A Formylated Hexapeptide Ligand Mimics the Ability of Wnt-5a to Impair Migration of Human Breast Epithelial Cells*

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Loss of Wnt-5a protein expression is associated with shorter recurrence-free survival in breast carcinoma patients and increased motility in mammary cell lines. Based on sequence analysis of Wnt-5a, we identified 14 peptide fragments and investigated their ability to mimic the effects of Wnt-5a on mammary cell adhesion and migration. Two of these peptides significantly increased adhesion and impaired migration in the non-tumorigenic HB2 breast epithelial cell line and in the MDA-MB-468 breast cancer cell line, both of which show little endogenous expression of the Wnt-5a protein. We removed two amino acids at a time from the N terminus of the shorter of these two peptides to identify the shortest peptide that still inhibited migration. The influence on tumor cell adhesion was gradually lost and was no longer detectable when only six amino acids remained. However, formylation of the N-terminal methionine of this hexapeptide restored its effect on adhesion and reduced tumor cell motility via a Frizzled-5 receptor-dependent mechanism, even at a low pH such as encountered in breast tumor tissue. This formylated hexapeptide ligand induced a rapid cytosolic calcium signal, whereas it did not affect the cellular levels of unphosphorylated β-catenin or active JNK. The novel formyl-Met-Asp-Gly-Cys-Glu-Leu peptide ligand is not only a valuable experimental tool but has also a potential role in antimetastatic treatment of the 50% of human breast cancer patients that have reduced endogenous Wnt-5a protein expression.

The Wnts are a family of secreted glycoproteins that have molecular masses of 39 – 46 kDa and participate in development and tumorigenesis via autocrine or paracrine routes (for reviews see Refs. 1–3). Secreted Wnt proteins bind to and activate G-protein-coupled receptors of the Frizzled (Frz) family (4, 5), and it is presumed that the low density lipoprotein (LDL) receptor-related proteins LRP5 and LRP6 act as coreceptors in that context (6, 7). Based on differences in the ability to transform mouse mammary epithelial cells (8), the Wnt proteins can in the present context be divided into the following three distinct classes: Wnt-1, Wnt-3a, and Wnt-7a have the greatest transforming capacity; Wnt-2, Wnt-5b, and Wnt-7b have an intermediate transforming capacity; and Wnt-4, Wnt-5a, and Wnt-6 are non-transforming.

We have previously reported that low level expression of Wnt-5a protein in primary invasive breast carcinomas is associated with higher histological grade (poor differentiation) and shortened recurrence-free survival because of more rapid development of distant metastases (9). This association cannot be caused by an effect of the protein on proliferation, because no correlation has been found between loss of Wnt-5a protein expression and presence of the proliferative marker Ki67 (10). Currently, the only available explanation for the ability of Wnt-5a to reduce the metastatic capacity of invasive breast cancer is that this protein enhances adhesion and thus reduces the migration of these tumor cells. However, it is possible that as yet undefined mechanisms also contribute to the mentioned clinical effect of Wnt-5a on tumor progression. Nevertheless, it has been shown that Wnt-5a-dependent activation of the adhesion receptor discoidin domain receptor 1 (DDR1) could be responsible for the decrease in the motility and the invasive potential of breast epithelial cells (11). Breast epithelial cells are normally firmly adherent, but reduced expression of Wnt-5a leads to decreased adhesion and thereby increases motility. In accordance with that, we have previously observed that introduction of a Wnt-5a expression vector into a MCF-7 breast cancer cell line, which exhibits little endogenous Wnt-5a expression, was required for collagen-induced activation of the adhesion receptor DDR1 (11). In good agreement, non-tumor mammary epithelial HB2 cells transected to express increased and decreased Wnt-5a protein levels, respectively, exhibited in the former case increased and the latter decreased adhesion to collagen I (11). It has not yet been established which of the different Frz receptors mediates the effects of Wnt-5a in human mammary cells. However, experiments using a Frz-5-specific blocking Ab have shown that this particular receptor is responsible for the effects induced by Wnt-5a in both fibroblast-like synoviocytes and in malignant melanoma cells (12, 13).

To develop a treatment that is based on the functional role of Wnt-5a and will target breast cancer metastasis, it is important to understand the cellular and molecular events that underlie the impact of this protein on such epithelial tumors. One of the critical and initial events in metastasis is when the transformed cells detach from each other and also from the ECM components of the basement membrane (14). In the presence of Wnt-5a, the adhesion receptor DDR1 in breast epithelial cells can be activated by collagen, hence this receptor is a likely candidate for mediating the Wnt-5a-induced increase in anchoring of these cells to collagen (11). It is plausible that the elevated metastatic activity seen in breast carcinomas that lack Wnt-5a is caused by reduced adhesion and a subsequent increase in the migratory capacity of these tumor cells. Therefore, one approach to reduce the metastatic activity in Wnt-5a-deficient breast carcinomas might be to find a way to overcome the translational defect responsible for the loss of Wnt-5a protein expression (10) and...
another to reconstitute the Wnt-5a-induced signaling and activation of the cells. Experiments have shown that adding recombinant Wnt-5a to differentiated thyroid carcinomas induces tumor suppressor activity (15). However, the fact that Wnt-5a is a relatively large protein (43 kDa) makes it attractive to search for a small molecule that can mimic the signaling and functional effects of Wnt-5a.

Based on sequence analysis of Wnt-5a, we identified and investigated 14 peptide fragments regarding their ability to mimic the effects of the native protein on mammmary cell adhesion and locomotion. Our goal was to find a substance that can serve as a possible model in the development of novel antimetastatic treatment strategies for breast carcinomas that exhibit reduced endogenous expression of Wnt-5a.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—We used the HB2 mammary epithelial cell line, which is a subclone of the MTSV-1.7 line developed in the laboratory of Dr. J. Taylor-Papadimitriou (16). HB2 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 5 units/ml penicillin, 0.5 units/ml streptomycin, 2 mM glutamine, 10 µg/ml bovine insulin, and 5 µg/ml hydrocortisone. The human mammary carcinoma cell lines MCF-7 and MDA-MB-468 were grown in DMEM supplemented with 10% fetal bovine serum, 5 units/ml penicillin, 0.5 units/ml streptomycin, and 2 mM glutamine. The pH experiments were done in MEM supplemented with 10 mM Tris, 0.5% BSA, 5 units/ml penicillin, 0.5 units/ml streptomycin, and 2 mM glutamine. The pH 7.4 medium, but not the pH 6.7 medium, was further supplemented with 4 mM sodium bicarbonate. All cells were incubated at 37 °C in 95% air and 5% CO₂ except for the fura-2-loaded cells from which we recorded the fluorescence response to Wnt-3a and a formylated hexapeptide (Fig. 9C).

**Chemicals and Reagents**—The following Abs and chemicals were used (sources within parentheses): anti-actin monoclonal Ab C4 (MP Biomedicals, Irvine, CA); anti-β-catenin dephospho amino acids 35–50 monoclonal Ab 7A7 (Alexis Biochemicals, Lausen, Switzerland); anti-β-catenin monoclonal Ab and collagen type I from rat tail (BD Biosciences, San José, CA); anti-β₁,-integrin monoclonal Ab P5D2 (Chemicon, Temecula, CA); anti-phospho SAPK/JNK Thr183/Tyr185 monoclonal Ab and anti-SAPK/JNK Ab (Cell Signaling Technology, Beverly, MA); anti-phosphotyrosine monoclonal Ab 4G10 (Upstate Biotech. Inc. Lake Placid, NY); recombinant mouse Wnt-3a and recombinant mouse Wnt-5a (R&D Systems, Abingdon, UK); SDS-PAGE reagents (Bio-Rad); fura-2/AM (Molecular Probes, Eugene, OR).

**Sequence Analysis**—Amino acid sequences of Wnt-5a from different species were investigated interactively and using multiple sequence alignment, secondary/solvent accessible surface predictions according to the PHD method (17) to identify potentially solvent-exposed loop segments.

**Peptide Synthesis**—Different peptide fragments derived from the Wnt-5a molecule that were used in the experiments are outlined in Table 1. Most of them were synthesized by Eurogentec (Seraing, Belgium), although the shorter peptides and the formylated peptide derived from the 12-amino acid long pentapeptide175 were from Pepscan systems (Leiden, Netherlands). The synthesized peptides were quality controlled by RP-HPLC and mass spectrometry. To reach different purity levels, peptides were purified by preparative RP-HPLC. The crude (60–70% pure) peptides were synthesized at two different occasions whereas the pure (>95% pure) peptides were synthesized three times. Regardless of the batch used, we obtained similar results with peptides derived from different syntheses. All other laboratory reagents were of analytical grade and obtained from Sigma Aldrich.

**Receptor-blocking Antibodies**—The peptide CPIKESHPLYNKVRT-GQVPN, corresponding to amino acids 198–217 in the ectodomain of the human Frz-5 receptor was used as previously described to generate a Frz-5 receptor-blocking Ab (12), and the peptide CPRVLKVPSYL-SYKFLGERD, corresponding to amino acids 202–220 in the ectodomain of the human Frz-2 receptor, was used to produce a control anti-Frz-2 Ab. (In the peptide designations, letters in italics indicate the Frz-5 and Frz-2 sequences, whereas the initial non-italic cysteine (C) in each peptide was added for subsequent purifications of the Abs.) The productions of rabbit anti-Frz-5 and anti-Frz-2 antisera were managed by AgriSera (Vännäs, Sweden; permit A69–04 from Umeå Animal Ethics Committee). The antisera were affinity-purified in our laboratory by coupling the respective peptide antigen to Sulfolink-Sepharose over which the relevant antisera was eluted.

**Immunoprecipitation and Western Blotting**—Western blot analysis was performed to evaluate expression of the proteins Wnt-5a, Frz-2, Frz-5, dephospho β-catenin, total β-catenin, actin, phospho-JNK, and total JNK in HB2 and/or MDA-MB-468 cells. The batch of MDA-MB-468 cells we used evidently expressed only a low level of Wnt-5a. The Wnt-5a Ab was produced in our laboratory as previously described (9, 11). The cells were lysed and boiled in 2X concentrated Laemmli buffer. The protein content in each sample was determined and adjusted to ensure loading of equal amounts of protein in each lane. Thereafter, 50 µM dithiothreitol were added, and the lysates were boiled for 10 min and then separated by SDS gel electrophoresis. The proteins were subsequently transferred to polyvinylidene difluoride membranes, which were blocked in 3% BSA or 5% nonfat milk for 45 min, incubated for 1 h with the primary Ab in 1% BSA or 1% nonfat milk (diluted 1:1,000 for phospho-JNK, total JNK, dephospho β-catenin, total β-catenin, and actin; 1:2,000 for Wnt-5a; 1:5,000 for phosphotyrosine, Frz-2 and Frz-5), and then for 1 h with a horseradish peroxidase-conjugated secondary Ab. Ab-antigen complexes were detected using an enhanced chemiluminescence kit from Amersham Biosciences. Before reprobing, the membranes were stripped with a Reblot Strong solution from Chemicon.

The phosphorylation status of DDR1 was analyzed as previously described (18), with the following modifications. The cells were subjected to serum depletion for 16 h and then detached with Versene and allowed to adhere to collagen I for 60 (MDA-MB-468 cells) or 90 (HB2 cells) min at 37 °C. The experiments were terminated by disrupting both adherent and non-adherent cells with a lysis buffer (50 mM Tris, pH 7.4, 1 mM Triton X–100, 5 mM EDTA, 5 mM EGTA, 50 mM NaCl, 42 µg/ml aprotinin, 2.5 mM benzamidine, 1 mM pefabloc, 10 µg/ml leupeptin, and 2 mM Na₃VO₄). In immunoblotting, an anti-DDR1 rabbit polyclonal Ab, sc-532 (Santa Cruz Biotechnology) was used at a dilution of 1:10,000. The Ab recognizes DDR1b/c, which is essential because collagen only induces phosphorylation of the b and/or c isoforms of DDR1 in mammary epithelial cells (11).

**Adhesion Assay**—The adhesion assay was carried out as previously described (18). Briefly, cells were serum-depleted for 16 h, detached with Versene, and allowed to adhere individually to collagen I for 60 (MDA-MB-468 cells) or 90 (HB2 cells) min at 37 °C. Non-adherent cells were removed by washing with phosphate-buffered saline. The adherent cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) in a dye reduction assay (Promega), and absorbance was measured at 490 nm using a Fluostar microplate reader from BMG LabTechnologies (GmbH, Offenberg, Germany). A single value was calculated for each experiment, based on the mean of adhesion from six separate wells after subtracting the adhesion to plastic alone. The Wnt-5a-con-
A Formylated Hexapeptide Impairs Breast Cell Migration

ditioned media (CM) used as a positive control in the adhesion assays was obtained from confluent stably transfected Wnt-5a-overexpressing HB2 cells (for experiments with Wnt-5a antisense HB2 cells) or Wnt-5a-overexpressing MCF-7 tumor cells (for use with MDA-MB-468 tumor cells). The CM was always used within 3 days of collection. The specificity of the CMs was checked by incubating the medium with either the anti-Wnt-5a Ab or a control Ab for 45 min at 37 °C. Subsequently, 10 μl of protein A-Sepharose was added, and 30 min later the protein A-bound antibodies were removed by centrifugation leaving the Wnt-5a-depleted supernatants to be used in experiments.

Cell Migration Assay—Cell migration was analyzed in a modified Boyden chamber (Transwell; Costar, Cambridge, MA) in which the two chambers were separated by a polycarbonate membrane (10-μm thick and with a pore diameter of 8.0 μm). In some experiments (as indicated in figure legends) the membrane was precoated with collagen by adding a collagen I solution (10 μg/ml in 0.25% acetic acid) to the upper chamber for 1 h at 37 °C and thereafter washing the membrane three times with phosphate-buffered saline. At the onset of each experiment, the cells were detached by treatment with Versene for 10–20 min at 37 °C, washed, and resuspended as single cells in serum-free DMEM medium supplemented with 0.5% BSA. A 0.2-ml aliquot of such a cell suspension (containing 25,000 MDA-MB-468 or 50,000 HB2 cells) was added to the upper transwell chamber together with the agonist of interest. The lower chamber was filled with 0.6 ml of serum-free, BSA-containing DMEM medium supplemented with the chemotactant IGF-I (1 ng/ml). As a control, IGF-I was excluded from some of the wells. The cells were allowed to migrate in the Boyden chamber for 18 h at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. Discarding the medium, using a cotton-tipped applicator to remove non-migratory cells and cut the membrane out of the chamber, terminated an experiment. The cells that had migrated through the membrane and were located on its lower surface were stained for 20 min with 0.5% crystal violet diluted in 20% methanol, 80% water. The membrane was subsequently washed, and the stained cells were counted (number per membrane) in an inverted microscope. The results for each experiment are presented as the median value for three separate wells.

Fluorescence Microscopy—MDA-MB-468 cells were detached from the tissue culture dishes by incubating with Versene for 10 min at 37 °C, washed, resuspended as single cells in serum-free DMEM medium supplemented with 0.5% BSA and 100 μM of the agonist of interest, and plated on a cover glass precoated with 10 μg/ml collagen-I in a cell culture dish. These experiments were terminated after 18 h by fixing the cells with 4% paraformaldehyde for 10 min. The cells were then permeabilized by incubation for 5 min with 0.5% Triton X-100 and blocked by a 45-min incubation in a 3% BSA solution supplemented with 0.3% Triton X-100. The cells were stained in the dark by incubating for 40 min with AlexaFluor® 488 phalloidin (diluted 1:500; Molecular Probes) in the presence of 1% BSA, and then washed extensively and mounted with Dako Cytomation fluorescent mounting medium. The samples were examined and photographed in a Nikon Eclipse 800 microscope using a ×60 oil immersion objective.

Determination of Cytosolic Free Calcium Levels—MDA-MB-468 cells grown on cover glasses were incubated with 4 μM fura-2/AM in culture medium for 30 min at 37 °C (19). After fura-2 loading of the cells, the cover glasses were washed and mounted in a specially designed chamber to which we added a calcium-containing medium (136 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 1.1 mM CaCl₂, 1.2 KH₂PO₄, 5 mM NaHCO₃, 5.5 mM glucose, and 20 mM Hepes, pH 7.4). The chamber was then placed in a system consisting of a Nikon Diaphot microscope connected to a Photon Technology International (PTI) imaging system. The cells were first allowed to rest for 10 min before any stimulation was performed. Fura-2 fluorescence was then recorded from the cells before and after stimulation with either Wnt-3a (100 ng/ml) or the formylated hexapeptide (100 μM) using an excitation wavelength rapidly alternating between 340 and 380 nm, whereas the emission was recorded at 510 nm. The fluorescence intensity ratios (340/380 nm) were subsequently calculated and analyzed using the PTI Image Master Software.

RESULTS

Peptide Sequences Based on PHD Prediction—We first predicted the most likely exposed amino acid residues by the PHD method (17). The results are shown in Fig. 1 together with the synthesized peptides and corresponding identification number (see also Table 1). Several segments are likely to be solvent-exposed because of the presence of several charged/polar/short residues and most were investigated.

Peptide-induced Effects on Adhesion and Migration of Wnt-5a Antisense HB2 Cells—Table 1 lists the initial 14 peptide fragments, designated pept167 through pept180, which were investigated regarding their ability to mimic the effects of the Wnt-5a protein on mammary cell adhesion and motility. All the peptides were derived from the amino acid sequence of Wnt-5a. In a previous study (18), we had shown that Wnt-5a is required for collagen-induced activation of the adhesion receptor DDR1 in mammary cells and for maximal adhesion of these cells to collagen. Therefore, in the current experiments, we analyzed DDR1 phosphorylation and adhesion as a means of screening for the ability of the peptides to mimic the effects mediated by Wnt-5a in Wnt-5a antisense HB2 cells. We chose these cells because they contained a low level of Wnt-5a, which made it easier to discern Wnt-5a mimicking effects of the peptides. The representative blots and the accumulated results of densitometric analysis shown in Fig. 2 demonstrate that pept168, pept169, pept170, and pept175 (100 μM) caused significant phosphorylation of DDR1. According to HPLC analysis (data not shown), the purity of these four peptides was ~65% in the initial screens (Figs. 2 and 3). Additionally, we conducted experiments to determine the percentage of cells adhering to collagen I in the presence of pept168, pept169, pept170, or pept175. We also included pept171 as a negative control and incubated the cells in Wnt-5a-CM as a positive control. The results show that adhesion was not affected by pept168, but it was significantly influenced by pept171, which we had presumed to be a negative control peptide (Fig. 3A). Inasmuch as there was no DDR1 phosphorylation in the presence of pept171 (Fig. 2A), we deduced that the mechanism underlying the impact of this peptide on cell adhesion does not require the activity of DDR1. Earlier studies had shown that β₁-integrins are not involved in activation of DDR1 (20) and that Wnt-5a-dependent adhesion of mammary cells is insensitive to preincubation with a blocking anti-β₁-integrin Ab (18). Therefore, we pretreated suspended HB2 cells with the previously used monoclonal anti-β₁-integrin Ab for 45 min at 37 °C to block the integrins. The results strongly suggested that the increased adhesion caused by the control pept171 was caused by β₁-integrin activation (Fig. 3B), which is in contrast to the effects of pept175 (Fig. 3B). In this context, it should be pointed out that, at 65% purity, none of the four peptides in question (pept168, pept169, pept170, and pept175) increased HB2 cell adhesion to the same extent as the CM did (Fig. 3A). To ascertain whether the effects of CM that are illustrated in Fig. 3 were actually mediated by Wnt-5a, we pretreated CM with an anti-Wnt-5a Ab and protein A to deplete the content of Wnt-5a, and then we tested the ability of the medium to induce cell adhesion. The results of these control experiments revealed that the Wnt-5a-depleted CM had no effect on adhesion (data not shown).
Having identified four Wnt-5a-derived peptides that had the capacity to increase adhesion of HB2 cells with low endogenous expression of Wnt-5a (Fig. 3A), we next performed a concentration-dependent analysis of how these peptides affected cell adhesion, but this time at higher purity (95%, determined by HPLC). The high purity peptide was most effective in augmenting HB2 cell adhesion to collagen I (Fig. 4, A–D). The effect of peptide was enhanced at higher purity, whereas high purity peptides had less consistent effects or no impact at all on adhesion. The lack of effect on adhesion with the 95% pure peptide indicates that the increase in adhesion with the 60–70% pure peptide was caused by an impurity in the preparation, which affected the integrin-dependent adhesion. Consequently, the pure peptide was subsequently used as a control/reference peptide since it lacks effect on both DDR1 activation and cell adhesion. Moreover, co-exposure to high purity peptide and peptide did not further increase the adhesion of HB2 cells caused by the high purity peptide alone (data not shown). At this stage of the screening, we also tested the effect of an 8-amino acid-extended version of peptide (peptide) on its ability to induce cell adhesion. We found that the 20-amino acid peptide (Table 1) was not as effective as the shorter peptide in increasing HB2 cell adhesion to collagen I (Fig. 4E).

Increasing Wnt-5a protein levels in HB2 cells has been shown to impair hepatocyte growth factor-induced migration in a collagen gel, presumably because of increased adhesion to the collagen (11).
ering the effects of pept169 and pept175 on cell adhesion in the present study, we proceeded to investigate the influence of these two peptide fragments (≥95% purity) on migration of Wnt-5a-low HB2 cells. As

Peptide-induced Effects on Adhesion and Migration of Wnt-5a-deficient MDA-MB-468 Tumor Cells—Based on available data, we have an interest in reconstituting Wnt-5a signaling in breast cancer cells and

| Peptide (pept) | Amino acid sequence | Position in the Wnt-5a sequence (amino acids) | No. of amino acids |
|---------------|---------------------|---------------------------------------------|------------------|
| 167           | SRAARPkDLPRD
| 168           | DAFRKerIK
| 169           | ADPRFKVD
| 170a          | VfAAKvKteIV
| 171           | SQgLAVSQQ
| 172           | G2NNIDY
| 173           | PGITQI
| 174a          | LotQaQRLC
| 175           | NNTSEQMD
| 176           | YQDbrNQI
| 177           | QYQFKRNA
| 178           | RNQIGSRE
| 179           | HNEAGR
| 180           | NSQKLV

*a* The three cysteines in the native Wnt-5a sequence were replaced with alanines (A) in the peptide.

*b* Extended sequence based on pept175.

A Formylated Hexapeptide Impairs Breast Cell Migration

![Figure 2](image_url)

**Figure 2.** Effects of crude Wnt-5a-derived peptides on collagen-induced DDR1 phosphorylation in Wnt-5a antisense HB2 cells. Single cells were allowed to adhere to collagen-I-coated plates in the presence of the indicated crude peptides (100 μM). Results are illustrated for experiments performed in the presence of pept167–173 (A) and pept175–180 (B). After 90 min, the cells were lysed, and DDR1 was immunoprecipitated. The immunoprecipitates were analyzed by Western blotting with an antiphosphotyrosine Ab, after which the membranes were stripped and reprobed with an anti-DDR1 Ab to ascertain equal loading. The diagrams depict densitometric ratios of DDR1 tyrosine phosphorylation normalized against the total amount of DDR1, expressed as percent of the control value obtained in the absence of any peptide. The blots shown are representative of three separate experiments, and the diagram outlines mean ± S.D. of the calculated ratios derived from three separate experiments.

![Figure 3](image_url)

**Figure 3.** Effects of crude Wnt-5a-derived peptides on adhesion of Wnt-5a antisense HB2 cells to collagen. A, single cells were allowed to adhere for 90 min in the absence or presence of the indicated crude peptides (100 μM). The extent of cell adhesion was determined as described under “Experimental Procedures,” and the results shown are means ± S.E. of seven separate experiments. Adhesion of Wnt-5a antisense HB2 cells to collagen was analyzed after pretreating the cells with an anti-β1-integrin Ab for 45 min and then allowing them to adhere for 90 min in the absence or presence of pept171 or pept175 (100 μM) or CM (Wnt-5a-containing medium). The extent of cell adhesion was determined as described under “Experimental Procedures,” and the results shown are means ± S.E. of six separate experiments; *, p < 0.05; **, p < 0.01; and ***, p < 0.001. ns, not significant.

![Image](image_url)
were harvested under the same conditions as applied in later experiments. Leptomycin B (50 nm) was added to the MDA-MB-468 cell culture at 80–90% confluence and pretreated with serum-free medium for 16 h prior to use, unless otherwise stated. The adhesion of MDA-MB-468 breast cancer cells to collagen I was significantly increased by exposure to pept175, another peptide with the same amino acid sequence but cysteine substituted for alanine (175Ala), as in the Wnt-5a protein expression in MDA-MB-468 cells in relation to both Wnt-5a antisense and HA-Wnt-5a-overexpressing HB2 cells. The results show that both the Wnt-5a antisense HB2 cells and the MDA-MB-468 cells contained low levels of Wnt-5a, whereas Wnt-5a-transfected HB2 cells expressed substantial amounts of this protein (Fig. 5A). It has been reported that Wnt-5a mRNA levels depend on culturing conditions and also on how confluent the cells are at the time of analysis (21). Consequently, the cells used in the experiments illustrated in Fig. 5A were harvested under the same conditions as applied in later experiments, i.e. at 80–90% confluence and pretreated with serum-free medium for 16 h prior to use, unless otherwise stated. The adhesion of MDA-MB-468 cells to collagen I was significantly increased by exposure to pept169 and pept175 and by incubation with CM, but not by the control pept171 (Fig. 5B). Peptides that contain one or more free cysteines, such as our pept175, may exert unspecific effects by participating in cysteine-cysteine interactions with other proteins/peptides, in this case, exposed on the cell surface. To ascertain whether the influence of pept175 on cell adhesion was related to its cysteine residue, we compared the impact of this peptide with that of a peptide that was identical except that its cysteine had been replaced with an alanine residue. We found that the alanine-containing pept175 substitute increased adhesion to at least the same extent as the cysteine-containing pept175 did (no statistically significant difference between the two; Fig. 5C).

It is known that cell migration is a prerequisite for cancer invasion and metastasis and that increased migration in a Boyden chamber is correlated with increased invasive properties of tumor cells in vivo (22). Therefore, we investigated the effects of the high purity pept171 (control), pept169, and pept175 on MDA-MB-468 cell motility in a Boyden chamber assay. Migration of the cells was markedly decreased by pept169 and pept175 but was not affected by the control pept171 (Fig. 6A). The same results were obtained when the wells and the membranes were coated with collagen I (10 μg/ml) at 37 °C for 1 h before initiating the experiments (data not shown). Incidentally, we observed that MDA-MB-468 cells exposed to pept175 contained a significantly higher level of filamentous actin (F-actin) compared with untreated cells, as visual
A Formylated Hexapeptide Impairs Breast Cell Migration

Effects of pept175 on Adhesion and Migration of MDA-MB-468 Tumor Cells

A Formylated Hexapeptide Impairs Breast Cell Migration

FIGURE 6. Effects of the selected and high purity Wnt-5a-derived peptides on the migration and F-actin content of MDA-MB-468 tumor cells. A, cells were allowed to migrate for 18 h in the absence or presence of the indicated more than 95% pure peptides (100 μM). The data in A are expressed as means ± S.E. of eight separate experiments, each performed in triplicate; *, p < 0.05. B, cells were allowed to adhere to collagen-I-coated cover glasses for 18 h in the absence or presence of high purity pept175 (100 μM) and were subsequently stained with Alexa488-phalloidin as described under “Experimental Procedures.” The graphs at the bottom outline higher magnifications of the indicated white-framed areas in their respective top graph. The illustrated micrographs are representative of six separate experiments.

D DISCUSSION

The present investigation, we derived a number of different peptides from the amino acid sequence of the Wnt-5a protein (presented in Table 1) and identified two that could mimic Wnt-5a in terms of inhibiting the motility of breast cancer cells. We reasoned that, like many coupled receptors and some of these bacterial peptides, like our hexapeptide, have a methionine residue in their N-terminus. Accordingly, we tested the effect of a formylated variant of our hexapeptide on cell adhesion. The results show that formylation of the N-terminal methionine of this hexapeptide restored, and possibly even potentiated, its impact on adhesion when compared with the original twelve amino acid long peptide (Fig. 7A). Control experiments revealed that the formylated tripeptide fMet-Leu-Phe, which is known to activate leukocytes (26), did not at 10 μM affect the percentage of adherent MDA-MB-468 cells (control 15.3 ± 3.6, fMet-Leu-Phe 15.7 ± 4.5; n = 6, mean ± S.E.). Nor was an effect seen when these tumor cells were exposed to 10 nM of fMet-Leu-Phe, a concentration with a significant effect on leukocyte motility (control 13.3 ± 2.6, fMet-Leu-Phe 14.6 ± 3.3; n = 6, mean ± S.E.). These findings indicate that the Wnt-5a hexapeptide sequence plays a specific role in mediating the effect of our formylated hexapeptide. In accordance with the influence of pept175 and the formylated hexapeptide on cell adhesion, we also found that, at a concentration as low as 10 μM, both these peptides significantly impaired the motility of MDA-MB-468 cells (Fig. 7, B and C).

To ascertain whether the formylated hexapeptide and recombinant Wnt-5a actually bind to the Frz-5 receptor in mammary cells, as previously shown for the Wnt-5a protein in synoviocytes and malignant melanoma cells (12, 13), we generated the Frz-5 receptor-blocking Ab described in the cited investigations. As a control, we raised an Ab against the same sequence in another Frizzled receptor expressed by these cells, the Frz-2 receptor. The two Abs were raised and isolated in parallel by identical methodology. The two affinity-purified Abs specifically recognize protein bands that perfectly match the molecular weights of the Frz-2 and the Frz-5 receptor, respectively (Fig. 8A). We also performed peptide blocking experiments in which the Abs were preincubated with the antigen peptides against which they were raised (1:10 molar ratio). In the subsequent Western blot analysis, the Frz-2 and Frz-5 receptors were undetectable even when the films were heavily overexposed (Fig. 8A, lanes 2 and 4, respectively). In agreement with reported data on malignant melanoma (13), we observed that the Wnt-5a-induced effect on MDA-MB-468 cell migration was mediated via the Frz-5 receptor, whereas the control Ab had no effect (Fig. 8B). Interestingly, we also noted that the influence of our formylated hexapeptide on MDA-MB-468 cell migration was mediated via the Frz-5 receptor (Fig. 8B). When inhibiting tumor cell migration in vivo, it should be kept in mind that the pH in solid tumor tissue is lower, ~6.7 (27, 28). Consequently, it is intriguing that the formylated hexapeptide was equally effective at inhibiting MDA-MB-468 cell migration at pH 6.7 as at pH 7.4 (Fig. 8C).

To further characterize the action of the formylated hexapeptide we investigated its ability to activate intracellular signaling events that have been suggested to be part of the major Wnt-induced signaling pathways (29). The formylated hexapeptide did not cause any detectable increase in the cellular level of unphosphorylated β-catenin or phosphorylated JNK (Fig. 9, A and B). Wnt-3a was used as a positive control in this context, because it has previously been shown to cause activation of these two signals in mammalian cells (30, 31). In contrast, the formylated hexapeptide triggered a prompt cytosolic free calcium signal, whereas Wnt-3a did not (Fig. 9C).
other protein ligands, Wnt-5a probably interacts with its receptor through at least one solvent-exposed loop protruding from its surface.

The three-dimensional structure of Wnt-5a is not known, and we could not apply homology modeling methods because of the lack of appropriate templates. Therefore, to surmise which segment of Wnt-5a could form a solvent-exposed loop, we performed secondary/solvent accessible surface predictions according to the PHD method (17). The initial results allowed us to focus on only a few peptides that were 8–15 amino acids long.

Several peptide ligands have been successfully identified, among them the well-established tripeptide Arg-Gly-Asp, which functions as an integrin receptor ligand (32), and two hexapeptides that specifically activate the G-protein-coupled protease-activated receptors 1 and 4 (33). Another example, but with an antagonizing effect, is a septapeptide that binds the G-protein-coupled receptor for thrombin (34). Analysis of peptide-induced effects on mammary cell adhesion was our primary screening approach in the current study. Using that strategy, we identified two peptides that, at high purity (>95%), caused a concentration-dependent increase in adhesion to collagen I in both Wnt-5a antisense HR2 cells and MDA-MB-468 cancer cells (two cell lines with very little endogenous Wnt-5a protein). We sequentially removed two amino acids at a time from the N terminus of the shorter of these two peptides (originally twelve amino acids long) to eventually identify the shortest peptide that still had an antimigratory effect similar to that of Wnt-5a. We found that the octapeptide was still able to increase adhesion, whereas the hexapeptide could not. However, the N-terminal amino acid in the hexapeptide was a methionine, which brought to mind the strong promoting effect that is exerted by the formyl group of the N-terminal methionine of the bacteria-derived fMet-Leu-Phe peptide (26). N-formylated methionine is the initial template in the synthesis of all bacterial proteins. During an infection, a large number of bacteria are disrupted in various ways and thereby release short N-formylated peptides, which very potently attract and activate leukocytes by binding with high affinity to the G-protein-coupled formyl peptide receptors on these cells (26). In mammalian cells, the hexapeptide sequence Met-Asp-Gly-Cys-Glu-Leu is present solely in the Wnt-5 proteins, whereas it occurs in two different bacterial proteins. However, in neither of those cases is it located in the N terminus, thus it cannot exist in a formylated state. Consequently, N-formylation of the hexapeptide described here is unique in that it cannot be found in either bacteria or mammalian cells.

We have previously shown that loss of Wnt-5a protein expression in breast tumor cells leads to decreased adhesion and thus increases motility (11). That finding, together with the knowledge that increased cell migration is associated with cancer metastasis (35), prompted us to investigate an antimigratory effect of the formylated hexapeptide on breast tumor cells. We performed these experiments in a modified Boyden chamber, because it has previously been shown that the migration of tumor cells in the Boyden system correlates well with the invasive behavior of such cells in vivo (22). We found that the formylated hexapeptide strongly impaired the migration of MDA-MB-468 tumor...
cells, which agrees well with the effect of this peptide on adhesion of these cancer cells. The minimum concentration of the formylated hexapeptide that had a significant effect on migration was 10 μM, which is the same concentration that Andersen et al. (33) found to be a minimal requirement for the function of their thrombin-derived hexapeptides in platelets. Our data demonstrating a significant peptide-induced increase in F-actin in parallel with decreased motility suggest that F-actin-mediated stiffness of the MDA-MB-468 cells is a possible explanation for the stronger adhesion and reduced motility. Interestingly enough, the ability of the formylated hexapeptide to impair breast cancer cell motility and increase their F-actin level in the present study is identical to the effects of recombinant Wnt-5a on motility and the F-actin content in a colon epithelial cell line (36). These data suggest that the formylated hexapeptide and Wnt-5a have the ability to activate one or more small GTPases. The present demonstration that JNK is not activated by the formylated hexapeptide suggest that small GTPases other than Rho are the most likely to be activated by this peptide ligand. Although important, we believe that an analysis and identification of the different GTPases involved in mediating the effect of the formylated hexapeptide on the actin network is beyond the scope of this study.

The novel finding that Wnt-5a affects breast cancer cell motility via the G-protein-coupled receptor Frz-5 also enabled us to explore if the described effects of the formylated hexapeptide were unspecific or if they reflected a true ligand-receptor interaction. In support of the latter,
A Formylated Hexapeptide Impairs Breast Cell Migration

a previously described Frz-5 receptor-blocking Ab abolished the effect of the formylated hexapeptide on breast cancer cell migration, whereas an identically produced control Ab directed against a corresponding ectodomain of the Frz-2 receptor did not. In this context, it should be mentioned that N-formyl-Met-Leu-Phe, the tripeptide that potently activates leukocytes, had no effect on adhesion of MDA-MB-468 cells, indicating that not just any formylated peptide can bind to and activate the Frz-5 receptor on these tumor cells. Furthermore, in agreement with previous reports on Wnt-5a signaling characteristics (29), the formylated hexapeptide triggered a prompt cytolic calcium signal whereas it had no significant effect on either canonical β-catenin or JNK signaling.

The potential clinical significance of the present findings is obvious. The fact that lack of endogenous Wnt-5a protein expression in mammary tumor cells is strongly associated with higher histological grade and reduced disease-free survival (9), underlines the need for a means to reconstitute Wnt-5a signaling in such cancer cells and thus potentially delay or inhibit the establishment of distal metastases in these patients. Furthermore, reconstitution of Wnt-5a signaling might offer a future therapeutic approach in other tumor forms as well, since this protein has been shown to have tumor suppressor activity in thyroid carcinoma (15), and loss of Wnt-5a expression is connected with high risk neuroblastoma (37) and shortened survival of patients diagnosed with a Duke’s B colon cancer (36).

Because of inefficient vascularization and increased production of lactate, the extracellular pH is lower in solid tumors than in normal tissue. However, it has also been suggested that Na⁺/H⁺ exchanger isoform 1 participates in the regulation of both extracellular and intracellular pH in human breast tumors (38). Therefore, it is essential that potentially metastasis-inhibiting substances are also effective at the low pH encountered in tumor tissue. The pH in solid tumor tissue in vivo has been estimated to be ~6.7 (27, 28), thus it is noteworthy that the formylated hexapeptide we synthesized inhibited the motility of MDA-MB-468 breast tumor cells at both a low and a normal pH (6.7 and 7.4, respectively).

In conclusion, we have established that a novel formyl-Met-Asp-Gly-Cys-Glu-Leu peptide ligand derived from the sequence of the Wnt-5a molecule: binding to the Frz-5 receptor, and impairing the motility capacity of a human breast tumor cell line with little endogenous expression of Wnt-5a. Accordingly, this peptide ligand can serve as a model substance for future development of an innovative antimetastatic treatment of the ~50% of human breast cancers that exhibit reduced endogenous expression of Wnt-5a.

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