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Cellular Interactions Mediated by Glyconectins

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Contents

There is an old legend that solids, especially crystalline solids having a structure that reflects the harmony of creation, are the "work of God", while the surfaces, often having a form that represents disharmony, fracture, and the end of order, are the "work of Devil". In the past, the societies have punished those attracted to study the works of Devil including, presumably, cellular interactions. Currently, in more modern and enlightened times, such

a "hindrance" no longer exists because it is recognized that studies of this kind are punishment enough themselves [Richardson, 1995].

1. SUMMARY

Cellular interactions involve many types of cell surface molecules and operate via homophilic and/or heterophilic protein-protein and protein-carbohydrate binding. Our investigations in different model-systems (marine invertebrates and mammals) have provided direct evidence that a novel class of primordial proteoglycans, named by us **glyconectins**, can mediate cell adhesion via a new alternative molecular mechanism of polyvalent carbohydrate-carbohydrate binding. Biochemical characterization of isolated and purified glyconectins revealed the presence of specific carbohydrate structures, acidic glycans, different from classical glycosaminoglycans. Such acidic glycans of high molecular weight, containing fucose, glucuronic or galacturonic acids, and sulfate groups, originally found in sponges and sea urchin embryos, may represent a new class of carbohydrate carcino-embryonal antigens in mice and humans.

Such interactions between biological macromolecules are usually investigated by kinetic binding studies, calorimetric methods, X-ray diffraction, nuclear magnetic resonance, and other spectroscopic analyses. However, these methods do not supply a direct estimation of the intermolecular binding forces that are fundamental for the function of the ligand-receptor association. Recently, we have introduced **atomic force microscopy** to quantify the binding strength between cell adhesion proteoglycans. Measurement of binding forces intrinsic to cell adhesion proteoglycans is necessary to assess their contribution to the maintenance of the anatomical integrity of multicellular organisms. As a model, we selected the **glyconectin 1**, a cell adhesion proteoglycan isolated from the marine sponge *Microciona prolifera*. This glyconectin mediates *in vivo* cell recognition and aggregation via homophilic, species-specific, polyvalent, and calcium ion-dependent carbohydrate-carbohydrate interactions. Under physiological conditions, an adhesive force of up to 400 piconewtons between two cell glyconectins was measured. This large cohesive force between two individual sponge glyconectins is theoretically able to hold the weight of approximately 1,600 cells in physiological solution. Thus the integrity of the multicellular sponge organism, with at least 1,000

glyconectin molecules per cell, may be maintained by the multiplicity of glyconectin-glyconectin interactions. Our results suggest that the strength and polyvalency of glycan-glycan interactions are essential for cell adhesion. At the same time, selective spatial and temporal expression of glyconectins governs both cell recognition and adhesion during morphogenesis, maintenance and renewal of adult tissue, metastasis, and immune recognition and rejection.

The specificity of homophilic glyconectin interactions in sponges approaches the degree of selectivity of the evolutionarily advanced heterophilic immunoglobulin superfamily recognition system. Glyconectin-glyconectin interactions may thus provide a new paradigm for molecular self-recognition. We propose that the evolution of glyconectin-like proteoglycan molecules, with the capacity for self-recognition and adhesion, may have been a fundamental requirement for the emergence of the first multicellular organisms, as well as for the further divergence of species, and the appearance of more complex multicellular forms of life.

2. CARBOHYDRATE STRUCTURES MEDIATE CELL RECOGNITION AND ADHESION

The cell surface is the "contact layer" utilized by cells to make contact with the outside world. Through the activities of cell surface molecules, cells recognize self from non-self, send and receive physico-chemical signals, and adhere to other cells. Cell recognition and adhesion are a cascade of multistep events involving the extracellular matrix glycoprotein, lectin, immunoglobulin, integrin and cadherin families, operating via homophilic and/or heterophilic protein-protein and protein-carbohydrate interactions. Carbohydrates in the form of glycolipids, glycoproteins, proteoglycans and mucins are principal components of many cell surfaces.

All living cells express surface carbohydrates that participate in cell-cell interactions. These cell-bound carbohydrates interact with a variety of molecules (antibodies, agglutinins, toxins, or transmitter substances), triggering cell surface modifications, signal transduction, or metabolic activities. The carbohydrates, as the most abundant molecules at the cell surface, are preferentially selected by some pathogens as attachment sites during infection. As receptors, carbohydrate structures are candidates for

participation in recognition, and are involved in specific adhesion. This form of adhesion depends upon **lectins** on the surface of microorganisms. Lectins are ubiquitous proteins/glycoproteins that exhibit a specific and reversible carbohydrate-binding activity [Liener *et al.*, 1986; Beuth and Uhlenbruck, 1995].

Bacterial agglutinins and/or hemagglutinins were discovered in 1902, one year after the discovery of human blood groups by Landsteiner. In the mean time, considerable evidence has accumulated showing the fundamental role of lectin-carbohydrate binding in cell-cell interactions, as well as in microbial pathogenicity. Many bacterial lectins have been isolated, purified, and characterized. A detailed knowledge of the binding sites of these lectins and their receptors should lead to the design of potent inhibitors of recognition and adhesion. By understanding the molecular mechanisms of adhesion and the different forms of nonspecific adherence it will be possible to find out which events, signals, and messengers follow the fixation and what happens after the recognition of the specific and/or nonspecific receptors [Beuth and Uhlenbruck, 1995].

Species-specific reaggregation of dissociated marine sponge cells was the first experimental system to provide direct evidence for the existence of cell recognition and adhesion [Wilson, 1907]. Later work with *Microciona prolifera* revealed that both cellular interactions are mediated by an adhesion proteoglycan molecule, however, without the quantitative and biochemical evidence about the underlying molecular mechanisms [Humphreys, 1963; Henkart *et al.*, 1973]. Further investigations provided for the first time the direct evidence that carbohydrate-carbohydrate interactions can mediate cell adhesion [Misevic and Burger, 1986; Misevic *et al.*, 1987; Misevic and Burger, 1993]. Immunological and biochemical studies showed that the functional carbohydrate structures belong to a new class of large, fucosylated acidic glycans different from the classical glycosaminoglycans [Misevic *et al.*, 1987; Misevic and Burger, 1993].

Biochemical and immunological analyses also indicated that the large acidic glycan molecules isolated from sea urchin embryos closely resembled the sponge adhesion carbohydrate structures [Papakonstantinou and Misevic, 1993; Papakonstantinou *et al.*, 1994].

Currently, two papers published in *Nature* remark the key role in signal transduction played by a cell surface heparan-sulfate-modified

proteoglycan, named Dally, isolated from *D. melanogaster*. Dally, encoded by the *division abnormally delayed (dally)* gene, is a glycosyl-phophatidyl inositol-linked glypican and may act as a co-receptor for Wingless (Wg). Wg is a member of the Wnt family of growth factors, secreted proteins that control cell proliferation and differentiation during development. [Tsuda *et al.*, 1999; Lin and Perrimon, 1999]. A few families of cell-cell signals dominate the decisions that cells make. Among these are members of the Wg signal-transduction pathway, inappropriate activation of which contributes to human cancers [Peifer, 1999].

Mammalian cell surfaces are also "ornamented" with complex carbohydrate molecules. These glycans display a remarkable degree of structural diversity, that is in turn a function of the cell type, or tissue, or particular stage of development of the cell or tissue. Observations demonstrating that cell surface glycan structure varies with the differentiation phase or lineage of a cell suggest that these molecules play important roles in mediating information transfer into and out of the cell. During mammalian development, for example, dynamic changes are observed in cell surface glycoconjugate expression. Such data suggest that complex mechanisms have evolved to regulate these processes, and that they have important functional correlates. The cell surface carbohydrate molecules may participate as ligands in adhesive processes mediated by mammalian lectins. An accurately studied class of mammalian lectins is represented by **selectins**. They recognize a specific group of surface oligosaccharide determinants during adhesive processes mediating leukocyte adhesion to vascular endothelium [Lowe, 1994].

Commonly, cancer cells lose their normal adhesion functions, allowing them to detach from their tissues of origin and migrate free to other tissues. Carbohydrate-carbohydrate interactions have also been proposed as the mediators of cell adhesion in different malignant tumors: Le^x-Le^x association in the case of teratocarcinoma cells [Eggens *et al.*, 1989] or G_{g3}-G_{M3} ganglioside association in the case of lymphoma and melanoma cells [Kojima and Hakomori, 1989; Kojima, 1992]. These later observations have confirmed the results obtained on *M. prolifera* by Misevic *et al.* [1987].

Our immunofluorescence and biochemical studies showed that carbohydrate structures, resembling sponge and sea urchin cell adhesion

molecules, are also expressed in humans, in normal colon and colon carcinoma cells [Misevic and Popescu, 1995].

3. EXPERIMENTAL APPROACH OF CELLULAR INTERACTIONS

A fundamental problem in studying cellular interactions is that available experimental methodologies do not allow us a close and direct examination of what occurs *in vivo*, in real time and with a required spatial resolution as we would like, for all the cells of interest. If we were able to watch accurately what occurs we would have direct cinematic and structural information about the adhesion process of cells [Richardson, 1995]. Theoretical models can be useful in many ways. They can focus attention on important aspects able to provide a reliable representation consistent with the main observations and measurements. The experimental designs, inspired by these theoretical models, are frequently chosen for testing, evaluating and understanding the cascade of cellular interactions.

Conceptually, cellular interactions particularly cell recognition and adhesion, like other surface phenomena, can be analyzed from at least three viewpoints:

i) macroscopic viewpoint, which does not benefit from any specific theory about the nature of matter;

ii) atomic-molecular viewpoint, which invokes certain laws of interactions; electric fields associated with the charge of protons and electrons are very important at this scale;

iii) cellular and subcellular viewpoint, which is intermediate in scale between previous two; this viewpoint refers mainly to the biochemical aspects.

Cellular interactions are not plainly biological phenomena; practically, they depend upon physical phenomena. At present, many of the recent advances have occurred at the scale of the cell, the membrane, and the receptors, and have focused on the identification and characterization of **cell adhesion molecules** (CAMs). In these circumstances, the accurate understanding of cell-cell and/or cell-extracellular matrix interactions at larger physical scale requires an interdisciplinary approach [Richardson, 1995; Zhu, 1995; Popescu, 1996].

4. THE ATOMIC FORCE MICROSCOPY (AFM)

The biological relevance of CAMs has been demonstrated using different functional assays. Such investigations provide data concerning two essential aspects:

- the biophysical definition of adherence, and
- the biochemical modifications of CAMs.

It is obvious that the biophysical basis for any functional assay is the mechanical strength of adhesiveness. The effects of biochemical manipulations can be compared quantitatively only when a valid and accurate estimation of adhesiveness is made in a controlled experiment. Generally, either the force or the energy of the interaction can define the mechanical strength. The adhesive force can be calculated at two levels: cellular (avidity) and molecular (affinity). At the cellular level, fracture stress is defined as contact stress (force per unit area of adhesion) at the point of detachment. The surface adhesion energy density is defined as the mechanical work done to separate a unit contact area. At the molecular level, bond strength is defined as the maximum force a single molecular crossbridge can sustain. Bonding energy is defined as the energy required to break a single crossbridge [Zhu, 1995]. Until recently the direct measurement of force or energy of the adhesive interactions *per se* was not possible.

The AFM is considered a relatively new tool suitable for measuring intermolecular forces between nanometer-scale objects. It was first developed as an imaging device but, at this time, is one of the most widely used instruments for measuring intermolecular forces. The local interactions can be measured in real time with a high spatial resolution, because the AFM uses a probe with a radius of curvature typically of the order of 10-100 nm [Dammer *et al.*, 1995; Heinz and Hoh, 1999].

Heinz and Hoh [1999] are trying to present the basic elements of the AFM, considering the "small sphere on a weak spring" model as the most adequate to explain how an AFM works. In such a model, a weak mechanical spring is used to measure the forces between a probe (microsphere) and a sample whose position may change relatively to the probe. In fact, the spring is represented by a cantilever and the microsphere by the tip at the free end of the cantilever. This approximation is appropriate for most interaction forces. But, in some cases, the specific geometry and

surface chemistry of the tip are critical. The cantilever tip and the substrate surface are moved relative to each other using piezoelectric ceramics. The movements of the cantilever are measured using sensitive optical methods.

Force is measured by collecting a force curve, which is a plot of cantilever deflection, d_c, as a function of sample position along the z-axis (the z-piezo position). It assumes a simple relationship between the force, F, and the cantilever deflection:

$$F = -k\, d_c$$

where k is the spring constant of the cantilever. The interpretation of AFM force curves is based on established force laws describing force as a function of the probe-sample separation distance (D) rather than as a function of the z-piezo position. Thus, to be useful, the force curves must be transformed into descriptions of force as a function of distance, $F(D)$ [Dammer *et al.*, 1995; Popescu *et al.*, 1996; Heinz and Hoh, 1999].

5. GLYCONECTIN 1 AS MODEL-SYSTEM FOR AFM STUDIES

Electron microscopy, X-ray diffraction studies and biochemical analyses showed that, beside mucins, proteoglycans are the largest macromolecules extending above the cell surface many times higher then any other cell adhesion glycoprotein [Varki, 1993; Laine, 1994; Dwek, 1996]. The fact that glycans are sterically the most exposed and accessible molecules on the cell plasma membrane and in the extracellular matrix implies that at least the initial cell-cell and cell-matrix contacts should take place through sugar-sugar interactions. Our initial investigations in marine invertebrates provided direct evidence that primordial proteoglycans can indeed mediate cellular interactions via a new alternative molecular mechanism of polyvalent carbohydrate-carbohydrate binding [Misevic *et al.*, 1987; Misevic and Burger, 1993]. The ability of this newly recognized molecular mechanism of cell recognition and adhesion is also supported by the following findings:

i) the oligomeric glycan structures are the biological molecules keeping the highest potential information, and

ii) the expression of specific glycan structures is timely and spatially regulated during both morphogenesis and in adult organism.

Long- and short-range contacts between biological macromolecules and macromolecular superstructures are extremely important for the dynamic behavior of biological systems. Such interactions are usually investigated using thermodynamic and kinetic approaches. However, these methods do not supply a direct estimation of the intermolecular binding forces that are fundamental for the function of the ligand-receptor association. Distinct measurement of the force (the derivative of energy with respect to separation distance) is not possible and, as a result, the direct information concerning the distribution of interaction energy between two biological structures is incomplete. Recently, we have introduced atomic force microscopy to quantify the binding strength between cell adhesion proteoglycans [Dammer *et al.*, 1995; Popescu *et al.*, 1996].

Measurement of binding forces intrinsic to cell adhesion proteoglycans is necessary to assess their contribution to the maintenance of the anatomical integrity of multicellular organisms. As a model, we selected the adhesion proteoglycan isolated from the marine sponge *Microciona prolifera*. This proteoglycan, named by us **glyconectin 1**, mediates *in vivo* cell recognition and aggregation via homophilic, species-specific, polyvalent, and Ca^{2+} dependent carbohydrate-carbohydrate interactions [Misevic *et al.*, 1987; Misevic and Burger, 1993; Dammer *et al.*, 1995; Popescu *et al.*, 1996; Popescu and Misevic, 1997; Popescu and Misevic, 1998].

To measure glyconectin-glyconectin interaction forces, we covalently attached glyconectins to an AFM sensor tip and a flat surface; the attachment process involves exclusively the protein moiety while the functional carbohydrate adhesion sites are not altered. The cantilever tip was carefully moved toward the substrate surface, and a sequence of approach-and-retract cycles was collected. The stability of binding events during the course of these experiments indicated that none or very few of the glyconectin functional adhesion sites were irreversibly damaged. Two typical approach-and-retract curves are shown in Fig. 1 (**a** and **b**). The adhesion peaks were retarded, indicating that there was no interaction during the surface approach, but on retraction the lever detected an attractive force at a distance from 0 to approximately 200 nm above the surface.

The shape of the approach - and - retract curves between glyconectins suggests the presence of long-range interactions, interpreted as the lifting and extensions of stringlike arms, followed by further stretching until the elastic force of the cantilever equals the strength of the binding and the lever "jump-off".

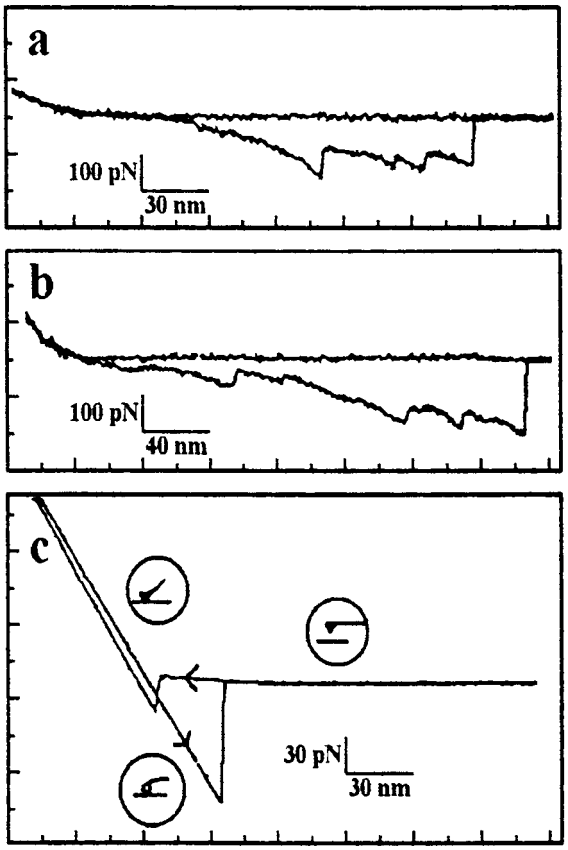

Figure 1. Typical AFM approach-and-retract cycles for glyconectin 1-glyconectin 1 interactions. The abscissa shows the vertical movement of the cantilever; the ordinate shows the bending of the cantilever and thus the force acting on it. **(a)** and **(b)** represent typical glyconectin-glyconectin interactions, whereas **(c)** is an example of the interaction between two gold surfaces covered with self-assembled monolayers (1-dodecanethiol).

By contrast, a typical approach-and-retract cycle taken between two control surfaces, without glyconectins, showed that adhesion took place directly at the surface; this measurement indicates the presence of short-range forces, and the slope of the adhesion curve shows that there was no elasticity in the sample itself (Fig. 1**c**). At the physiological Ca^{2+} concentration of 10 mM in seawater, multiple jump-offs were frequently observed, indicating polyvalent binding with an average adhesive force of 40 ± 15 pN (Fig. 1) [Dammer *et al.*, 1995].

Characterization of glyconectin-glyconectin adhesion was done also by measuring both the force necessary for final jump-offs (separation of glyconectin-functionalized sensor tip from the glyconectin surface) and the percentage of interaction events under different ionic conditions. These two indicators of glyconectin activity varied reversibly with Ca^{2+} concentration, accordingly to previous qualitative data [Humphreys, 1963; Misevic *et al.*, 1987; Misevic and Burger, 1993; Spillmann *et al.*, 1993]. At a Ca^{2+} concentration of 10 mM, the average force between glyconectins was 125 pN, ranging up to 400 pN, with a high probability of binding (60 ± 10%). At a Ca^{2+} concentration of 2 mM, cell adhesion was sharply reduced and the force (40 ± 15 pN) and probability (12 ± 5%) were also decreased [Dammer *et al.*, 1995].

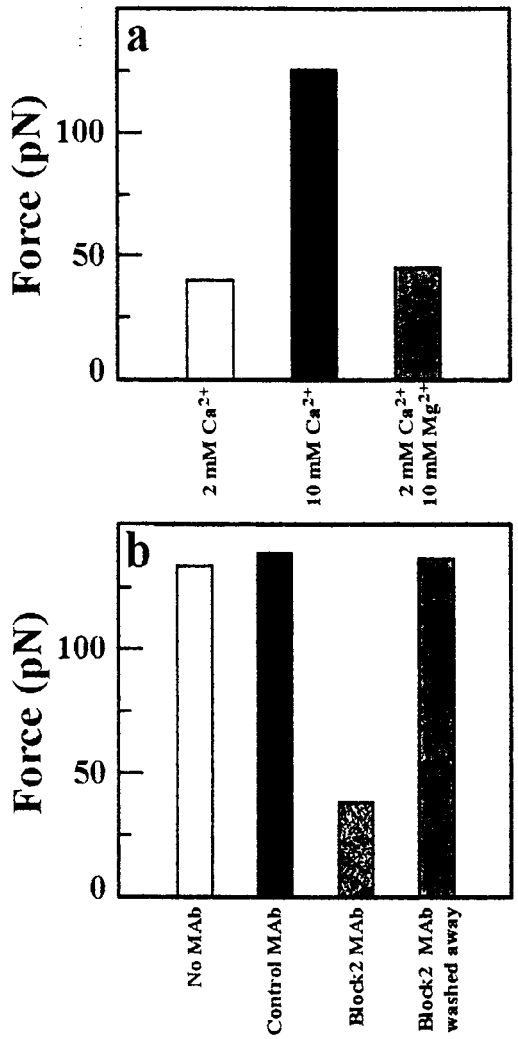

Figure 2. AFM measurements of glyconectin 1-glyconectin 1 binding strength. Sequential measurements were carried out with the specified cation or monoclonal antibody (MAb). **(a)** Ca^{2+} and Mg^{2+} dependence of the adhesive force in artificial seawater. **(b)** Effect of antibodies on adhesive force. Fab fragments of MAb Block 2 or control MAb (both immunoglogulins G2b) were used at 20 μg/ml in artificial seawater containing 10 mM Ca^{2+}.

The interaction between glyconectins is Ca^{2+}-selective since 10 mM Mg^{2+} could not replace Ca^{2+} (Fig. 2a). Use of monoclonal antibody (MAb) Block 2 (Fab fragments), recognizing a carbohydrate epitope, provided accurate evidence that the AFM-measured interactions originate from glyconectin-glyconectin binding. This MAb reduced the interactive force close to the level measured at 2 mM Ca^{2+}. Under equivalent conditions, a control MAb did not prevent glyconectin-glyconectin interaction (Fig. 2b). Thus, during AFM measurements in all tested experimental conditions, glyconectin-glyconectin interactions resemble cell-cell adhesion events observed *in vivo* [Dammer *et al.*, 1995].

6. MOLECULAR MECHANISM OF GLYCAN-GLYCAN INTERACTIONS IN *M. prolifera*

By using AFM imaging, we observed glyconectin 1 rings with a diameter of 200 nm and about 20 irradiating arms, each 180 nm long [Dammer *et al.*, 1995]. These arms are composed of glycans with a relative molecular weight of 200 000 D (g200) [Misevic *et al.*, 1987]. The functional assays provide direct evidence that homophilic carbohydrate-carbohydrate interactions of the g200 glycans mediate recognition and adhesion. The glass aminopropyl beads were coated with either glyconectin 1 or g200 glycan and their aggregation was monitored following addition of a physiological concentration of $CaCl_2$ (10 mM). Aggregation of coated glass beads occurred as glyconectin 1 promoted cell or latex-amidine beads aggregation in the presence of 10 mM $CaCl_2$, but not with 2 mM $CaCl_2$ [Misevic and Burger, 1993; Popescu and Misevic, 1997].

Such calcium-dependent aggregation of g200 beads suggests the g200 glycan is capable of mediating recognition and adhesion exclusively through homophilic sugar-sugar interactions. Also, the AFM experiments showed that stringlike structures, the g200 glycans, were responsible for polyvalent glyconectin-glyconectin interactions. This possibility is further supported by the fact that the length (180 nm) and the number (20 copies) of the g200 glycan per glyconectin molecule are similar to the length and number of glyconectin arms as measured by AFM and electron microscopy. At last, the inhibitory MAb Block 2 is directed against a self-association epitope located on the g200 glycan [Misevic and Burger, 1993; Dammer *et al.*, 1995]. Thus, highly polyvalent g200-g200 binding represents the basis

for glyconectin 1-glyconectin 1 association, which by itself promotes cell recognition and adhesion.

Fab fragments of the Block 2 monoclonal antibody showed a concentration-dependent inhibition of glyconectin 1 and g200 coated glass bead agglutination. This antibody, recognizing a sulfated carbohydrate epitope, appears to preclude cell adhesion through a direct inhibition of glyconectin 1 self-interaction, as shown previously [Misevic and Burger, 1993].

Our biophysical, biochemical and immunological data make clear that carbohydrate-carbohydrate binding provides the essential strength for cell-cell adhesion in the marine sponge *M. prolifera*. The molecular mechanisms for such interactions is based on a Ca^{2+}-dependent highly polyvalent associations of a sulfated, fucose-containing, glycan epitopes located in the g200 glycan [Misevic *et al.*, 1987; Misevic and Burger, 1993; Spillmann *et al.*, 1993; 1995].

Although the primary structure of the g200 glycan remains to be determined, our data indicate that this N-linked highly fucosylated and acidic polysaccharide, containing glucuronic acid, mannose, galactose, N-acetyl glucosamine, sulfate and pyruvate, belongs to a novel class of acidic glycans distinct from the classical glycosaminoglycans.

The cross-reactivity of the *Lytechinus pictus* polysaccharides with the Block 1 and Block 2 MAbs indicated similarity with the sponge glycans and thus could also be classified in the same group of large fucosylated acidic glycans [Papakonstantinou and Misevic, 1993; Papakonstantinou *et al.*, 1994]. In addition, preliminary studies with sea urchin acidic glycans showed that these structures also mediate cell adhesion via polyvalent and Ca^{2+}-dependent homophilic binding. The functional studies revealed that these glycans mediate cell adhesion during embryonal development. The important regulatory function of these carbohydrate molecules in cell adhesion and movement during gut morphogenesis was indicated by the spatial and temporal regulation of their expression as evaluated by immunofluorescence microscopy with Block 1 and Block 2 MAbs [Misevic, unpublished results].

Immunofluorescence light microscopy of human colon carcinomas and healthy colon samples with Block 1 and Block 2 MAbs established that the carbohydrate structures resembling the invertebrate acidic glycan

adhesion molecules are also expressed in humans. The Block 1 MAb labeled basal and apical lamina of tumor cells, whereas the Block 2 MAb bound exclusively to the apical part of the epithelium. In normal tissue whole goblet cell membrane was stained with both antibodies indicating that transformation leads to spatial rearrangement of glycan antigens. Immunodot assays with the same MAbs revealed that tumor cells have elevated expression of both carbohydrate structures. These results suggest that the acidic glycan adhesion molecules, originally found in sponges and sea urchin embryos, may represent a new class of carbohydrate carcino-embryonal antigens involved in cellular interactions associated with morphogenesis, metastasis, and maintenance and renewal of adult tissue [Misevic and Popescu, 1995].

This sugar-sugar interaction is distinct from the higher affinity low valency protein-carbohydrate or protein-protein binding described for lectin-carbohydrate, integrin-extracellular matrix, immunoglobulin-immunoglobulin, and cadherin-cadherin adhesion molecules. In this context many reports are relevant, *i.e.* a system which utilize hyaluronic acid-heparan sulfate-mediated bead adhesion [Turley and Roth, 1980], or hyaluronic acid-chondroitin sulfate heteroaggregation [Scott, 1992], and other systems utilizing homophilic binding of heparan sulfate chains [Fransson *et al.*, 1981; Fransson *et al.*, 1983], or hyaluronic acid chain association [Scott, 1992]. Although direct evidence for the role of carbohydrate-carbohydrate interactions in cell adhesion was not given, the data of these authors indirectly support the hypothesis that a variety of low affinity anionic polysaccharides may mediate this process. The idea would be logical since carbohydrates are the most exposed and highly polyvalent components of the cell surface and extracellular matrix and are thus qualified as the most suitable mediators of the initial cellular interactions.

An open question concerning the role of carbohydrate-carbohydrate associations during cellular interactions is whether such an interaction provides the degree of specificity required for cell recognition. Our knowledge of noncovalent bonding suggests that many parameters determine selectivity in the binding of neighboring carbohydrate structures [Misevic and Burger, 1993; Popescu and Misevic, 1998].

7. A POSTULATED MODEL OF GLYCAN-GLYCAN INTERACTIONS

The AFM observations are consistent with a model in which the glycan arms are responsible for glyconectin 1-glyconectin 1 cohesion. Because of their polysaccharide nature, the glycan arms are not involved in the glyconectin 1 crosslinking to AFM surface and thus remained free to irradiate into the buffer. During each approach-and-retract cycle, multiple noncovalent bonds between arms attached to the sensor tip and arms connected to the substrate were formed and broken. Because the radius of curvature of a typical functionalized AFM tip is about 50 nm and the glyconectin backbone ring is approximately 200 nm in diameter, only a single glyconectin molecule from the tip could participate in the measured interaction. The observation that approach-and-retract curves often exhibited multiple jump-off steps (Fig. 1) indicates that binding was polyvalent. Each step of 40 ± 15 pN corresponds to the unbinding of a pair of glycan arms; deviation is caused by a varying degree of mutual overlap. The maximal measured adhesion force of 400 pN and the average force of 125 pN are thus interpreted as the binding between 10 and 3 pairs of glycan arms. Our data also provide information about the behavior of glycan arms during homophilic interactions. First, upon stretching, glycan arms did not behave as ideal springs; instead, their stiffness increased gradually (Fig. 1). Second, although the rupture force of a single covalent C-C bond is about 10 nN, the strongest measured noncovalent glyconectin-glyconectin binding forces are about 25 times weaker (400 pN). These findings explain why the glyconectins remained intact throughout the AFM measurements and how they allow cell dissociation without being destroyed [Dammer *et al.*, 1995; Popescu *et al.*, 1996].

The absolute configuration of the majority of monosaccharide residues in a glycan chain is the $^4C_1$ D-configuration, except fucose, which exists in the $^1C_4$ L-configuration [Homans, 1994]. The fucose represents more than 60% of total carbohydrate content of g200 glycan. Because of its particular configuration, fucose could be also an important factor, which may determine the specificity and selectivity of glyconectin molecule interactions. In the same time, the presence of Ca^{2+} (at physiological concentration of 10 mM) is very important for this carbohydrate-carbohydrate interaction; calcium ions are essential for cell recognition and adhesion in *M. prolifera* sponge. Recently, it has been demonstrated that

calcium ions also mediate interaction between dextran sulfate and dimyristoyl-*sn*-glycero-3-phosphocholine via calcium bridges. Attractive forces between negatively charged polyelectrolytes and zwitterionic phospholipids arise from the assembly of calcium bridges [Huster and Arnold, 1998].

In this regard, the model for homophilic glyconectin-glyconectin interaction proposed by Simon [1998a and b] is very appropriate. Intercellular adhesion requires physiological Ca^{2+} concentration, and this suggests that pairs of saline bonds are formed between anionic groups localized on opposite g200 glycan arms belonging to two different glyconectin molecules. Glycans should stem towards the exterior of each cell membrane and the model for the homophilic, autocomplementary interaction should explain the formation of large numbers of saline bonds in homophilic interaction, while only small numbers of such bonds should be possible in heterophilic interactions. The positioning and spacing between charged groups on such chains is essential. Returning to molecular symmetry concepts, the term of C_2-autocomplementarity seems adequate to this situation [Simon, 1998a and b]. This model is almost supported by our experimental data.

8. GLYCONECTIN-GLYCONECTIN INTERACTIONS: A PATHWAY TO MULTICELLULARITY

The emergence of a multicellular organism required the concurrent development of cell adhesion and recognition properties. In attempting to solve the basic question of what may have been the molecular support for early self-recognition and nonself discrimination we focused our interest on the role of proteoglycans in *Porifera* xenogeneic cellular interactions, as the most compatible experimental system for ancestors of Metazoans. Structural and biochemical analyses demonstrated that, beside mucins, proteoglycans are the largest macromolecules extending above the cell surface. Based on these facts we advanced the hypothesis that proteoglycans as the most peripheral cell surface environment sensors may have provided the first key recognition and adhesion functions during the emergence of multicellular forms of life. If so, the simplest Metazoans alive today, such as Porifera, should have preserved, at least in part, proteoglycan recognition and adhesion mechanisms guiding the beginning phase of xenogeneic selectivity

of cellular interactions [Popescu and Misevic, 1997]. We have demonstrated, by using atomic force microscopy (AFM), that proteoglycan to proteoglycan binding strength supplies fundamental cell adhesion forces in the marine sponge *Microciona prolifera* [Dammer *et al.*, 1995; Popescu *et al.*, 1996]. This new evidence has confirmed previous functional investigations showing the adhesion role of *M. prolifera* proteoglycans [Humphreys, 1963; Jumblatt *et al.*, 1980; Misevic *et al.*, 1987; Misevic and Burger, 1993]. This cell adhesion proteoglycan, **glyconectin 1**, underlies the molecular mechanism of self-recognition and adhesion [Popescu and Misevic, 1997].

Under physiological conditions, an adhesive force of up to 400 piconewtons between two cell glyconectins was measured. This large cohesive force between two individual sponge glyconectins is theoretically able to hold the weight of approximately 1,600 cells in physiological solution. Thus, the integrity of the multicellular sponge organism, with at least 1,000 glyconectin molecules per cell, may be maintained by the multiplicity of glyconectin-glyconectin interactions [Dammer *et al.*, 1995].

The specificity of homophilic glyconectin interactions in sponges approaches the degree of selectivity of the evolutionarily advanced heterophilic immunoglobulin superfamily recognition system. Glyconectin-glyconectin interactions may thus provide a new paradigm for molecular self-recognition. We proposed that the evolution of glyconectin-like proteoglycan molecules, with a capacity for self-recognition and adhesion, may have been a fundamental requirement for the emergence of the first multicellular organisms, as well as for the further divergence of species, and the appearance of more complex multicellular forms of life [Popescu and Misevic, 1997].

9. CONCLUDING REMARKS AND FUTURE PROSPECTS

Cellular interactions are cardinal biological processes involved in the morphogenesis of multicellular organisms, tissue maintenance and renewal, and homeostasis of the immune system. In many pathological situations there is a strong relationship between distinctive modifications of surface carbohydrate structures and inappropriate functioning of cell adhesion and recognition. Identification, isolation and purification of functional glycans along with quantitative estimation of their adhesive/antiadhesive forces and

their specificity could improve our comprehension of the cell-cell and cell-extracellular matrix interaction complexity.

Our results provide the first and essential evidence that a novel molecular mechanism of homophilic, specific, polyvalent, and Ca^{2+}-dependent glycan-glycan interactions mediate cellular interactions in invertebrates. Such a carbohydrate-carbohydrate interaction can perform the cell recognition and adhesion functions that we have assigned to it. At the same time, our results prompt us to advance the hypothesis that the human acidic glycan adhesion molecules, originally found in sponges and sea urchin embryos, may represent a new class of carbohydrate carcino-embryonal antigens involved in cellular interactions associated with morphogenesis, metastasis and renewal of adult tissue. Future studies using a similar approach may verify whether carbohydrate-carbohydrate binding mediates cell recognition and adhesion during multistep processes of cell-cell or cell-extracellular matrix interactions in other Metazoans.

Further experiments and theoretical modeling are required to demonstrate the generality of our paradigm of glycan-glycan interactions involved in cell adhesion and recognition. At the conceptual and theoretical levels, it is fundamental to improve the current description of the molecular-scale properties of cell surfaces, and glyconectin surfaces, respectively. Theoretical approach of the surface interactions at short-range requires that the surfaces be treated, not just as hard or soft walls, but with the same molecular detail as are the intervening liquid molecules, including a correct balancing of the interplay between the long-range and short-range intermolecular forces [Israelachvili and Wennerstrom, 1996].

It is obvious that the spatial distribution of intermolecular forces controls macromolecular interactions. In this context, the AFM can be used to obtain essential data about charge density, adhesion, and stiffness of a determined biological surface. The present assumption of a biological structure delineated by a van der Waals surface must be achieved by considering all interactions involving such a structure. This approach will reach the age as a novel concept: **the interaction surface**.

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REZUMAT (Interacţiuni celulare mediate de gliconectine)

Adeziunea celulară şi recunoaşterea celulară sunt mediate activ de interacţiuni moleculare succesive la care participă mai multe categorii de molecule ale suprafeţei celulare: caderine, integrine, proteine din superfamilia imunoglobulinelor, lectine, glicoproteine din matricea extracelulară. Aceste interacţiuni selective, caracteristice organismelor multicelulare, pot fi de natură homofilică sau heterofilică (proteină-proteină sau proteină-poliglucid). Studiile noastre pe diferite modele experimentale (nevertebrate marine: spongieri şi arici de mare, şi mamifere: şoarece şi material uman) au evidenţiat că o nouă clasă de molecule de adeziune celulară, denumite de noi **gliconectine**, pot media adeziunea celulară printr-un nou mecanism molecular care are la bază interacţiuni poliglucid-poliglucid, polivalente şi dependente de ionii de calciu. Caracterizarea biochimică a gliconectinelor izolate şi purificate a revelat existenţa unor structuri poliglucidice specifice, glicani acizi, care diferă de glicozaminoglicanii clasici. În cazul mamiferelor luate în studiu, aceşti glicani acizi, de masă moleculară mare, bogaţi în fucoză, acid glucuronic sau galacturonic şi grupări sulfat, ar putea reprezenta o nouă categorie de antigene carcino-embrionare.

Pentru investigarea mecanismelor moleculare specifice interacţiunilor responsabile de adeziunea celulară sunt folosite diferite tehnici calorimetrice, cinetice, difracţie cu raye X, rezonanţă magnetică nucleară sau alte tehnici spectroscopice. Însă, nici una din aceste tehnici nu oferă posibilitatea determinării sau estimării directe a forţelor de legare dintre macromoleculele implicate în adeziunea celulară. Recent, grupul nostru a demonstrat că **microscopia de forţă atomică** (atomic force microscopy) poate fi utilizată cu succes pentru măsurarea directă a acestor forţe de interacţiune. S-a folosit ca model **gliconectina 1**, proteoglicanul de adeziune celulară izolat de la buretele de mare *Microciona prolifera*. Acest proteoglican mediază *in vivo* recunoaşterea şi agregarea celulelor de spongieri prin interacţiuni poliglucid-poliglucid specifice speciei, homofilice, polivalente şi dependente de ionii de calciu. În condiţii fiziologice, s-a constatat că forţa de adeziune dintre două gliconectine ajunge la o valoare de 400 piconewtoni. Teoretic, această forţă poate susţine o greutate echivalentă cu 1600 de celule de spongier, în soluţie izoosmotică cu apa de mare. Deoarece fiecare celulă posedă cel puţin 1000 de molecule de gliconectină 1 este evident că

integritatea anatomică a buretelui de mare *M. prolifera* este asigurată de forţe considerabile. Măsurarea acestor forţe este necesară pentru a determina contribuţia fiecărui tip de moleculă de adeziune celulară la menţinerea integrităţii anatomice a organismelor multicelulare. Rezultatele obţinute sugerează că intensitatea şi polivalenţa interacţiunii glican-glican sunt esenţiale pentru adeziunea celulară. În acelaşi timp, exprimarea spaţio-temporală selectivă a gliconectinelor controlează atât recunoaşterea celulară cât şi adeziunea celulară. Aceste procese biologice complexe sunt asociate cu dezvoltarea embrionară, menţinerea şi regenerarea ţesuturilor în organismul adult, dezvoltarea şi funcţionarea sistemului imunitar precum şi cu transformarea malignă şi metastaza organ-specifică.

Specificitatea acestor interacţiuni moleculare homofilice se apropie de nivelul de selectivitate caracteristic sistemului heterofilic de recunoaştere, mult mai evoluat, al superfamiliei imunoglobulinelor. Astfel, interacţiunile de tip gliconectină-gliconectină reprezintă un model nou de auto-recunoaştere la nivel molecular. Existenţa proteoglicanilor asemănători gliconectinelor având capacitatea de auto-recunoaştere şi adeziune ar fi putut fi una din cerinţele fundamentale pentru apariţia primelor organisme multicelulare. De asemenea, gliconectinele ar fi putut juca un rol important şi în diversificarea ulterioară a speciilor şi evoluţia spre organisme multicelulare mult mai complexe.