Figure 1. Structure of the *Wnt*-1 protein, showing residues targeted for mutagenesis. The stippled box represents the 27 amino acid signal peptide, inverted Ys indicate the predicted N-linked glycosylation sites, the positions of dibasic residues that are potential sites for cleavage by serine proteases are indicated by Vs and cysteine residues are shown as vertical lines. Mutations are designated according to wild-type amino acid, followed by residue number and mutant amino acid (e.g., A27V changes alanine 27 to valine). ΔL refers to a deletion of residues 2-26.

a mouse *Wnt*-1 cDNA clone: those coding for the signal peptide, the signal peptidase cleavage site, other potential proteolytic cleavage sites, the four sites for N-linked glycosylation, and some cysteine residues (Figure 1, Tables 1–3). In addition, some of these mutations were combined to generate N29T/A27V, −3N, and −4N, providing a total of 15 mutant alleles.

The mutant alleles were initially screened for production of stable proteins by transient transfection of the quail fibroblast line QT6. As is evident from the radiolabeled immunoprecipitates shown in Figure 2, all of the mutants directed synthesis of *Wnt*-1 proteins that accumulated to levels similar to those observed with the wild-type allele. Proteins made by all but one of the mutants (the signal peptide deletion mutant, ΔL) appeared to enter the endoplasmic reticulum; this was judged by association with the 78-kDa protein BiP and by changes in mobility ascribed to glycosylation (Figure 2). The wild-type and most mutant alleles produced four readily detected species of protein: an unglycosylated form lacking its N-terminal signal peptide (36 kDa), and species glycosylated successively at one (38 kDa), two (40 kDa), or three (42 kDa) positions. (A fifth species [44 kDa], glycosylated at all four sites, is not readily detected in the analysis of proteins from QT6 cells.) In some cases, such as mutants lacking glycosylation sites, changes in the gel mobility of the protein species were found; these are described below in the presentation of individual mutants.

Transformation Assays. Recombinant murine leukemia virus vectors carrying each of the mutant *Wnt*-1 alleles were generated by passage through helper cells (see MATERIALS AND METHODS). Virus stocks were used to infect the *Wnt*-1 responsive, mouse mammary epithelial cell line C57MG (Vaidya *et al.*, 1978). Pools of stably infected cells, selected for resistance to the antibiotic G418, were examined to determine the effects of each mutant allele. Morphological change—from flat, cuboidal cells to elongated, densely-packed, highly refractile cells—is the most obvious sign of the incomplete transformation of C57MG cells produced by wild-type *Wnt*-1 (Brown *et al.*, 1986), and it was the major criterion by which the biological activity of the mutant alleles was judged (Figure 3). In this assay, *Wnt*-1 proteins can conceivably act within protein-producing cells, through interactions at the surface of those cells or through actions on adjacent cells. For convenience, however, we

Table 1. Secretion mutations

Mutation	Transforming activity		Comments
	Autocrine	Paracrine	
ΔL	−	−	Inactive in *Xenopus* assay (McMahon and Moon, 1989)
A27V	+	+	Major cleavage site probably shifted to A28

Table 2. Glycosylation mutations

Mutation	Transforming activity		Comments
	Autocrine	Paracrine	
N29T	+	ND	
N316Q	+	+	Mutant protein hypersecreted
N346Q	+	+	Site inefficiently glycosylated in wt protein
N359Q	+(ts)	+(ts)	Inactive at 40°C in both assays
N29TA27V	+	+	
−3N	+	ND	Not ts
−4N	+	+	Not ts

Table 3. Cysteine mutations

Mutation	Transforming activity		Comments
	Autocrine	Paracrine	
C121S	+	+	Cysteine residue unique to *Wnt*-1 orthologs
C143S	–	–	
C151S	+ (ts)	–	Inactive at 40°C in both assays
C369W	–	–	Inactive in *Xenopus* assay (McMahon and Moon, 1989); impaired secretion

will refer to tests of C57MG cells that express *Wnt*-1 alleles as autocrine assays, although paracrine effects may also contribute to the phenotype.

To test specifically for biological effects believed to require secretion of *Wnt*-1 proteins, the virus stocks were also used to generate pools of stably infected, G418-resistant colonies of NIH3T3 cells. Like other fibroblast cell lines that have been tested, these cells show no evidence of a physiological response to *Wnt*-1, but they produce *Wnt*-1 proteins and can induce morphological changes in adjacent cocultivated C57MG cells (Jue *et al.*, 1992). This secretion-dependent assay, referred to here as the paracrine assay, was used to determine whether mutant alleles could produce *Wnt*-1 proteins capable of inducing transformation of neighboring uninfected cells (Figure 4).

In a bid to identify temperature-sensitive (ts) mutants of *Wnt*-1, cultures of C57MG cells infected with each mutant were grown at several temperatures (generally 32, 37, and 40°C); as described below, two ts alleles were discovered in this manner (Figure 5). In addition, the two ts alleles were tested for temperature-dependent activity in the paracrine assay (Figure 5).

Although all of the mutant alleles were demonstrated to encode stable *Wnt*-1 proteins in transfected QT6 cells (Figure 2), we have also examined the synthesis, processing, and secretion of *Wnt*-1 proteins in the infected C57MG cells (Figure 6) and NIH3T3 cells (Figure 7) used for biological assays. This was done for two reasons: 1) we were concerned that the products of some mutant alleles might behave differently in the various host cell lines, 2) we wished to document that adequate levels of mutant *Wnt*-1 proteins, at or above some threshold for morphological transformation by wild-type protein, were present in the pools of infected cells, particularly those carrying alleles that scored negative in biological assays. This proved to be an important maneuver, because, for reasons we cannot explain, infected C57MG or NIH3T3 cells often display precipitous loss of *Wnt*-1 protein production, despite continued resistance to G418. Consequently, unless otherwise noted, all of the transformation assays presented here have been validated by direct concordant measurements of *Wnt*-1 proteins.

Characterization of Mutants

Mutations Intended to Affect Secretion Demonstrate a Requirement to Enter the ER and Presumptive Alternate Sites for Cleavage by Signal Peptidase. As described above, *Wnt*-1 protein is known to be secreted when ectopically expressed in cell lines. Also, the *Drosophila* homologue, *wingless*, is known to act in a cell nonautonomous fashion (Morata and Lawrence, 1977; van den Heuvel *et al.*, 1989). Thus, *Wnt*-1 protein is assumed to be secreted to exert its effects. To test this directly, we designed mutants expected to alter the secretion profile of *Wnt*-1 protein (Table 1).

1) ΔL. ΔL lacks the entire hydrophobic signal peptide sequence (Figure 1). As would be predicted, the mutant protein does not enter the ER, as evidenced by its failure to associate with BiP, to become glycosylated, and to

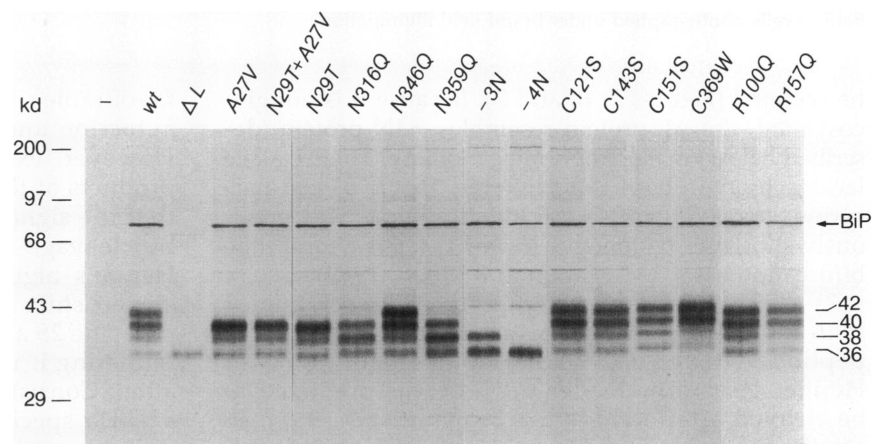

Figure 2. Immunoprecipitation of protein products of mutant *Wnt*-1 alleles expressed in transiently transfected QT6 cells. QT6 cells transiently transfected with pON plasmids bearing each of the mutant alleles of *Wnt*-1, as well as the wild-type cDNA (wt), were labeled with [^{35}S]methionine and [^{35}S]cysteine, and *Wnt*-1-specific proteins were immunoprecipitated from cell lysates with anti-*Wnt*-1-peptide A monoclonal antibodies. The apparent molecular weights of the observed *Wnt*-1 species are marked, as is the position of the binding protein BiP, determined with reference to molecular weight standards.

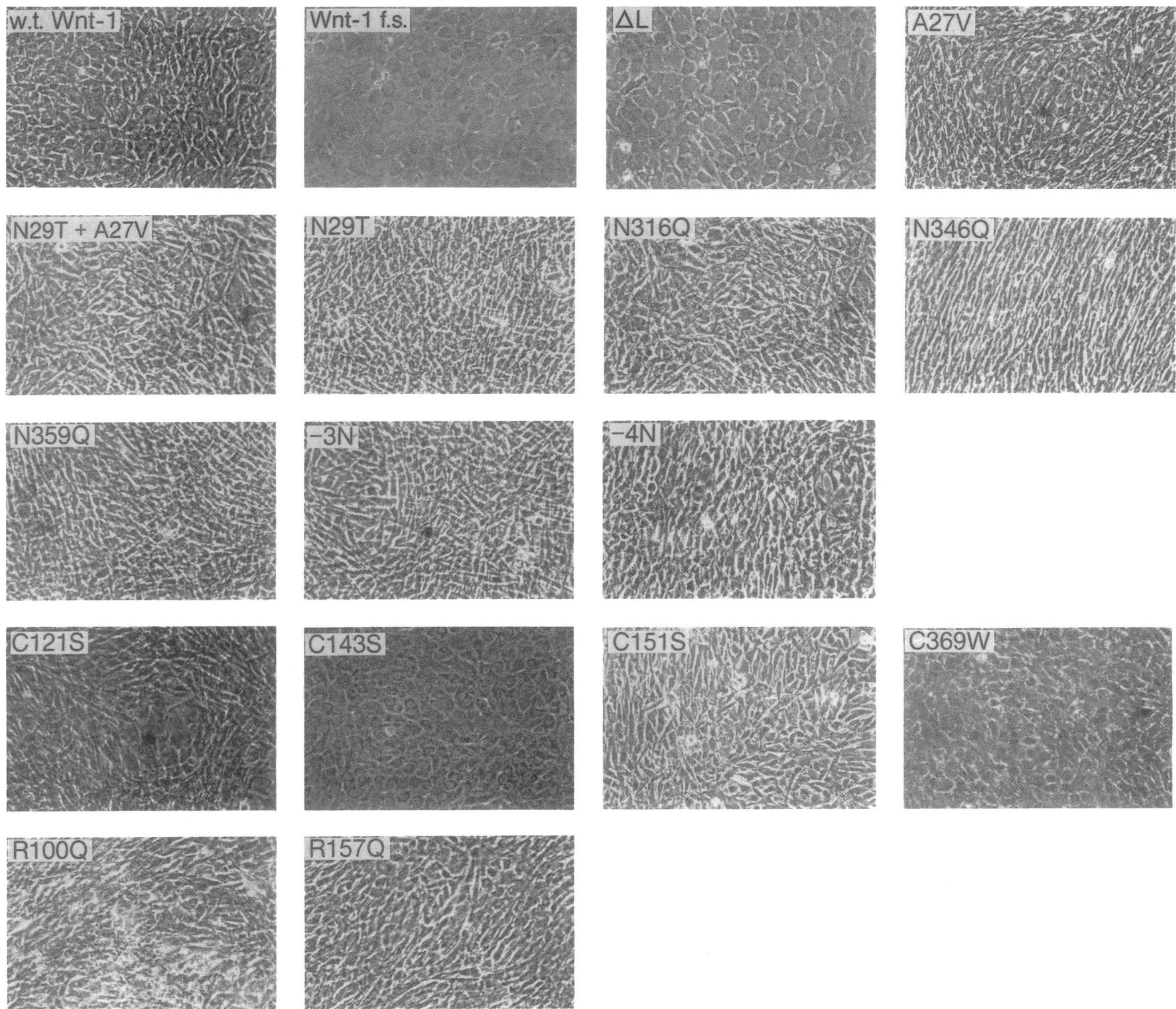

Figure 3. Autocrine transformation of C57MG cells by mutant *Wnt*-1 alleles. Pools of >50 G418-resistant C57MG colonies expressing each mutant allele, a frameshift mutant (Wnt-1 f.s.) and wild type *Wnt*-1 (w.t. Wnt-1), were plated at a density of $10^4/cm^2$ and allowed to grow to confluence in the serum-free medium HB-CHO (usually 3–5 d). Plates were photographed at confluence. Each panel shows a representative field of cells photographed under bright field illumination.

be secreted (Figures 2, 6, and 7). Because it is not glycosylated, the ΔL protein resembles −4N protein (described below) in electrophoretic mobility. The ΔL allele lacks activity in both the autocrine (Figure 3) and paracrine assays (Figure 4). An identical allele was previously shown to be inactive in the *Xenopus* neural tube bifurcation assay (McMahon and Moon, 1989).

2) A27V. We also attempted to alter secretion of *Wnt*-1 protein by blocking cleavage of the leader peptide. With the use of von Heijne's algorithm (von Heijne, 1986), the leader peptide was predicted to be cleaved after alanine 27 (Brown *et al.*, 1987), so we changed alanine 27 to valine to make this an unfavourable site. A27V was found to be active in both autocrine and paracrine assays (Figures 3 and 4). However, examination of the sizes of the protein products of this allele (Figures 2, 6, and 7) indicates that the signal peptide is in fact removed, probably by cleavage after alanine 28, again based on von Heijne's algorithm (von Heijne, 1986). This presumed shift in the cleavage site would expose asparagine 29 at the N-terminus of the protein, thereby rendering it unlikely to be a substrate for glycosylation. Consistent with this prediction, the prominent 42-kDa species of *Wnt*-1 protein is not evident in cells expressing A27V. Moreover, only one form of

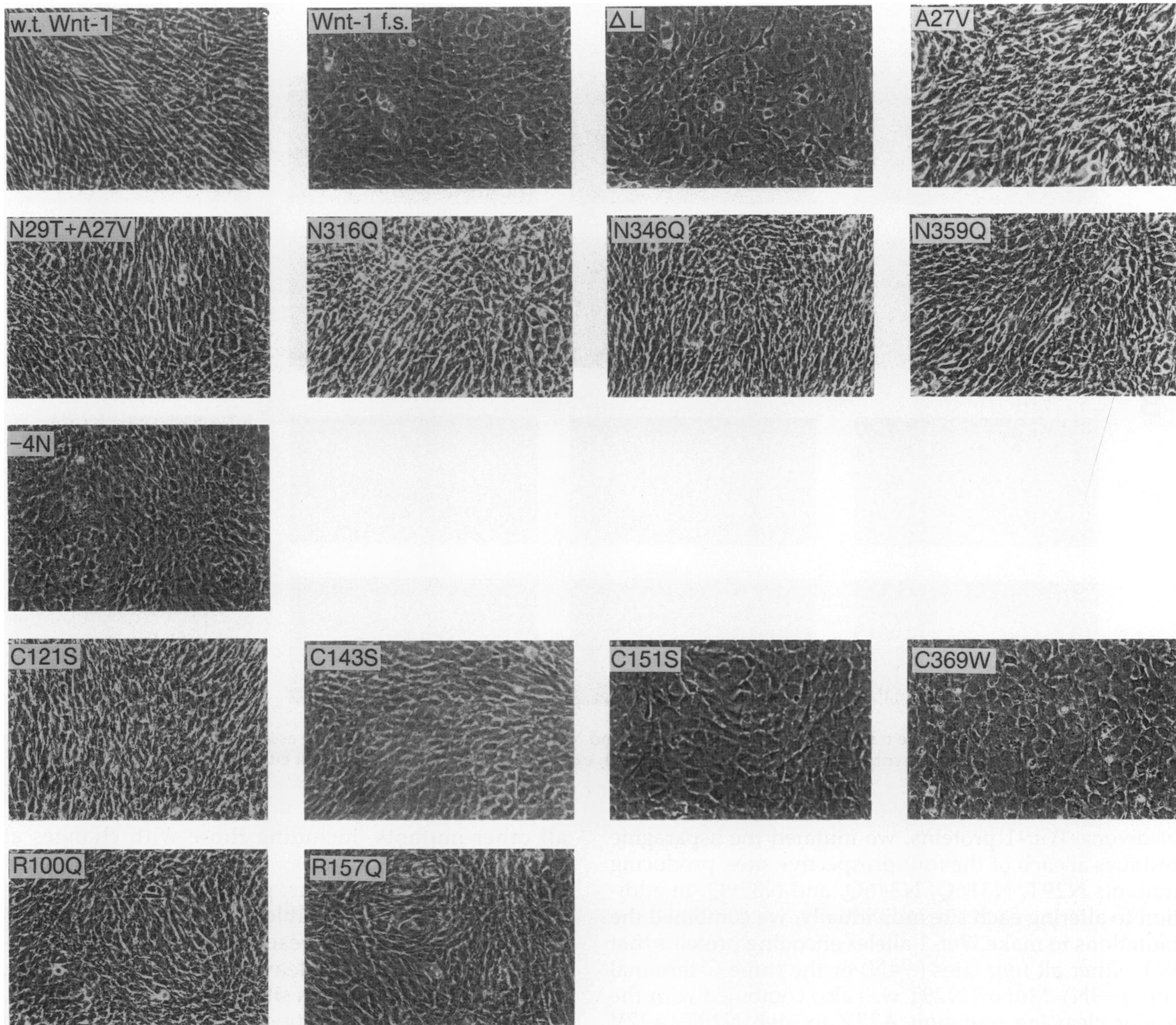

Figure 4. Paracrine transformation of C57MG cells by mutant *Wnt*-1 alleles. Pools of NIH3T3 cells expressing each mutant and control alleles were co-cultivated with an equal number of uninfected C57MG cells. After 24 h, serum-supplemented medium was replaced with the defined medium HB-CHO, and cells were grown for a further 2 d and then photographed.

the protein, the 40-kDa species, is now abundant, rather than two species. This observation could be readily explained if signal peptidase normally cleaves *Wnt*-1 protein after alanine 28 nearly as frequently as it cleaves at the favored position, alanine 27; then glycosylation would be prevented at asparagine 29 in a large fraction of *Wnt*-1 proteins, producing an abundant doublet of glycosylation products. This suggestion is strengthened by analysis of glycosylation mutants, including the double mutant N29T/A27V, described in the next section.

Mutations That Eliminate One or More Glycosylation Sites Retain Transforming Activity in the Autocrine Assay, Allow Identification of Sites for Efficient Glycosylation, and Provide One ts Mutant (N359Q). There are four potential N-linked glycosylation sites in *Wnt*-1 protein (Figure 1), and sequential, incomplete glycosylation at those positions is likely to account for the four or five species of *Wnt*-1 protein observed in cultured cells (Figures 2, 6, and 7; Papkoff *et al.*, 1987). To test the importance of glycosylation for the transforming activity of *Wnt*-1 and to assess its role in the generation

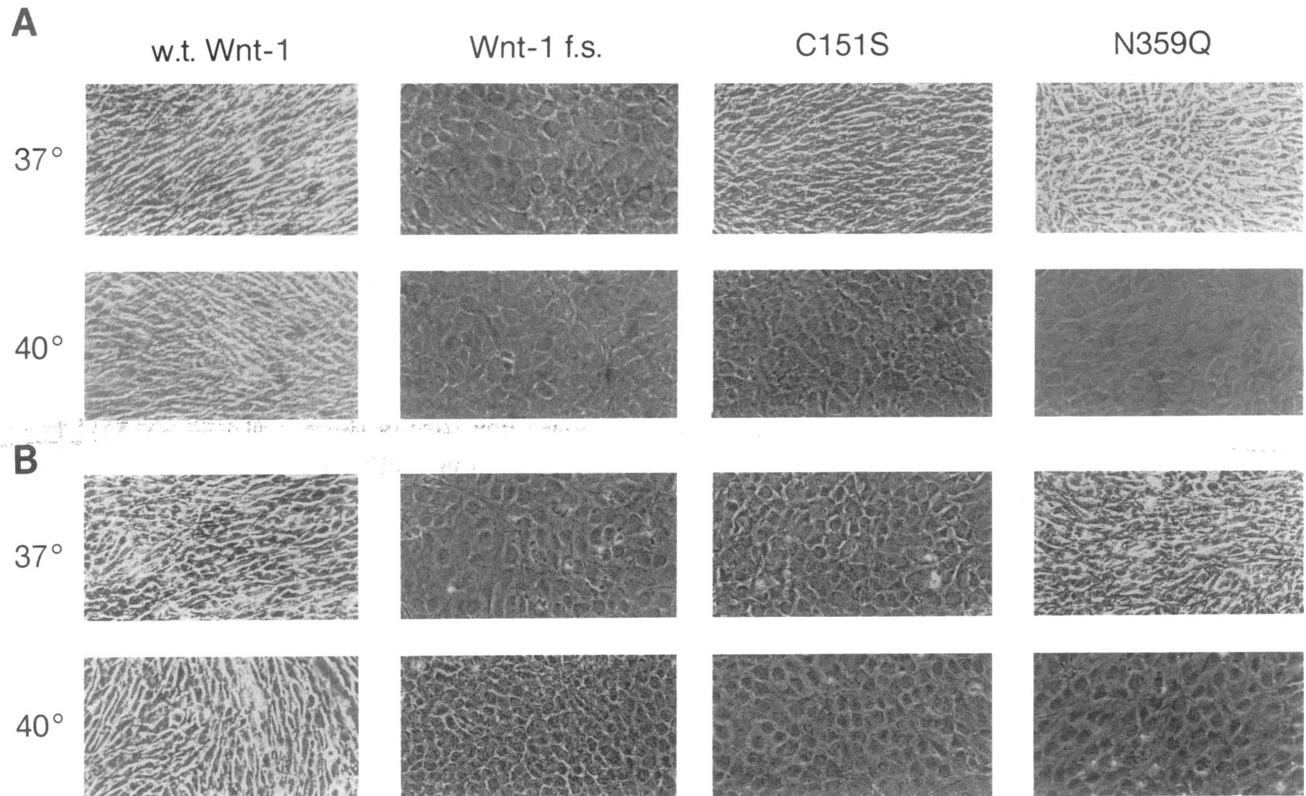

Figure 5. Temperature-sensitive transformation by alleles C151S and N359Q in autocrine and paracrine assays. Autocrine (A) and paracrine assays (B) were performed as described in legends to Figures 3 and 4, except that cells were maintained at either 37 or 40°C.

of diverse *Wnt*-1 proteins, we mutated the asparagine residues at each of the four prospective sites, producing mutants N29T, N316Q, N346Q, and N359Q. In addition to altering each site individually, we combined the mutations to make *Wnt*-1 alleles encoding proteins that lack either all four sites (−4N) or the three C-terminal sites (−3N). Mutant N29T was also combined with the leader cleavage mutation A27V to give N29T/A27V (Table 2).

1) N29T and N29T/A27V. N29T and N29T/A27V remove the glycosylation site near the amino terminus of *Wnt*-1 protein; the double mutant also eliminates the favored site for cleavage by signal peptidase (see above). The mutant proteins retain autocrine activity, as evidenced by their ability to transform C57MG cells (Figure 3). We were unsuccessful in obtaining infected NIH3T3 cells expressing detectable levels of protein from N29T, so we could not determine its paracrine activity directly; however, mutant alleles containing the N29T lesion in combination with other changes (e.g., N29T/A27V and −4N) are active in the paracrine assay. The predominant form of *Wnt*-1 protein in cells expressing N29T or N29T/A27V is a single species of 40 kDa (Figures 6 and 7). In contrast, wild-type *Wnt*-1 and all other mutants, including those with changes at other glycosylation sites, produce a predominant doublet of *Wnt*-1 proteins, provided that they retain N29. We believe the doublet to be caused by partial glycosylation at N29, a result of variation in the position of signal peptide cleavage, as discussed above. Loss of this glycosylation site eliminates the doublet.

2) N316Q. A glycosylation site at the position of the N316Q mutation is found in a large number of proteins encoded by *Wnt* family members in several species. Nevertheless, the mutant allele retains both autocrine and paracrine activities (Figures 3 and 4). As expected for a glycosylation site that is used on most or all molecules, the sizes of the major *Wnt*-1 proteins are reduced from 42 and 40 kDa to 40 and 38 kDa by the N316Q mutation (Figures 2, 6, and 7). Comparison of the intracellular and secreted proteins produced by this allele in C57MG cells suggests that they are more efficiently secreted than wild-type *Wnt*-1 proteins (Figure 6); alternatively, this difference may reflect increased stability of the extracellular mutant proteins or increased affinity for heparin. In addition, the major secretory products migrate atypically in polyacrylamide gels, with M_rs of ~39 and 41 kDa, rather then the expected 38 and 40 kDa (Figures 6B and 7B). We have not attempted to define the modifications responsible for these changes

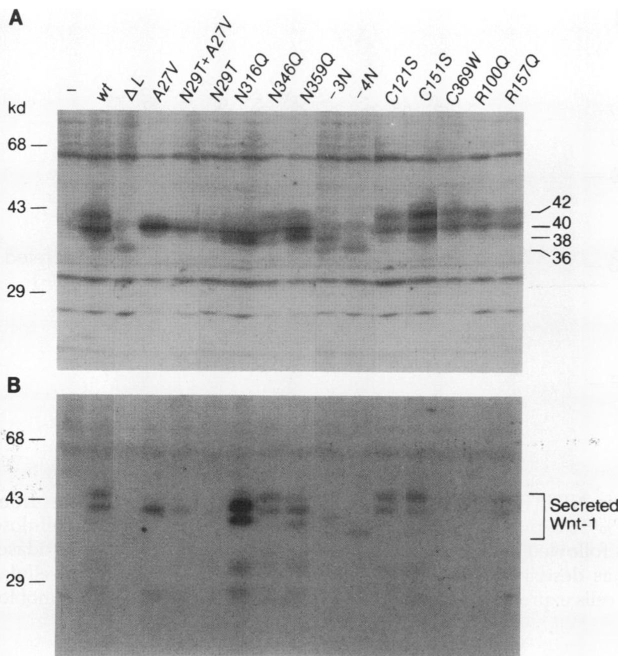

Figure 6. Immunoblots of mutant *Wnt*-1 proteins produced and secreted in C57MG cells. (A), intracellular proteins. Detergent lysates from C57MG cells expressing mutant *Wnt*-1 proteins, were fractionated on a 10% SDS-polyacrylamide gel and then transferred to a nitrocellulose filter. The filter was probed with anti-*Wnt*-1-peptide A monoclonal antibodies followed by [^{125}I]anti-mouse IgG antibodies as described in MATERIALS AND METHODS. (B), extracellular proteins. Heparin-bound proteins were collected from the media of C57MG cells expressing mutant *Wnt*-1 proteins and subjected to immunoblot analysis as described in Figure 6A.

in mobility, but note that they are unique to the secreted forms of the mutant proteins.

3) N346Q. N346Q is also active in both the autocrine and paracrine assays (Figures 3 and 4). Glycosylation must occur infrequently at the site of this mutation in wild-type *Wnt*-1 proteins, because the pattern of *Wnt*-1 proteins is indistinguishable from that of wild-type proteins when N346Q is expressed in QT6, C57MG, and NIH3T3 cells (Figures 2, 6, and 7). We speculate that occasional glycosylation at this site may be responsible for production of the 44-kDa species of *Wnt*-1 protein (Papkoff *et al.*, 1987). It is notable that N346 is bounded on either side by cysteine residues; if these are involved in disulfide bonds, access of glycosylation machinery to the site may be impeded.

4) N359Q. N359Q removes the most C-terminal of the glycosylation sites. The pattern of intracellular *Wnt*-1 proteins produced by this allele is similar to that produced by N316Q, suggesting that the site is normally fully glycosylated (Figures 2, 6, and 7). However, the proteins produced by this allele in C57MG cells include a 43-kDa species (Figure 6B) that presumably has been glycosylated at three sites, implying increased glycosylation at N346 in the N359Q background. Like the other glycosylation mutants, N359Q is active in the autocrine and paracrine assays at 37°C (Figures 3 and 4), but, unexpectedly, it is inactive in both assays at 40°C (Figure 5). The temperature sensitivity cannot, of course, represent differential glycosylation at this site at the two temperatures, because the site is unconditionally destroyed. Instead, the functional thermolability must be attributable either to the amino acid change at N359 or to the loss of a protective modification. This question might be further resolved by changing T361, thereby inactivating the glycosylation signal without altering N359. There are, however, precedents for a mutation that affects glycosylation nonconditionally and produces a heat-sensitive phenotype (Gibson *et al.*, 1979; Ng *et al.*, 1990).

5) −3N. −3N combines mutations N316Q, N346Q, and N359Q retaining only the glycosylation site at N29. This allele was active in the autocrine assay (Figure 3) but has not been adequately tested for paracrine activity. Surprisingly, despite the inclusion of the N359Q lesion, the −3N allele is not temperature sensitive when introduced into C57MG cells. Augmented secretion of *Wnt*-1 protein was also not observed, even though N316Q was included in the −3N allele.

6) −4N. −4N lacks all four N-linked glycosylation sites, yet it is active in both the autocrine and paracrine assays (Figures 3 and 4), providing the best evidence that glycosylation of *Wnt*-1 proteins is not required for biological function. Morphological transformation in paracrine assays with −4N was less complete than with wild-type *Wnt*-1, perhaps reflecting the lower levels of *Wnt*-1 protein produced by cells expressing this allele. As with −3N, transformation is not temperature sensitive, and secretion is not augmented. As expected, only a single 36-kDa species of *Wnt*-1 protein is found in cells expressing −4N (Figures 2, 6, and 7). This protein is probably identical to the product of ΔL, save for the four asparagine substitutions and different N-terminal residues; nevertheless, the ΔL protein is completely inactive, apparently because it is inappropriately located within the cytoplasm.

Most Mutations that Eliminate a Cysteine Residue Impair Transforming Activity in Both Autocrine and Paracrine Assays, and One (C151S) Creates Another ts Allele. Conservation of cysteine residues is a hallmark of the *Wnt* gene family: 22 of the 23 cysteines in *Wnt*-1 protein are encoded by most of the *Wnt* genes sequenced to date. In the absence of evidence for oligomerization of *Wnt* proteins, it is assumed that the cysteine residues are essential to ensure the proper folding and stability of the proteins (e.g., by formation of intramolecular disulfide bonds) or to facilitate interactions with *Wnt* receptor(s) or components of the extracellular matrix.

As a first step in analyzing the significance of cysteine residues in *Wnt*-1 protein, we examined the effects of altering individual cysteine residues (Figure 1, Table 3).

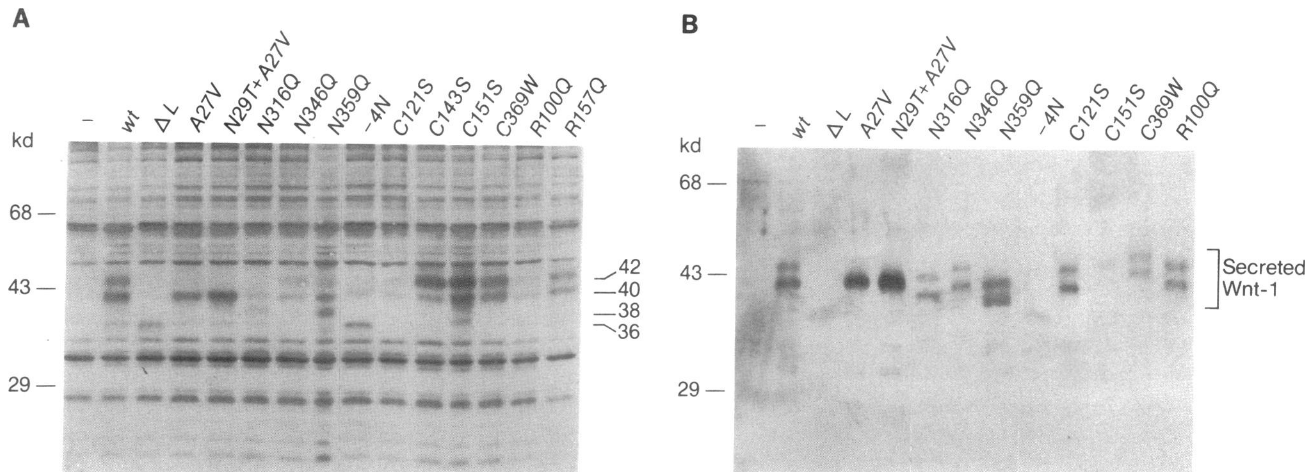

Figure 7. Immunoblots of mutant *Wnt*-1 proteins produced and secreted in NIH3T3 cells. (A), intracellular proteins. Detergent lysates from NIH3T3 cells expressing mutant *Wnt*-1 proteins were fractionated on a 10% SDS-polyacrylamide gel and then transferred to a nitrocellulose filter. The filter was probed with anti-*Wnt*-1-peptide monoclonal antibodies followed by anti-mouse IgG conjugated to horseradish peroxidase. Bound antibodies were detected by enhanced chemiluminescence (ECL) as described in MATERIALS AND METHODS. (B), extracellular proteins. Heparin-bound proteins were collected from the media of NIH3T3 cells expressing mutant *Wnt*-1 proteins and subjected to immunoblot analysis as described in Figure 7A.

1) C121S. C121S changes a cysteine residue found in *Wnt*-1 protein and its orthologs in other species (including the *wingless* protein, and *Wnt*-1 proteins from *Xenopus*, zebrafish, and man) but not in the other *Wnt* family proteins. The mutant is fully active in both autocrine and paracrine assays (Figures 3 and 4) thus failing to identify a function for this *Wnt*-1-specific residue.

2) C143S. C143S exchanges a universally conserved cysteine residue for serine, generating a protein that lacks transforming activity in both autocrine and paracrine assays (Figures 3 and 4), despite apparently normal levels of production in 3T3 cells (Figure 7A) and in C57MG cells (data not shown).

3) C151S. C151S also substitutes serine for a universally conserved cysteine but retains the ability to transform C57MG cells in the autocrine assays at 37°C (Figures 3 and 4). When cultures are grown at 40°C, however, the mutant fails to induce morphological transformation in the autocrine mode despite continued production and secretion of *Wnt*-1 protein; when C151S-expressing C57MG cells are shifted to 37°C or below, they reacquire a transformed phenotype. Thus this mutant, like N359Q, is temperature-sensitive for morphological transformation. However, unlike N359Q, the C151S allele is unable to transform C57MG cells in the paracrine assay at 35, 37, or 40°C. In the experiment shown in Figure 7, the C151S protein was poorly secreted; however, in two other experiments, it was secreted with approximately the same efficiency as wild-type protein. Remarkably, substitution of serine for cysteine also explains a temperature-sensitive mutant of *wingless* (R. Nusse, personal communication); in the *wingless* allele, however, the change is at position 104 (equivalent to position 107 in mouse *Wnt*-1).

4) C369W. C369W changes the penultimate residue in *Wnt*-1, another universally conserved cysteine, to the chemically unrelated residue, tryptophan; the same mutation has been previously reported to inactivate the ability of exogenous *Wnt*-1 to duplicate neural structures during *Xenopus* development (McMahon and Moon, 1989). As shown here, C369W is also inactive in transformation assays, both direct and paracrine (Figures 3 and 4). Moreover, secretion of the mutant protein from C57MG cells is impaired (Figures 6B and 7B), suggesting that the protein may fail to fold properly in the secretory pathway. Alternatively, the reduced levels of extracellular C369W proteins may reflect changes in the stability of the extracellular mutant proteins or their ability to bind heparin. Thus the C369W mutation differs from a potentially analogous mutation of a penultimate cysteine residue in another BiP-associated, secretory protein, the IgM heavy chain: the mutant heavy chain is hypersecreted and binds poorly to BiP (Sitia *et al.*, 1990), whereas the C369W *Wnt*-1 protein is poorly secreted and binds normally to BiP (Figures 2, 6, and 7). In 3T3 cells, C369W *Wnt*-1 proteins are secreted, but the glycosylation pattern of the extracellular forms is altered to more slowly migrating species, suggesting differences in the way the carbohydrate additions are processed.

Taken together, this group of mutations demonstrates the importance of at least some of the highly conserved cysteine residues. Three of the four mutations are partially or completely impaired in transformation assays; the only allele to retain full activity has lost a cysteine residue that is not conserved among other *Wnt* proteins. Although we do not know the function of the cysteine residues, precedents with cysteine mutants of PDGF-A (Mercola *et al.*, 1990) suggest that the mutants might

behave as dominant negative alleles, a possibility now being tested.

Mutations that Alter Possible Targets for Cleavage by Proteases (Other Than Signal Peptidase) Do Not Affect Transforming Activity or Protein Processing. The mouse *Wnt*-1 protein is predicted to contain one tribasic and three dibasic peptides (Figure 1), potential recognition sites for proteolytic cleavage by serine proteases. Although there is no direct evidence for cleavage at these sites, or at sites other than the signal peptidase recognition site, internal proteolytic processing is known to be required for the activation of other secretory proteins such as TGF-β (Wakefield *et al.*, 1988), insulin, and insulin-like growth factors (Blundell and Humble, 1980). Two mutations were therefore engineered at potential cleavage sites to address the possibility that an undetected product of proteolysis is responsible for the transforming activity of *Wnt*-1.

1) R100Q eliminates the dibasic peptide RR at positions 100–101, and 2) R157Q interrupts the tribasic peptide RRR at positions 156–158. Both mutant alleles are fully transforming in the autocrine and paracrine assays (Figures 3 and 4), without apparent change in the production, processing, or secretion of *Wnt*-1 proteins in any of the three cell types in which the mutants were tested (Figures 2, 6, and 7). We can detect small amounts of proteolytic fragments of *Wnt*-1 proteins, 28–35 kDa in size, in media from C57MG and 3T3 cells (Figures 6B and 7B). However, these species are also detectable in the media of C57MG cells expressing R100Q and R157Q. From these two mutants we draw the provisional conclusion that proteolytic processing at the two altered sites is not required to generate biologically active protein.

CONCLUDING REMARKS

The site-directed mutants of mouse *Wnt*-1 presented here are informative about several aspects of the conserved group of cysteine-rich, secretory proteins encoded by the *Wnt* gene family.

Synthesis and processing of *Wnt*-1 proteins are difficult to study in those settings in which *Wnt*-1 is normally expressed—restricted areas of the mid-gestational embryonic central nervous system and in round spermatids. By expressing wild-type and mutant alleles of *Wnt*-1 ectopically in cultured cell lines, we are beginning to assemble a coherent picture of the biogenesis of *Wnt*-1 proteins. A hydrophobic, aminoterminal signal sequence is essential for entry of nascent *Wnt*-1 protein into the ER and consequently for glycosylation and biological activity. Cleavage by signal peptidase can probably occur after either alanine 27 or alanine 28. There is no physical or biological evidence for further proteolysis (e.g., at the di- and tribasic peptides in the aminoterminal half of *Wnt*-1 protein).

The first available site for N-linked glycosylation, N29, is normally glycosylated on about one-half of the *Wnt*-1 translation products. Based on the behavior of mutant A27V, presumptive cleavage at position 28 inhibits glycosylation at N29, suggesting that the choice of cleavage site determines whether this glycosylation occurs. Normally all, or almost all, of the protein chains are glycosylated at two potential sites near the C-terminus: N316, the most highly conserved glycosylation site in the *Wnt* family, and N359. We suggest that only a few chains are glycosylated at the fourth site, N346, to account for the low level of the previously reported 44 kDa species (Papkoff *et al.*, 1987). No single glycosylation is required for biological activity or secretion, because mutant proteins lacking any one or all four sites are active in the autocrine and paracrine assays and secreted at levels detected by immunoblotting. One glycosylation site mutant (N359Q) is temperature-sensitive in biological assays, and another (N316Q) alters the mobility and secretory pattern of *Wnt* proteins. However, these properties should not necessarily be ascribed to loss of sugar modifications *per se*, so a functional role for glycosylation of *Wnt* proteins remains to be established.

The high proportion of conserved cysteine residues in *Wnt* proteins suggests that cysteines are critical determinants of the final structure of the protein, most likely through formation of intramolecular disulfide bonds. (Thus far there is no evidence for ordered oligomerization of Wnt proteins.) The importance of the cysteine residues is documented here by the detrimental effects of the loss of single cysteines upon *Wnt*-1 transforming activity: only one of four such mutants retains full activity, and the active mutant lacks the only cysteine that is not conserved throughout the *Wnt* family.

During its passage through the ER, *Wnt*-1 proteins are associated with BiP, a molecular chaperone that may regulate folding or secretion (Kitajewski *et al.*, 1992). However, our mutants have not shed much light on this interaction. Save for the mutant protein that fails to enter the ER (ΔL), all appear to associate normally with BiP, including those with abnormal patterns of secretion (N316Q and C369W).

The development of a paracrine assay for *Wnt*-1 demonstrates that the gene can act in a secretion-dependent manner (Jue *et al.*, 1992). However, in the direct assay, it is not certain whether *Wnt*-1 proteins are acting within C57MG cells, by contacting receptors during passage through the secretory apparatus or only on the cell surface after secretion. The question is important because it addresses whether there is any requirement for formation of complexes between *Wnt*-1 proteins and components of the extracellular matrix to generate an effective signal, as is known to be the case for other growth factors such as FGFs (Yayon *et al.*, 1991) and PDGF (La Rochelle *et al.*, 1991). In an effort to address this question more directly, we attempted to restrict *Wnt*-

1 proteins to the ER by adding six amino acids, including the ER retention signal KDEL, to the carboxy terminus of *Wnt*-1 protein. However, to our surprise, the KDEL mutant was active in both autocrine and paracrine assays and was secreted from C57MG and NIH3T3 cells as efficiently as wild-type protein. Additional mutants are currently being tested.

Receptors for *Wnt* proteins have not been identified, and it therefore remains uncertain whether they can respond to proteins encoded by one or more members of the *Wnt* gene family. Studies with *Xenopus* embryos indicate that at least two kinds of *Wnt* proteins, those encoded by X-*Wnt*-5A and X-*Wnt*-8, are likely to use different receptors (Olson *et al.*, 1991). In the mouse, *Wnt*-1 and *Wnt*-3 have been implicated in mammary tumorigenesis (Nusse *et al.*, 1984; Roelink *et al.*, 1990), but only *Wnt*-4, *Wnt*-5A, *Wnt*-5B, and *Wnt*-6 appear to be expressed in normal mammary tissue, and only *Wnt*-4 and *Wnt*-5A are expressed in the C57MG line used for our biological assays (Gavin *et al.*, 1992). Thus it is possible that we have been testing our mutants for their ability to stimulate heterologous receptors. In theory, mutant alleles could be tested for their ability to complement *Wnt*-1 deficiencies in genetically manipulated mice (McMahon and Bradley, 1990; Thomas and Capecchi, 1990), but this approach is impractical at present because we and others have been unable to target transgene expression specifically to *Wnt*-1 expressing cells (J.O. Mason, unpublished observations; A.P. McMahon, personal communication).

One of the most significant results of our analysis has been the isolation of two temperature-sensitive mutants of *Wnt*-1. We expect these conditional mutants to be useful for defining physiological responses to *Wnt*-1 and for devising strategies to identify *Wnt* receptors.

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