Carbohydrate–carbohydrate interaction provides adhesion force and specificity for cellular recognition

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The very first experimental demonstration of cellular recognition and adhesion phenomena in the animal kingdom came from an invertebrate system, i.e., from marine sponges (Wilson, 1907), and was later assigned to cell surface proteoglycans (Humphreys, 1963). Dissociated sponge cells from two different species have the capacity to reaggregate through surface proteoglycans (Fernandez-Busquets and Burger, 2003) in a Ca2+-rich environment (10 mM, i.e., physiologic for seawater) by sorting out according to their origin, i.e., the same way as live sponge cells did. Live cells also demonstrated species selective binding to glycans coated on surfaces. These findings confirm for the first time the existence of relatively strong and species-specific recognition between surface glycans, a process that may have significant implications in cellular recognition.

Quantitative measurements of adhesion forces between glycans from identical species versus glycans from different species confirmed the species specificity of the interaction. Glycan-coated beads aggregated according to their species of origin, i.e., the same way as live sponge cells did. Live cells also demonstrated species selective binding to glycans coated on surfaces. These findings confirm for the first time the existence of relatively strong and species-specific recognition between surface glycans, a process that may have significant implications in cellular recognition.

Introduction

One of the fundamental features of a living cell is a prompt and adequate behavior during formation, maintenance, and pathogenesis of tissues. Short-term adhesion events, e.g., leukocyte recruitment (Robinson et al., 1999), development of the nervous system (Stipp and Hemler, 2000), or microbial pathogenesis (Feizi and Loveless, 1996) require reversible, but still specific molecular surface interactions, rather than tight and stable adhesions between stationary cells (Spillmann and Burger, 1996). Carbohydrates, the most prominently exposed structures on the surface of living cells, with flexible chains and many potential binding sites are ideal to serve as important players in these events. Molecular interactions where carbohydrates are involved are usually considered as weak interactions (Varki, 1994; Spillmann and Burger, 1996), and therefore, biological relevance of carbohydrate–carbohydrate interactions is often questioned. Hakomori’s group has been first to show glycosphingolipid self-interactions to occur by way of Lewisx determinant (Galβ1→4[Fucα1→3]GlcNAcβ1→3Galβ1→4Glcβ) (Le3) to Le4 carbohydrate-dependent cell adhesion in the compaction of mouse embryo (Eggens et al., 1989) and autoaggregation of human embryonal carcinoma cells (Song et al., 1998). Extended studies revealed specific cellular recognition between lymphoma and melanoma cells based on gangliotriaosylceramide (GalNAcβ1→4Galβ1→4Glcβ1→1Cer)–sialosyllactosylceramide (NeuAcα2→3Galβ1→4Glcβ1→1Cer) interaction, and sialosyllactosylceramide (NeuAcα2→3Galβ1→4Glcβ1→1Cer)-dependent adhesion of melanoma cells, which led to spreading and enhancement of cell motility (Kojima and Hakomori, 1989; Iwabuchi et al., 1998).

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Abbreviations used in this paper: AFM, atomic force microscopy; CSW, Ca2+- and Mg2+-free artificial seawater buffered with 20 mM Tris, pH 7.4, supplemented with 2 mM CaCl2; Le, Lewis determinant (Galβ1→4[Fucα1→3]GlcNAcβ1→3Galβ1→4Glcβ); pN, piconewtons.

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species of origin, in the same way as dissociated embryonic cells from two different vertebrate tissues sort out according to their tissue of origin. Consequently, this simple and highly specific cellular recognition phenomenon in sponges has been used for almost a century as a model system to study recognition and adhesion events in multicellular organisms. Sponge cell–cell aggregation involves Ca\(^{2+}\)-independent binding of proteoglycans to a cell surface and Ca\(^{2+}\)-dependent self-association of proteoglycans (Turner and Burger, 1973; Jumblatt et al., 1980). A monoclonal antibody raised against the purified proteoglycan from Microciona prolifera sponge inhibited the proteoglycan self-association and the epitopes were identified as short carbohydrate units of the 200-kD glycan (Misevic and Burger, 1993); a sulfated disaccharide (Spillmann et al., 1995) and a pyruvylated trisaccharide (Spillmann et al., 1995). Recently, Vliegenthart’s group could demonstrate self-interactions of the sulfated disaccharide using surface plasmon resonance (Hasley et al., 2001).

However, species-specific interactions between 200-kD glycans from different sponge species have not yet been demonstrated in order to prove the existence of species-specific carbohydrate–carbohydrate recognition system.

200-kD glycan moieties from adhesion proteoglycans from four different marine sponge species were purified here and the species specificity of a glycan–glycan interaction was investigated in aggregation and adhesion assays. Atomic force microscopy (AFM) measurements were performed to measure the binding strength between single interacting glycan molecules and to demonstrate quantitative differences in binding forces between different species of 200-kD glycans. Results confirm the concept of the relatively strong and species-specific carbohydrate–carbohydrate interaction as an important player in cellular recognition.

**Results**

**Live cells specifically aggregate with glycan-coated beads**

In the classical assay for specific cell–cell recognition, live sponge cells can recognize their own kind and form big homogeneous aggregates on a shaker at the right shear forces, i.e., rotor speed, as shown for red cells of Microciona prolifera (Fig. 1 A). At too high rotation speed no cell aggregates are formed because shear forces are too high. At too low rotation speed unspecific, faulty initial contacts will remain because such cells do not get a second or third chance to form higher affinity adhesions with other cells. When cells from two different sponge species were shaken together in suspension (red cells, Microciona prolifera; yellow cells, Suberites fuscus), they sorted out into separate aggregates consisting of cells from the same species only (Fig. 1 B), and no heterotypic mixtures consisting of cells from different species would form. Specific cellular recognition could be inhibited by the absence of Ca\(^{2+}\) ions (Fig. 1 C). Recognition between Microciona (red) cells could be inhibited by the antibody directed against the carbohydrate epitope of the Microciona proteoglycan (Fig. 1 D), and much smaller cell aggregates could be seen as compared with aggregation in physiological Ca\(^{2+}\) (Fig. 1 A). There was no visible effect of the antibody on homotypic interactions between cells from other species (unpublished data).

In a cell–glycan recognition assay, live cells were allowed to aggregate with glycan-coated red beads (1-μm diam) similar in size to small sponge cells (2-μm diam), under the same shear forces, i.e., rotor speed as for cell–cell aggregation. Yellow cells (Suberites) specifically recognized red beads coated with their own glycans and formed large mixed aggregates (Fig. 1 E). Yellow cells, however, did not mix but separated from aggregates of red beads coated with glycans from a different species, namely Microciona (Fig. 1 F). As in cell–cell recognition, the absence of Ca\(^{2+}\) ions (Fig. 1 G) inhibited the cell–glycan recognition. The antibody directed against the carbohydrate epitope of Microciona proteoglycan could only inhibit the homotypic interaction between red Microciona cells and their glycans coated on red beads (Fig. 1 H). Aggregation between cells from other species and their glycans coated on red beads could not be inhibited by that antibody (unpublished data).

**Aggregation of glycan-coated beads mimics species-specific cellular aggregation**

An assay for glycan–glycan recognition was designed, which mimics the classical assay for specific aggregation of sponge cells. Glycan-coated red and green beads the size of small sponge cells were allowed to aggregate under identical shear
forces, i.e., rotor speed as used for cell–cell recognition assays. Beads coated with glycans from identical proteoglycans formed 63–79% yellow aggregates, which are the result of intermingling of red and green beads (Fig. 2). In stark contrast, beads coated with glycans derived from proteoglycans from different species did separate into red and green aggregates. In this case yellow aggregates, i.e., heterotypic mixtures of glycans originating from different species, never formed >12% of aggregated patches.

There were two possible modes for the color distribution in the glycan-coated bead–bead aggregation: either it was random or species specific. Random distribution, which can be described by Pascal’s triangle (Pickover, 2001), applies to the homotypic glycan-coated bead–bead aggregation, i.e., between glycans from the same species. In this case, red and green beads coated with identical glycans were mixing in a casual manner leading to a high number of yellow patches. However, based on Pascal’s triangle analysis, the color distribution in the heterotypic glycan-coated bead–bead aggregation, i.e., between glycans from two different species, was not random but species specific. Red and green beads coated with glycans from two different species were specifically sorting out into separate red and green aggregates.

As in cell–cell and cell–glycan recognition, the absence of Ca\(^{2+}\) ions inhibited the glycan–glycan recognition. The antibody directed against the carbohydrate epitope of the Microciona proteoglycan inhibited the homotypic interaction between Microciona glycans coated on red and green beads. There was no visible effect of the antibody on homotypic interactions between other species glycans (unpublished data). Results obtained with glycan-coated beads (Fig. 2) reflect thus the same results obtained with live cells (Fig. 1, A–D).

It has been reported previously that 400 molecules of Microciona proteoglycan bound per cell cause live cells to aggregate (Jumblatt et al., 1980). The number of 200-kD glycan copies per proteoglycan molecule was determined from the mass of total carbohydrate recovered in 200-kD glycan fractions either after gel electrophoresis or gel filtration (Misevic and Burger, 1993). Because 37% of the total carbohydrate content of the proteoglycan molecule occurred in the form of 200-kD glycan (∼70% of the proteoglycan mass is carbohydrate; proteoglycan \(M_r = 2 \times 10^8\)), one proteoglycan carries ∼26 copies of this glycan. Therefore, ∼10,400 glycan molecules (400 proteoglycan molecules) per cell cause living cells to aggregate. In our experiments, binding measurements indicated that ∼2,500 molecules of 200-kD glycan per bead specifically aggregated glycan-coated beads. The number was calculated from the specific absorbance of stained glycans after reversing the binding to beads (which gave the number of moles: \(0.192 \times 10^{-5}\)) and Avogadro’s number, and was divided by the number of beads (4.5 \(\times\) 10^8). Surface areas of the cell and the bead were calculated from diameters, and they were 12.56 \(\mu\)m\(^2\) and 3.14 \(\mu\)m\(^2\) accordingly. This led to the final assessment that the glycan density per cell and per bead causing species-specific live cell and glycan-coated bead recognition and aggregation is similar: 828 molecules/\(\mu\)m\(^2\) for cell–cell aggregation and 810 molecules/\(\mu\)m\(^2\) for glycan-coated bead–bead aggregation.

### Live cells and cell surface glycans adhere species specifically to glycans coated on a plastic surface

The binding of live cells to glycans from their surface proteoglycans coated onto a solid polystyrene phase was assessed (Fig. 3, A–D). The binding to glycans from proteoglycans from different species of origin was three to five times lower. Cell adhesion showed clear dependence on the quantity of the glycan coated, and could be abolished in the absence of Ca\(^{2+}\) ions. Pretreatment of Microciona cells with the antibody directed against the carbohydrate epitope of their surface proteoglycan inhibited 86% of these cells from adhesion to their own glycan (Fig. 3 E). In this assay, little cross-reactivity of the antibody (Misevic et al., 1987) could be detected because it blocked only 23% or less adhesion between other cells and their 200-kD glycans.

Similarly to cell–glycan adhesion, 200-kD glycans adhered strongly to surface-bound glycans from identical pro-
teoglycans in a dose-dependent manner (Fig. 4, A–D). The adhesion to glycans from different species was 2.5–6 times lower. This specific glycan–glycan adhesion could only be observed in the presence of Ca\(^{2+}\)/\(\mathrm{H}^{11001}\) ions. The antibody against the carbohydrate epitope of Microciona proteoglycan blocked 93% of the adhesion between its 200-kD glycans and produced little cross-reactivity (Misevic et al., 1987) by blocking 12% or less of the adhesion between glycans from other proteoglycans (Fig. 4 E).

The glycan–glycan adhesion force is in the piconewton range

Intermolecular adhesive force measurements between 200-kD glycan molecules coated on a probe tip and a surface (Fig. 5 A) were performed using AFM (Rief et al., 1997; Alonso and Goldmann, 2003). Based on fluorescence imaging with labeled glycans, no clustering of glycans was observed either on the probe tip or the surface. During retraction of the tip the glycan structure was lifted, stretched and finally noncovalent bonds between two molecules were being broken one by one. The existence of multiple noncovalent bonds between carbohydrates of glycan molecules is suggested by the presence of multiple peaks on the force curves, as recorded in the presence of Ca\(^{2+}\) (Fig. 5 B, lines 3–8). There was no interaction recorded between gold–gold (Fig. 5 B, line 1) or in the absence of Ca\(^{2+}\) (Fig. 5 B, line 2). Force measurements taken directly at the surface (<10 nm) were due to nonspecific interactions between the cantilever tip and the surface (Carrión-Vázquez et al., 2000), e.g., the first rupture peak in Fig. 5 B (lines 3, 5, and 8) and the first two in Fig. 5 B (lines 4 and 7). Only those measured at distances >10 nm from the surface were considered as direct interactions between glycan molecules. Strong multiple interactions were observed between glycans from the same species and noncovalent bonds between two interacting glycan molecules of the same origin (one peak) were ruptured at the forces between 190 and 310 piconewtons (pN; Fig. 6 A). The strength of the attachment of 200-kD glycans through S to the Au surface of the probe tip and the substrate is much stronger: 1.4 nanonewtons (Grandbois et al., 1999).
Single glycan–glycan adhesion force is species specific

In stark contrast to strong binding forces between glycans from the same species, clearly reduced forces were recorded between glycans from different species (Fig. 6 A). The single noncovalent bond between two interacting glycan molecules from two different species was ruptured at the forces between 110 and 210 pN. In a statistical analysis, the binding forces between glycans from the same species were always stronger than those between glycans from different species. P values for the difference in binding force between the two, calculated from Mann-Whitney test, were clearly below 0.01 and showed that the difference is statistically significant.

Specificity of the carbohydrate–carbohydrate interaction is also reflected in the polyvalence

The characteristic feature of the glycan–glycan interaction is the repetition of interactive sites along the glycan chain, which further increases the strength of the interaction and thus the specificity. The distance between the peak numbers 1 and 2 (2 and 3, etc.) on force curves was measured to produce a histogram of the peak periodicity (Fig. 6 B). 80% of force curves between glycans from the same species of cells showed more than one interaction peak, with a distance between binding motifs of ~20 nm. In contrast, <35% of force curves between glycans from different species of cells showed multiple interaction peaks, demonstrating the preference for one rather unspecific binding event during the interaction.

The glycan molecules used here have chain-like structures of an average folded length of ~40 nm as imaged by AFM (Jarchow et al., 2000), whereas the extended structure has a length of up to 180 nm (Dammer et al., 1995). 75% of the total lengths of the force curves for the same species glycans were 20–50 nm, and in some cases the curves showed extensions up to 130 nm (Fig. 6 C). This then indicates that the interaction sites are located along the carbohydrate chain and not only at its end. In contrast, 70% of the force curves for glycans from two different species showed total interaction lengths of 10–30 nm only.

Pronase digestion of glycans is essentially complete

Proteoglycan molecules were subjected to an extensive pronase digestion in order to obtain protein-free glycans.
The total 200-kD glycans were separated from free amino acids and peptides by gel filtration and ion-exchange chromatography (Misevic et al., 1987). The 200-kD glycan has an apparent $M_r = 200 \times 10^3 \pm 40 \times 10^3$, and electrophoretic and chromatographic separation techniques indicated that the glycan is a single molecular species with possible charge and size microheterogeneities (Misevic et al., 1987). There have been essentially no losses of carbohydrates during the purification procedures because the carbohydrate yield of the glycan fractions was $\sim 97\%$. Amino acid analysis of glycans from the four sponge species used showed that there was from 0.5 ($Cliona celata$) to 0.9 ($Microciona prolifera$) mole of linker aspartate/mol of glycan (Table I). Only trace amounts of a few other amino acids were detected. This indicates that the digestion was complete and that the purification pro-
procedure for the 200-kD glycans led to essentially pure glycan fractions, free of any protein contaminations.

Discussion

Virtually all animal cells produce proteoglycans, which vary greatly in structure, expression, and functions (Kjellen and Lindahl, 1991). Nevertheless, they do have a general propensity to be ECM components and to mediate specific interactions related to different aspects of cell adhesion phenomena through their protein or carbohydrate portions (Truant et al., 2003). In contrast to the rapid progress in studies of cell recognition and adhesion through protein–protein or protein–carbohydrate interactions (Feizi, 2000; Hynes and Zhao, 2000), the number and progress of studies on the possible role of carbohydrate–carbohydrate interactions in these events is still very small. Cell recognition and adhesion processes controlling the remarkable ability of sponge cells to species specifically aggregate after mechanical dissociation to finally reconstitute a functional sponge with canals, mineral skeleton, and collagen fibrils and fibers (Wilson, 1907; Galtsoff, 1925) is mediated by proteoglycans and this function may reside in the glycan portion. Data presented here significantly broaden the current views on the role of carbohydrates in cellular recognition by a novel demonstration of the species-specific character of the glycan–glycan interaction based on relatively strong single binding forces in the range of several hundred pN, providing an adequate affinity and avidity to mediate specific cell–cell recognition.

The examination of carbohydrate–carbohydrate interactions at the atomic level is critical in understanding the nature of these interactions and their biological role. Measured adhesive forces between identical glycans (190–310 pN) compare well with the range of forces between the entire proteoglycan molecules, which vary from 50 to 400 pN, depending on the number of binding sites ruptured (Dammer et al., 1995; Popescu et al., 2003). Similar values are also reported for other biologically relevant forces, e.g., for single protein–glycan interactions (Fritz et al., 1998; Hanley et al., 2003), when interaction of P-selectin from leukocytes with its carbohydrate ligand from endothelial cells was measured (165 pN), or for single antibody–antigen recognition (Hinterdorfer et al., 1996; Saleh and Sohn, 2003) with rupture forces of 244 pN. The force spectra shown in Fig. 5 exhibit the general shape anticipated for simple entropic polymers that extend until the carbohydrate–carbohydrate interaction ruptures. Considering molecular compliance it should be noted that the rupture forces measured here are below those between two different species of glycans could still be recorded in AFM measurements, though of lower stability than those between glycans from the same species. Similarly, some amount of heterotypic aggregates consisting of different species of glycans was present in glycan-coated bead aggregation experiments.

Ca2+ ions or other divalent cations are crucial in carbohydrate–carbohydrate interactions. Here, the presence of Ca2+ ions was essential. No interaction between cells, cells and surface glycans, and between surface glycans could be observed in the absence of Ca2+. Also no adhesion forces between single glycan molecules could be detected during AFM measurements. However, it has been reported that the presence of Ca2+ ions did not contribute significantly to the adhesion force in Le–Le interaction (Tromas et al., 2001). On the other hand, self-aggregation of Le molecules in aqueous solution, where the molecules move freely, occurred only in the presence of Ca2+ ions (de la Fuente et al., 2001). On the molecular level, Ca2+ ions probably provide coordinating forces (Haseley et al., 2001), though ionic forces cannot be excluded. These Ca2+ interactions are thought to stabilize conformations and can thereby lead to hydrogen bonds and hydrophobic interactions elsewhere in the glycan molecule (Spillmann and Burger, 1996). Further studies are required to resolve the exact role of Ca2+ and other divalent cations in carbohydrate–carbohydrate interactions.

Inhibition of sponge cell recognition and aggregation by species-specific carbohydrate epitope antibodies as shown earlier (Misevic et al., 1987; Misevic and Burger, 1993) do not prove glycan–glycan interactions to be relevant because they leave the option of glycan–protein interactions open. The same interpretation holds for two of the approaches presented here: species specificity for the glycan-coated bead interaction with live sponge cells (Fig. 1, E–H) and for the live cells binding to glycan-coated plastic surfaces (Fig. 3). The specificity found here for glycan–glycan interaction (Fig. 4), for glycan-coated bead sorting (Fig. 2), and the force and specificity shown in the AFM measurements (Fig. 6) make, however, a role for carbohydrate–carbohydrate in
sponge cell recognition and adhesion likely. The fact that the outermost cell surface is made up primarily of a dense layer of hydrophilic glycans supports the notion that upon first contact between cells such reversible and flexible glycan–glycan interactions may play a pivotal role in cell recognition processes.

Materials and methods

Sponges, live cells, cell surface proteoglycans, and glycans

Sponges, i.e., *Microciona prolifera*, *Halichondria panicea*, *Sabaterius fuscus*, and *Chiona celata* were collected by the Marine Biological Laboratory Marine Resources Dept. Live sponge cells were isolated as described previously (Misevic et al., 1987). Isolation of cell surface proteoglycans and prorian digestion of the core protein 200-kD glycans were performed as described previously (Misevic et al., 1987).

Analytical methods

For amino acid analyses, dry gycan samples were diluted in 1-ml of ultra pure water and the aliquots of 25 µl were lyophilized and hydrolyzed during 22 h. After hydrolysis, the black residues were suspended in 50 mM HCl containing 50 µmol/ml Sar and Nva each while ultrasonicated for 15 min. After a 15-min centrifugation, the transparent solutions were transferred into new reagent tubes and analyzed on a Hewlett-Packard AminoQuant II analyzer.

Aggregation assay

4.5 X 10^4 freshly sonified amine-modified beads (1-µm diam; Molecular Probes) were coupled with isolated glycans (1.5 mg/ml) by incubation in CaCl_2- and MgCl_2-free artificial seawater buffered with 20 mM Tris, pH 7.4, supplemented with 2 mM CaCl_2 (CSW), and 2 mg of 5,5'-dithiobis-(2-nitrobenzoic acid) (Molecular Probes) overnight at RT. Coupling efficiency was determined by measuring the glycan concentration (by staining with 1% benzoic acid) (Molecular Probes) overnight at RT. Coupling efficiency was determined by measuring the glycan concentration (by staining with 1% benzoic acid) (Molecular Probes) overnight at RT. Coupling efficiency was determined by measuring the glycan concentration (by staining with 1% benzoic acid) (Molecular Probes) overnight at RT.

Binding of cells and glycans to glycan-coated plates

Solutions of 200-kD glycans in CSW were placed in each well of a 96-well plate preincubated for 2 h at RT. Afterwards, plates were immersed in CSW with 100 µl of pure water and the aliquots of 25 µl of glycans to coated wells for 4 h after the addition of 10 mM CaCl_2. Images of aggregates were acquired with a confocal laser-scanning microscope (Leica) equipped with an argon/krypton laser and a 10× objective (PL Fluorat, N.A. 0.3). Image processing was performed using Adobe Photoshop version 6.0. Quantifications were performed using LUTHCSA Image Tool version 2.0 Alpha.

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