Glycosaminoglycans Can Modulate Extracellular Localization of the *wingless* Protein and Promote Signal Transduction

Frieda Reichsman, Laurie Smith, and Susan Cumberledge

Department of Biochemistry and Molecular Biology, University of Massachusetts, Amherst, Massachusetts 01003

Abstract. *wingless*, the *Drosophila* homologue of the proto-oncogene *Wnt*-1, encodes a secreted glycoprotein that regulates differentiation and proliferation of nearby cells. Here we report on the biochemical mechanism(s) by which the *wingless* signal is transmitted from cell to cell. When expressed in S2 cells, the majority (~83%) of secreted *wingless* protein (WG) is bound to the cell surface and extracellular matrix through specific, noncovalent interactions. The tethered WG can be released by addition of exogenous heparan sulfate and chondroitin sulfate glycosaminoglycans. WG also binds directly to heparin agarose beads with high affinity. These data suggest that WG can bind to the cell surface via naturally occurring sulfated proteoglycans. Two lines of evidence indicate that extracellular glycosaminoglycans on the receiving cells also play a functional role in WG signaling. First, treatment of WG-responsive cells with glycosaminoglycan lyases reduced WG activity by 50%. Second, when WG-responsive cells were preincubated with 1 mM chlorate, which blocks sulfation, WG activity was inhibited to near-basal levels. Addition of exogenous heparin to the chlorate-treated cells was able to restore WG activity. Based on these results, we propose that WG belongs to the group of growth factor ligands whose actions are mediated by extracellular proteoglycan molecules.

COMMUNICATION between cells is an integral part of development and differentiation. Cells determine their fates, in part, by where they are located relative to other cells. Work in *Drosophila* has shown that this positional information is often provided by the distribution of specific extracellular ligands such as the *wingless*, *hedgehog*, and *decapentaplegic* proteins (for reviews see Klingensmith and Nusse, 1994; Siegfried and Perrimon, 1994). Anterior/posterior, dorsal/ventral, and proximal/distal axes provide the framework for subsequent development of many tissue and organs. Loss of *wingless* activity alters many of these positional axes, severely disrupting epidermal patterning, appendage formation, and CNS development in *Drosophila* (Siegfried and Perrimon, 1994).

Similar signaling systems are also present in vertebrates. For example, *Wnt-1* is required for fetal brain development in mice (McMahon and Bradley, 1990) and body axis specification in *Xenopus* (McMahon and Moon, 1989). Several lines of evidence indicate that *wingless* protein (WG)[1] and Wnt-1 are both structural and functional homologues (Nusse and Varmus, 1992). The two proteins share 54% amino acid sequence identity; both are glycosylated, cysteine-rich, and have a hydrophobic signal sequence. Both WG and Wnt-1 can regulate proliferation of specific neuronal cells during CNS development (Dickinson et al., 1994; Kaphingst and Kunes, 1994). *wingless* (*wg*) can even substitute for *Wnt-1* in some functional assays in vitro; for example, RAC mammary epithelial cells can be transformed via expression of either Wnt-1 protein or WG (Ramakrishna and Brown, 1993).

Much of the WG/Wnt-1 pathway downstream of signal reception has also been conserved across species. Genetic screens in *Drosophila* have revealed other genes in the *wg* pathway, such as *dishevelled*, *armadillo*, and *zeste-white 3*, which have vertebrate counterparts (Siegfried and Perrimon, 1994). The most striking example is the *armadillo* protein (ARM), which is 70% identical to vertebrate β-catenin (McCrea et al., 1991). Candidate receptor(s) for WG/Wnt-1 have been identified (Bhanot et al., 1996), and some of the downstream steps in the pathway have now been ordered. In response to the *wg* signal, intracellular *dishevelled* protein inhibits the activity of the *zeste-white 3* protein kinase (ZW3) (Siegfried et al., 1992). Inhibition of

Address all correspondence to Susan Cumberledge, Department of Biochemistry and Molecular Biology, University of Massachusetts, Amherst, MA 01003. Tel.: (413) 545-4031. Fax: (413) 545-3291. E-mail: susanc@biochem.umass.edu

1. *Abbreviations used in this paper*: ARM, *armadillo* protein; ECM, extracellular matrix; GAG, glycosaminoglycan; S2HSWG(+), *Drosophila* Schneider Line 2 (S2) cells transformed with a *wg* cDNA minigene; S2HSWG(−), *Drosophila* Schneider Line 2 (S2) cells transformed with a *wg* cDNA minigene in the antisense orientation; *wg*, *wingless* gene; WG, *wingless* protein.

ZW3 allows activation of ARM/β-catenin, prompting an increase in the dephosphorylation of ARM and an increase in the amount of cytoplasmic ARM (Peifer et al., 1994*a*,*b*). These changes in the state of ARM ultimately result in regulation of gene expression, although the mechanism by which the signal is transmitted to the nucleus is not known.

In vitro experiments have shown that WG/Wnt-1 modulation of ARM/β-catenin activity can also affect cadherin activity, cell adhesion, cell movement, gap junctions, and perhaps the function of adherens junctions in some cell types (Moon et al., 1993*a*,*b*; Peifer, 1995). Recently, Hartenstein et al. (1994) have proposed that the delamination and division of certain *Drosophila* neuroblasts can be triggered by cytoskeletal changes that occur in response to the WG signal. It is not known whether WG can regulate cytoskeletal structure simply by modulating ARM and cadherin activity, or if these effects are more indirect. The *wg/Wnt-1* pathways may also interact with other intercellular signaling systems, including the *Notch* (Couso and Martínez Arias, 1994), *noggin* and *follistatin* (McGrew et al., 1995), *FGF* (e.g., Pan et al., 1995; Parr and McMahon, 1995), and *decapentaplegic/TGFβ* pathways (Pankratz and Hoch, 1995). Such interactions could occur downstream of signal reception, or extracellularly at the level of ligand and receptor interactions.

Significant progress has been achieved in our understanding of the intracellular steps involved after cells receive the signal, but what are the extracellular events leading to signal transduction? WG and Wnt-1 are much larger (468 and 370 amino acids, respectively) than most growth factors. How these ligands are transmitted from cell to cell and the molecules that govern their extracellular localization are not known. During early embryogenesis, extracellular WG is restricted to within one or two cell diameters of the WG-secreting cells in the epidermis, and the protein is distributed symmetrically. But later, during germ band retraction, the distribution becomes quite asymmetrical (Bejsovec and Martínez Arias, 1991). At that time, WG is found several cell diameters anterior to the secreting cells, but is not detected on the posterior side. These observations indicate that localization of extracellular WG may be regulated in a dynamic fashion.

Work in vitro (Bradley and Brown, 1995) suggests that Wnt-1 activity may require an accessory molecule that is secreted by C57MG epithelial cells, but not by Rat-2 fibroblast cells. Extracellular WG and Wnt-1 are both associated tightly with the cell surface (Papkoff and Schryver, 1990; van den Heuvel et al., 1993; Smith, L.A., X. Wang, and S. Cumberledge, manuscript submitted for publication) and extracellular matrix (Bradley and Brown, 1990; Gonzalez et al., 1991; van den Heuvel et al., 1993). The nature of these interactions is not known. However, Wnt-1 can be released from the cell surface by treatment with exogenous heparin (Bradley and Brown, 1990), suggesting that Wnt-1 protein may interact with cell surface associated proteoglycans. Proteoglycans are a distinct class of glycoproteins that contain covalently linked glycosaminoglycans (GAGs) such as heparan sulfate and chondroitin sulfate (Kjéllan and Lindahl, 1991). They can bind to a variety of growth factors, and in some cases function as coreceptors (Klagsbrun and Baird, 1991; Schlessinger et al., 1995). For example, secreted bFGF associates with the cell surface proteoglycan syndecan-1 (Bernfield and Hooper, 1991). Both syndecan-1 and the FGF receptor are required for transmembrane signaling (Yayon et al., 1991). Similarly, betaglycan binds to TGFβ and promotes TGFβ binding to its high affinity receptor (López-Casillas et al., 1993).

We are interested in the mechanism by which extracellular WG becomes localized, how the ligand travels from cell to cell, and how the WG signal is transmitted across the membrane. Here, we show that secreted WG can bind to glycosaminoglycans with high affinity. These interactions are specific and are mediated, in part, by carbohydrate moieties found on the WG protein. Extracellular diffusion of WG in vivo may be restricted by binding to cell surface proteoglycans. Furthermore, we show that interactions with glycosaminoglycans can promote WG signal transduction. Proteoglycans are known to function as coreceptors for certain vertebrate growth factors; we propose that transmission of the WG signal may occur via a similar mechanism.

Materials and Methods

Cell Culture

The WG-expressing *Drosophila* Schneider Line 2 (S2) cell line (S2HSWG(+)) contains the *wg* cDNA under the control of the heat shock promoter (Cumberledge and Krasnow, 1993). S2HSWG(−) cells are identical except that the *wg* cDNA is in the antisense configuration. S2 cells were cultured in Schneider's Drosophila Medium (GIBCO BRL, Gaithersburg, MD) supplemented with 10% FCS (GIBCO BRL) and penicillin/streptomycin (25 U/ml and 25 μg/ml, respectively; GIBCO BRL) at 24°C. Clone-8 cells were grown in Shields and Sang M3 Insect Medium (Sigma Chemical Co., St. Louis, MO) supplemented with insulin (0.125 IU/ml; Sigma Chemical Co.), 2% FCS (GIBCO BRL), penicillin/streptomycin (as above; GIBCO BRL), and 2.5% fly extract (Currie et al., 1988).

Preparation of Conditioned Medium

S2HSWG(+) cells were grown for 24–48 h, to a density of 6–8 × 10^6 cells/ml in flasks or 10–12 × 10^6 cells/ml in suspension. The cells were then heat shocked for 45–60 min at 37°C, washed twice, and allowed to recover at 20 × 10^6 cells/ml in serum-free M3 medium at 24°C for 2–3 h. The conditioned medium was separated from cells by centrifugation at 2,000 *g* for 5 min and then cleared by centrifugation at 20,000 *g* for 15 min at 4°C. In control experiments when cells were pulsed with [^{35}S]Met, there was a 1 h lag after heat shock before soluble [^{35}S]WG could be detected in the medium (not shown).

For release of WG from the cell surface using GAGs, 10 μg/ml of heparin (mol wt 6,000; Sigma Chemical Co.), heparan sulfate (mol wt 7,500; Sigma Chemical Co.), or chondroitin sulfate (contains 70% chondroitin sulfate A and 30% chondroitin sulfate C, which differ solely in the position of the single sulfate per disaccharide; Sigma Chemical Co.) was added to S2HSWG(+) cells after heat shock and harvest, at the beginning of the 2-h recovery period. Conditioned media were concentrated in Centriprep-30 concentrators (Amicon, Beverly, MA), and media from equal numbers of cells were resolved by SDS-PAGE and transferred to nitrocellulose. Ponceau S protein stain (Sigma Chemical Co.) was used to verify equal loading of total protein in each lane. Blots were probed with polyclonal rabbit α-WG and goat α-rabbit HRP antibodies (BioRad Labs, Hercules, CA), and protein bands were visualized using enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL) or Super Signal (Pierce, Rockford, IL). For concentration dependence experiments, conditioned medium was diluted with M3 medium to the specified concentrations and applied to clone-8 cells as described below. No WG was detected in Western analyses of conditioned media from S2HSWG(−) cells (not shown).

Preparation of F(ab)$_2$ Fragment for Immunoprecipitation

To differentiate between antibody and WG antigen during immunoprecipitation experiments (WG and the IgG heavy chain have very similar apparent molecular masses, 55 kD), we used biotinylated rabbit α-WG F(ab)$_2$ fragment (25 kD) for all immunoprecipitations. F(ab)$_2$ fragment was generated from whole rabbit α-WG antibody by digesting with 20 μg pepsin (Sigma Chemical Co.) per mg of antibody in 100 mM sodium citrate, pH 3.5, for 6 h at 37°C. The reaction was stopped with 1/10 vol of 3.0 M Tris, pH 8.0, and the sample was dialyzed against 0.1 M sodium borate, pH 8.8. To biotinylate the F(ab)$_2$, 250 μg *N*-hydroxysuccinimide ester (10 mg/ml in dimethyl sulfoxide) was added per mg of antibody and the solution was incubated at room temperature for 4 h. The reaction was stopped by adding 20 μl 1 M NH_4Cl per 250 μg ester and incubating at room temperature for 10 min. Free biotin was removed by exhaustive dialysis against PBS. Avidin agarose beads (Pierce) were used to immunoprecipitate the F(ab)$_2$–protein complexes. The efficiency of immunoprecipitation from the medium was measured by quantitative Westerns (see below). >85% of the soluble WG protein found in the starting material (conditioned medium) was recovered after immunoprecipitation (not shown).

Quantitation of WG Protein

The relative amounts of WG protein on the cell surface, medium, and ECM were measured by Western blotting using rabbit α-WG primary antibody followed by ^{125}I-labeled goat α-rabbit antibody (ICN) and phosphorimager analysis. S2HSWG(+) cells (8 × 10^6 cells/ml) were heat shocked and allowed to recover for 2 h, and then the whole cells, medium, and ECM were separated. For immunoprecipitation from the cell surface, cells were washed and resuspended in M3 medium + 1% BSA. α-WG F(ab)$_2$ (1:500 dilution) was added and incubated with gentle mixing for 45 min at 4°C. Cells were harvested by centrifugation, washed twice in PBS, and lysed in 1 ml RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) by incubating for 15 min on ice. The lysate was centrifuged at 14,000 *g* to remove particulate matter. Avidin conjugated agarose beads (Sigma Chemical Co.) preabsorbed with 1% BSA were added to the supernatant and incubated for 1 h at 4°C with gentle mixing. Beads were then sedimented at 14,000 *g* for 15 s, washed three times in RIPA buffer, resuspended in SDS sample buffer (50 mM Tris, 2% SDS, 10% glycerol), and boiled 5 min. For immunoprecipitation from the medium, α-WG F(ab)$_2$ antibody was added to the medium (1:500), and the mixture was incubated with gentle mixing for 45 min at 4°C. Avidin-agarose beads were then added and incubated for 1 h at 4°C with gentle mixing. Beads were sedimented at 14,000 *g* for 15 s, washed three times in RIPA, resuspended in SDS sample buffer, and boiled 5 min. The ECM fraction was prepared after the cells were removed. First, the flask was washed five times with PBS, incubated for 5 min with 4 ml of RIPA, and then washed five times with RIPA buffer. The ECM was then extracted from the flask by adding 2× SDS sample buffer, boiling for two minutes, and scraping with a cell scraper. Samples were electrophoresed on a 10% SDS gel and transferred to nitrocellulose (Schleicher & Schuell, Inc., Keene, NH). The filter was blocked with 3% milk PBST (PBS, 0.05% Tween 20), probed with rabbit α-WG antibody (1:15,000) for 1 h, washed, and then probed with ^{125}I-labeled goat α-rabbit antibody (1 μCi/ml) for 2 h. The nitrocellulose was washed and then exposed to a Phosphor Screen. The screen was subsequently scanned on a Storm Phosphorimager (Molecular Dynamics, Sunnyvale, CA), the image analyzed, and the amount of WG protein in each lane quantitated using Imagequant software (Molecular Dynamics).

The absolute amount of WG in the medium was measured by metabolic labeling of cells with [^{35}S]methionine followed by immunoprecipitation of WG from the medium. Log phase S2HSWG(+) cells were washed and then resuspended (8 × 10^6 cells/ml) in M3 medium without methionine for 30 min. [^{35}S]methionine was added at 350 μCi/ml for 3 h including heat shock and recovery. For immunoprecipitation from the medium, whole rabbit α-WG antibody was added and incubated with gentle mixing for 45 min at 4°C. Protein A beads were then added and incubated for 1 h at 4°C with gentle mixing. Beads were sedimented at 14,000 *g* for 15 s, washed three times in RIPA, resuspended in SDS sample buffer, and boiled 5 min. The sample was electrophoresed on a 10% SDS gel. For fluorography, the gel was treated with Entensify according to the manufacturer's instructions (DuPont/New England Nuclear, Wilmington, DE), and then dried and exposed to film. After developing, the WG bands were excised and quantitated by scintillation counting. The efficiency of [^{35}S]Met incorporation, or specific activity of each preparation, was determined by measuring the total amount of radioactivity incorporated (quantitated as the TCA precipitable counts), and the protein concentration was determined by BCA assay (Sigma Chemical Co.). The amount of WG protein in the medium was calculated by dividing the cpm incorporated (in the WG band) by the specific activity of the total protein. This number estimates the amount of WG present but is influenced by the number of Met residues in WG compared to the average number of Met residues in the total protein preparation. A typical specific activity of the total protein was 1.6 × 10^4 cpm/μg protein.

Heparin-Agarose Affinity Chromatography

Conditioned medium from S2HSWG(+) cells was gently mixed with heparin-4% agarose beads (Sigma Chemical Co.) overnight at 4°C. Beads were collected by centrifugation at 2,000 *g* for 2 min, washed with 20 vol of 50 mM NaCl/10 mM Hepes, and eluted at room temperature for 5–15 min in the specified concentrations of NaCl/10 mM Hepes.

Activity Assays

Clone-8 cells were plated into 6-well plates (Corning Glass, Inc., Corning, NY and Falcon Plastics, Cockeysville, MD), grown to 70–90% confluency, and then incubated with conditioned media from S2HSWG(+) cells for 2.5–3 h. After WG or control treatment, medium was removed by aspiration and the clone-8 cells were harvested in PBS and lysed in lysis buffer (50 mM Tris, 2% SDS). The total protein concentration was measured by BCA assay (Sigma Chemical Co.), and equal amounts of protein were loaded in each lane of an SDS polyacrylamide gel and resolved by SDS-PAGE. The proteins were transferred to nitrocellulose or polyvinyl difluoride (PVDF) membrane and stained with Ponceau S (Sigma Chemical Co.) to verify equal loading of each lane on the gel. Blots were then probed with mouse monoclonal α-ARM antibody N27A1 and goat α-mouse-HRP (BioRad Labs). Immunoreactive protein bands were visualized by treating the blots with enhanced chemiluminescence (Amersham Corp.) or Super Signal (Pierce) as per the manufacturer's instructions and then exposing the blot to X-ray film. The film was analyzed by densitometry scanning, and the amount of ARM was quantitated using NIH Image software. WG activity was quantified as the increase in dephosphorylated ARM. As a third, independent control for equal protein loading, some blots were also probed with α-HSP70 antibody. Data normalized using the HSP70 loading controls were not significantly different from nonnormalized data. No change in ARM amount or distribution was detected in assays of conditioned media from S2HSWG(−) cells (not shown).

For enzyme treatments, clone-8 cells were incubated with heparin lyases I (heparinase) and III (heparatinase) and chondroitin sulfate ABC lyase (Seikagaku America Inc., Rockville, MD) at 1.0 mIU/ml overnight before and during WG treatment. Conditioned medium from S2HSWG(+) cells was treated with the same concentration of the lyases for 30 min immediately before use in the assay. For sodium perchlorate treatment, clone-8 cells were incubated with 1 mM sodium perchlorate (final concentration) 48 h before as well as during the incubation with WG in conditioned medium. Heparin and chondroitin sulfate (see Preparation of Conditioned Medium, above) were added at 10 μg/ml where indicated.

Results

Extracellular Localization of WG

S2HSWG(+) cells secrete active WG (Cumberledge and Krasnow, 1993; van Leeuwen et al., 1994). Fig. 1 (lanes *1* and *2*) shows that three distinct electrophoretic forms of WG with apparent molecular masses of 52, 55, and 57 kD can be immunoprecipitated from S2HSWG(+) whole cell lysates; no WG is detected in control cells. The two larger species (forms II and III) are N-linked glycoproteins, and the fastest migrating form is unglycosylated (form I) (Smith, L.A., X. Wang, S. Cumberledge, manuscript submitted for publication). van den Heuval et al. (1993) have shown previously that one WG species can be found in the medium, on the cell surface and on the extracellular matrix (ECM). By comparison, when Wnt-1 is expressed in

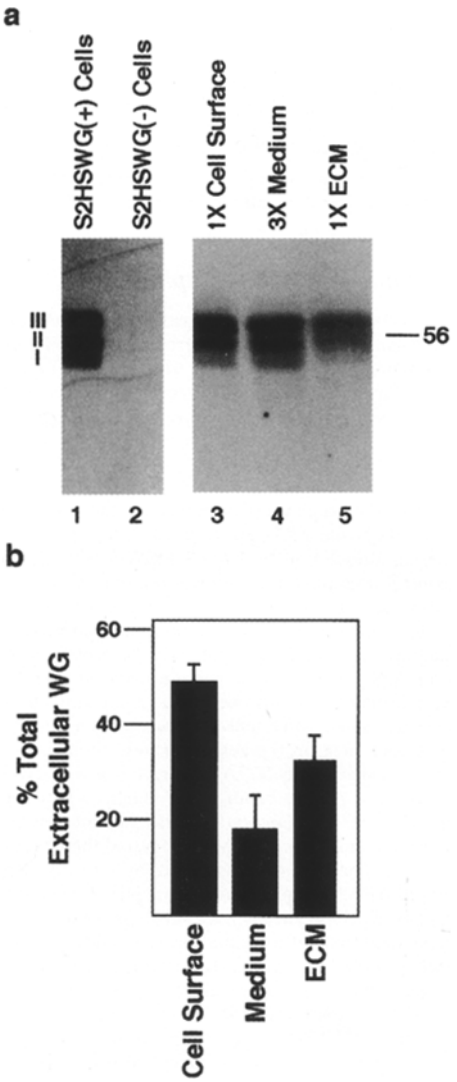

Figure 1. Most secreted WG is bound to the cell surface and extracellular matrix. (*a, Left*) Intracellular WG was immunoprecipitated from S2HSWG(+) whole cell extracts (lane *1*) and S2HSWG(−) whole cell extracts (lane *2*). The immunoprecipitates were analyzed using 10% SDS-PAGE and Western blotting techniques. Three WG isoforms are labeled. (*Right*) Immunoprecipitated WG from the cell surface (lane *3*), medium (lane *4*), and total ECM extract (lane *5*) were analyzed using 10% SDS-PAGE and Western blotting techniques. The nitrocellulose blots were probed with rabbit α-WG antibody followed by ^{125}I-labeled goat α-rabbit secondary antibody. Filters were analyzed using a Phosphorimager (Storm model 660; Molecular Dynamics). All immunoprecipitations were carried out with biotinylated rabbit α-WG $F(ab)_2$ antibody. 1× is equivalent to material from 10×10^6 cells. Control experiments show >85% of the soluble WG in the medium can be recovered after immunoprecipitation with α-WG $F(ab)_2$ antibody and avidin-agarose beads (not shown). (*b*) Relative distribution of extracellular WG. Data from *a* and two similar experiments were quantified using Imagequant (Molecular Probes, Inc., Eugene, OR).

various cell lines, the secreted protein is usually restricted to the cell surface and ECM. Of the cell types that have been examined thus far, only C57MG/Wnt-1 cells secrete soluble Wnt-1 into the medium (Bradley and Brown, 1990, 1995; Papkoff and Schryver, 1990). Multiple glycoforms of Wnt-1 have also been detected, and different transgenic cell lines express different forms. These observations raise several questions: Are all three forms of WG secreted, where is the majority of the extracellular WG, and does glycosylation affect the extracellular localization of WG?

Fig. 1 *a* (lanes *3* and *4*) shows that all three WG isoforms are also present on the cell surface and in the medium. Both the glycosylated and the unglycosylated forms are secreted. Furthermore, the relative abundance of each form does not vary significantly between locations: most of the protein is found as form III, with only a small amount of form I present. Multiple WG isoforms can also be detected in the ECM fraction (Fig. 1 *a*, lane *5*). These isoforms resolve poorly on SDS–polyacrylamide gels, making it difficult to identify unequivocally which glycoforms are present. We do not know if the aberrant behavior reflects a change in the composition of the WG protein, or if it is an artifact of the method used to prepare the ECM fraction. We also quantitated the relative amount of WG protein present in each extracellular location (Fig. 1 *b*). Of the total extracellular WG, 50% was localized to the cell surface, 17% to the medium, and 33% was found associated with the ECM. We conclude that the majority of extracellular WG is tethered to the cell membrane and ECM and a small fraction is soluble in the medium.

Next, we examined how WG is bound to the cell surface. We tested several factors for their ability to release WG. When S2HSWG(+) cells were treated with 0.5 M NaCl or 0.005% Triton X-100, no WG was released from the cell surface (data not shown). Previous work by Bradley and Brown (1990) has shown that addition of exogenous heparin, an abundant and naturally occurring sulfated glycosaminoglycan (SO_4-GAG), can release Wnt-1 from the cell surface. Therefore, we treated heat-shocked S2HSWG(+) cells with medium plus exogenous SO_4-GAGs. In contrast to NaCl treatment, addition of as little as 10 μg/ml heparin, heparan sulfate, or chondroitin sulfate releases bound WG from the cell surface (Fig. 2). After a 2-h incubation in the presence of SO_4-GAGs, there is a 5–10-fold increase in soluble WG in the medium as compared to the control treatment. This competition is quite specific and cannot be accounted for simply by differences in charge density, as heparin and heparan sulfate preferentially release WG form III, whereas chondroitin sulfate releases WG form II. Thus, WG binds to the cell surface through specific, noncovalent interactions.

WG is a Heparin-binding Protein

Can SO_4-GAGs mediate the release of WG by competing directly for binding sites on WG, or is the effect indirect? To address this question, we tested whether WG can bind directly to heparin. When conditioned medium containing WG is incubated with heparin-agarose beads, WG binds to the beads quantitatively and with high affinity (Fig. 3). WG is eluted from the column only after washing with 1.0 M NaCl. Form III appears to bind with somewhat stronger avidity to heparin agarose than either form I or II. This is consistent with our observation that heparin and heparan sulfate are more effective at releasing form III from the cell surface. Together, these results suggest that WG may

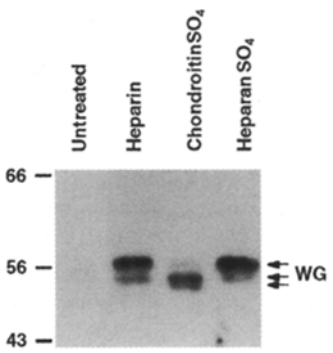

Figure 2. Release of WG from the cell surface by exogenous SO_4-GAGs. After heat shock, S2HSWG(+) cells were harvested into centrifuge tubes, washed, and resuspended in fresh, serum-free M3 medium. The cells were allowed to recover in the tubes for 2 h in the absence or presence of 10 μg/ml heparin, chondroitin sulfate, or heparan sulfate. The conditioned media, containing the released WG, were concentrated, and material from 7 × 10^6 cells was loaded in each lane of a 10% SDS–polyacrylamide gel. Immunoblotting was performed as described in Fig. 1.

associate with specific cell surface proteoglycans. Is WG also bound to SO_4-GAGs in the conditioned medium and on the ECM? Many proteoglycans found on the cell surface are shed into the medium, and would therefore be available for binding; however, we have not tested this directly. In the case of the ECM, preliminary results suggest that WG bound to the matrix can also be released by the addition of heparin and chondroitin sulfate (data not shown).

Sulfated Glycosaminoglycans Can Promote WG Signaling

Before testing the role of glycosaminoglycans in WG signaling, we first examined normal WG activity using the ARM assay described by van Leeuwen et al. (1994). Previous genetic studies have shown that *zw(3)*, a ser/thr kinase, promotes the phosphorylation and inhibition of ARM protein. WG inhibits *zw(3)* activity, thereby activating ARM. When clone-8 cells, a *Drosophila* cell line derived from imaginal discs (Currie et al., 1988), are incubated with soluble WG in conditioned medium from S2HSWG(+) cells, there is a large increase in the ratio of dephosphorylated to phosphorylated ARM and a concomitant increase in the total amount of cellular ARM protein. We have quantitated WG activity in this assay by measuring the increase in the dephosphorylated (faster migrating) form of ARM. Fig. 4 shows a concentration depen-

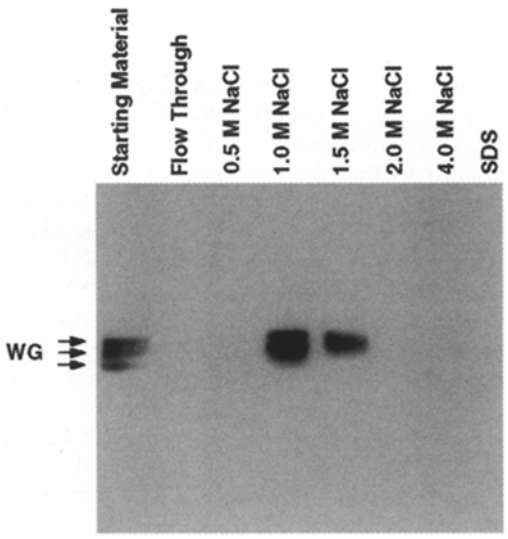

Figure 3. Heparin affinity chromatography. Conditioned medium from S2HSWG(+) cells was incubated with heparin-4% agarose beads. The column was washed with 50 mM NaCl and fractions were eluted with 0.5, 1.0, 1.5, 2.0, and 4.0 M NaCl, followed by 2% SDS. Material from 15 × 10^6 cells was loaded in each lane of an SDS gel and then subjected to Western analysis as in Fig. 1.

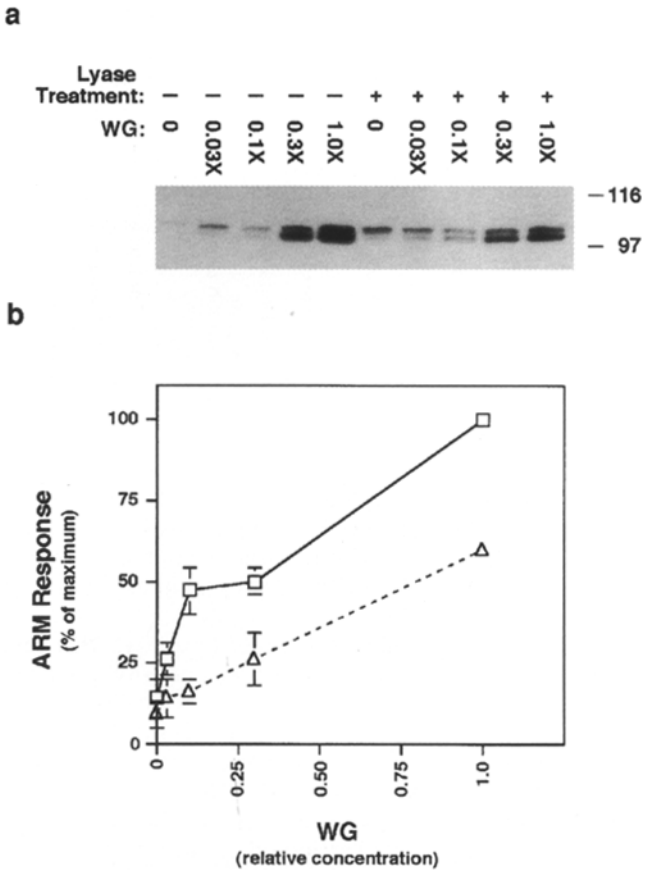

Figure 4. Enzymatic degradation of SO_4-GAGs inhibits WG activity. (*a*) Western analysis of the concentration dependence of WG activity in the absence or presence of SO_4-GAG lyases. Control (untreated) and lyase-treated conditioned media from S2HSWG(+) cells were assayed for WG activity on control or lyase-treated clone-8 cells, respectively. 1× medium = ~4 nM WG, as measured by immunoprecipitation of [^{35}S]Met-labeled WG (see Materials and Methods). Lysates of the clone-8 cells were subjected to 8% SDS-PAGE (70 μg protein/lane) and Western analysis. The blots were probed with N27A1 mouse α-ARM and goat α-mouse–HRP antibodies, and immunoreactive proteins were visualized using enhanced chemiluminescence. In lighter exposures, the phosphorylated and dephosphorylated forms are clearly resolved as two separate bands in all lanes. (*b*) Data from *a* and two similar experiments are represented graphically. The increase in the amount of dephosphorylated (faster migrating) ARM was used to quantitate WG activity in control (*squares*) vs. lyase-treated (*triangles*) clone-8 cells. Densitometry was performed using NIH Image. No ARM response was observed in clone-8 cells exposed to conditioned medium from S2 cells transfected with the *wg* cDNA minigene in the antisense orientation (S2HSWG(−) cells, not shown).

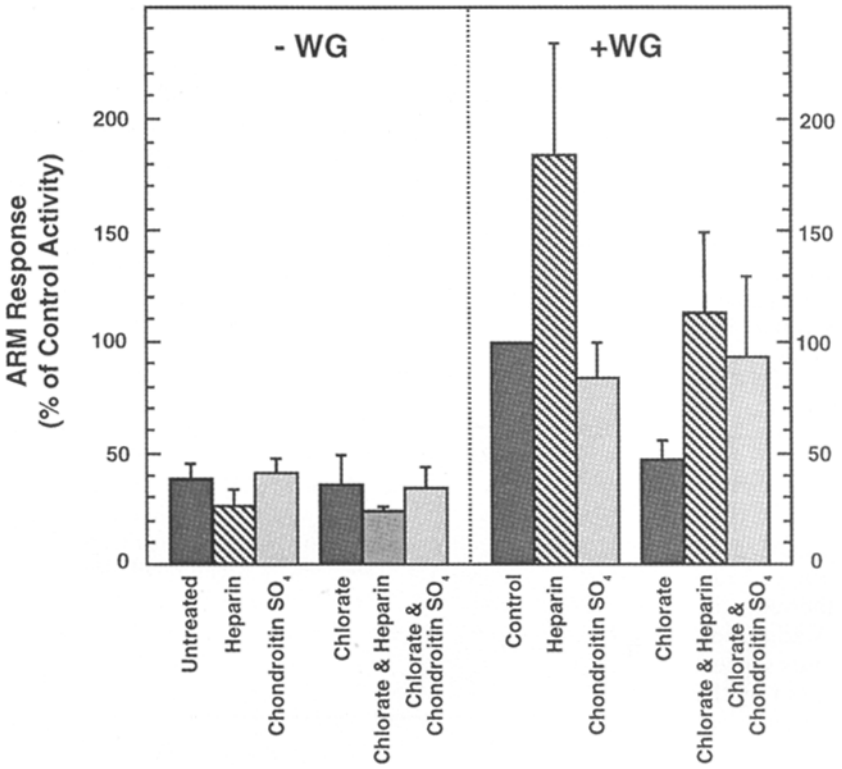

Figure 5. Chlorate treatment blocks WG activity. Clone-8 cells grown in the absence or presence of 1 mM chlorate (as indicated) were mock treated (*left panel*) or treated with WG-conditioned medium from S2HSWG(+) cells (*right panel*). The concentrations of WG added (0.1–0.3×) were those that typically elicit ~50% of WG activity at 1× (see Fig. 3). Heparin and chondroitin sulfate were applied, where indicated, at 10 μg/ml. The amount of dephosphorylated ARM in each lane of Western blots of the clone-8 cell lysates was quantified using NIH Image. All treatments are shown as a percentage of the control +WG value (100%). Bars indicate standard errors; $n \geq 3$ for all treatments except chlorate + chondroitin sulfate in the absence of WG, $n = 2$.

dence curve for WG protein present in the conditioned medium of S2HSWG(+) cells. Using the ARM assay, we can detect WG activity at concentrations in the pM range (Fig. 4). This range of activity suggests an interaction with a high affinity receptor.

We used two independent methods to test whether loss of specific SO_4-GAGs would affect WG activity. First, we used glycosaminoglycan lyases to enzymatically remove SO_4-GAGs from clone-8 cells and from S2HSWG(+) conditioned medium and then tested WG activity. Heparinase, heparatinase, and chondroitin ABC lyase cleave the SO_4-GAGs heparin, heparan sulfate, and chondroitin sulfates, respectively, while leaving other carbohydrate moieties and sulfate groups intact (Linhardt, 1994). When clone-8 cells and conditioned medium were treated with these three SO_4-GAG lyases, there was a 50% loss of activity as compared to untreated control cells (Fig. 4). No differences in proliferation, morphology, or cell adhesion were noted between control and enzyme-treated clone-8 cells. Thus, enzymatic removal of extracellular SO_4-GAGs significantly impairs WG activity. Are the SO_4-GAGs supplied by the S2HSWG(+) conditioned medium or the clone-8 cells? Endogenous SO_4-GAGs can be found on the cell surface or be shed into the medium as proteoglycans. Preliminary studies treating only the clone-8 cells with the lyases yielded similar results, indicating that conditioned medium from S2HSWG(+) cells is probably not a sufficient source of SO_4-GAGs.

We also looked at the role of cell surface SO_4-GAGs using a second, independent approach. We inhibited sulfation of proteoglycans in vivo by treating cells with sodium perchlorate, a reversible, competitive inhibitor of ATP-sulfurylase (Farley et al., 1976, 1978). Rapraeger et al. (1991) have shown that using chlorate treatment to block sulfation of proteoglycans effectively blocks FGF-induced DNA synthesis and mitogenesis, as well as the binding of FGF to its high affinity receptor. If SO_4-GAGs promote WG activity, then chlorate treatment of the clone-8 cells should lower WG activity at submaximal WG concentrations. Fig. 5 shows that after incubation with 1 mM sodium perchlorate, clone-8 cells are considerably less responsive to WG. In fact, at the submaximal concentrations of WG used, the response of the chlorate treated cells is inhibited to a near-baseline level. The chlorate inhibition can be abrogated by the addition of heparin. When chlorate-treated cells are exposed to WG in the presence of 10 μg/ml heparin, they respond like untreated cells (Fig. 5). Chondroitin sulfate is also able to restore activity. The effect of chlorate on the cells is not simply a generally debilitating one, since it is unlikely that the addition of GAGs would overcome an overall decline of cell function. Rather, the loss of WG activity in chlorate-treated cells is most likely due to the specific effect of blocking sulfation of GAGs on these cells, since supplying sulfated GAGs restores activity. Together, these results argue that sulfated GAGs can play an important role in promoting WG signaling.

Can the addition of exogenous SO_4-GAGs stimulate WG activity directly? In the case of FGF, addition of exogenous heparin can induce oligomerization of the ligand and promote FGF receptor dimerization and signal transduction. In contrast, sucrose octasulfate, a persulfated disaccharide that binds FGF without ligand oligomerization, has no effect on FGF signaling (Spivak-Kroizman et al., 1994). We have found that supplementing WG conditioned medium with 10 μg/ml heparin increases WG activity by as much as 80% (Fig. 5). The addition of heparin alone has no effect on ARM, indicating that heparin activation is mediated through WG, and that WG and heparin

act synergistically. Addition of chondroitin sulfate does not enhance WG activity (Fig. 5).

Discussion

Posttranslational Modification and Localization of WG

We find that ~17% of the extracellular WG secreted by S2HSWG(+) cells is in an active, soluble form. Characterization of WG expression in these cells suggests that glycosylation of WG is not required for secretion in vitro, nor does it affect the distribution of the protein to the cell surface, ECM, or medium. However, glycosylation may influence WG interactions with extracellular proteoglycans as discussed below. Once secreted, most of the extracellular WG is associated with the ECM and cell surface, probably via interactions with sulfated glycosaminoglycans. This tethering may account for the fact that in vivo extracellular WG protein can usually be detected only one to two cell diameters away from the secreting cells.

WG Interacts with Cell Surface Sulfated Glycosaminoglycans

We have shown that WG can be released from the cell surface by addition of the SO_4-GAGs heparin, heparan sulfate, and chondroitin sulfate. In addition, WG binds to heparin-agarose beads with high affinity, suggesting that exogenous SO_4-GAGs probably promote WG release by direct competition for binding sites on WG. The interactions between WG and the SO_4-GAGs are specific: chondroitin sulfate preferentially releases WG form II, while heparin and heparan sulfate release form III. In addition, form III binds to heparin-agarose with a stronger affinity than form II. The biological effects of the SO_4-GAGs are also specific: low concentrations of heparin stimulate WG activity, but chondroitin sulfate does not.

What factors might account for this specificity? The differences between these three GAGs are somewhat subtle. All are highly negatively charged, being composed of irregularly repeating disaccharide units that are N-acetylated, and N- and O-sulfated (Silbert et al., 1995). Heparin is the most highly sulfated of the three, averaging 2–2.5 sulfates per disaccharide vs. 1 for chondroitin sulfate and <1 for heparan sulfate (Silbert et al., 1995). Therefore, it is unlikely that the degree of sulfation is responsible for the specificity of the interactions. The sugar makeup of the three GAGs correlates well with respect to their actions on WG. While all three contain *N*-acetyl-glucosamine, they differ in that heparin and heparan sulfate contain *N*-acetyl-iduronic acid, whereas chondroitin sulfate contains *N*-acetyl-galactosamine (Silbert et al., 1995). This suggests that the hexosamine composition of the GAGs is an important binding determinant for specificity. Different glycosylation forms of WG could bind to different proteoglycans or to different GAGs on the same proteoglycan.

Sulfated Glycosaminoglycans Play a Functional Role in WG Signal Transduction In Vitro

What is the biological significance of these interactions? Proteoglycans are expressed by most vertebrate cells and homologues have also been identified in *Drosophila* (Spring et al., 1994; Nakato et al., 1995). They often mediate cell–cell and cell–matrix interactions (Silbert et al., 1995). Some, such as syndecan-1 (Bernfield et al., 1993) and glypican (Vaughan et al., 1994), interact with growth factors (e.g., FGF, TGFβ) during signal transmission (Silbert et al., 1995). Others, such as perlecan, bind to cell adhesion molecules (Hayashi et al., 1992).

Our results show that GAGs can also participate in WG signaling. The enzymatic digestion of SO_4-GAGs in conditioned medium and on the clone-8 cell surface results in a ~50% loss of WG activity. The specificities of the lyases for the GAGs heparin, heparan sulfate, and chondroitin sulfates A and C suggest that the loss of activity is due directly to the loss of these GAGs. This idea is further collaborated by the finding that chlorate-treated cells respond poorly to WG. The loss of activity in response to chlorate treatment is most likely a specific result of decreased sulfation of GAGs, as addition of soluble chondroitin sulfate or heparin restores activity to the chlorate-treated cells. These results are also consistent with the notion that clone-8 cells, and not S2 cells, express the necessary GAGs. Only the clone-8 cells, and not the S2HSWG(+) conditioned medium, were subjected to chlorate treatment.

How might extracellular proteoglycans modulate WG activity? Of the growth factors that interact with cell surface proteoglycans, FGF is probably the best characterized. FGF requires both a "low affinity" syndecan receptor ($K_d \sim 10^{-9}$ M), and a high affinity FGF receptor ($K_d \sim 10^{-11}$ M) for mitogenic activity (Moscatelli, 1987; Klagsbrun and Baird, 1991). How does the low affinity receptor promote signaling if, at equilibrium, the high affinity receptor will be saturated well before a significant fraction of the low affinity receptor is bound? Two models have been proposed to explain FGF signaling (see Klagsbrun and Baird, 1991 and Schlessinger et al., 1995, for discussion). Either of these can be adapted to WG signaling as shown in Fig. 6. In the first model, WG binding to cell surface proteoglycans limits diffusion of the ligand to two dimensions. If the on/off rates of the low affinity receptors are high, the net effect will be to increase the local concentration of ligand available for binding to the high affinity receptors. We suggest that WG signaling may occur in an analogous manner. In the second model, ligand binding to the proteoglycan induces ligand oligomerization. This, in turn, promotes receptor clustering and transmembrane signaling. The data in this paper are consistent with either of these models. With the recent identification of *fz-2* as a potential WG receptor (Bhanot et al., 1996), it will now be possible to test these models using a more defined system.

Do proteoglycans interact with other WNT proteins? Wnt-1 binds to heparin agarose (Bradley and Brown, 1990), and recent work by Burrus and MacMahon (1995) has shown that several other WNT family members are released from the cell surface by the addition of exogenous heparin. Jue et al. (1992) have found that addition of 50–200 μg/ml heparin can inhibit Wnt-1 transformation in a coculture assay. Nevertheless, heparin effects on FGF activity have been shown to vary from enhancement to inhibition depending on experimental conditions and the concentrations of heparin tested (Schlessinger et al., 1995). It will be interesting to determine if lower concentrations of heparin can stimulate Wnt-1 activity. Our studies provide

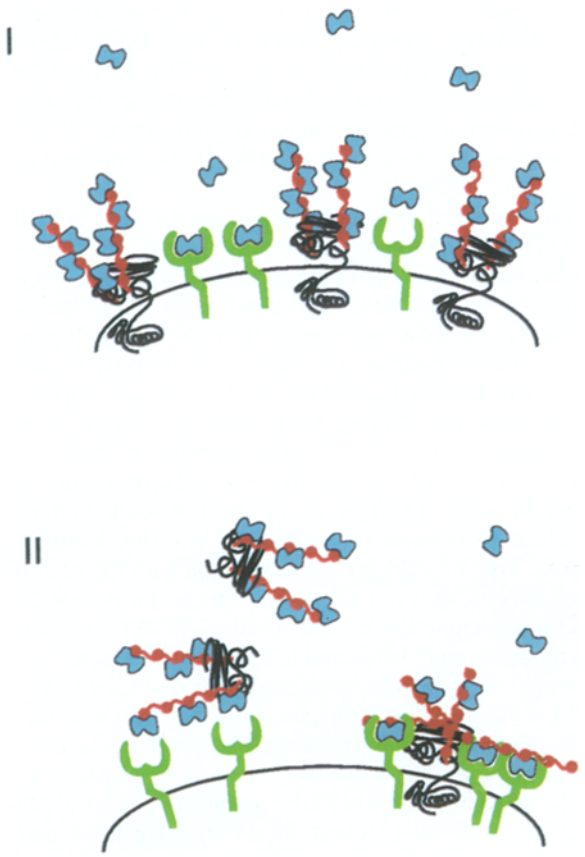

Figure 6. Models of WG interaction with cell surface accessory proteoglycans and high affinity receptors. Two hypotheses for activation of WG signaling by SO_4-GAGs are illustrated. (*I*) Localization of WG (*blue*) to the cell surface via SO_4-GAG chains (*red*) of a low affinity receptor (core protein in *black*) reduces the number of dimensions for diffusion of WG from three to two, increasing the chances that WG will interact with a high affinity receptor (*green*). (*II*) Binding of WG to SO_4-GAG chains on a low affinity receptor on or shed from the cell surface induces oligomerization of WG and receptor clustering.

the first evidence that sulfated GAGs participate in WG signaling pathways. Given the remarkable degree of conservation between the WG and Wnt-1 signaling pathways, we speculate that GAGs may also play a functional role in Wnt-1 activity.

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