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A Soluble Form of Wnt-1 Protein with Mitogenic Activity on Mammary Epithelial Cells

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The proto-oncogene *Wnt*-1 plays an essential role in fetal brain development and causes hyperplasia and tumorigenesis when activated ectopically in the mouse mammary gland. When expressed in certain mammary epithelial cell lines, the gene causes morphological transformation and excess cell proliferation at confluence. Like other members of the mammalian *Wnt* family, *Wnt*-1 encodes secretory glycoproteins which have been detected in association with the extracellular matrix or cell surface but which have not previously been found in a soluble or biologically active form. We show here that conditioned medium harvested from a mammary cell line expressing *Wnt*-1 contains soluble Wnt-1 protein and induces mitogenesis and transformation of mammary target cells. By immunodepletion of medium containing epitope-tagged Wnt-1, we show that at least 60% of this activity is specifically dependent on Wnt-1 protein. These results provide the first demonstration that a mammalian Wnt protein can act as a diffusible extracellular signaling factor.

The *Wnt* gene family constitutes a set of 15 or more related genes that play important roles in both tumorigenesis and embryonic development (28, 32). *Wnt* genes have been highly conserved in evolution, and examples have been described in a wide variety of both vertebrate and invertebrate species (19, 28, 37, 39, 54). Vertebrate *Wnt* genes are implicated in a wide variety of crucial developmental processes, including early mesodermal patterning, brain morphogenesis, somite formation, limb bud development, nephrogenesis in the kidney, and regulation of mammary gland proliferation (13, 24, 28, 32, 42, 44, 51, 56). In mice, at least 10 different *Wnt* genes have so far been characterized and have been shown to encode secreted proteins that are believed to act in cell-cell communication (1, 3, 11, 30, 40). However, these proteins have proved difficult to isolate in soluble or biologically active form, and many details of their mechanism of action have therefore remained obscure.

The best-characterized mammalian *Wnt* gene is mouse *Wnt*-1, which was first identified as a proto-oncogene activated in mammary carcinomas induced by the mouse mammary tumor virus (26). In a majority of these tumors, insertions of mouse mammary tumor virus proviral DNA at the *Wnt*-1 locus activate expression of wild-type Wnt-1 protein, which is not normally expressed in mammary tissue (17, 27). The consequences of this have been duplicated in transgenic mice designed to express *Wnt*-1 in mammary tissue: both male and female transgenic mice rapidly develop mammary hyperplasia and subsequently adenocarcinoma (46). In addition, expression of *Wnt*-1 in tissue culture cells has shown that it causes transformation and deregulated growth of the mammary epithelial cell lines C57MG and RAC311 (7, 36). The transformed phenotype is characterized by an elongated and refractile cell morphology, apparent loss of contact inhibition at confluence, and growth to higher cell densities (7, 36). In fibroblastic cell lines, however, the transforming potential of *Wnt*-1 is very weak, and in many cases, it has no obvious phenotypic effect (2, 7, 15). In normal mouse tissues, *Wnt*-1 is expressed only in the embryonic neural tube during mid-gestation and in spermatids of the adult testis (38, 52). The significance of *Wnt*-1 expression in the testis is unclear, but targeted gene disruption experiments have demonstrated that the gene is essential for correct fetal development of the midbrain and cerebellum (23, 45). Recent experiments have suggested that *Wnt*-1 may exert a mitogenic effect on neuroectodermal cells in vivo (9).

The homolog of *Wnt*-1 in *Drosophila melanogaster* is the segment polarity gene *wingless*, a gene required for correct cellular patterning within the embryo and imaginal discs (8, 18, 35, 43). In the embryonic ectoderm, *wingless* is expressed in narrow stripes of epidermal cells at the posterior boundary of each parasegment, where it functions to modulate the phenotype and developmental fate of neighboring cells (18, 33). *wingless* encodes a protein that is 54% identical to Wnt-1 (35), and like *Wnt*-1, the gene is capable of causing transformation of mouse mammary cells (34). In *Drosophila* embryos, extracellular Wingless protein has been detected by immunoelectron microscopy (12, 49), and secretion of the protein has recently been demonstrated in cell culture systems (34, 48, 50).

The protein products of mouse *Wnt*-1 are cysteine-rich glycoproteins with a molecular mass of 36 to 44 kDa which undergo cleavage of an amino-terminal signal peptide and enter the secretory pathway (5, 29). In cell lines expressing exogenous *Wnt*-1, much of the protein remains within the endoplasmic reticulum or Golgi apparatus, but low-abundance secreted forms of Wnt-1 have also been described (3, 17, 30). These have an apparent molecular mass of 41 to 44 kDa. Rather than accumulating in the cell culture medium, the extracellular forms of Wnt-1 have mostly been found associated with cell surfaces or the extracellular matrix (ECM) (3, 30). Except when anionic compounds such as heparin or suramin are used to displace the protein from these sites, little or no Wnt-1 has been detected in cell culture supernatants (3, 30).

The secretory nature of Wnt-1 and other mammalian Wnt proteins has clearly suggested that they function as extracellular signaling molecules. Strong support for this model has also been provided by coculture experiments which exploit the target cell specificity of *Wnt*-1 transformation (15). These experiments show that Rat-2 fibroblasts expressing *Wnt*-1, although not themselves transformed, are able to elicit morphological

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transformation of neighboring C57MG mammary cells (15). This indicates that the *Wnt*-1 gene can mediate transformation via a paracrine mechanism. However, definitive evidence that Wnt-1 protein itself acts as an extracellular signaling factor has been lacking, largely because none of the Wnt-1 protein products has yet been shown to have biological activity.

In this report, we have used *Wnt*-responsive mammary epithelial cell lines to develop an assay for biologically active Wnt-1 protein. While no activity was detected in conditioned medium (CM) from Rat-2 fibroblasts expressing *Wnt*-1, mitogenic activity was detected in medium harvested from C57MG mammary cells expressing *Wnt*-1 cDNA. When applied to quiescent cultures of C57MG or RAC311 target cells, this medium induced transformed cell morphologies and stimulated DNA synthesis. We show that this mitogenic activity is dependent on soluble Wnt-1 protein secreted by C57MG/*Wnt*-1 cells and hence that Wnt-1 can act as a diffusible extracellular factor.

MATERIALS AND METHODS

Cell lines and retroviral vectors. Rat-2 fibroblasts (American Type Culture Collection, Rockville, Md.) and E86 retroviral packaging cells (20) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics. C57MG and RAC311c mammary epithelial cells (41, 47) were maintained in the medium described above with the addition of 10 μg of insulin per ml (Sigma).

The retroviral vector pMV*Wnt*-1 contains mouse *Wnt*-1 cDNA expressed from the viral long terminal repeat, together with a bacterial neomycin phosphotransferase gene (*neo*) expressed from an internal promoter (15). pMV7, the parental vector, expresses *neo* alone (16). pMVWHA is similar to pMV*Wnt*-1, except that the *Wnt*-1 coding region contains 18 amino acids added after the carboxy-terminal leucine residue (tagged allele kindly provided by J. Kitajewski). The sequence of additional amino acids is (L)SMAYPYDVPDYASLGPGP, which includes a nine-amino-acid epitope from influenza virus hemagglutinin (HA) (underlined [53]). Helper-free virus stocks were generated from the plasmids described above by transfection of E86 packaging cells and were used to infect Rat-2 or C57MG cells according to procedures previously described (6, 15). Approximately 50 to 100 G418-resistant colonies were pooled to generate the infected cell lines used in these experiments. Rat-2 and C57MG cells infected with MV*Wnt*-1 virus are designated Rat-2/*Wnt*-1 and C57MG/*Wnt*-1, respectively.

Mitogenesis assays. To obtain CM from cells infected with MV*Wnt*-1 or MV7, recently infected cells were plated at a density of 10^5/10-cm-diameter dish and grown to confluence (approximately 6 days), at which time the CM was harvested, centrifuged for 10 min at 2,000 × *g* to remove debris, and frozen at −80°C until used. For mitogenesis assays, C57MG or RAC311 target cells were plated at 10^5 cells per 6-cm-diameter dish and allowed to grow for 6 days or until confluent, at which time the cell culture medium was removed and replaced with 2 ml of CM to be tested. At the same time, [^{3}H]thymidine was added to the CM at 10 μCi/ml. After 24 h of labeling, the cells were rinsed in phosphate-buffered saline, fixed in 3.7% formaldehyde for 10 min, and washed sequentially with 5% trichloroacetic acid, distilled water, and ethanol, all at 4°C. For autoradiography, dishes were dried, coated with Kodak NTB2 photographic emulsion, and left in the dark for 2 days, after which the emulsion was developed in Kodak D19 developer. The average number of cells undergoing DNA replication was determined from counting the total number of labeled nuclei in 10 random fields from dishes treated in parallel. Where indicated, 200 μg of heparin (porcine intestinal mucosa [Sigma]) per ml was added to the CM at the time of transfer to the target cell monolayer. Also where indicated, CM was protease treated by incubation at 37°C for 20 min in 0.01% trypsin (Sigma), followed by inactivation with 0.01% soybean trypsin inhibitor (Sigma), prior to addition of the medium to target cells. To fractionate CM into soluble and insoluble components, samples precleared by centrifugation at 1000 × *g* for 5 min were spun at 100,000 × *g* for 3 h at 4°C. Supernatants were decanted, and pellet fractions were resuspended in DMEM.

Immunodepletion of C57MG/*Wnt*-1 CM. For immunodepletion of C57MG/*Wnt*-1HA CM, 25 μl of mouse 12CA5 anti-HA ascites fluid (Berkeley Antibody Co., Richmond, Calif.) was preincubated with 400 ml of protein A-Sepharose at 4°C for 30 min, after which the beads were washed twice in serum-free DMEM by brief centrifugation. Beads were then resuspended in 5 ml of CM and incubated at 4°C with mixing. After 2 h, the beads were removed and replaced with a fresh aliquot of antibody-bound protein A-Sepharose. After three rounds of immunodepletion, the CM was removed and tested for mitogenic activity. Where indicated, antibody was blocked by preincubation for 15 min with 1 mg of HA peptide per ml prior to addition of protein A-Sepharose.

Immunoblot analysis of Wnt-1. For immunoblot analysis, C57MG CM was brought to a concentration of 1× Laemmli sample buffer, boiled for 3 min, and analyzed on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel. After electrophoresis, proteins were transferred to nitrocellulose by electroblotting, and the blot was incubated overnight at 4°C in blocking buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.05% Tween 20, 1% bovine serum albumin [BSA]). Mouse monoclonal antibody against Wnt-1 peptide A (5) (hybridoma supernatant) was diluted 1:10 in blocking buffer without BSA and incubated with the blot for 12 h at 4°C. The blot was then washed in blocking buffer for 30 min with three changes and incubated for 1 h at 4°C in a 1:5,000 dilution of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Amersham, Arlington Heights, Ill.), followed by a second wash. The blots were then incubated for 1 min in enhanced chemiluminescence detection reagents (Amersham) and exposed to X-ray film for 10 min.

RESULTS

CM from Rat-2 cells expressing *Wnt*-1 shows no specific mitogenic activity. Cell cocultivation experiments have previously shown that Rat-2 fibroblasts expressing *Wnt*-1 are able to induce transformation of C57MG mammary cells in a paracrine manner, suggesting that they produce Wnt-1 proteins in a functional form (15). Initially, therefore, we sought to identify functional Wnt-1 protein in cultures of Rat-2/*Wnt*-1 cells. We have previously shown that these cells secrete 44- and 41-kDa forms of Wnt-1, which are mostly found associated with the ECM, although small amounts are detectable in the cell culture medium (reference 15 and unpublished data). To test CM for Wnt-related biological activity, we devised an assay based on the transforming effects of the *Wnt*-1 gene when expressed in C57MG mammary cells. If this transformation is due to autocrine actions of Wnt-1 protein, we reasoned that exogenous Wnt-1 protein applied to quiescent C57MG cells would induce mitogenesis and morphological transformation.

Rat-2/*Wnt*-1 cells, generated by infection with the retrovirus vector MV*Wnt*-1, and control Rat-2/MV7 cells infected with vector alone were grown to confluence, and their CM was harvested for mitogenesis assays. CM was applied to confluent and quiescent monolayers of C57MG mammary epithelial cells for 24 h, during which time the cells were labeled with [^{3}H]thymidine. Cultures were then processed for in situ autoradiography, and the numbers of labeled nuclei per unit area were determined. As shown in Fig. 1, treatment of the target cells with CM from Rat-2/*Wnt*-1 cells produced no significant mitogenic effect relative to control dishes treated with Rat-2/MV7 CM. In contrast, C57MG cells treated in parallel with fresh DMEM containing 10% FCS showed a significant increase in labeled nuclei, indicating that the target cells were competent to enter S phase (Fig. 1). Similar assays in which C57MG cells were plated on ECM fractions of Rat-2/*Wnt*-1 cultures also yielded negative results (data not shown). Therefore, despite the ability of Rat-2/*Wnt*-1 cells to cause paracrine transformation of C57MG cells in coculture experiments, we were unable to demonstrate Wnt-1-dependent mitogenic activity produced by these cells.

CM from C57MG cells expressing *Wnt*-1 contains mitogenic activity. In view of the inability to detect mitogenic activity associated with Wnt-1 protein secreted from Rat-2 cells, we next attempted similar experiments with C57MG mammary cells infected with MV*Wnt*-1 as a source of CM. Medium was harvested from confluent cultures of both C57MG/*Wnt*-1 and C57MG/MV7 cells and tested for biological activity as described above, with uninfected C57MG cells as targets. Unlike the negative results described above, when quiescent C57MG cells were incubated with CM harvested from C57MG/*Wnt*-1 cells, morphological transformation was observed within 24 h (Fig. 2B). The cells became more refractile and showed elongated morphology at high magnification. In contrast, C57MG cells treated with CM from C57MG/MV7 control cells remained morphologically unchanged (Fig. 2A). Autoradiogra-

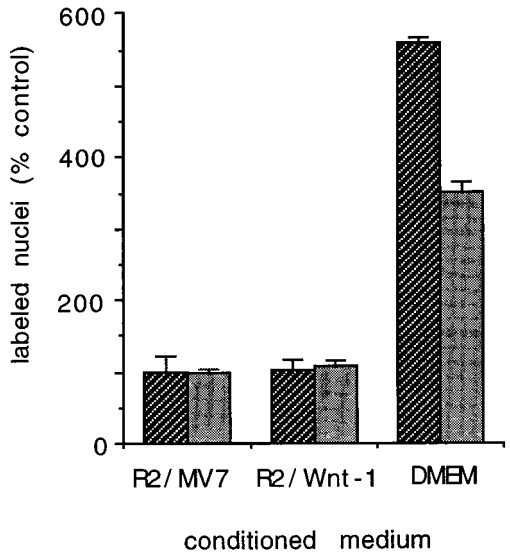

FIG. 1. CM from Rat-2 cells expressing *Wnt*-1 is not mitogenic for C57MG mammary cells. CM harvested from Rat-2 fibroblasts infected with control retrovirus vector MV7 (R2/MV7) or with a similar vector expressing *Wnt*-1 (R2/Wnt-1) was applied to quiescent monolayers of C57MG cells for 24 h in the presence of [^{3}H]thymidine. Cultures were then analyzed by in situ autoradiography. The mean number of labeled nuclei per unit area of cells treated with Rat-2/*Wnt*-1 CM is expressed as a percentage relative to cells treated with Rat-2/MV7 CM. As a positive control for mitogenesis, cells were treated with fresh DMEM containing 10% FCS (DMEM). The results shown are from two independent experiments.

phy of cells labeled with [^{3}H]thymidine during this period showed many more labeled nuclei in dishes receiving C57MG/*Wnt*-1 CM than in dishes treated with CM from control cultures (Fig. 2C and D). CM from C57MG/*Wnt*-1 cells is thus mitogenic for C57MG cells. Quantitation of this effect in several experiments gave values similar to those obtained from treating the cells with fresh 10% serum (Fig. 3). In the experiments shown in Fig. 3, there was a threefold stimulation above the negative control, but in later experiments four- to sixfold more labeled nuclei were typically observed (see Fig. 5 to 7 below).

C57MG/*Wnt*-1 cells secrete soluble Wnt-1 protein. Previous studies were not able to demonstrate Wnt-1 protein in the medium of C57MG/*Wnt*-1 cells unless the cells were grown in the presence of either of the anionic compounds heparin and suramin (3, 21, 30), which themselves inhibit transformation mediated by *Wnt*-1 (15). Having observed that C57MG/*Wnt*-1 cells produce mitogenic activity when grown in the absence of these compounds, we wished to determine whether the CM from these cells contained detectable levels of Wnt-1 protein. By immunoblot analysis with enhanced chemiluminescence reagents for improved sensitivity, we were able to detect secreted Wnt-1 proteins with molecular masses of 44 and 41 kDa in the CM from C57MG/*Wnt*-1 cells (Fig. 4A). The electrophoretic mobilities of these proteins are similar to those reported for secreted Wnt-1 proteins in other cell lines (3, 15). As expected, the two proteins were not detected in CM from C57MG/MV7 control cells (Fig. 4A). To verify that the Wnt-1 proteins in

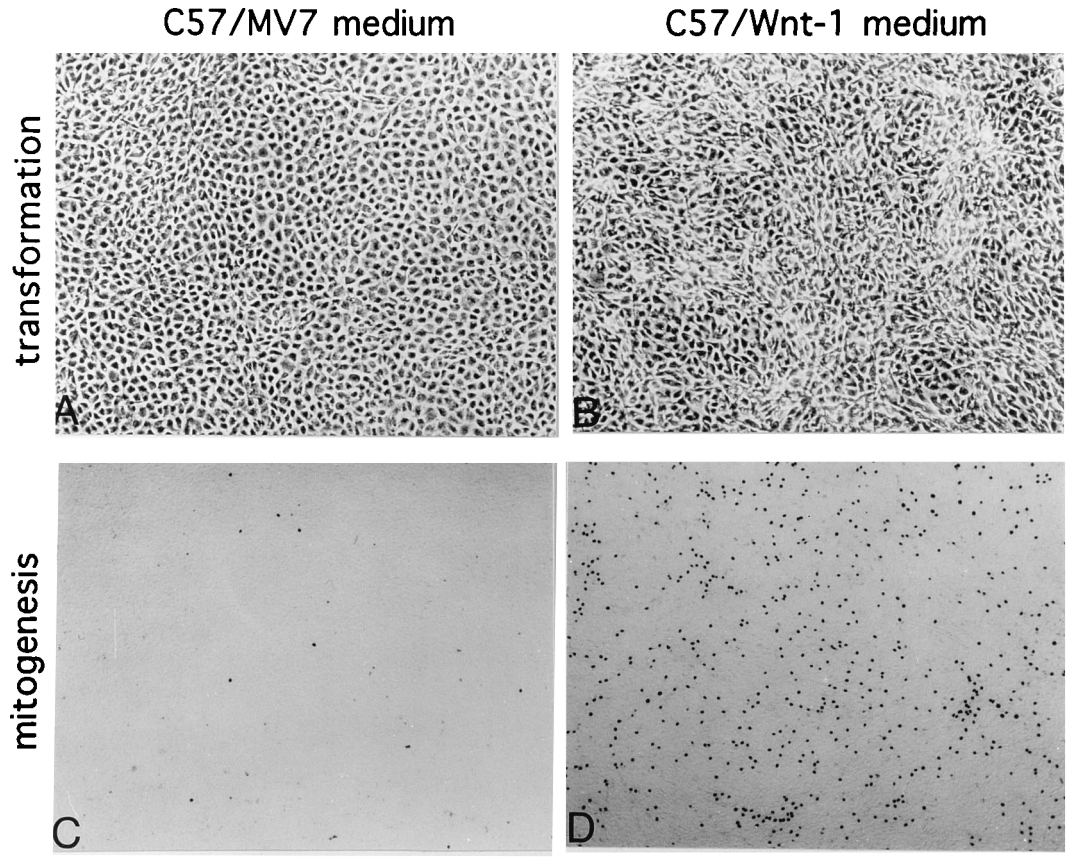

FIG. 2. CM from mammary cells expressing *Wnt*-1 induces transformation and mitogenesis of C57MG mammary cells. Quiescent monolayers of C57MG cells were incubated with CM harvested from C57MG/*Wnt*-1 cells (B and D) or from control C57MG/MV7 cells (A and C), and [^{3}H]thymidine was added to label cells undergoing DNA synthesis. After 24 h, cultures treated with medium from *Wnt*-1-expressing cells had a refractile and transformed morphology (B), and autoradiography revealed areas with increased numbers of [^{3}H]labeled nuclei (D).

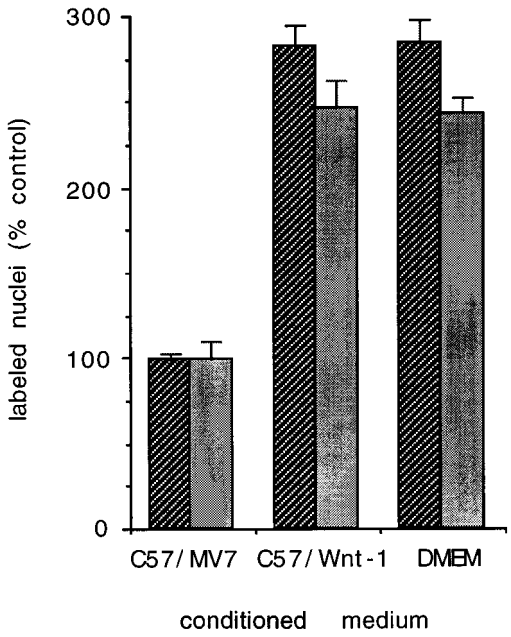

FIG. 3. Quantification of the mitogenic effect of C57MG/*Wnt*-1 CM on quiescent C57MG monolayers. Mitogenesis assays were performed as described in the legend to Fig. 2 with medium harvested from C57MG cells expressing vector alone (C57/MV7) or expressing *Wnt*-1 (C57/Wnt-1). After autoradiography, labeled nuclei were counted and the mean number per unit area was expressed relative to the number in negative control dishes. As a positive control, cells were treated in parallel with fresh medium (DMEM) containing 10% FCS. Results shown are from two independent experiments.

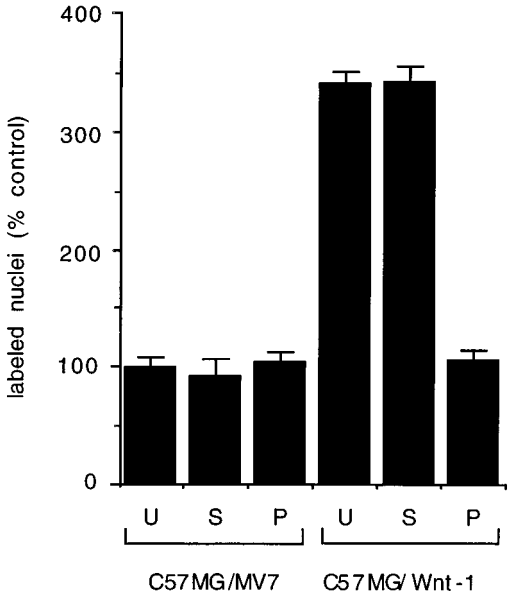

FIG. 5. Mitogenic activity of C57MG/*Wnt*-1 CM is found in the supernatant fraction after high-speed centrifugation. CM from C57MG/MV7 and C57MG/*Wnt*-1 cells was centrifuged at 100,000 × *g* (as described in the legend to Fig. 4). The unfractionated media (U), supernatant fractions (S), or pellets resuspended in serum-free DMEM (P) were assayed for mitogenic activity on quiescent mammary epithelial cell monolayers as described in the legends to Fig. 1 and 2. The numbers of labeled nuclei per unit area are expressed relative to the number observed in dishes receiving C57MG/MV7 CM. While resuspended pellet fractions were not significantly mitogenic, the supernatant fraction of C57MG/*Wnt*-1 CM had mitogenic activity comparable to that from unfractionated CM, indicating that the activity is attributable to soluble factors.

C57/*Wnt*-1 CM are indeed soluble, we centrifuged the CM at 100,000 × *g* to remove ECM components and other partially soluble material. Immunoblot analysis showed approximately 50% of the Wnt-1 protein in the supernatant fraction after this treatment, indicating that a significant proportion of the protein is soluble by these criteria (Fig. 4B). Mitogenesis assays performed as described above showed that at least 90% of the activity detected in C57/*Wnt*-1 CM is associated with the soluble fraction (Fig. 5).

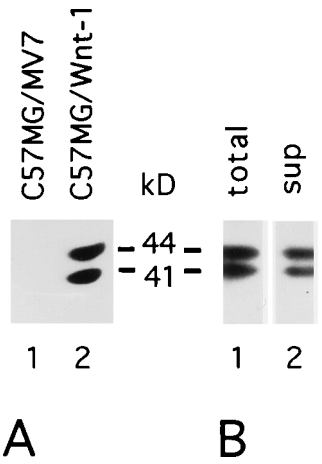

FIG. 4. Medium conditioned by C57MG/*Wnt*-1 cells contains soluble Wnt-1 proteins. (A) CM from C57MG/MV7 cells (lane 1) and C57MG/*Wnt*-1 cells (lane 2) was analyzed by immunoblotting with a monoclonal antibody against Wnt-1. Secreted Wnt-1 proteins with molecular masses of 44 and 41 kDa were detected in the medium from C57MG/*Wnt*-1 cells (lane 2). (B) Secreted Wnt-1 proteins remain soluble after high-speed centrifugation. CM from C57MG/*Wnt*-1 cells was analyzed by immunoblotting before (lane 1) and after (lane 2) centrifugation at 100,000 × *g*. Approximately 50% of the Wnt-1 protein remained in the supernatant (sup) after this treatment.

Mitogenic activity of C57MG/*Wnt*-1 CM is protease sensitive and inhibited by heparin. C57MG/*Wnt*-1 cells are morphologically transformed and exhibit altered growth parameters relative to control cells (7). Although soluble Wnt-1 protein was detected in the medium of these cells, it is possible that other growth factors might be secreted as a secondary consequence of transformation. To investigate whether the mitogenic activity in C57MG/*Wnt*-1 CM might be due to Wnt-1 protein rather than a secondary factor released from the cells, we first tested whether the activity had properties consistent with Wnt-1. Trypsin treatment of the CM showed that the mitogenic activity was protease sensitive, establishing that it is dependent on protein factors (Fig. 6). In addition, the activity in C57MG/*Wnt*-1 CM was completely abolished when mitogenesis assays were performed in the presence of heparin (Fig. 6). Since heparin binds secreted Wnt-1 and is known to suppress *Wnt*-1-mediated transformation (3, 15), this result was compatible with involvement of Wnt-1 protein in the mitogenic activity of C57MG/*Wnt*-1 CM. The target cell specificity of the activity was also consistent with this notion, since the CM was mitogenic for both C57MG and RAC311 cells, two lines that can be transformed by expression of the *Wnt*-1 gene (7, 36 [Fig. 3 and 7]), but had no significant effect on NIH 3T3 fibroblasts or MOMMA mammary epithelial cells, neither of which show obvious morphological effects in response to *Wnt*-1 expression (references 7 and 15 and data not shown). Thus, the mitogenic activity has properties consistent with those predicted for Wnt-1 protein.

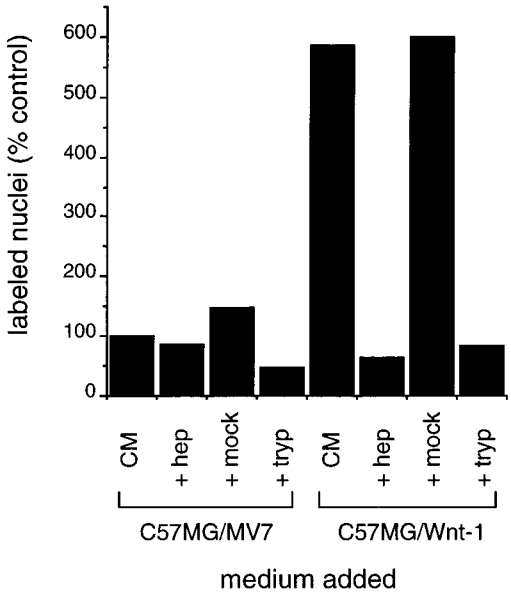

FIG. 6. Mitogenic activity in CM from C57MG/*Wnt*-1 cells is abolished by protease treatment or addition of heparin. CM harvested from C57MG/MV7 or C57MG/*Wnt*-1 cells was assayed for mitogenesis on C57MG monolayers as described above. Treatment of CM with 0.01% trypsin (+ tryp) destroyed the mitogenic activity, while incubation in the absence of enzyme (+ mock) had no effect. Addition of 200 μg of heparin per ml (+ hep) to C57MG/*Wnt*-1 CM completely abolished the mitogenic effect, as would be predicted for an activity dependent on Wnt-1 protein.

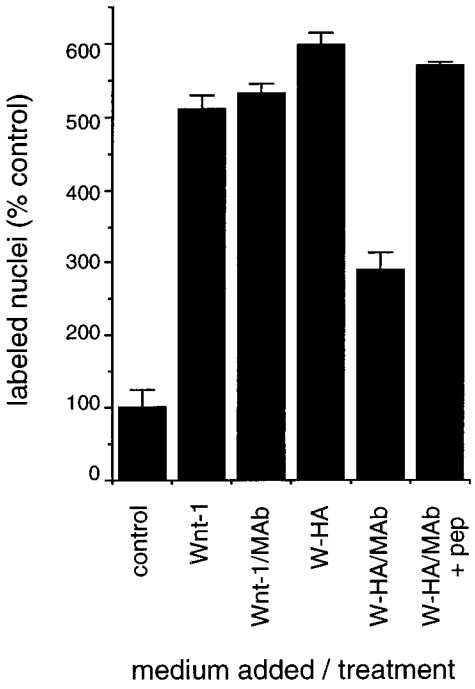

FIG. 7. Mitogenic activity is diminished by immunodepletion with antibodies specific for an epitope-tagged Wnt-1 protein. CM samples from C57MG cells expressing wild-type *Wnt*-1 or Wnt-1 tagged with HA epitope (W-HA), were assayed for mitogenic activity on quiescent RAC311 cells. CM from cells expressing W-HA showed activity comparable to those expressing wild-type Wnt-1 protein. However, when immunodepleted by incubation with immobilized anti-HA antibody, the activity of CM from W-HA-expressing cells was reduced by approximately 60% (W-HA/MAb). No reduction was observed when the antibody was preincubated with 500 ng of HA peptide per ml (W-HA/MAb + pep). Moreover, anti-HA antibody had no significant effect on CM from cells expressing wild-type Wnt-1 (Wnt-1/MAb). The specificity of immunodepletion indicates that at least 60% of the activity in W-HA CM is dependent on Wnt-1 protein. MAb, monoclonal antibody.

Mitogenic activity is diminished by immunodepletion of Wnt-1 protein. To determine directly whether mitogenic activity was dependent on soluble Wnt-1 protein, we performed immunodepletion of CM samples. For these experiments, we made use of an epitope-tagged allele of *Wnt*-1, designated *Wnt*-1HA, whose protein product carries an influenza HA epitope at its carboxy terminus. The secretory properties of this protein are similar to those of wild-type Wnt-1 (unpublished data). We first verified that an MV7-based retrovirus vector carrying *Wnt*-1HA was fully competent to transform C57MG cells and that CM harvested from such infected cells showed mitogenic activity comparable to that obtained from expression of wild-type *Wnt*-1 (Fig. 7). Immobilized monoclonal antibody for the HA epitope was then used to immunodeplete the CM samples. As shown in Fig. 7, approximately 60% of the mitogenic activity was consistently removed from C57MG/*Wnt*-1HA CM by this treatment. Depletion was prevented by preincubating the antibody with HA peptide. Most importantly, however, the anti-HA antibody caused no significant depletion of mitogenic activity in the CM from C57MG/*Wnt*-1 cells expressing the untagged protein (Fig. 7). The specificity of this immunodepletion indicates that at least 60% of the mitogenic activity detected in CM from C57MG cells expressing *Wnt*-1 is dependent on soluble Wnt-1 protein. We therefore conclude that under these circumstances, mouse Wnt-1 protein can act as a soluble extracellular signaling factor.

DISCUSSION

A long-standing difficulty in studying the mechanism of action of mammalian *Wnt* genes has been the inability to isolate their protein products in soluble form or to identify a fraction of Wnt protein that has demonstrable functional activity. In this study, we have developed a functional assay for mouse Wnt-1 protein based on the transforming effects of *Wnt*-1 in certain mammary epithelial cell lines and have used this to identify a secreted form of Wnt-1 that is biologically active. Our results provide the first demonstration that a mammalian Wnt protein can act externally on target cells as a soluble secreted factor and imply that Wnt-1 protein has the potential to act in either an autocrine or paracrine manner. In view of the similar structural and physical properties among different members of the *Wnt* family (28), it is very likely that other Wnt proteins will also be active in soluble form.

When applied to confluent cultures of C57MG and RAC311 mammary epithelial cells, CM containing soluble Wnt-1 protein induced continued cell division and a refractile and elongated cell morphology. These effects closely resemble the partial transformation observed upon expression of *Wnt*-1 in these cell lines via DNA transfection or retroviral infection (7, 36). Several other observations were consistent with a role for Wnt-1 protein in the observed activity: the mitogenicity was protease sensitive, was inhibited by heparin, and showed a target cell specificity similar to that of transformation mediated by expression of the *Wnt*-1 gene. Thus far, however, we have detected this activity only in medium conditioned by C57MG/*Wnt*-1 cells. Since these producer cells are themselves transformed while the control C57MG/MV7 cells are not, it was crucial to establish that mitogenic activity was specifically as-

sociated with soluble Wnt-1 protein secreted by these cells. This was confirmed by immunodepletion experiments in which more than 60% of the mitogenic activity resulting from expression of an epitope-tagged *Wnt*-1 allele was removed by incubation with a monoclonal antibody specific for the epitope. Since the antibody had no depleting effect on CM from cells expressing untagged Wnt-1, these results strongly argue that the majority of the mitogenic activity measured by these assays is directly dependent on soluble Wnt-1. The inability to deplete 100% of the activity may be due to either incomplete removal of the Wnt-1 protein or, potentially, the presence of additional heparin-sensitive mitogens released by the cells as a secondary consequence of *Wnt*-1 expression. Although the latter cannot be excluded, we currently favor the former explanation, since immunoblot analysis detects significant residual amounts of Wnt-1HA protein in the CM after immunodepletion (unpublished data). It seems likely that the antibody-antigen interaction does not go to completion under these conditions, even when high antibody concentrations are employed.

A surprising aspect of our results was the inability to demonstrate Wnt-1-dependent mitogenic activity in CM from Rat-2 fibroblasts expressing *Wnt*-1. Coculture experiments show that these cells can induce paracrine transformation of C57MG cells and that this phenomenon is dependent on their expressing *Wnt*-1 (15). Moreover, CM from Rat-2/*Wnt*-1 cells contains at least as much secreted Wnt-1 protein as C57MG/*Wnt*-1 CM, and the proteins show similar electrophoretic mobilities in SDS-polyacrylamide gels (unpublished data). One model to explain these observations would be that C57MG cells, but not Rat-2 cells, produce an accessory molecule which is required for full activity of secreted Wnt-1 protein. This might work by facilitating the binding of Wnt-1 to specific receptors (22, 55) or possibly by preventing the protein from binding to ECM components which may inactivate it. We are currently seeking direct evidence of a possible accessory factor in order to reconcile the negative results for Rat-2/*Wnt*-1 cells presented here with the ability of these cells to function in paracrine transformation assays (15). In support of this model, preliminary results suggest that mitogenic activity can be detected in CM from Rat-2/*Wnt*-1 cells when the cells are grown in medium previously conditioned by C57MG (2a).

The mitogenic effect of soluble Wnt-1 protein produced by C57MG cells has so far been observed only when quiescent monolayers of C57MG and RAC311 mammary cells are used as targets. The effect on these cells may reflect the oncogenic potential of *Wnt*-1 in vivo, because the gene induces hyperplasia and tumorigenesis when expressed in the mouse mammary gland (26, 46). Although *Wnt*-1 itself is not normally transcribed in mammary tissue, at least six other *Wnt* genes are expressed during mammary gland development (10, 51). The differential expression of these genes during periods of mammary gland growth and differentiation, together with the mitogenic effects of Wnt-1 on mammary cells, strongly suggests that mammalian Wnt proteins normally act to regulate mammary cell proliferation in vivo. Evidence of a mitogenic effect of *Wnt*-1 has also been observed in the embryonic neural tube, where ectopic expression of the gene results in overproliferation of spinal cord precursor cells (9). Other reports have suggested that *Wnt*-1 may act via modulation of cell-cell adhesion mechanisms (4, 14, 25), and it is intriguing to speculate that in some cases this may be linked to the control of cell proliferation.

Consistent with the notion that other Wnt proteins can act as soluble extracellular factors, Van Leeuwen et al. (50) have recently described a *Drosophila* cell line that secretes a soluble and biologically active form of Wingless protein, the *Drosophila* homolog of Wnt-1. Although no mitogenic activity was observed, when applied to an imaginal disc cell line, CM from these cells induced rapid stabilization of Armadillo protein, a downstream component of Wingless signaling in the epidermis of *Drosophila* embryos (18, 50). Since expression of Wnt-1 in mammalian cell lines has been shown to regulate protein levels of the mammalian Armadillo homologs plakoglobin and β-catenin (4, 14), it will now be interesting to see whether these proteins are targets of rapid modification in response to extracellular Wnt-1 protein.

In mammalian cell culture, the majority of secreted Wnt-1 has previously been found associated with the ECM or cell surface, and relatively little accumulates in the medium (3, 15, 30). Nevertheless, we have been unable to demonstrate mitogenic activity associated with Wnt-1 protein in ECM fractions. This may be for technical reasons, but it is also possible that Wnt-1 protein bound to the ECM is inactive or unavailable for its receptor. Previous descriptions of localized phenotypic effects elicited by cells expressing *Wnt*-1 have implied that paracrine effects may be limited to short distances only, as if active Wnt-1 protein is restricted to the ECM or cell surfaces (15, 31). In contrast, our present data imply that functional forms of Wnt-1 can be freely diffusible. Given the propensity of Wnt-1 protein to associate with ECM, it is possible that partition of the protein between diffusible and immobilized phases, and the protein's activity in vivo, may be governed by the nature and local distribution of particular proteoglycans or other matrix constituents.

Although Wnt proteins are assumed to act via specific cell surface receptors, no such molecules have yet been identified in any species, and their signaling mechanisms remain obscure. The identification of functional Wnt-1 protein should now facilitate studies of early cellular responses to a mammalian Wnt signal and should also be an important step in the quest for Wnt-1 receptors.

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