Wheat Germ Agglutinin Binds to the Epidermal Growth Factor Receptor of Artificial Caco-2 Membranes as Detected by Silver Nanoparticle Enhanced Fluorescence

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Purpose. The purpose of this study was to identify one of the ligands that mediate carbohydrate-specific cytoadhesion and cytoinvasion of wheat germ agglutinin (WGA)-containing drug delivery systems.
Methods. The receptor–ligand studies were performed with isolated epidermal growth factor (EGF) receptors as well as biomimetic membranes prepared from Caco-2 and A-431 cells. The binding of fluorescent labeled WGA was detected by the silver nanoparticle enhanced fluorescence technique.
Results. The binding of WGA to isolated EGF receptors is saturable and the equilibrium is reached within 1 min. The interaction between WGA and isolated EGF receptors is fully inhibited by the complementary carbohydrate and at least 85% of WGA binding to artificial Caco-2 membranes is caused by protein–carbohydrate interactions involving the tetrasialo-binding motif. The integrity and the presence of EGF-receptors in artificial Caco-2 membranes as well as their WGA-binding capacity were confirmed by immunoblot detection.
Conclusions. The glycosylated extracellular domain III of the EGF receptor is involved in the WGA–Caco-2 cell interaction. Accordingly, receptor mediated endocytosis is the basic mechanism for internalization of WGA. As the EGF receptor is overexpressed in a high number of tumors but also occurs in non-malignant tissue at considerable density, WGA-mediated drug delivery opens exciting possibilities for specific binding and uptake of poorly absorbable drugs.

KEY WORDS: Caco-2; EGF; lectin-mediated drug delivery; silver nanoparticle-enhanced fluorescence; wheat germ agglutinin.

INTRODUCTION

In search of new strategies for successful delivery of even poorly absorbable drugs, the lectins, termed as "bioadhesives of the second generation," were found to mediate specific adhesion as opposed to nonspecific mucoadhesives (1). Lectin-mediated drug delivery relies on the specific interaction between the glycocalyx covering the surface of most epithelial cells and the carbohydrate binding site of the lectin. Among the dietary lectins, the wheat germ agglutinin (WGA) from *Triticum vulgare* interacts specifically with *N*-acetyl-D-glucosamine and sialic acid containing oligosaccharides. Moreover, the WGA not only binds to the cell membrane of Caco-2 cells and human enterocytes but is also taken up into the cells (2). Because of proteolytic stability and fecal recovery of high amounts of intact WGA after peroral administration to rats, this lectin is an appealing candidate for peroral administration of conjugated drugs or drug delivery systems (3,4). But prior to use of WGA as a "shoehorn" for drug delivery in practice, the whys and hows of cytoadhesion and cytoinvasion need to be elucidated.

There are some subtle hints in the literature that WGA interacts with the epidermal growth factor (EGF) receptor: After preincubation of PC12 cells with either WGA or concanavalin A, the binding of I^{125}-EGF decreased to less than 50% as the result of lowering the affinity of the receptor. The inhibition was reversed by addition of complementary carbohydrates (5). Additionally, the tyrosine kinase activity of isolated EGF-receptors was activated by concanavalin A and WGA to a similar extent as induced by EGF. The activation of WGA was partially prevented by *N*-acetylglucosamine (6).

To get evidence whether the EGF-receptor is involved in binding and uptake of WGA, the interaction of WGA with isolated EGF-receptors as well as artificial cell membranes prepared from Caco-2 and A-431 cells is characterized. The Caco-2 cell line is a well-established model for the intestinal epithelium and A-431 cells contain EGF-receptors at high density. To avoid hazardous handling of radioactivity, a new technique for fluorescence enhancement is used. The silver nanoparticle enhanced fluorescence (SNEF) system relies on the phenomenon that an electromagnetic nearfield is generated upon illumination on the surface of silver nanoparticles. The interaction of this field with nearby fluorophores results in fluorescence enhancement and allows discrimination from fluorophores in the bulk solution (7–9).

MATERIAL AND METHODS

Materials

Sucrose, 3-aminopropyltrimethoxysilane, bovine serum albumin (BSA), fetuin, alkaline phosphatase (AP)-labeled streptavidin, anti-sheep IgG antibody–AP conjugate (in donkey), and leupeptin were purchased from Sigma (Vienna, Austria). Skim milk powder, perchloric acid 20%, dodecylamine, soybean trypsin inhibitor, *N,N′N″*-triacetylchitotriose (TCT) and the buffer chemicals were from Fluka (Vienna, Austria). 96 well polystyrene microplates were from Greiner (Kremsmünster, Austria). Fluorescein labeled wheat germ agglutinin (F-WGA) from Triticum vulgare (molar ratio fluorescein/protein 3.2) and biotinylated wheat germ agglutinin were from Vector Laboratories (Burlingame, CA, USA).

Cell Culture

A-431 cells and Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were grown in 75-cm^2 flasks in RPMI-1640 supplemented with 10% fetal calf serum, 4 mM L-glutamine, and 150 μg/mL gentamycin at 37°C in a humidified 5% CO_2/95% air atmosphere and subcultured by trypsination. For plasma membrane preparation passages 4 to 40 of A-431 cells or Caco-2 cells were used.

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Interaction between F-WGA and Isolated EGF Receptor in the SNEF System

To investigate the contribution of the EGF receptor on F-WGA binding the SNEF system was coated with isolated EGF-receptor (Fig. 1). The preparation of silver colloid-coated microplates used for the SNEF system and the theoretical background were described in detail previously (7). For coating the SNEF wells, the EGF receptor was adsorbed to the silver colloid surface by incubation with 100 μL of an EGF receptor solution (0.8 U/μL, diluted 1:1000 in phosphate-buffered saline [PBS]; Upstate, Buckingham, UK) at room temperature for 1 h. After washing twice with 200 μL of PBS each, nonspecific binding sites were blocked with 200μl 3% (w/v) nonfat milk powder in PBS for 30min. After washing again with PBS the EGF receptor-coated SNEF system was ready for use.

Real-Time Monitoring of the F-WGA–EGF Receptor Interaction

To plot the mean velocity of the lectin/EGF receptor interaction vs. increasing amounts of the lectin, 40 μL of a 3, 14, 28, 69, 139, 278, or 694 nM F-WGA solution in PBS were added to the EGF receptor-coated SNEF plate followed by real time monitoring for 10 min. Negative controls containing PBS instead of the lectin were included in all experiments.

Specificity and Reversibility of the F-WGA–EGF Receptor Interaction

To estimate the specificity as well as the reversibility of the lectin–receptor interaction the EGF receptor coated surfaces were incubated with 40 μL F-WGA solution (3, 14, 28, 69, 139, 278, 694 nM in PBS) for 40 min at room temperature. Excessive F-WGA was removed by washing twice with 200 μL of PBS. After addition of 40 μL TCT solution (159 μM in PBS) the detachment of F-WGA from the EGF-receptor was monitored in real time. Negative controls were included in every experiment. As a positive control PBS was used instead of the carbohydrate solution.

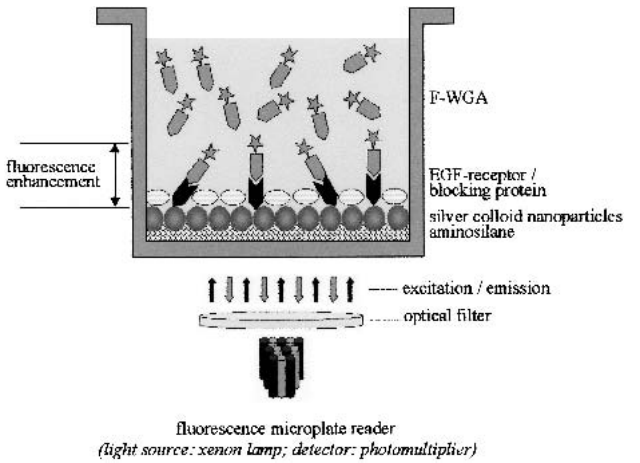

Fig. 1. Scheme of the assay setup consisting of the silver nanoparticle-enhanced fluorescence system coated with epidermal growth factor receptors for real-time monitoring of receptor–ligand interactions.

Interaction between F-WGA and Artificial Cell Surfaces Overlying the SNEF System

Plasma Membrane Preparation

Confluent A-431 or Caco-2 cells were harvested and broken up by Potter homogenization in 1 mM Tris/HCl-buffer pH 7.4 containing 75m M sucrose, 0.5 mM dithiothreitol, 45 μM leupeptin, and 0.5 mg/mL soybean trypsin inhibitor under cooling with ice. Most of the cytoplasmic constituents were removed by centrifugation (1 h, 122,000 g, 4°C) and the membrane pellet was further purified by sucrose-density centrifugation (16 h, 113,000 g, 4°C) in a 10–60% discontinuous gradient (10–12). To identify the fractions rich in plasma membranes the fractions (750 μL) were assayed for their content of RNA, protein, and AP.

For estimation of the RNA content both, DNA and RNA were precipitated by addition of 6% perchloric acid and the pellet was dissolved in 1 N NaOH. The DNA was precipitated by 3% perchloric acid and the amount of RNA remaining in the supernatant was determined by reading the OD at 260nm. The protein content of the fractions was determined by the Bradford assay (Bio-Rad protein assay, Bio-Rad, Vienna, Austria). The activity of AP was estimated by cleavage of colorless nitrophenylphosphate yielding yellow nitrophenolat at alkaline pH (AP optimized kit, Sigma, Vienna, Austria).

Building Up Artificial Cell Surfaces

For spreading and stable anchoring of the lipophilic membrane vesicles on the polar SNEF-coated microplates an intermediate layer consisting of amphiphilic aminoalkanes is necessary (13). This self-assembled monolayer (SAM) was built up by adding 100 μL of 1 mM dodecylamine in ethanol to each silver colloid coated well and incubating for 18 h at 4°C. After washing twice with 200 μL of ethanol the SAM-coated surface was dried with compressed air. Artificial cell surfaces were obtained by fusion of 100 μL pooled and 1:2 diluted plasma membrane vesicles with the SAM-coated silver colloid microplates overnight at 4°C. After washing, blocking with skim milk powder, and repeated washing with PBS the artificial cell surface SNEF system was used for the assays.

Interaction between F-WGA and Artificial A-431 or Caco-2 Cell Surfaces

The assay was performed as described above but using SNEF plates coated with biomimetic membranes.

Effect of N,N′,N″*-Triacetylchitotriose on F-WGA Binding to Artificial Cell Surfaces*

The specificity of the interaction between WGA and the glycocalyx of artificial cell surfaces was investigated by competitive inhibition with TCT. Thus, 25 μL of TCT solution (20, 159, or 318 μM in PBS) were added to the membrane coated wells of the SNEF system followed by addition of 25 μL F-WGA solution (278 nM in PBS). After immediate monitoring of the F-WGA binding kinetics, the endpoint of F-WGA binding was detected 1 h later as described above.

To elucidate the potential reversibility of WGA binding,

the artificial Caco-2 surfaces were loaded with 50 μL of F-WGA solution (139 nM in PBS) for 1 h. After removal of excessive lectin by washing twice with 200 μL of PBS, 50 μL of TCT solution (10, 79.5, or 159μM in PBS) were added. The artificial cell surfaces were further incubated for 70 min at room temperature followed by fluorimetric detection of surface bound F-WGA. Negative controls were included in every experiment. Using PBS instead of the carbohydrate solution served as a positive control representing the maximum F-WGA binding to the artificial cell surfaces.

Gel Electrophoresis and Western Blotting of EGF Receptors and Caco-2 Plasma Membrane Vesicles

To get evidence for the involvement of EGF-receptor in F-WGA binding, the interaction between the lectin and the EGF-receptor as well as the Caco-2 plasma membrane vesicles was further elucidated by gel electrophoresis and western blotting.

Discontinuous polyacrylamide gel electrophoresis was performed in a Mini-Protean®II Electrophoresis Cell (Bio-Rad, Austria) under native conditions on 7.5% acrylamide. But the samples were prepared under reducing conditions in presence of 5% β-mercaptoethanol and 2% sodium dodecyl sulfate (95°C, 5 min). The EGF receptor, the Caco-2 plasma membrane vesicles as well as bovine serum albumin and fetuin as controls were stained with colloidal gold. For estimation of the molecular weight prestained Precision Protein standards (Bio-Rad, Vienna, Austria) were used.

For immunoblotting, the separated proteins were electrotransfered to polyvinylidene diflouride membranes (PVDF) using a Mini Trans Blot®Electrophoretic Transfer Cell (Bio-Rad, Vienna, Austria) at 30 V for 20 h.

Detection of WGA Binding to the Blotted EGF Receptor and Caco-2 Plasma Membrane Vesicles

After protein transfer the membrane was washed three times with TBS buffer (50 mM Tris/HCl, pH 7.2, 27 mM NaCl) for 10 min and blocked for 30 min with blocking solution (Immun-Blot Kit for glycoprotein detection, Bio-Rad, Austria) in TBS buffer. After additional washings with TBS the blot was incubated with 10 mL of biotinylated WGA (250 μg/mL) for 1 h at room temperature followed by removal of unbound lectin by washing three times with TBS. The EGF receptor or Caco-2 plasma membrane-bound biotinylated WGA was detected by incubation with AP–streptavidin for 1 h followed by incubation with color development solution (Bio-Rad, Vienna, Austria) containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphat.

Detection of Blotted EGF Receptors

After blocking and washing the membrane as described above, the blotted EGF-receptor was detected by a sandwich consisting of anti-EGF receptor polyclonal antibody (raised in sheep, 10 μg/mL TBS; Upstate, Buckingham, UK) and AP-conjugated anti-sheep IgG antibody raised in donkey (1: 1000 in TBS) as the second antibody by incubating each for 1 h on a shaker. Followed by three washings with TBS, the AP-containing bands were stained as described above.

RESULTS

The isolated EGF-receptor as well as the EGF receptor present in artificial plasma membranes prepared from Caco-2 or A-431 cells were screened for possible interaction with WGA. To improve the detection limit of F-WGA after binding to the artificial surfaces, microplates coated with silver nanoparticles were used for the assays throughout.

Interaction of WGA with Isolated EGF Receptors

To enhance the fluorescence intensity of the fluorescent labeled lectin upon binding to the receptor, the commercially available EGF receptor was directly adsorbed to the polar surface of silver colloid coated microplates. Successful immobilization of the EGF receptor was confirmed by sandwich ELISA. Anti-EGF-receptor antibodies developed in sheep were used as first and AP-labeled anti-sheep antibodies as second antibody followed by color development with *p*-nitrophenylphosphate. Upon EGF receptor coating an absorbance of 0.624 ± 0.073 was observed which was significantly higher as compared with 0.274 ± 0.025 in case of the blank using PBS instead of EGF receptor for coating. Additionally, non-specific lectin binding sites were blocked with skim milk powder prior to incubation with F-WGA.

Real-Time Binding Kinetics of F-WGA to EGF Receptors

The F-WGA binding kinetics to isolated EGF receptors overlying the SNEF system were elucidated by real time monitoring using serial dilutions of the lectin. Up to 278 nM F-WGA the mean velocity of the interaction increased linearly ($r = 0.998$, $p < 0.0001$) concurrent with increasing amounts of F-WGA yielding a mean velocity of 8664 ± 1578 mFU/s (Fig. 2). At higher F-WGA concentrations the EGF-receptors were saturated as indicated by flattening of the curve. According to the slope of the curve the lectin binds rapidly to the EGF-receptor reaching the equilibrium within 1 min.

Specificity and Reversibility of the F-WGA - EGF-Receptor Interaction

The specificity of the interaction between F-WGA and EGF receptors was determined by displacement of EGF-

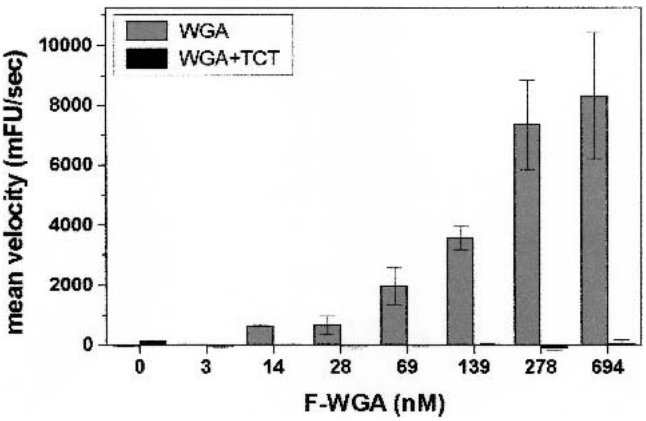

Fig. 2. Real-time binding kinetics of F-WGA in the presence (WGA + TCT) and in the absence of *N, N′, N′′*- triacetylchitotriose (WGA) to EGF receptors immobilized on the silver nanoparticle-enhanced fluorescence system (mean ± SD, n = 3).

receptor bound lectin with the free complementary carbohydrate (TCT). Upon addition of a thousandfold excess of chitotriose the receptor bound lectin was entirely displaced from the oligosaccharide moieties of the receptor protein (Fig. 2). Moreover, this indicates full reversibility of the interaction between WGA and isolated EGF-receptors.

WGA Binding to the Blotted EGF-Receptor

Surprisingly, the EGF receptor protein was only transferable to the PVDF membrane after electrophoresis under native conditions and sample preparation under reducing conditions. In accordance with the literature the isolated EGF receptor protein exhibited a molecular weight of 170 kDa (Fig. 3). As the blotted receptor was immunologically detectable by formation of a colored precipitate after incubation with EGF receptor antibody, AP-conjugated anti-antibody, and nitroblue tetrazolium/indolylphosphate as a substrate, it is likely that the native structure of the receptor was preserved. The binding of WGA to the blotted EGF receptor was visualized by use of avidin/biotin and enzymatic detection (Fig. 4). Whereas BSA as a negative control was not detectable, the fetuin representing a glycoprotein rich in sialic acid residues as well as the EGF receptor yielded bands colored deeply violet. This result confirms binding of WGA to the EGF receptor qualitatively.

Interaction between F-WGA and Artificial Cell Surfaces Overlying the SNEF System

To get an idea of the contribution of the EGF receptor on lectin binding to the cell membrane, artificial plasma membrane vesicles were prepared from A-431 and Caco-2 cells. For most sensitive detection of lectin binding to the membrane, the vesicles were fused with the SNEF system coated with the self-assembled monolayer.

Characterization of A-431 and Caco-2 Plasma Membrane Vesicles

After Potter homogenization and purification by sucrose density gradient centrifugation the fractions were assayed for

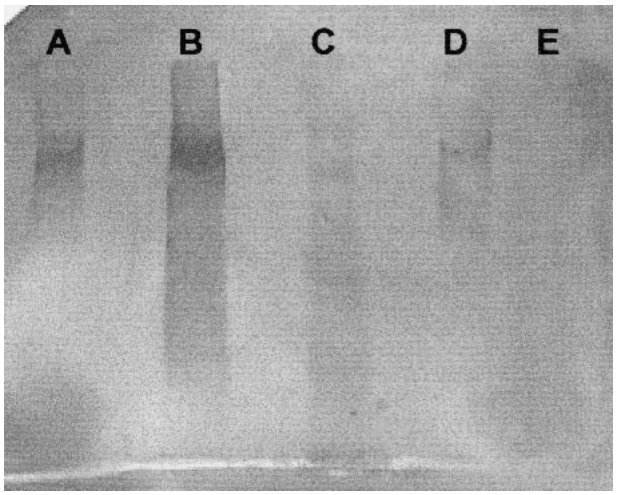

Fig. 3. Caco-2 plasma membrane vesicles (lanes A, D), isolated epidermal growth factor receptor (lane B), prestained molecular weight marker (lane C), and bovine serum albumin (lane D) detected with anti-epidermal growth factor receptor antibody/anti-IgG-antibody–alkaline phosphatase conjugate after Western blotting.

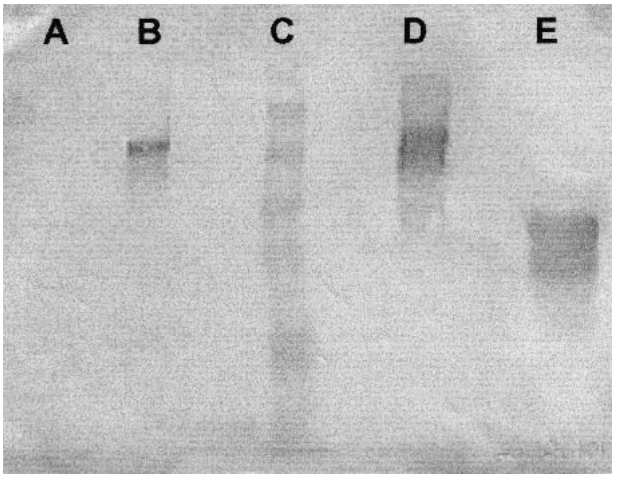

Fig. 4. BSA (lane A), isolated epidermal growth factor receptor (lane B), prestained molecular weight marker (lane C), Caco-2 plasma membrane vesicles (lane D), and fetuin (lane E) detected with biotinylated WGA/streptavidin–alkaline phosphatase conjugate after Western blotting.

their content of RNA, protein, and AP-activity. The fractions 1–4 contained only low amounts of AP, protein, and RNA, which indicates lack of plasma membrane vesicles (Fig. 5). Concurrent with fraction number and sucrose density the protein content and the AP-activity increased indicating presence of plasma membrane vesicles. Independent from the cell line used, the maximum AP-activity was detected in fraction 8. But the maximum enzyme activity was significantly higher in case of Caco-2 cells as compared to A-431 cells yielding 22.0 dE/min/mg protein (Caco-2) and 0.8 dE/min/mg protein (A-431), respectively. The fractions 9–16 are characterized by a decreasing and rather low AP-activity concurrent with an increase in protein and RNA content, which points to presence of cytosolic vesicles. Thus, the fractions 5–9 corresponding to 25–39% sucrose density were pooled and used to build up artificial cell surfaces.

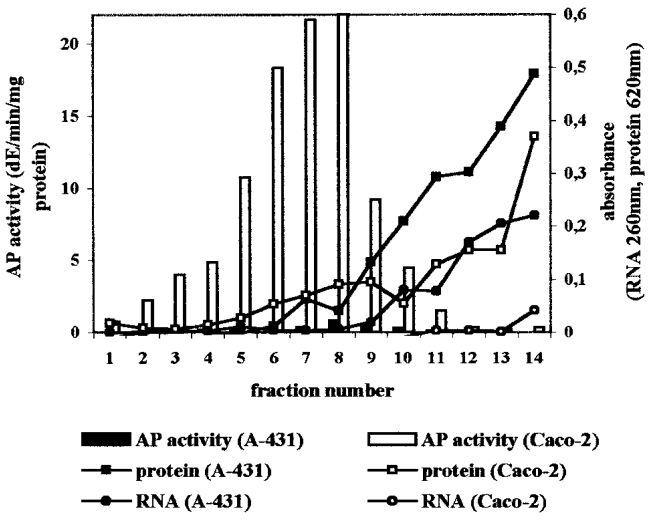

Fig. 5. Characteristics of the fractions collected after sucrose density centrifugation of homogenates prepared from Caco-2 and A-431 cells.

Interaction between F-WGA and Artificial A-431 or Caco-2 Cell Surfaces

After fusion of Caco-2 plasma membrane vesicles with the self assembled monolayer coated SNEF-system, the real time binding kinetics of F-WGA to artificial Caco-2 cell surfaces were investigated. Within the concentration range of F-WGA tested, a linear relationship between lectin concentration and mean velocity was observed ($r = 0.991$, $p < 0.0001$) yielding 448 ± 16 mFU/s at 347 nM F-WGA (Fig. 6). According to the ascent of the curve, the interaction is rather rapid and the equilibrium is reached within 4min.

To examine the WGA binding capacity of A-431 and Caco-2 cell membranes, the artificial surfaces were incubated with 0.6μg F-WGA for 40min. The comparison of the endpoint detection data amounting to 3325 ± 447 FU (A-431) and 1108 ± 132 F.U. (Caco-2) revealed that a 3-fold higher amount of F-WGA was bound to A-431 membranes.

Effect of the Complementary Carbohydrate on F-WGA Binding to Artificial Cell Membranes

As lectins preferably bind to carbohydrate moieties located at the cell membrane, the interaction between WGA and Caco-2 cell surfaces was examined with respect to its specificity and reversibility even in case of artificial membranes.

The specificity of lectin binding to artificial Caco-2 cell membranes was investigated by competitive inhibition of the lectin binding to the Caco-2 membrane (Fig. 7). Upon addition of increasing amounts of the complementary carbohydrate the amount of membrane bound F-WGA decreased to 13.7 ± 4.2% at 159 μM chitotriose. Thus, at least 86.4 ± 4.2% of F-WGA is bound to artificial Caco-2 membranes via N-acetyl-D-glucosamine moieties.

The reversibility of the lectin–Caco-2 membrane interaction was examined by displacement of membrane-bound lectin by means of subsequent exposure to the competitive carbohydrate. In accordance with the results of the specificity assay, 83.4 ± 7.8% of the membrane-bound lectin were displaced by 159 μM chitotriose. Consequently, at least 85% of the F-WGA is bound to oligosaccharide moieties of the Caco-2 cell membrane in a specific and fully reversible manner. Moreover, the lectin binding sites remain unaffected by the vesicle preparation procedure.

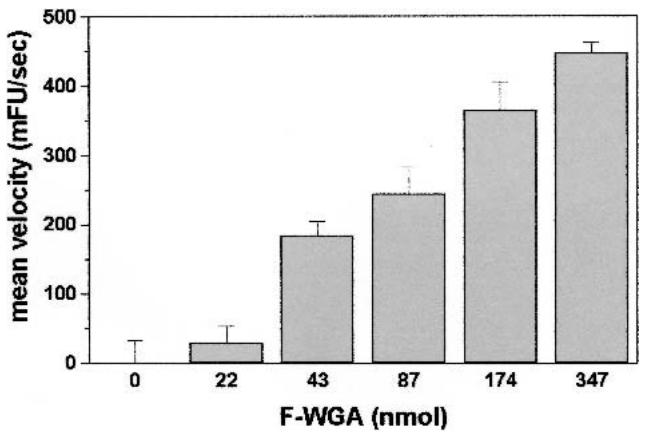

Fig. 6. Real-time binding kinetics of F-WGA to artificial Caco-2 cell membranes overlying the silver nanoparticle-enhanced fluorescence system (mean ± SD, n = 3).

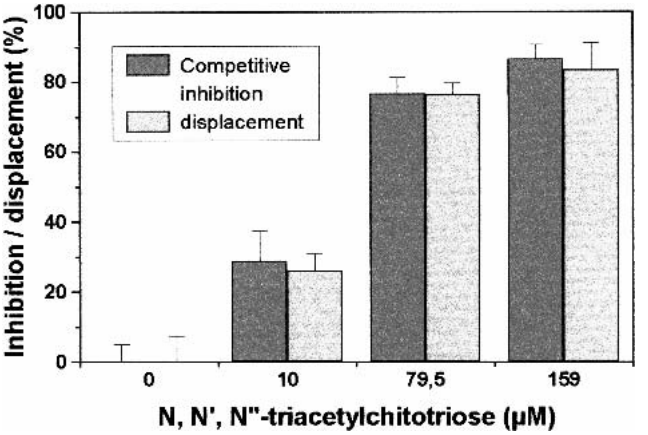

Fig. 7. Competitive inhibition and displacement of F-WGA binding to artificial Caco-2 cell membranes overlying the silver nanoparticle-enhanced fluorescence system (mean ± SD, n = 3).

Presence of EGF Receptors in Caco-2 Plasma Membrane Vesicles

To confirm the presence of the EGF receptor in plasma membrane vesicles prepared from Caco-2 cells, the vesicles were blotted on a PVDF membrane after electrophoresis. On the western blot, the EGF receptor protein was localized by binding of polyclonal anti-EGF receptor antibodies, which were detected by conversion of soluble substrate to an insoluble one by means of secondary AP-labeled anti-antibodies. The Caco-2 plasma membrane vesicles exhibited an intensely stained band with an electrophoretic mobility corresponding to the isolated EGF receptor (Fig. 3). Thus, the EGF receptor protein expressed by Caco-2 cells corresponds to the isolated EGF receptor with a molecular weight of 170 kDa. Furthermore, the structure of the EGF receptor is not altered by the vesicle preparation procedure due to immunologic detectability.

Moreover, the WGA-binding to the EGF receptor in Caco-2 plasma membrane vesicles was examined by incubation with biotinylated WGA and detection with AP-labeled streptavidin. According to the marker proteins and the isolated EGF receptor protein, the vesicles contain a similar glycoprotein of about 170 kDa (Fig. 4). The staining of this band after incubation with biotinylated WGA indicates the binding of WGA to EGF-receptors in Caco-2 membrane vesicles. As compared to the EGF receptor as a control, the broadening of the band might be due to occurrence of other sialic-acid containing glycoproteins in the glycocalyx of Caco-2 membrane vesicles. Additionally, nonspecific staining was excluded by invisibility of BSA as a negative control and carbohydrate specific detection was confirmed by staining of fetuin as a positive control.

DISCUSSION

Usually receptor–ligand studies require radiolabeling but immobilization of the receptor on silver nanoparticles featured a detection limit sufficient to follow the interaction between F-WGA and EGF receptors. Moreover, washing steps

were not required because of discrimination between bound and free ligand and real time monitoring of the F-WGA–receptor interaction was possible. Despite of adsorptive immobilization, the native structure of the receptor was retained as confirmed by detection with anti-EGF antibodies.

The EGF-receptor is a 170-kDa glycoprotein comprising an extracellular domain that binds EGF, a single membrane-spanning domain, and a cytoplasmic domain that has intrinsic tyrosine kinase activity. Ligand binding to the extracellular domain results in autophosphorylation of the catalytic domain, which opens potential pathways for signal transduction. Subsequently, the tyrosine-phosphorylated EGF-receptor is internalized in its activated form (14).

In our experiments, the isolated as well as the membrane associated EGF-receptor of Caco-2 and A-431 cells was used to characterize the interaction with WGA. As determined by SNEF and immunologic detection on western blots, the interaction between F-WGA and the isolated EGF-receptor is characterized by rapid and specific binding of WGA involving N-acetyl-D-glucosamine or sialic acid moieties of the receptor glycoprotein (Fig. 2).

In cells, the binding of ligands to the EGF-receptor is followed by internalization and down regulation of the receptor. To avoid any interference with cellular uptake, the binding of WGA to the EGF receptor was examined by means of artificial cell membranes prepared from Caco-2 and A-431 cells. As the cell membrane is free of RNA, contains moderate amounts of protein, and the AP-activity as a marker for differentiation is most abundant in the outer leaflet of Caco-2 membranes, the cell membrane vesicles were identified by these markers (Fig. 5). According to the molecular weight and immunoblot detection, intact EGF receptors formed part of the vesicles. The binding of biotinylated WGA to these EGF receptors was confirmed qualitatively by AP-labeled streptavidin. The interaction between WGA and artificial cell membranes was further characterized by fluorimetry using vesicles fused with the SNEF system. Considering the AP activity, the differentiation of Caco-2 vesicles was considerably higher than that of A-431 cells but the WGA binding capacity was about a third. As determined by ^{125}I-EGF-binding and uptake, Caco-2 cells possess a single EGF-binding site and about 7×10^3 receptors/cell whereas A-431 cells exhibit about 1.8×10^6 receptors/cell (15,16). The higher density of EGF-receptors concurrent with higher WGA-binding was another hint toward EGF-receptor binding of WGA.

Inhibition studies with the complementary carbohydrate revealed that about 90% of the WGA – Caco-2 membrane interaction were mediated by oligosaccharide moieties of the glycocalyx (Fig. 7). At one hand this confirms proper orientation of the artificial membrane after fusion with the SNEF system. Consequently, the Caco-2 model is not only useful for transport studies but also might be appropriate to screen the binding, specificity and affinity of new chemical entities as demonstrated by this new assay set-up combining artificial membranes and silver nanoparticle enhanced fluorescence. On the other hand, the lectin binding capacity is due to *N*-acetyl-D-glucosamine or sialic acid containing oligosaccharides stemming from the EGF receptor and other, yet unknown components of the glycocalyx. As results from F-WGA binding to the blotted receptor, it is most likely that at least part of lectin binding is mediated by the EGF receptor. Furthermore, the displacement of initially membrane bound WGA by chitotriose underlines the chitotriose motif of the WGA-binding site on artificial cell membranes. Accordingly, neuraminidase treatment of EGF-receptor expressing cells indicated specific interaction of EGF with terminal sialic acid residues of the receptor (17).

The carbohydrate specific binding of WGA to the EGF receptor suggests that glycosylated structures of the receptor represent the binding site of WGA. The extracellular region of the EGF receptor contains 12 potential sites for *N*-glycosylation in four domains (18). Of the four domains, domain III was identified to play a critical role in ligand binding involving oligosaccharide side chains linked to Asn-328, Asn-337, Asn-389, and Asn-420 (19–21). When the corresponding Asn residues were replaced by Gln, the Asn 420-linked sugar chain was found to be essential for binding of EGF as well as controllable dimerization of the occupied receptor (22). Because characterization of the carbohydrate side chains on human EGF receptor revealed that the multiantennary carbohydrates contain sialylated motifs (up to tetrasialo), the WGA-binding site on the EGF-receptor seems to involve the Asn-420 oligosaccharide chain (23).

The identification of the EGF receptor as a target protein for WGA binding also helps to explain the cellular fate of the lectin: 1) Similar to the binding of EGF to the EGF receptor, the sole cytoadhesion of WGA is independent from temperature, inhibited by *N*-acetylglucosamine, and fully reversible (2). 2) The occupied EGF-receptor complex is taken up rapidly by receptor mediated endocytosis which is characterized by accumulation in coated pits and energy dependence. By comparison, the enrichment of WGA in coated pit regions was not confirmed until now but WGA is rapidly taken up into Caco-2 cells by active transport. Additionally, there is high similarity of the binding, uptake and intracellular distribution of fluorescent-labeled EGF or WGA in A-431 cells or Caco-2 cells as observed by microscopy (24,25). According to the observation that all the WGA initially bound to Caco-2 membranes is internalized, it is likely that the EGF receptor plays an essential role in binding and uptake of WGA. 3) Finally, the internalized EGF receptor complex accumulates in the lysosomal compartment like WGA (26). Although the uptake mechanism of WGA and EGF via the EGF-receptor exhibit high similarities, the impact of EGF receptor bound WGA on signal transduction remains to be investigated.

The pivotal role of the EGF receptor in lectin binding opens two attractive areas for WGA-mediated drug delivery: First, the EGF receptor is overexpressed in a high number of tumors including those of the liver, the breast, the lung, and the bladder. Thus, prodrugs or drug-delivery systems containing WGA are expected to be appropriate for targeted delivery of anticancer drugs (27). Second, EGF receptor-containing cells were found to exist in most tissues of the body with 2×10^4–2×10^5 receptor molecules per cell (28). Consequently, the cytoadhesive and cytoinvasive characteristics of WGA open a promising pathway for improved binding and uptake of poorly available drugs including peptides, proteins and DNA.

ACKNOWLEDGMENTS

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