Murine Wnt-1 with an Internal c-myc Tag Recombinantly Produced in Escherichia coli Can Induce Intracellular Signaling of the Canonical Wnt Pathway in Eukaryotic Cells*

Received for publication, March 23, 2004, and in revised form, August 25, 2004
Published, JBC Papers in Press, August 26, 2004, DOI 10.1074/jbc.M403207200

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Wnt-1 belongs to the Wnt family of secreted glycoproteins inducing an intracellular signaling pathway involved in cell proliferation, differentiation, and pattern formation. The canonical branch is one of three known branches. This is also valid in vitro, and Wnts can be considered beneficial for culturing primary cells from organs, provided Wnts are available and applicable even with cells of different species. It was shown here that internally c-myc-tagged murine Wnt-1 produced in the heterologous host Escherichia coli was appropriate for inducing intracellular signaling of the canonical Wnt pathway in eukaryotic cells via stabilization of cytosolic β-catenin. The pioneering injection of the protein into the blastocoels of Xenopus laevis embryos led to axis duplication and suppression of head formation. Applying the recombinant murine Wnt-1 to metanephric mesenchyme activated the tubulogenic program. The signal-inducing activity of the recombinant protein was also positively demonstrated in the TOPflash reporter assay. Although Wnts were purified recently from the growth media of stably transfected eukaryotic cell lines, the production of active Wnt proteins in pro- or eukaryotic microorganisms reportedly has never been successful. Here soluble production in E. coli and translocation into the oxidizing environment of the periplasm were achieved. The protein was purified using the internal c-myc tag. The effect on the eukaryotic cells implies that activity was retained. Thus, this approach could make recombinant murine Wnt-1 available as a good starting point for other Wnts needed, for example, for maintaining and differentiating stem cells, organ restoration therapy, and tissue engineering.

The Wnt family consists of secreted and extracellular matrix-associated glycoproteins binding to frizzled seven-transmembrane span receptors (1). There are two functional classes (Wnt-1 and Wnt-5a) within this family. The canonical intracellular signaling pathway induced by members of the Wnt-1 class leads to stabilization and nuclear translocation of cytosolic β-catenin that associates with the transcription factors lymphoid enhancer factor/T-cell transcription factor (LEF/TCF)3 for activating the target genes (2, 3). The Wnt-5a class can induce two different pathways and can block the activity of the Wnt-1 class. Within the planar cell polarity pathway Jun N-terminal kinase is activated, and cytoskeletal organization and cell polarization are both activated and coordinated. In the Wnt/Ca2+ pathway, intracellular calcium is released most likely via G-proteins (4–6). The complex signaling network contributes to cell proliferation, differentiation, and pattern formation. Canonical Wnt pathway activation (e.g. by Wnt-1, -3a, -7b, and 8b but not by members of the Wnt-5a class) mediates the ectopic neural crest and secondary body axis formation in Xenopus embryos (5–7). The antagonists have dose-dependent effects. Low levels of frizzled-related proteins potentiate Wnt signaling, whereas high levels inhibit it (5). In vitro, the ligands induce signaling in cells of the respective species as well as in others. Drosophila Wingless and Xenopus Wnt-8 bind to several mouse frizzled receptors (8), and heterologous inducers (e.g. members of the wnt gene family) trigger tubulogenensis in metanephric mesenchyme cultures (9, 10).

Nineteen wnt genes have been identified already in the murine and human genome.2 The sequences of the human genes3 have matches of 42–52%, and the proteins have matches of 26–39% if aligned with an 80% similarity significance value. Human Wnt-4 is the most similar to human Wnt-1, sharing 44% of the amino acid sequence similarly aligned.3 One of the best characterized mammalian Wnts is murine Wnt-1 (13). It has four potential N-linked glycosylation sites and 23 conserved cysteines and is almost identical to human Wnt-1. There is only an isoleucine at position 40 in the mature murine protein (38.3 kDa) instead of valine. Both amino acids are neutral and hydrophobic. The aliphatic chain of isoleucine is longer just by a –CH2. The protein has an unprocessed pre-cursor (41.1 kDa) including the signal sequence for the endoplasmic reticulum. The internal c-myc tag situated after the mature murine Wnt-1 and Wnt-5a within this family. The canonical intracellular signaling pathway induced by members of the Wnt-1 class leads to stabilization and nuclear translocation of cytosolic β-catenin that associates with the transcription factors lymphoid enhancer factor/T-cell transcription factor (LEF/TCF) for activating the target genes (2, 3). The Wnt-5a class can induce two different pathways and can block the activity of the Wnt-1 class. Within the planar cell polarity pathway Jun N-terminal kinase is activated, and cytoskeletal organization and cell polarization are both activated and coordinated. In the Wnt/Ca2+ pathway, intracellular calcium is released most likely via G-proteins (4–6). The complex signaling network contributes to cell proliferation, differentiation, and pattern formation. Canonical Wnt pathway activation (e.g. by Wnt-1, -3a, -7b, and 8b but not by members of the Wnt-5a class) mediates the ectopic neural crest and secondary body axis formation in Xenopus embryos (5–7). The antagonists have dose-dependent effects. Low levels of frizzled-related proteins potentiate Wnt signaling, whereas high levels inhibit it (5). In vitro, the ligands induce signaling in cells of the respective species as well as in others. Drosophila Wingless and Xenopus Wnt-8 bind to several mouse frizzled receptors (8), and heterologous inducers (e.g. members of the wnt gene family) trigger tubulogenensis in metanephric mesenchyme cultures (9, 10).

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Wnt-1 class members, in concert with the antagonists, are considered to be beneficial for regulating stem cell development (e.g. hematopoietic, intestinal epithelium, epidermal, and hair follicle stem cells) and for maintaining them (5, 14). Future applications for this are found in regenerative tissue processes for human therapy. However, the availability of the proteinaceous reagents is limited. Production in eukaryotic cell cultures often results in accumulation of misfolded protein in the endoplasmic reticulum. Moreover, the proteins are associated with the extracellular matrix or the cell surface, and there is only a small soluble amount secreted. It is biologically active and useful in vitro.2 Murine Wnt-1 has been used, for instance, from conditioned medium but has not been purified (13) because it has proven difficult to retain activity during the purification. This might be largely attributed to the high degree of insolubility/hydrophobicity (15) and instability.3 Only recently, Wnts were successfully purified from the growth media of stably transfected eukaryotic cell lines, and active murine Wnt-3a from mouse L cells and Drosophila Wnt-8 from Drosophila S2 cells were obtained (15). However, the production of active Wnt proteins in Escherichia coli, in yeasts, or with the baculovirus system has never been proven successful.4

Here, internally c-myc-tagged murine Wnt-1 could be purified using the tag after gaining soluble expression in E. coli and translocation into the oxidizing periplasm. Activity was retained as axis duplication and suppression of head formation in Xenopus laevis embryos, activation of the tubulogenenic program in murine metanephric mesenchyme, expression of the luciferase gene in the TOPflash reporter assay, and stabilization of cytosolic β-catenin indicate the induction of the canonical Wnt pathway.

Recombinant murine Wnt-1 could be made available for tissue engineering by the approach presented here and by the effects shown on eukaryotic cells. This might be the starting point for the use of E. coli as a recombinant host for the production of other Wnts.

**EXPERIMENTAL PROCEDURES**

**Construction of Plasmids—** To construct pBSwnt1myc (5543 bp) the mwnt-1myc gene of pgem4int-1mycropyA (16) was amplified by means of PCR using the following oligonucleotide primers: 5′-ctagtggtatgctgcaagcttgaattcgagctc (uppercase letters represent the native sequence, and the HindIII site is underlined, and the start codon is in boldface type). The resulting 1176-bp fragment was digested with Stul and HindIII and inserted between the same restriction sites of pBF005 (17). The plasmid (see Fig. 1C) encodes the mwnt-1myc precursor gene (with the corresponding signal sequence) with an lpp termination sequence under the control of ptac additionally regulated by lpp from the StuI site is underlined, and the start codon is in boldface type) and the stop codon is in boldface type). The resulting 1176-bp fragment was digested with Stul and HindIII and inserted between the same restriction sites of pBF005 (17). The plasmid (see Fig. 1C) encodes the same genes as pBSwnt1myc, but instead of the mwnt-1myc precursor gene (with the corresponding signal sequence) there is the sequence of the mature mwnt-1myc gene behind the signal sequence of the E. coli ompA gene. The production of other Wnts.

Target Protein Synthesis and Purification—The E. coli strain RV308 (18, 19) harboring either pBSwnt1myc or pBSwnt1myc for protein production or RV308 harboring pBF005 (plasmid without the mwnt-1myc gene; Ref. 17) as negative control was grown in Superbroth supplemented with 100 μg ml⁻¹ ampicillin at 37 °C overnight. This preculture was used to inoculate the main culture in the same medium to a starting optical density of 0.1 at 550 nm. The main culture was performed at 37 °C; protein production was induced with 1 mM IPTG when an optical density of 0.5 at 550 nm was reached, and cultivation was continued for 4 h. The cells were then harvested (8,500 × g, 5 min, 4 °C). 0.9 g of cells were resuspended in 1.5 ml of PeriPreps™ periplasing buffer (PeriPreps™ periplasing kit, Epicentre) and incubated for 5 min at room temperature. 2.25 ml of ice-cold purified water of the same type was added, and everything was kept on ice for 10 min before being centrifuged (8,000 × g, 15 min, 4 °C). The supernatant was centrifuged again at the same parameters. Alternatively, the periplasmic fraction was extracted as follows for preparations not used in bioactivity assays. 650 mg of cells were resuspended in 50 ml of 20% Sucrose, 30 mM Tris, pH 8.0, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 1 mM benzamidine-HCl (all from Sigma) and incubated for 10 min at room temperature before being centrifuged for 15 min at 8,000 × g and 4 °C. The pellet was resuspended in 50 ml of ice-cold 5 mM MgSO₄, 1 mM phenylmethanesulfonyl fluoride, 1 mM benzamidine-HCl and then incubated for 10 min on ice before being centrifuged for 15 min at 8,000 × g and 4 °C. Then the pH of the supernatant was adjusted to 7.4. 1 ml of anti-c-Myc-agarose conjugate (Sigma) was used according to the manufacturer with some modifications for the purification. The slurry was packed in Polyprem chromatography columns (Bio-Rad) and equilibrated with 3 × 5 ml of PBS. After loading the sample, the column was washed with 3 × 5 ml of PBS, and the elution was performed with 9 × 1 ml of NH₄OH (pH 11.5). For neutralization of 1 M acetic acid were needed for the first and 40 μl for the other fractions. The acid had been provided in the tubes for collecting the fractions. Centricon YM-50 centrifugal filter units (Millipore) were used for buffer exchange (5 × 2 ml of PBS) of the eluted fractions 1–3 and for concentration according to the manufacturer. Roti-Nanoquant (Carl Roth) was used for determining protein concentrations as described by the manufacturer.

**Immunoblot—** Samples were electrophoresed in SDS-polyacrylamide gels and transferred onto Immobilon-P transfer membrane (Millipore). The low range SDS-PAGE molecular weight standard from Bio-Rad was used. The membranes were blocked with 3% skimmed milk powder in PBS with 0.1% Tween 20 and probed with monoclonal anti-c-Myc antibodies (clone 9E10, mouse ascites fluid, Sigma). Horseradish peroxidase conjugated secondary antibodies against mouse IgG (Amersham Biosciences) were used and visualized by enhanced chemiluminescence on Hyperfilm (Amersham Biosciences).

**Hydrophobicity Assay—** The soluble fraction of the whole cell extract in PBS was prepared by centrifugation after ultrasonication and kept on ice. Then 750 μl of the extract were mixed with 750 μl of ice-cold Triton X-114 (Amersham Biosciences) before the mixture was incubated at 22 °C. Complete phase separation took place almost instantly. Distances of both phases were then taken. Additionally, a sample from the Triton phase was precipitated using aceton (80% final concentration) overnight at −20 °C. After centrifugation, the pellet was dried and resuspended in PBS.

**Distribution in the Cellular Compartment—** After preparing the probe as described above, an amount of the bacterial culture corresponding to an optical density of 10 at 600 nm was harvested and resuspended in 500 μl of PeriPreps periplasing buffer (PeriPreps™ periplasing kit, Epicentre). The fractions of the cellular compartments were prepared according to the manufacturer using ultracentrifugation (138,000 × g, 1 h) at the end for separating the cytoplasmic fraction from membranes. The pellet membranes were then resuspended in 0.5% N-lauroylsarcosine, 10 mM Tris, pH 8.0, 5 mM EDTA.

**Injection Experiments in X. laevis—** X. laevis embryos were obtained by in vitro fertilization and raised in 25% modified Marc’s Ringer (20) at 18 °C. Embryos at the four-cell stage were injected with 18 nl of the samples of protein to be tested or the negative control (see above) in the ventral blastomeres. Alternatively, embryos at stage 7–8 were injected with 50 nl into the blastocoel (as indicated in Fig. 5A (adapted from Ref. 21)). All of the embryos were kept for 6 h in 25% modified Marc’s Ringer with 3% Ficoll (Amersham Biosciences) at 18 °C, then were raised in 25% modified Marc’s Ringer at 18 °C, and were fixed at tail bud stage in 100 mM Mops, pH 7.4, 2 mM EGTA, 1 mM MgSO₄, 4% paraformaldehyde before being analyzed for the formation of a secondary axis. Embryos were staged according to Nieuwkoop and Faber (22).

**Mouse Mesonephric Mesoderm—** Mouse mesonephric mesoderm (E 11.5) were collected into PBS on ice. To obtain timed embryos the vaginal plug was considered a criterion for mating. The kidneys were dissected and kept in PBS. N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid buffer (HEPES) were added at 37 °C until complete phase separation took place. Samples from both phases were then taken. Additionally, a sample from the Triton phase was precipitated using aceton (80% final concentration) overnight at −20 °C. After centrifugation, the pellet was dried and resuspended in PBS.
ric mesenchyme, and the mesenchymes were transferred from the medium onto Nuclepore filters supported by a stainless steel grid in a culture dish (1 mesenchyme/well). Mesenchyme cultures were performed according to Vainio et al. (23) in 300 μl of the same medium with serum and penicillin/streptomycin (Invitrogen) and 5% CO₂ in humidified air at 37 °C for 24 h. Before cultivation, the purified samples of recombinant protein to be tested or the negative control (see above) to a final concentration of 1:10–1:30, or the positive control (15 μM LiCl) were added directly to the medium and mixed gently, avoiding bubbles.

**TOPflash Assay**—MDCK cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 0.1 mM nonessential amino acids (BioWhittaker), and 1 mM sodium pyruvate (BioWhittaker) with 5% CO₂ in humidified air at 37 °C. Cells (140,000 in 500 μl of medium/well) were seeded in 24-well plates the day before transfection and were transfected at about 95% confluency with 300 ng of pTop-Flash (luciferase reporter plasmid; Refs. 24 and 25) and 200 ng of pB-gal (transfection efficiency control/well) using 2 μl/well LipofectAMINE 2000 (Invitrogen) according to the manufacturer. The culture medium was changed after 6 h of incubation against 500 μl of the same medium plus 100 units/ml penicillin and 100 μg/ml streptomycin sulfate. Then 10 μl of the purified samples of recombinant protein to be tested (sample dilutions of 1:1, 1:5, 1:10, and 1:20 resulting in final concentrations of 1:50, 1:250, 1:500, and 1:1,000), the positive control (20 μM LiCl) were added in duplicate, and the plates were incubated for 18 h. The wells were washed twice with PBS, and then the cells were lysed using 1× cell culture lysis reagent (Promega) for 15 min. Luciferase activities were measured by means of a luminometer using 20 μl of the cell lysate and 100 μl of luciferase assay reagent (Promega) according to the manufacturer. β-Galactosidase activities were measured spectrophotometrically using 2 μl of the cell lysate and the substrate chloromethyl red-β-D-galactopyranoside following the instructions of the high sensitivity β-galactosidase assay kit (Strategene).

**β-Catenin Stabilization Assay**—MDCK cells were cultured as described above (TOPflash assay). The cells (130,000 in 500 μl of medium/well) were seeded in 24-well plates the day before the assay. At a confluency of 80–90% the medium was changed against 500 μl of the same medium with serum and penicillin/streptomycin (Invitrogen), 0.1 μM nonessential amino acids (BioWhittaker), and 1 mM sodium pyruvate (BioWhittaker) with 5% CO₂ in humidified air at 37 °C. Cells (140,000 in 500 μl of medium/well) were seeded in 24-well plates the day before transfection and were transfected at about 95% confluency with 300 ng of pTop-Flash (luciferase reporter plasmid; Refs. 24 and 25) and 200 ng of pB-gal (transfection efficiency control/well) using 2 μl/well LipofectAMINE 2000 (Invitrogen) according to the manufacturer. The culture medium was changed after 6 h of incubation against 500 μl of the same medium plus 100 units/ml penicillin and 100 μg/ml streptomycin sulfate. Then 10 μl of the purified samples of recombinant protein to be tested (sample dilutions of 1:1, 1:5, 1:10, and 1:20 resulting in final concentrations of 1:50, 1:250, 1:500, and 1:1,000), the positive control (20 μM LiCl) were added in duplicate, and the plates were incubated for 18 h. The wells were washed twice with PBS, and then the cells were lysed using 1× cell culture lysis reagent (Promega) for 15 min. Luciferase activities were measured by means of a luminometer using 20 μl of the cell lysate and 100 μl of luciferase assay reagent (Promega) according to the manufacturer. β-Galactosidase activities were measured spectrophotometrically using 2 μl of the cell lysate and the substrate chloromethyl red-β-D-galactopyranoside following the instructions of the high sensitivity β-galactosidase assay kit (Strategene).

**RESULTS**

Internally c-myc-tagged Murine Wnt-1 Can Be Produced Solubly in E. coli—Wnt proteins have been purified from conditioned medium of eukaryotic cell lines and found to be active as stem cell growth factors (15) but have not been produced in E. coli. Internally c-myc-tagged Murine Wnt-1 Can Be Produced Solubly in E. coli—Wnt proteins have been purified from conditioned medium of eukaryotic cell lines and found to be active as stem cell growth factors (15) but have not been produced in E. coli. We intended to study whether a member of the Wnt family produced in E. coli was active in the oxidizing periplasm favors correct folding of cysteine-rich proteins such as Wnt-1 (23 cysteines). The two different signal sequence variants were chosen to study their impact on production, solubility, and folding. The signal sequence of eukaryotic proteins can act as a steric chaperone supporting the correct folding, and in some cases it also signals in E. coli (17, 26). The signal sequence of the homologous OmpA was used because it could possibly be more efficient in avoiding bottlenecks in translocation and by this prevent aggregation in the cytoplasm. The p_tac was used because its strength can be fine tuned by 1mM IPTG and an induction time of 4 h. However, some unspecific proteolysis could not be circumvented. Whole cell extract was separated into soluble and insoluble fractions by centrifugation after ultrasonication. Up to 50% of the produced target protein was soluble. The samples were analyzed by means of Western blotting. (The first antibody was anti-c-Myc from mouse, and the secondary antibody was anti-mouse-horseradish peroxidase.) The migration positions of molecular weight markers are shown on the left. The target protein is indicated by an arrow. C, both expression vectors have the same backbone (p_ompA, lacI*, p_tac, lpp termination sequence, fl intergenic region, ampicillin resistance marker, and ColE1 origin). The only difference is the signal sequence upstream of the mature wnt-1/myc gene. In the case of pBFsswt1myc (5543 bp) the signal sequence of the wnt-1/myc gene is encoded. The signal sequence of the E. coli ompA gene is used in the case of pBFompAwnt1myc (5569 bp).

The most stable product was obtained by growing the cells in rich medium (Superbroth) at 37 °C with an inducer concentration of 1 mM IPTG and an induction time of 4 h. However, some unspecific proteolysis could not be circumvented. Whole cell extract was separated into soluble and insoluble fractions by centrifugation after ultrasonication and was analyzed by means of Western blotting to assess the solubility of the product. In Fig. 1, it is shown that up to 50% of the produced target protein was soluble. Although more recombinant protein was produced using the plasmid pBFompAwnt1myc (up to 3× the amount produced using pBFsswt1myc), the final yield in the soluble product was not much higher because it was less soluble (up to 17%).

**Soluble Murine Wnt-1 Extracted from the E. coli Periplasm Can Be Purified Using the Internal c-myc Tag**—Translocation into the periplasm was achieved with both vector systems, and soluble target protein could be extracted from this compartment for further purification using the internal c-myc tag. In Fig. 2, it is shown that target protein specifically bound to anti-c-Myc-conjugated agarose, whereas a smaller proteolysis product did not. Excessive protein was washed off. The bound protein could be eluted here mainly in the second and third fraction using 0.1 M NH₄OH (pH 11.5). Some protein stayed bound to the resin. After elution, the protein was contained in an ammonium acetate solution inappropriate for later application as a cytokine, and thus the buffer had to be exchanged for PBS. This could be done by means of centrifugal filter units with a cutoff of 50 kDa. It was found that the protein accumulated exclusively in the retentate even though it is smaller than 50 kDa. On the average, 0.8 μg of protein were contained in the retentate after processing 900 mg of cells. The content varied depending on culture conditions and lot of the resin. The amount of active protein was presumably lower because the
Murine Wnt-1 with an Internal c-myc Tag Recombinantly Produced in E. coli Is Hydrophobic—It was published recently that Wnt proteins are palmitoylated in their active forms increasing membrane association (15). The murine Wnt-1 produced here in E. coli was subjected to temperature-induced Triton X-114 phase extraction to assess its hydrophobicity. At temperatures below 20 °C, Triton X-114 dissolves in aqueous buffers. Above 20 °C the mixture separates into a detergent and an aqueous phase. Hydrophobic proteins partition to the organic phase, and hydrophilic ones accumulate in the aqueous phase (27). The soluble fraction of the whole cell extract including the produced target protein was mixed with Triton X-114. After separation the phases were analyzed by means of Western blotting. Recombinant target protein was found in the detergent phase as depicted in Fig. 3. This indicated hydrophobicity. Fractions of the cellular compartments were prepared to find out whether the protein also associates with membranes. In Fig. 4, the distribution of the produced protein in the cell is shown. There were only traces of protein in the cytoplasm. Most of it accumulated in the periplasm or associated with the membranes. There was less target protein free in the periplasm than associated with the membrane when it was translocated due to its own signal sequence. It was vice versa for the product translocated using the E. coli ompA signal sequence.

Injection of Purified Recombinant Internally c-myc-tagged Murine Wnt-1 from E. coli into the Blastocoels of X. laevis Embryos Leads to Axis Duplication—Studying the effect of murine Wnt-1 on eukaryotic cells could be approached after murine Wnt-1 was made available, found to be hydrophobic, and associated with membranes. The secondary axis assay in X. laevis embryos was used to test the bioactivity of the protein in vivo. This assay is a well established readout for the activity of the canonical Wnt pathway. It has been demonstrated that the ectopic expression of Wnt-1, -2, -3a, -7b, -8, and 8b can induce a secondary body axis following injection of the corresponding RNA in ventral blastomeres of cleavage stage embryos (5–7, 16–28–31). Thus, the protein produced here was subjected to this assay. The purified protein was injected into both ventral blastomeres of four-cell stage embryos. This treatment led to only a very low percentage of embryos developing a secondary axis (Fig. 5B, lanes 1–3). Most likely this was because of the injection of the mature protein into the cytoplasm of the blastomeres. The mature protein cannot be secreted any longer, which would be needed for signal induction by binding to the receptor extracellularly. To circumvent this problem, embryos at stage 7–8 (i.e., before midblastula transition) were injected into the blastocoel with 50 nl of the protein, raised, and analyzed for the formation of a secondary axis. There were two outcomes possible here. The first was that if the injected protein was distributed randomly within the blastocoel regarding outcomes possible here. The first was that if the injected protein was distributed randomly within the blastocoel regarding the developing dorsal/ventral and anterior/posterior axes of the embryos, the effects on the different cell types surrounding the blastocoel could be rather different leading to different phenotypes. On the other hand, localized effects on axis formation might be expected if the protein remained rather localized itself because of the presence of abundant extracellular polysaccharides (e.g. hyaluronan) within the blastocoel (32). Here the injection of 50 nl of purified recombinant protein (product of pBFompAwnt1myc) resulted in formation of secondary axes in 18.8% (n = 16) of the embryos, whereas the product of pBFswnt1myc resulted in formation of secondary axes in 42% (n = 19) in the formation of secondary axes (Fig. 5B). In the embryos showing two axes neither the ectopic nor the endogenous axis contained anterior structures such as eyes and cement glands (Fig. 5C). Thus, the recombinant Mwnt-1 injected into the blastocoel affected both early and late canonical Wnt signaling resulting in a combination of induction of a secondary axis and inhibition of head formation (33). The induced secondary axes could be expected...
Applying the Recombinant Murine Wnt-1 with an Internal c-myc Tag to Metanephric Mesenchyme Activates the Tubulogenic Program—Wnt-4 is required for tubule formation in the kidney but can be replaced by other members of the Wnt gene family including Wnt-1. This effect can be seen also in vitro in isolated metanephric mesenchyme (9, 10). The recombinant murine Wnt-1 was assessed in this assay. Both variants of the protein (1:20 average final concentration) and the positive and negative controls were applied to mouse metanephric mesenchyme. The positive control was LiCl because this has been reported to activate the Wnt signaling pathway because of inhibition of glycogen synthase kinase-3β resulting in the cytoplasmic accumulation of β-catenin. In the presence of LiCl, condensations became apparent within isolated E 11.5 mesenchyme (34–36). This was also seen here. The positive control and the protein-containing samples maintained an intact mesenchyme. Moreover, differentiation foci, light areas indicating early tubulogenesis, were found (see Fig. 6). Even red blood cells developed from progenitors were seen. In the negative control, the mesenchyme underwent apoptosis. This is visible as gray areas in Fig. 6. The results were the same for both production variants (pBFsswnt1myc and pBFompAwnt1myc), and the findings were reproducible. Protein from two independent productions (from cell growth to buffer exchange) gave the same positive results.

Recombinant Murine Wnt-1 from E. coli Induces Stabilization of Cytosolic β-Catenin within the Canonical Wnt Pathway, Leading to Expression of the Luciferase Gene in the TOPflash Reporter Assay—The recombinant protein was tested in the TOPflash reporter assay to get more quantifiable information about its activity. For this assay MDCK cells were transfected with the luciferase reporter plasmid (and pB-gal for transfection efficiency control). Multimeric TCF-binding sites are situated to be incomplete as well because the injected protein could be effective only after the endogenous axis formation already had started. The injected protein obviously remained active for a certain time because the endogenous axis did not form a proper head leading to defects similar to those seen after wnt-1-DNA injection (30). In every experiment a purified extract from E. coli harboring the plasmid without the wnt-1 gene was used as a negative control. The strain was grown exactly the same way as the producing strain, and all extraction and purification steps were performed the same way. This was done to make sure nothing originating from E. coli or the protein purification process would induce any signaling in the cells of the embryo. As depicted in Fig. 5C, injecting this negative control resulted in embryos appearing as wild-type embryos. All findings were reproducible. Protein from three independent productions (covering the whole process from cell growth to buffer exchange) gave positive results (data not shown). After 3 weeks of storage at 4 °C, an impairment of bioactivity of the purified recombinant murine Wnt-1 protein dissolved in PBS was seen. 2.2% (n = 45) of the injected embryos showed a secondary axis (Fig. 5B, lanes 7–9).

FIG. 5. Recombinant Wnt-1 injected into the blastocoel of X. laevis embryos can induce axis duplication. A, the three prospective germ layers surround a cavity, the blastocoel, at blastula stages of Xenopus sp. embryos. Purified murine Wnt-1 produced in E. coli or the negative control (plasmid without the wnt-1 gene used for the whole production process) was injected into the blastocoel of X. laevis embryos as indicated in the drawing (adapted from Ref. 21) in the case of the results shown in B, bars 4–9, and C, B, lanes 1–3. Injection of 18 nl of purified product of pBFompAwnt1myc into the two ventral blastomeres of four-cell stage embryos resulted in one embryo with an ectopically induced secondary axis (3.2%, n = 30). Lanes 4–6, injection of 50 nl of purified product of either pBFompAwnt1myc or pBFsswnt1myc into the blastocoel of stage 7–8 embryos resulted in 18% (n = 16) and 42% (n = 19) ectopic body axes, respectively. Lanes 7–9, purified product stored for 3 weeks at 4 °C led to induction of a secondary body axis in 2% of the injected embryos (n = 45). Lanes 3, 6, and 9, injection of 50 nl of the negative control did not induce any ectopic body axis. C, top, embryo injected with the negative control, stage 35/36, showing eye and cement gland (c.g.). Middle and bottom, embryos injected with 50 nl of purified recombinant protein showing primary and secondary axes (arrows) lacking anterior structures (eye and cement gland).
Wnt-1 from *E. coli* Purified internally c-nephric mesenchyme. According to Vainio of mouse E 11.5 metanephric mesenchyme cultures (performed according to Vainio et al. (23) for 23 h). In the negative control (A) apoptosis (gray areas) was found. Only the positive control (B) and the protein-containing samples (C, protein translocated using the *E. coli* ompA signal sequence; D, protein translocated because of its own signal sequence) maintained an intact tissue and led to the formation of differentiation foci (marked by arrows) being an indicator for early tubulogenesis.

FIG. 6. Effect of recombinant Mwnt-1 on isolated mouse metanephric mesenchyme. Purified internally c-myc-tagged murine Wnt-1 from *E. coli* (1:20 average final concentration), the negative control (plasmid without the wnt-1 gene used for the whole production process), or the positive control (15 mM LiCl) was added to the medium of mouse E 11.5 metanephric mesenchyme cultures (performed according to Vainio et al. (23) for 23 h). In the negative control (A) apoptosis (gray areas) was found. Only the positive control (B) and the protein-containing samples (C, protein translocated using the *E. coli* ompA signal sequence; D, protein translocated because of its own signal sequence) maintained an intact tissue and led to the formation of differentiation foci (marked by arrows) being an indicator for early tubulogenesis.

**DISCUSSION**

In this work the question was addressed as to whether a member of the Wnt family produced in *E. coli* was appropriate for inducing intracellular signaling of the canonical Wnt pathway in eukaryotic cells. Although Wnts have been purified from conditioned medium of eukaryotic cells and have been found active as stem cell growth factors (15), they had not been produced successfully in a prokaryotic host yet. Thus, the challenging production had to be tackled first. Wnts are not easy to produce because of their molecular features (cysteine-rich, hydrophobic, unstable, basic pl (15)). That is why misfolded protein often accumulates in the host, or it is difficult to retain...
Mainly C14–C18 fatty acids are integrated in bacterial fats. The requirement for activity (besides correct folding) was met. The phobicity and was partly membrane-associated. Thus, one re-requirement for activity (besides correct folding) was met. The translocation into the periplasm prevented the aggregation. The amount overstraining the transport remained in the cytoplasm and aggregated. The internal c-myc tag actually intended to aid the purification also decreases the PI slightly. This might be a positive side effect enhancing the solubility of the product in the host environment.

Wnts have been found to be more hydrophobic than expected from the primary sequence because of posttranslational palmitoylation on a conserved cysteine (1, 15). The lipid is considered important for signaling because enzymatic palmitoyl removal or mutations of the modified cysteine result in loss of activity (1, 15). However, the conditions of the enzymatic treatment and the non-modified or missing cysteine could have impaired the correct folding. As the lipid is considered to increase the local Wnt concentration on membranes, its absence can be overcome by high amounts of ligand (1, 15). Here it could be shown that Rmwnt-1 accumulated in the detergent phase (implying hydrophobicity) and was partly membrane-associated. Thus, one requirement for activity (besides correct folding) was met. The physiology of E. coli would even allow for palmitoylation of Rmwnt-1 at the correct site. Many lipoproteins in bacteria have palmitate as their lipid covalently attached to the N-terminal cysteine residue (40). In prokaryotes, lipoprotein precursors are translocated across the inner membrane by a Sec-mediated mechanism (the E. coli signal sequence of OmpA used here is also Sec-dependent). On the periplasmic side the N-terminal cysteine of the mature protein forms a thioether bond with diacylglyceride catalyzed by a diacylglyceryl transferase. Then, the signal peptide is cleaved, and the cysteine is aminoa-lylated. The protein is then localized to the inner (if aspartate is next to the lipid-modified cysteine) or outer membrane (40–43). Lacking this aspartate, Wnt-1 would associate with the outer membrane. The transacylase Lnt has a broad specificity to acyl chain donors and to species of phospholipids and fatty acids (various lengths and saturation degrees) in E. coli (41). Mainly C14–C18 fatty acids are integrated in bacterial fats. The lipid A of the outer membrane contains C12, C14, and C16 (palmitate) fatty acids (44). However, it might be irrelevant what lipid species is linked with the protein and where it is linked because the lipid is only supporting membrane association and not directly entailing bioactivity (1, 15).

However, the question of whether the conditions in the prokaryotic host E. coli would be sufficient for producing active Wnt-1 had to be answered by appropriate bioactivity tests. As Drosophila and Xenopus Wnts can bind to several mouse receptors (8), the murine protein produced here could be subjected to the secondary axis induction assay in Xenopus embryos. Both the formation of secondary axes and suppression of head formation were observed after the injections. The results were comparable with Wnt-8 overexpression in the frog embryo (45). Ectopic activation of the early Wnt pathway in Xenopus induces secondary axes, whereas late signaling (after midblasto-tula transition) leads to embryos without heads (30, 46). Activity of the injected Rmwnt-1 was obviously maintained for several h in the blastocoel because the embryos had phenotypes with both features. Moreover, those effects were achieved for the first time with injected protein. To our knowledge only nucleic acid-based molecules had been injected before. The impairment of protein activity during storage is most likely because of aggregation. To prevent hydrophobic proteins from aggregating usually detergents such as CHAPS are added. This is done in the case of the purification of Wnt-3a (15). Rmwnt-1 is stored here in PBS only to avoid any negative impact on the activity assays. Finding a suitable additive in an appropriate concentration or even a cryoprotectant for lyophilization requires a considerable amount of the protein and bioactivity assays. Besides stabilizing the folding and preventing aggregation, it must not affect or impair any later application.

Positive regulators (e.g. heparan-sulfate proteoglycans and low density lipoprotein receptor-related proteins) and antagonists (e.g. the Dickkopf family of proteins) influence the signaling in a dose-dependent manner. Low levels of the Wnt antagonists frizzled-related proteins, for example, potentiate Wnt signaling, whereas high levels inhibit it (5). Hence, the concentra-tion ratios of all involved molecules are important for the result. This could be a reason, in addition to the injection time, to why the degree of secondary axis formation is sometimes varying in the samples.

The same might apply to the effects seen in the tubule induction experiments. Isolated metanephric mesenchyme undergoes apoptosis unless provided with a permissive stimulus (e.g. several Wnts) supporting survival and leading to differentia-tion of metanephric mesenchyme (10, 11). Tubule induction is a multistep process and may require the action of several signals. Sulfated glycosaminoglycans might, for instance, act as cofactors for binding the Wnt protein to the responsive cell (10). Both variants of the recombinant protein produced here maintained an intact tissue and led to the formation of differentiation foci when applied to metanephric mesenchyme. The effect was the same for the positive control LiCl (34) and comparable with Wnt-3a (10). Even though the recombinant protein from E. coli could induce signaling of the Wnt pathway in this assay when directed to the periplasm, this activity was not found when the product was accumulated in the oxidative cytoplasm of mutant strains (OrigamiTM and Rosetta-gamiTM, both from Novagen).4 This implies that the oxidative environment is not the only contributing folding factor.

The signal-inducing activity of the recombinant protein was tested in a reporter assay to achieve a quantifiable interpreta-tion. Multimeric TCF-binding sites (TCF activates target genes in the nucleus at the end of the canonical Wnt signaling) upstream of a c-fos promoter drive luciferase expression in the TOPflash assay (8, 24, 25). Normally DNA encoding a gene whose effect on the signaling is to be tested is transfected. As far as it is known, proteins have not been assayed this way. All other components of the signal cascade including the receptor have to be expressed and accessible to obtain significant results. Antagon-ists must not be present in amounts exceeding the ligand in question. Thus, a good positive control in the form of evidently active ligand in the same amount would be needed. However, this is not available. Instead, LiCl was used, as it is known to poten-tiate TCF activity by inhibiting glycogen synthase kinase-3-β. This is detectable because of the reporter gene expression (37). Without LiCl, β-catenin is degraded by glycogen synthase kinase-3-β in association with a multiprotein complex (12). This makes

4 J. Veijola, unpublished data.
it a positive control for endogenous signaling only because LiCl affects signaling downstream of the receptor depending on the LiCl concentration and the target cells (37). Here, very high signal intensities compared with the samples were measured. Rmwnt-1 produced in E. coli and for the first time assessed in this kind of assay could induce intracellular signaling of the canonical Wnt pathway proven by expression of the reporter gene. Activity of Rmwnt-1 had been shown already by inducing the differentiation of eukaryotic cells. With the TOPflash assay a concentration dependence could be demonstrated. The concentration dependence also was shown in a direct assay of the stabilization of cytosolic β-catenin.

All data presented here provide a new opportunity for research and therapeutic approaches involving Wnt-1 and other members of this family. Not only was the protein made available by recombinant production in a prokaryotic host, but also intracellular signaling of the canonical Wnt pathway in different eukaryotic cells was induced by the product and resulted in various differentiation processes. The technology might be applicable to related proteins. In the future an up-scale of the production will be needed and approached as well as optimizing the storage of the protein to get more material for all of the possible applications. Then even the target protein can be characterized further.

Acknowledgments—We thank Regina Casteleijn-Osorno for language revision, and we are very grateful to the reviewer for the helpful comments.

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