

Self-Recognition and Ca²⁺-Dependent Carbohydrate–Carbohydrate Cell Adhesion Provide Clues to the Cambrian Explosion

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The Cambrian explosion of life was a relatively short period approximately 540 Ma that marked a generalized acceleration in the evolution of most animal phyla, but the trigger of this key biological event remains elusive. Sponges are the oldest extant Precambrian metazoan phylum and thus a valid model to study factors that could have unleashed the rise of multicellular animals. One such factor is the advent of self-/non-self-recognition systems, which would be evolutionarily beneficial to organisms to prevent germ-cell parasitism or the introduction of deleterious mutations resulting from fusion with genetically different individuals. However, the molecules responsible for allorecognition probably evolved gradually before the Cambrian period, and some other (external) factor remains to be identified as the missing triggering event. Sponge cells associate through calcium-dependent, multivalent carbohydrate–carbohydrate interactions of the g200 glycan found on extracellular proteoglycans. Single molecule force spectroscopy analysis of g200–g200 binding indicates that calcium affects the lifetime (+Ca/–Ca: 680 s/3 s) and bond reaction length (+Ca/–Ca: 3.47 Å/2.27 Å). Calculation of mean g200 dissociation times in low and high calcium within the theoretical framework of a cooperative binding model indicates the nonlinear and divergent characteristics leading to either disaggregated cells or stable multicellular assemblies, respectively. This fundamental phenomenon can explain a switch from weak to strong adhesion between primitive metazoan cells caused by the well-documented rise in ocean calcium levels at the end of Precambrian time. We propose that stronger cell adhesion allowed the integrity of genetically uniform animals composed only of “self” cells, facilitating genetic constitutions to remain within the metazoan individual and be passed down inheritance lines. The Cambrian explosion might have been triggered by the coincidence in time of primitive animals endowed with self-/non-self-recognition and of a surge in seawater calcium that increased the binding forces between their calcium-dependent cell adhesion molecules.

Introduction

The Cambrian explosion is widely regarded as one of the most relevant episodes in the history of life on Earth (Conway-Morris 2006), when about half of living animal phyla first appear in the fossil record, and yet, its origins and causes remain controversial (Marshall 2006). It is not obvious why a greater speed in evolution could be beneficial by itself, and more likely it was a consequence of some other factor directly linked to evolutionary pressure. One of the phenomena more generally accepted as a principal force of selection is the “selfish” competition for passing down one’s genotype (Dawkins 1976) and not that of others. The development of self-/non-self-recognition in primitive metazoans might have been a necessary innovation to prevent interindividual cell parasitism, at the expense of reducing the resistance to environmental variations that results from increased genetic variability (Buss 1982). Somatic and germ-cell parasitism is a natural transplantation reaction of certain colonial ascidians that results alternatively in colony fusion or inflammatory rejection in a process regulated by a highly polymorphic histocompatibility locus (Stoner and Weissman 1996). Because this allorecognition system limits fusion to close relatives, it may have evolved as a mechanism to minimize the fitness costs

of chimera formation (Buss 1982). Fusion with conspecifics could confer benefits by gaining wider ranges of physiological resistance and increasing the size of the resulting chimera, which in turn limit susceptibility to the impact of ecological processes and favor reproductive output. However, because all genotypes in a chimera have access to the production of gametes, one genotype can functionally parasitize other members of the chimera by contributing disproportionately to gamete production (Buss 1982; Feldgarden and Yund 1992). In analogy to this contemporary ascidian allogeneic recognition, a situation can be envisaged where a fertilized egg from a primitive metazoan carrying a new gene that would impact negatively upon organismal fitness divides to form a free-swimming larva that settles down on the sea floor. In the absence of individual recognition, developing larvae that make contact through growth or crawling can merge and develop as chimeras. In this scenario, the detrimental effect would be maintained in the offspring through fusion with individuals lacking the negative mutation (fig. 1A). Viable chimeras have been observed to form between juveniles of the sponge *Amphimedon queenslandica*, provided that fusion takes place during the first 2 weeks after hatching (Gauthier and Degnan 2008), a process that could be the remnant of an ancient system permitting intraspecific somatic cell intermingling. On the other hand, if allorecognition exists, encounters between genetically different clones will result in nonfusion (fig. 1B), thus preventing the incorporation of new fitness-reducing genes. This might lead to an accelerated evolutionary rate by favoring the existence of individuals better

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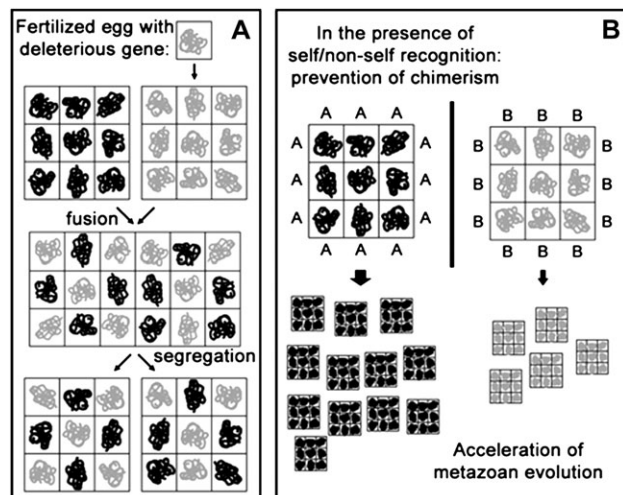


FIG. 1.—Self-/non-self-recognition in an evolutionary context. (A) Scheme representing the fate of encounters between multicellular aggregates in the absence of self-/non-self-recognition. (B) Scheme representing how self-/non-self-recognition could speed up metazoan evolution.

adapted to the environment that could have more abundant offsprings.

In marine sponges, the oldest extant Precambrian metazoan phylum, dissociated cells have the capacity to species specifically sort out and reaggregate through calcium-dependent associations of cell-surface proteoglycans termed aggregation factors (AFs, fig. 2) (Fernández-Busquets and Burger 2003), similarly as mixtures of dissociated vertebrate embryonic cells sort out according to their tissue of origin (Steinberg and Garrod 1975). In the marine sponge *Microciona prolifera*, the AF proteins MAFp3 (*M. prolifera* AF) and MAFp4 are encoded by a highly polymorphic gene, and grafting experiments revealed that histoincompatible *Microciona* individuals invariably have dissimilar MAFp3 and MAFp4 composition according to Southern blot data (Fernández-Busquets and Burger 1997). This led us to suggest that the AF, or a closely related molecule, could be the allogeneic determinant of sponge self-/non-self-recognition (Fernández-Busquets and Burger 1999, 2003) that arguably was one of the first allorecognition systems to evolve in animals given that sponges may be at the basis of the metazoan phylogenetic tree (Dunn et al. 2008). However, the first sponges preceded the Cambrian explosion by at least a 100 million years (My) (Budd 2008; Love et al. 2009). This time expanse was long enough to develop a rich palette of genes that genetic studies are identifying in sponges, which rival the complexity of vertebrate genomes (Müller 2001; Müller et al. 2001; Sakarya et al. 2007; Fernández-Busquets 2008). Sponges possess elements involved in transplantation immunity that are key components of mammalian innate immunity (Müller and Müller 2003), including molecules containing scavenger receptor cysteine-rich domains (Pahler et al. 1998), Toll-like receptors (Wiens et al. 2007), the (2',5')oligoadenylate synthetase (Kuuskalu et al. 1995), cytokine-like molecules (Müller et al. 1999), and also factors similar to those synthesized by cytokine-responsive macrophage molecules such as the allograft inflammatory factor 1 (Kruse et al. 1999). The

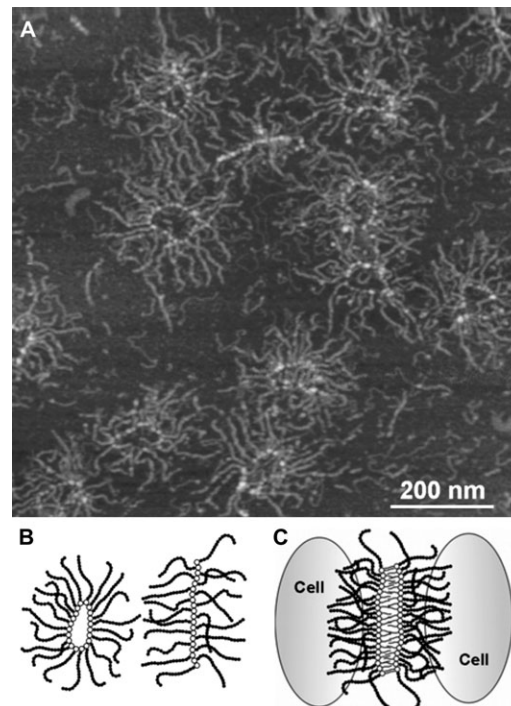


FIG. 2.—Species-specific cell adhesion in sponges is based on calcium-dependent carbohydrate-carbohydrate interactions of sponge proteoglycans. (A) AFM image of MAFs. (B) In its native form, MAF has the structure of a sunburst where the ring is formed by ~20 units of the MAFp3 protein (empty circles), each noncovalently linked to a unit of the MAFp4 protein (a MAF “arm”). If the ring of MAF were open, the resulting structure is analogous to a classical proteoglycan, with MAFp3 and MAFp4 in place of link protein and proteoglycan monomer, respectively. (C) Model of the MAF interactions responsible for species specificity of cell adhesion: Carbohydrates on MAFp4 bind receptors on the cell membrane, whereas the g200 glycan on MAFp3 self-interacts in a calcium-dependent manner. For clarity, both MAF molecules are represented linearized.

immunoglobulin-like domain, a hallmark of adaptive immune systems, has been identified in sponges (Blumbach et al. 1999), in some cases exhibiting intraspecific polymorphism (Pancer et al. 1998). Finally, allogeneic recognition in sponges has been found to be mediated by a specific cell type through mechanisms similar to those governing similar processes in vertebrates (Fernández-Busquets et al. 2002; Sabella et al. 2007). Hardly could such a refined machinery have appeared almost overnight in the geological timescale. Thus, another event was necessary in addition to the advent of self-/non-self-recognition for the Cambrian explosion to occur. Some proposed factors such as the origin of bilaterian body plans and the development of vision were in all likelihood later events. Sudden changes in the biosphere are often related to environmental factors, several of which have been proposed as the trigger event of the Cambrian explosion (Conway-Morris 2006; Marshall 2006), including a worldwide massive glaciation, an increase in the sophistication of predatory strategies, ecological niche saturation, or alterations in the chemistry of the oceans such as a rise in oxygen levels or a modification in the composition of seawater, but none of them has provided so far a sufficiently satisfactory explanation.

Sponge AFs are bifunctional molecules exhibiting a Ca^{2+} -independent interaction with cell membrane

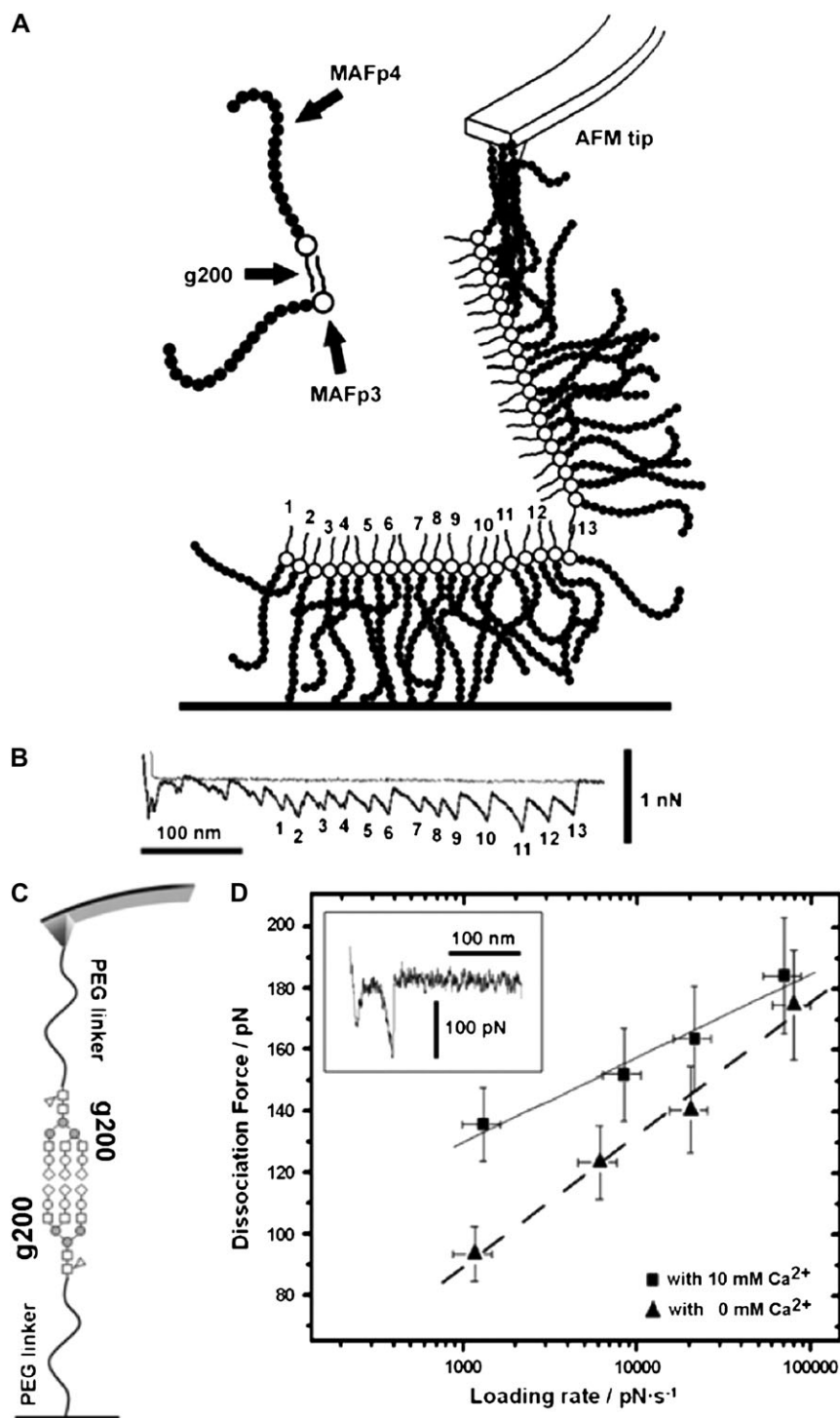


FIG. 3.—SMFS analysis of the effect of calcium in g200–g200 interactions. (A) Scheme of an SMFS experiment to study the adhesive interactions between two MAF molecules. (B) Typical SMFS approach–retract curve obtained in experiments performed with native MAF, consistent with the cartoon representation from A. The numbers from 1 to 13 correspond to the unbinding of individual g200–g200 interactions as the AFM tip retracts from the surface. Adapted from Garcia-Manyes et al. (2006). (C) Experimental setup showing g200 bound through a polyethylene glycol linker to the AFM tip and surface. (D) Dynamic force spectroscopy graph where the measured binding forces in the presence and in the absence of calcium are plotted against the loading rate. The inset shows a typical g200–g200 retracting force curve. Controls performed with linker alone did not reveal significant interactions (<0.5% binding probability) either in the absence or in the presence of calcium.

receptors and a Ca²⁺-dependent self-binding activity. In *M. prolifera*, the AF self-binding site has been tracked down to g200, a complex glycan of 200 kDa (Misevic et al. 1987; Jarchow et al. 2000; Bucior et al. 2004). The poly-

valency of g200 on an AF molecule permits the integration of multiple individual binding sites into a much stronger bond (fig. 3A and B). Most functional studies of sponge cell adhesion have been done at the current seawater

Ca^{2+} concentration of 10 mM, but little attention has been placed on investigating possible implications of adhesion forces in low calcium (Rice and Humphreys 1983). To quantitatively explore the significance of changing Ca^{2+} concentrations on the association of primitive metazoan cells, we have performed a comparative single molecule force spectroscopy (SMFS) study of g200 self-binding in low and high Ca^{2+} , analyzing the data in the context of a multivalent system model. Our results are discussed within the framework of the ongoing discussion about which factor(s) triggered the Cambrian explosion of life.

Materials and Methods

Atomic Force Microscope (AFM) Imaging

AFM images were acquired with the commercial instrument Nanoscope III (Multimode and Bioscope AFM, Digital Instruments, Santa Barbara, CA). Imaging was done in tapping mode of operation under ambient conditions with standard Si cantilevers (Nanoprobe, Wetzlar, Germany). Sample preparation was always according to our standard protocols (Fritz et al. 1997) or according to the protocols using NH_2 -functionalized mica substrates (Lyubchenko et al. 1993).

SMFS Assays

A solution containing 1 μM of purified g200 (Garcia-Manyes et al. 2006) and 1 μM *N*-hydroxysuccinimide-poly(ethylene glycol)-maleimide (NHS-PEG-MAL 3,400, Nektar Therapeutics) in deionized water was incubated for 5 h at 4 °C. Si_3N_4 cantilevers (Microlever, Veeco Instruments) and mica surfaces (Provac AG) were silanized with aminopropyltriethoxysilan (Sigma Aldrich) in an exsiccator and then overlaid with the g200-PEG linker solution at 4 °C overnight. After washing with H_2O , cantilevers and surfaces were ready for use in force spectroscopy experiments. Several cantilevers and surfaces were prepared simultaneously to ensure maximum immobilization homogeneity. Freshly prepared tips and surfaces were used in each experiment. SMFS measurements were performed with a commercial AFM (MFP3D, Asylum Research) at room temperature in calcium- and magnesium-free artificial seawater prepared according to the standards of the Marine Biological Laboratory (www.mbl.edu), with or without 10 mM CaCl_2 . The spring constant of the cantilever was calibrated by the thermal fluctuation method (Hutter and Bechhofer 1993) and the same cantilever was used in each experiment for both plus and minus calcium conditions. Typically 2,000–3,000 force curves were recorded at four different retracting velocities while the approach velocity was kept constant. The order in which the velocities were applied did not influence the results. When the same tip and surface were used with and without calcium (in either order), the results were equivalent to those obtained with a different tip-surface combination for each condition. The curves were analyzed with a Matlab-based analysis software (Math Works), corrected to display the actual molecular extension calculated from the *z* piezo extension and

plotted in force histograms (supplementary fig. S1, Supplementary Material online) where the maximum of the force probability distribution is referred to as dissociation force (F_{max}).

Calcium Uptake Assays

Live sponge cells and g200 glycan preparation and aggregation inhibition by Block 2 monoclonal antibody were done as previously described (Misevic et al. 1987). Coupling of g200 to amine-modified 1- μm latex beads (Molecular Probes), and cell- and bead-aggregation assays were done as specified before (Bucior et al. 2004). Calcium uptake was estimated for cell-cell and g200-coated bead-bead aggregation. *Microciona prolifera* cells (5×10^3 cells/ml) or 9×10^7 g200-coated amine-modified beads/ml were allowed to aggregate on a rotary shaker at 60 rpm in calcium- and magnesium-free Tris-buffered seawater containing 10 mM CaCl_2 labeled with ^{45}Ca (74 MBq, 2 mCi, Amersham Pharmacia Biotech). After different times of incubation, cells or beads were spun down ($1,000 \times \text{g}$, 5 min). The amount of ^{45}Ca remaining in solution was quantified in a liquid scintillation counter (Beckmann LS 3801) and subtracted from the initial radioactivity to determine the percentage of Ca^{2+} taken by aggregating cells or glycan-coated beads.

Results

We performed g200-g200 AFM SMFS assays using a well-established method for the immobilization of biomolecules via a heterobifunctional polyethylene glycol linker (Hinterdorfer et al. 1996). Such a strategy introduces a distance between interacting molecules and surfaces (fig. 3C), adds steric flexibility for the binding partners, and guarantees an almost complete reduction of unspecific binding events (Eckel et al. 2005). The results obtained in calcium-free artificial seawater indicate the existence of relatively strong forces between 100 and 200 piconewton (pN) for the g200-g200 interaction (supplementary fig. S1, Supplementary Material online). These forces being of the same order of magnitude as those obtained for the same molecule in the presence of 10 mM Ca^{2+} , one would expect that AFMs could aggregate cells in the absence of calcium. This, however, contradicts the well-established fact that the multicellular integrity of contemporary seawater sponges is lost below a critical calcium concentration threshold (Rice and Humphreys 1983; Fernández-Busquets and Burger 2003). Thus, absolute binding forces cannot be used as the dominating factor to explain the carbohydrate-mediated interaction between sponge cells.

According to the standard model of thermally driven dissociation under external force (Evans and Ritchie 1997), the measured dissociation forces (F_{max}) depend on the experimental retract velocity and should be represented against the corresponding molecular loading rates in a dynamic force spectroscopy (DFS) plot. To obtain molecular loading rates, the retract velocity is multiplied by the elasticity of the system, which is determined by fitting the slope of every single force curve just before

dissociation. Generally, the measured F_{\max} obeys the following law:

$$F_{\max} = \frac{k_B T}{x_\beta} \ln \frac{x_\beta r}{k_B T k_{\text{off}}}, \quad (1)$$

where $k_B T$ and r denote thermal energy and the loading rate, respectively. x_β is a length parameter along the reaction coordinate representing the distance between the minimum of the binding potential and the transition state separating bound and free states, which is commonly referred to as the reaction length. k_{off} is the thermal off-rate constant under zero external load and can be deduced by linearly extrapolating the experimental data in the DFS plot to zero external force ($F = 0$). It has already been shown earlier (Schwesinger et al. 2000) that estimated k_{off} in single molecule experiments can unequivocally be assigned with kinetic data extracted from molecular ensemble experiments. The inverse relation of k_{off} to the average lifetime of the complex, τ ($k_{\text{off}} = \tau^{-1}$), allows a direct way of evaluating the stability of the complex. The logarithmic DFS plot of our measured SMFS data (fig. 3D) yields the expected linear dependence according to equation (1). A clear and significant difference between the experiments with (10 mM) and without (0 mM) Ca^{2+} can be inferred. For the single molecule process, quantitative analysis yields dissociation rate constants of $k_{\text{off,s.m.}}(+\text{Ca}^{2+}) = 1.47 \pm 1.23 \times 10^{-3} \text{ s}^{-1}$ and $k_{\text{off,s.m.}}(-\text{Ca}^{2+}) = 0.38 \pm 0.24 \text{ s}^{-1}$, and reaction lengths of $x_\beta(+\text{Ca}^{2+}) = 3.47 \pm 0.44 \text{ \AA}$ and $x_\beta(-\text{Ca}^{2+}) = 2.27 \pm 0.15 \text{ \AA}$.

Thus, in 0 mM calcium (fig. 4A), the mean g200–g200 reaction length falls below the hydrogen bond distance in biomolecules, which is around 2.9 \AA between the centers of donor and acceptor atoms (Mathews and van Holde 1990), whereas in the presence of 10 mM calcium (fig. 4B), the mean reaction length increases to well above the hydrogen bond range. When sponge cells or g200-coated beads are allowed to aggregate in 10 mM CaCl_2 , there is a significant calcium uptake from the medium by the glycan structures (supplementary fig. S2, Supplementary Material online), which is abolished in the presence of a monoclonal antibody against a species-specific g200 self-adhesion epitope (Misevic and Burger 1993). This sequestering of calcium is not immediate, suggesting that the cation becomes gradually incorporated into the glycan structure in a time-dependent process. Because the reciprocal effect of calcium leakage from g200 in a calcium-free environment is also expected to be gradual, glycan-bound Ca^{2+} ions are likely responsible for a significant fraction of the interaction measured by SMFS at the nominal concentration of 0 mM Ca^{2+} . The known inability of g200 to aggregate cells in the absence of calcium might lead to the conclusion that calcium is required simply to provide forces stronger than hydrogen bonds between the carbohydrate chains. However, other cations such as Na^+ and Mg^{2+} cannot substitute for Ca^{2+} (Rice and Humphreys 1983), suggesting additional binding elements other than purely ionic interactions. Calcium ions could be responsible for the approach and organization of the sugar moieties that provide the adequate surfaces for interaction or may also enhance the adhesion between complementary surfaces through

a suitable configuration of hydroxyl groups. Single hydroxyl groups in sugars are too weak to coordinate cations in the presence of water molecules, although with the combination of two to three adequately positioned hydroxyls on one sugar residue or over two adjacent residues, calcium ions can become coordinated to electron pair donor groups on the carbohydrates (Spillmann and Burger 1996), either directly or through intervening water molecules, all of which would result in increased reaction lengths (fig. 4B).

There is a second parameter that can be derived from SMFS data having profound evolutionary implications related to the polyvalent calcium-dependent association of sponge cells to form a multicellular animal. The mean lifetime of the g200–g200 binding, $\tau_{\text{s.m.}}$ ($1/k_{\text{off,s.m.}}$), is much increased in the presence of 10 mM calcium: 680 versus 2.6 s in its absence. For a given binding force between two ligands, the time during which the interaction takes place will have dramatic consequences on its outcome. A rapid on–off switching between bound and unbound states will be more prone to dissociation than a much more stable association. In addition, slow on–off rates will favor the adequate positioning of coordinating lock-and-key epitopes that might account for the specificity of the interaction. Marine sponges like *M. prolifera* can keep for years ($>10^7 \text{ s}$) their multicellular integrity in seawater containing 10 mM Ca^{2+} and start to dissociate within minutes ($<10^2 \text{ s}$) in the absence of Ca^{2+} , whereas the corresponding Ca^{2+} -dependent g200 interaction on a single molecule level only exhibits lifetimes of about 10^3 and 1 s, respectively. This disparity in lifetimes by several orders of magnitude can be explained by the multivalency of g200 on AF molecules and of these on sponge cell surfaces. This polyvalency can be treated from a theoretical viewpoint within the framework of stochastic dynamics of adhesion clusters in the presence of molecular rebinding (Erdmann and Schwarz 2004) (fig. 5). In the limit of a vanishing force ($F = 0$), the adhesion cluster lifetime T can be calculated as

$$T = \frac{1}{1 + k_{\text{on}}/k_{\text{off}}} \times \left[\sum_{n=1}^{N_0} \left\{ \left(\frac{N_0!}{n!(N_0 - n)!} \right) \frac{(k_{\text{on}}/k_{\text{off}})^n}{n} \right\} + H_{N_0} \right], \quad (2)$$

where N_0 and H_{N_0} denote bond number and harmonic numbers (sum of the reciprocals of the first N_0 natural numbers), respectively. We applied this formula that is based on a stochastic version of Bell's dissociation rate theory (Bell 1978) to extract multivalent lifetimes from straightforward assumptions and our experimental data. Upon assuming a conservative overall number of bonds between two sponge cells of $N_0 = 20\text{--}100$ (Garcia-Manyes et al. 2006), a typical rebinding rate constant of $k_{\text{on}} = 0.01 \text{ s}^{-1}$ (Erdmann and Schwarz 2004) and using our experimental single molecule dissociation rate constants of $k_{\text{off,s.m.}}(+\text{Ca}^{2+})$ and $k_{\text{off,s.m.}}(-\text{Ca}^{2+})$, we can deduce a multivalent cell aggregation lifetime $\tau_{\text{cell}}(+\text{Ca}^{2+})$ of $>10^{15} \text{ s}$ for a calcium-rich (10 mM) and a reduced lifetime $\tau_{\text{cell}}(-\text{Ca}^{2+})$ of $<10 \text{ s}$ for a calcium-deficient (0 mM) environment. This simulation result is robust against large variations of k_{on} and systemic modulations. The

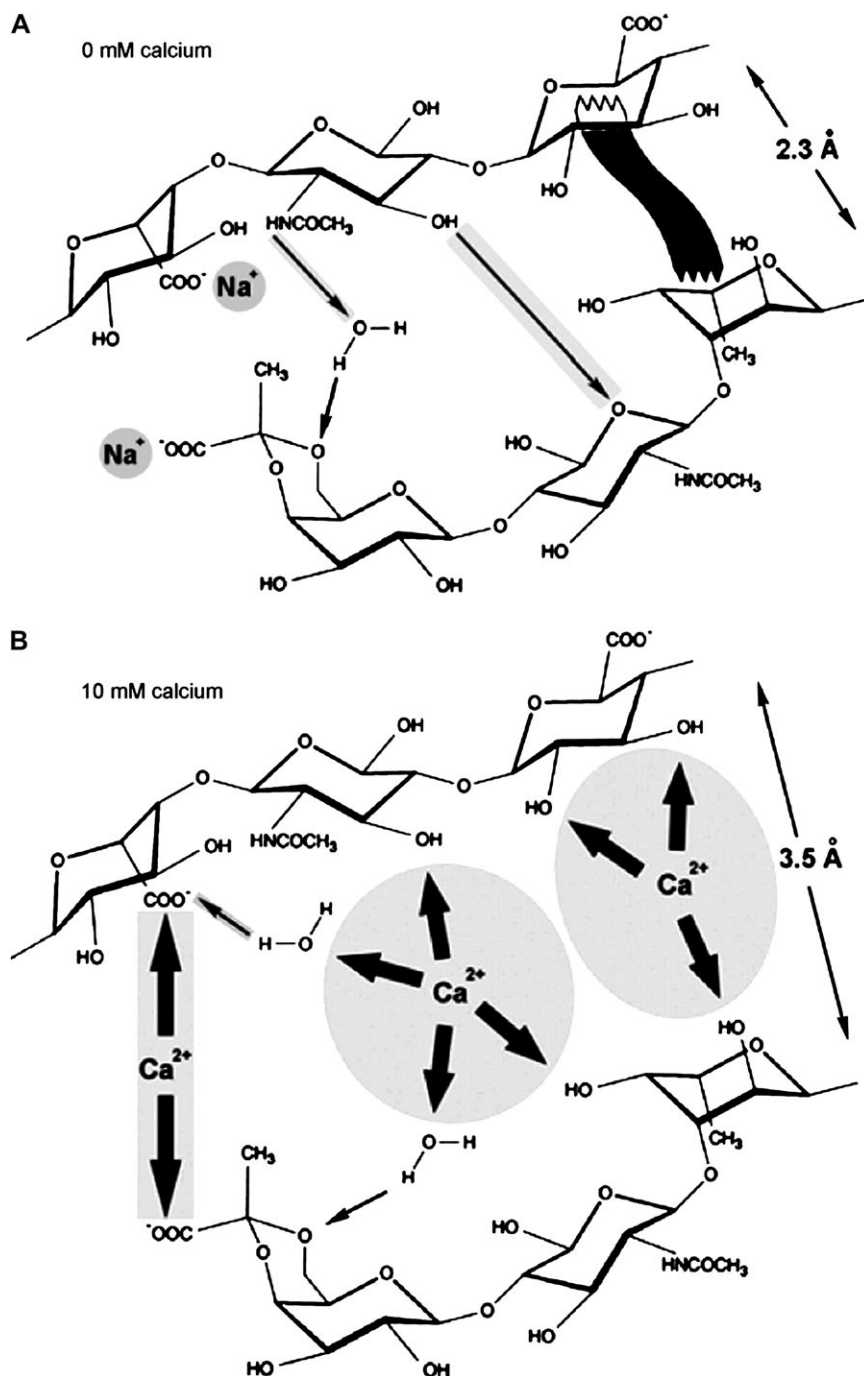


FIG. 4.—Models summarizing the proposed forces participating in carbohydrate-carbohydrate interactions in the absence (A) and in the presence of calcium (B). In 0 mM Ca^{2+} , excess Na^{+} ions contained in seawater efficiently neutralize the repulsive forces expected from the high negative charge of carboxyl and sulfate groups in g200. Hydrogen bonds are represented by thin arrows and hydrophobic interactions by a ribbon. Adapted from Spillmann and Burger (1996).

assumption to use similar rebinding rate constants for calcium-deficient and calcium-rich situations is justified and supported by the experimental fact that under both conditions the overall (re)binding probabilities remain almost constant (supplementary fig. S1, Supplementary Material online).

Discussion

Calcium binders with biological activity are mainly considered to be proteins, but carbohydrates are emerging

as important players in the field, foreseeing the upcoming age of glycomics. In particular, carbohydrate-carbohydrate interactions are being accepted as a novel and highly versatile mechanism for cell adhesion due to the extraordinary plasticity of glycan chains, the low affinity and reversibility of individual binding sites, and the capacity to form multivalent complexes leading to increased association forces (Bucior et al. 2009). Embryogenesis, metastasis, and other cellular proliferation processes are mediated by

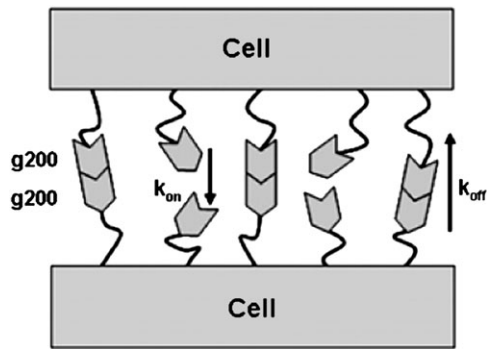


FIG. 5.—Multivalent calcium-dependent carbohydrate-carbohydrate cell adhesion in metazoan evolution. Scheme of the theoretical model for treating a polyvalent binding mechanism within the framework of stochastic dynamics of adhesion clusters in the presence of molecular dissociation and rebinding.

carbohydrate self-recognition events based on van-der-Waals contacts, hydrogen bonds, and electrostatic forces provided by cations (Eggens et al. 1989; Hakomori 1991; Yu et al. 2002; Rinaudo 2006). Carbohydrate-based cell adhesion evolved early, as exemplified by the essential role played by glycan-glycan interactions in sponges, the oldest extant metazoans.

SMFS analysis of intact AF molecules and of g200 glycans has revealed a dependence of their adhesion forces on calcium concentration. In 10 mM Ca^{2+} , an elevated average force with high probability of binding between AFs was measured (Dammer et al. 1995), whereas in 2 mM Ca^{2+} , where sponge cell adhesion is strongly diminished (Humphreys 1963; Cauldwell et al. 1973; Jumblatt et al. 1980; Misevic et al. 1987; Misevic and Burger 1993), the force and probability of binding events were also reduced. SMFS measurements of the adhesion forces between single g200 glycans (Bucior and Burger 2004) showed a significant increase from 310 to 375 pN when the Ca^{2+} concentration was raised from 10 to 100 mM. More recent studies (Garcia-Manyes et al. 2006) revealed a strong Ca^{2+} -dependent cooperative binding in intermolecular adhesion domains of native MAF molecules. Although the calculated mean sponge cell disaggregation time of 10^{15} s (~ 30 My) in the presence of 10 mM Ca^{2+} possibly does not represent a meaningful number for a sponge individual, it dramatically proves the nonlinear and divergent characteristics of the different asymptotic final states upon using physiological parameters in this cooperative model for carbohydrate-carbohydrate binding, which leads to either weakly aggregated cells or stable multicellular assemblies as a function of Ca^{2+} concentration in the extracellular environment. Apparently small differences in adhesion force between the individual binding sites result in clearly different outcomes when placed in the context of a multivalent system. This suggests that even small variations of oceanic Ca^{2+} amounts could have had significant effects on the aggregative competency of early metazoan cells. At present, the most generally accepted candidate for causing—or allowing—the Cambrian explosion is a rise in oxygen levels at the end of the Proterozoic (Nursall 1959). But although oxygen was likely important to permit the existence of large animals, this does not imply that it

was also the trigger for their evolution, and we have to consider a complex geochemical setting which should also include other factors such as temperature and salinity (Knauth 2005).

The data presented here acquire a deep evolutionary significance if one realizes that the content of calcium in seawater has oscillated throughout the different geological periods. One characteristic of the Cambrian explosion fauna is the abundance of animals with calcareous shells (Kerr 2002), a phenomenon that reflects a well-referenced substantial increase in seawater calcium levels around the beginning of the Cambrian period (Elkinton 1957; Hardie 1996; Berner 2004; Brennan et al. 2004; Petrychenko et al. 2005). Although there is some discrepancy in the literature concerning the precise ion concentration in the oceans at the Precambrian-Cambrian boundary, the generalized calcium surge is an undisputed fact. Many developmental and other physiological processes in animals are calcium-sensitive, from those having calcium as second messenger to biocalcification and cell adhesion. Cellular ion concentrations are under strict control of plasma membrane ion channels that should ward off fluctuations in environmental calcium levels. Nevertheless, during the early Cambrian, the ambient oceanic calcium may have risen to such a degree that some cells could no longer effectively exclude or expel the Ca^{2+} ions, causing intracellular calcium in certain marine organisms to reach toxic levels (Simkiss 1977). The advent of biomineralization (Kerr 2002; Brennan et al. 2004) might have been a metabolic detoxification process induced by such increases in intracellular Ca^{2+} (Simkiss 1977). Opportunistic organisms may have modified existing cellular waste-removal systems by binding Ca^{2+} as relatively insoluble minerals, which could have been the starting point of the complex process that eventually led to shell production (Simkiss 1977; Brennan et al. 2004). However, of all cellular functions, cell adhesion was probably the most strongly modulated by even small changes of the calcium concentration in seawater.

We have characterized the adhesion forces of g200 at the contemporary extremes of 0 and 10 mM calcium, although the AFs of 600 My ago might have been adapted to work optimally in different salinity conditions. SMFS assays have revealed that AFs from different sponge species having different carbohydrate structures self-bind with similar forces (Bucior et al. 2004). This suggests that carbohydrate interactions have sufficient plasticity to maintain their function by adapting to changing environmental conditions and therefore that the calcium-dependent binding is a property of AFs throughout the geological eras. So far, orthologues of sponge AF genes have not been identified in other phyla and the closest sequence homology is with a region of the Na-Ca exchanger, an ion channel regulating intracellular calcium levels. Thus, the evolutionary role proposed here for AFs was played in primitive sponges or in the stem leading the crown metazoans, and later consolidation of protein-based cell adhesion could explain their absence from more modern animals. Neither is evidence yet for AF precursors in the sister group to the Metazoa, the choanoflagellates, but future research might uncover them. Although protein sequence homologies can be difficult to spot due to the high variability detected for AF genes

(Fernández-Busquets and Burger 1997), a carbohydrate-based cell aggregation can be expected to be discovered in choanoflagellates. This prediction is founded on the striking morphological and functional resemblance between choanoflagellates and the sponge cell type responsible for food intake, choanocytes, which associate to form choanocyte-feeding chambers through AF-mediated cell adhesion (Simpson 1984).

One of the characteristics of the Cambrian explosion is an increased evolution speed, but it is not clear why this evolutionary acceleration should have been favored. Factors such as extreme competition for limited resources or to escape from predators are more likely scenarios after the new phyla occupied most available ecological niches. Alternatively, faster evolution could be the mere by-product of a necessary innovation such as self-/non-self-recognition. It had been suggested that the capacity of fusion with both kin and genetically unrelated conspecifics to form chimeras is evolutionary retained in several phyla because the resulting organism obtains some selective advantages (Buss 1982). However, experimental data derived from sponges and colonial marine invertebrates indicate that chimerism does not affect (Maldonado 1998) or even reduces fitness by causing a decrease in growth rates, reproduction, and survivorship (Chadwick-Furman and Weissman 1995). The extensive allorecognition allele polymorphism commonly observed in natural populations of colonial ascidians and the reported instability of chimeras has led to argue that selection for fusion with self, rather than fusion with kin, may be a major selective force acting on allorecognition systems (Buss 1982; Feldgarden and Yund 1992). Self-/non-self-recognition could have evolved to prevent the costs of germ cell or somatic cell parasitism that can result from the fusion of different individuals while keeping some demographic benefits such as increased size in the event of self-fusion (Stoner and Weissman 1996). Perhaps tighter links between cells was an important factor in preventing fusion in the first place, a strategy that could have worked in more primitive animals. In agreement with this view, the AFs responsible for species-specific cell adhesion in sponges are so polymorphic that they have been suggested to be closely related to the molecules that determine graft rejection and acceptance in these animals (Fernández-Busquets and Burger 1997, 1999; Fernández-Busquets et al. 1998, 2002). The correlation between AF protein polymorphism and the outcome of sponge grafts (Fernández-Busquets and Burger 1997) hints at an evolutionary link between early metazoan calcium-dependent cell adhesion and primitive allorecognition molecules that could have preceded histocompatibility systems.

We propose two innovations were necessary for metazoan evolution: First, allorecognition was likely to have evolved as a way of preventing the negative effects of chimerism, for example, germ-cell parasitism or the intrusion of fitness-reducing mutations from a genetically distinct individual. Data from living sponges indicate that sponge larvae may habitually fuse to form viable, genetically hybrid adults (McGhee 2006; Gauthier and Degnan 2008), whereas adult sponges possess a precise allorecognition system that efficiently prevents the fusion of different indi-

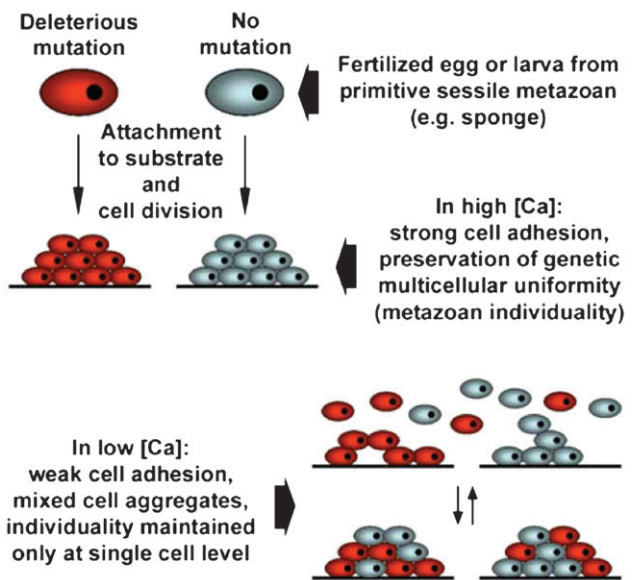


FIG. 6.—Cartoon summarizing the effect of high and low calcium concentrations on the integrity of primitive sessile metazoans having calcium-dependent cell adhesion molecules. Early metazoans capable of self-/non-self discrimination could have developed carbohydrate-based adhesion that at the low calcium content of Precambrian seas was too short lived to keep cells together for a time long enough to permit the stable persistence of multicellular individuals.

viduals (Fernández-Busquets and Burger 1997; Sabella et al. 2007). This ontogenetic shift might reflect the adoption of improved self-/non-self discrimination at some point during primitive sponge evolution prior to the Cambrian explosion. We must assume that the bilaterian lineages started to adopt the mechanism of self-/non-self-recognition more strictly than sponges do. As a second necessary factor, we suggest that the calcium increase in Cambrian oceans coincided with the existence of primitive sessile metazoans, endowed with allogeneic recognition, and carrying cell adhesion molecules that had significantly longer dissociation times at the new calcium concentrations (fig. 6). Only when calcium levels rose, polyvalent intercellular adhesion became strong enough to allow individual surface-bound metazoans such as sponges to maintain their integrity until reproduction. This scenario would permit genetic constitutions to remain within the multicellular individual and be passed down inheritance lines.

Recent evidence clearly places the appearance of sponges during the Cryogenian period, >635 Ma (Love et al. 2009), whereas the major bursts in animal diversification took place in the next and last Precambrian period, the Ediacaran (Peterson et al. 2008; Shen et al. 2008). However, the body plans characteristic of modern phyla are thought to have evolved gradually somewhat after the time of the Cambrian explosion (Budd 2008), in an accelerated evolution of the lineages that had already started to diverge earlier (Valentine 2004). According to current estimates, oceanic calcium rose from very low levels around 2 mM (Elkinton 1957) to reach approximately 10 mM at the Precambrian–Cambrian boundary (Berner 2004; Brennan et al. 2004) and continued increasing up to concentrations that were higher in early Cambrian oceans than they are at

present (Hardie 1996; Berner 2004; Brennan et al. 2004; Petrychenko et al. 2005). Thus, with the currently available data presented above on AF function, the effect proposed here for calcium-dependent cell adhesion on the evolution of early metazoans would have been mainly exerted during the Precambrian diversification phase.

Supplementary Material

Supplementary figures S1 and S2 are available at *Molecular Biology and Evolution* online (<http://www.mbe.oxfordjournals.org/>).

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