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# Involvement of a Highly Polyvalent Glycan in the Cell-Binding of the Aggregation Factor From the Marine Sponge *Microciona prolifera*

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A proteoglycan-like aggregation factor from the marine sponge *Microciona prolifera* (MAF) mediates cell-cell recognition via a cell-binding and a self-association domain. After repetitive and prolonged treatment of MAF with glycopeptide-N-glycosidase (PNGase) the specific binding of MAF to homotypic cells was decreased by 72%. Polyacrylamide gel electrophoresis and gel filtration analysis of such PNGase digests showed that: 1) the enzyme released a single glycan type of $M_r = 6 \times 10^3$ (G-6) from MAF, 2) 1 mole of MAF contains at least 830 moles of N-linked chains of G-6 glycan. The correlation between the loss of the binding activity of MAF and the extent of the release of the repetitive G-6 polysaccharide strongly suggests its involvement in MAF-cell association via highly polyvalent interactions.

**Key words: glycans, cell recognition**

The first experimental demonstration of cell-cell recognition phenomenon was provided by reaggregation experiments with dissociated sponge cells [1,2]. The possibility of using a simple functional assay, together with the fact that sponge cells could be dissociated without use of proteolytic enzymes, thus leaving their cell surface intact, has allowed the molecular mechanism of aggregation to be elucidated [1–12]. At least three components have been shown to play important roles during cell recognition/adhesion in the marine sponge *Microciona prolifera:* 1) $Ca^{2+}$ [1–3,8,13], 2) proteoglycan-like aggregation factor (MAF) of $M_r = 2 \times 10^7$ [3–6], and 3) specific cell surface receptor for MAF [7,9].

We have shown recently that two functional domains, namely a cell-binding and a self-association site on MAF molecules, mediate cell-cell recognition via highly polyva-

Abbreviations used: CSW, $Ca^{2+}$,$Mg^{2+}$-free seawater supplemented with 2 mM $CaCl_2$ and buffered with 20 mM Tris pH 7.4; MAF, *Microciona prolifera* aggregation factor.

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lent low-affinity interactions [8,10–12]. Here we present the evidence that the major part of the cell-binding activity of MAF is lost by enzymatic removal of a highly repetitive glycan of $M_r = 6 \times 10^3$ (G-6).

## MATERIALS AND METHODS

### Sponges

Live specimens of *Microciona prolifera* were collected by authors and by the Supply Department of the Marine Biological Laboratory, Woods Hole, MA, USA. *Haliclona occulata* was obtained through Marine Research Associates, Ltd., St. Andrews, New Brunswick, Canada.

### Purification and Radioiodination of MAF

MAF was isolated according to a previously described method [4,5]. Final purification was achieved by CsCl gradient centrifugation [10–12]. The chloramine-T procedure was used for radioiodination of intact or glycosidase-treated MAF [8,10,11].

### Enzymatic Treatment of MAF

Glycopeptide-N-glycosidase treatment of MAF was performed after dialysis of the molecule against 50 mM EDTA in 0.1 M NaCl buffered with 20 mM Tris pH 7.4. Typically 25 units of PNGase obtained from Boehringer-Mennheim GmbH (25,000 units/mg, 100 units/0.5 ml) dissolved in the same EDTA/NaCl/Tris buffer was added to 500 $\mu$g of MAF (50% of the MAF mass is represented by neutral hexose [4,5,10,11]). After 24 h incubation at room temperature an additional 25 units of the enzyme was supplied. The reaction was carried out again for 24 h under the same conditions. The identical procedure was repeated one more time. After the total incubation time of 72 h, equilibration of samples with either $Ca^{2+}$,$Mg^{2+}$-free seawater supplemented with 2 mM $Ca^{2+}$ and 20 mM Tris pH 7.4 (CSW) or distilled water was achieved by using a Bio-Gel P-2 (200–400 mesh) (0.5 × 9 cm) column. The control samples were incubated and processed without the enzyme under the identical conditions as the digested MAF. Iodination of samples was performed after enzymatic treatments.

### Cell-Binding Assay

Binding of the iodinated control and PNGase-treated MAF was performed in Eppendorf tubes in 400 $\mu$l of CSW by using $2 \times 10^7$ glutaraldehyde-fixed cells per ml. After 20 min incubation at room temperature, with regular rotation of tubes to prevent settlement of cells, samples were centrifuged at 500*g* for 2 min. Whole tubes were then counted in a gamma spectrometer for determination of total radioactivity. After aspiration of the supernatants radioactivity of pellets was measured and efficiency of the cell binding was quantified.

### Analytical Methods

Polyacrylamide gel electrophoresis of MAF glycans was performed essentially as previously described with some modifications [14–16]. The gels were stained with solution of 0.5% Alcian blue in 25% isopropyl alcohol and 1% acetic acid for 12 h and destained by using the same solution in the absence of dye [11].

The determination of neutral hexose concentration and amino acid analysis were performed as described earlier [11,12,17].

## RESULTS

### Effect of PNGase on the Cell-Binding Activity of MAF

In order to determine whether the putative cell-binding site of MAF was associated with the core protein or carbohydrate moieties of the molecule, the effect of glycopeptide-N-glycosidase treatment of MAF on its association with the homotypic cells was examined. Typically 500 μg of MAF was incubated with 25 units of PNGase for 24 h followed by two further additions of the same amount of enzyme at 24 h intervals (see also Materials and Methods). The control and the PNGase-treated MAF samples obtained after 72 h treatment were first iodinated and then their binding specificity was tested with homotypic and heterotypic cells. The results of Table I show that the control undigested MAF samples bound species-specifically, whereas the PNGase-treated MAF displayed very low binding to homotypic cells, and practically no binding to heterotypic cells.

Species-specific cell-binding activity of iodinated samples obtained after the first and third cycles of digestion as well as of the corresponding controls incubated under identical condition for the same period of time without the enzyme was measured. As presented in Table II there was a decrease in binding of PNGase treated samples to the homotypic cells which correlated with incubation time and the amount of enzyme used. After the third and final PNGase addition, MAF binding to the homotypic cells was 72% lower than that of the control, thus showing that such extensive treatment leads to almost complete loss of the cell-binding function (Tables I and II).

### Identification of a Polysaccharide Released From MAF by PNGase

In order to identify the polysaccharide released from MAF by PNGase treatment, control MAF and samples obtained after prolonged 72 h digestion were analyzed by electrophoresis on linear gradient (7.5–20%) polyacrylamide gels. Alcian blue staining and autoradiography demonstrated that only a single glycan migrating slightly slower then phenol red was released by PNGase digestion of the $^{125}$I-MAF subunits (Fig. 1). No covalently bound radioactivity was associated with the cleaved glycan (Fig. 1).

**TABLE I. Binding Specificity of PNGase-Digested and Control MAF***

| Ligand | % bound to fixed cells of | |
|---|---|---|
| | *Microciona prolifera* | *Haliclona occulata* |
| EDTA-treated MAF | 23.6 | 4.3 |
| EDTA- and PNGase-treated MAF | 9.2 | 3.9 |

*Binding of 1 μg ($1.2 \times 10^6$ cpm/μg neutral hexose) of 72 h EDTA-treated control MAF and of 1.4 μg ($3.7 \times 10^5$ cpm/μg neutral hexose) of 72 h EDTA- and PNGase-digested $^{125}$I-MAF to *Microcina prolifera* and *Haliclona occulata* glutaraldehyde-fixed cells was performed in 400 μl CSW for 20 min at room temperature as described under "Materials and Methods." After centrifugation and aspiration of the supernatant the amount of bound radioactivity was measured. Percent of MAF associated with cells was obtained by subtracting the background binding of MAF to tubes from that obtained with cells. The data represent means of duplicate determination within the same cell and MAF and PNGase preparation, and standard deviation was less than ±12% of each value.

TABLE II. Cell-Binding Characteristics of MAF After PNGase Treatment*

| Ligand | Amount of PNGase per 100 μg MAF (units) | Time of incubation (hours) | MAF bound to *M. prolifera* cells (%) | Amount of G-6 released from 200 μg MAF (μg neutral hexose) |
|---|---|---|---|---|
| EDTA treated MAF | 0 | 24 | 25.9 | 0 |
| EDTA and PNGase treated MAF | 5 | 24 | 20.9 | 15 |
| EDTA treated MAF | 0 | 72 | 19.3 | 0 |
| EDTA and PNGase treated MAF | 15 | 72 | 5.3 | 50 |

*Binding of PNGase-treated and control $^{125}$I-MAF to *Microciona prolifera* glutaraldehyde-fixed cells was performed in CSW as described under "Materials and Methods." Incubation of 1.4 μg of MAF ($3.7 \times 10^5$ cpm/μg neutral hexose) following digestion for 24 h and 72 h (represents three equivalent additions of PNGase in intervals of 24 h) and 1 μg of corresponding controls of undigested MAF ($1.2 \times 10^6$ cpm/μg neutral hexose) with 400 μl of $2 \times 10^7$ *M. prolifera* cell/ml of CSW was performed for 20 min at room temperature (for details see "Materials and Methods"). The amount of bound radioactivity was determined upon cell centrifugation. Percent of species-specifically associated MAF with cells was obtained after subtraction of heterotypic binding to *Haliclona occulata* cells. The quantity of G-6 glycan released was measured by neutral hexose determination after its purification by gel filtration or by densitometric scanning of Alcian blue-stained gels. The data represent means of duplicate determination within the same cell and MAF and PNGase preparation, and standard deviation was less than ±12% of each value.

The same samples were also analyzed on a TCK G2000 SW (0.8 × 30 cm) column equipped with a TCK SW precolumn (0.8 × 4 cm). Measurements of optical density at 206 nm, the refractive index, and neutral hexose content of the fractions confirmed our findings by electrophoretic analysis that indeed only a single species of glycan with $M_r = 6.3 \times 10^3$ (G-6) was released by enzymatic digestion from MAF under the conditions described (Fig. 2).

Amino acid analysis of the G-6 molecule isolated by gel filtration after PNGase treatment showed the complete absence of amino acids (less than 0.2 moles per 1 mole of G-6), thus indicating that G-6 is purely polysaccharide in content.

## Correlation of MAF Cell-Binding With the Release of G-6

The levels of G-6 released from MAF by relatively mild (25 units for 24 h) and extensive (75 units for 72 h) enzymatic digestion were determined either by measurements of the neutral hexose content of samples isolated by gel filtration or by densitometric scanning of Alcian blue–stained gels. As shown in Table II the amount of G-6 released correlated with the time and the quantity of the enzyme used for digestion. Furthermore, as already discussed, there was a similar dependent decrease in binding of MAF to cells following its exposure to PNGase. Since the extensive digestion by PNGase of MAF obliterated its cell-binding activity, and since the G-6 molecule was the only component of MAF released by such treatment, we concluded that this glycan was directly implicated in the process of MAF association with cell surface receptors.

## G-6 Is a Highly Repetitive Glycan of MAF

Determination of the amount of neutral hexose in the isolated G-6 glycan obtained after prolonged PNGase treatments of MAF showed that 50% of the total MAF carbohydrate content could be accounted by this polysaccharide (Figs. 1, 2, and Table II). Since the neutral hexose content of MAF is already known (50% by weight) and the

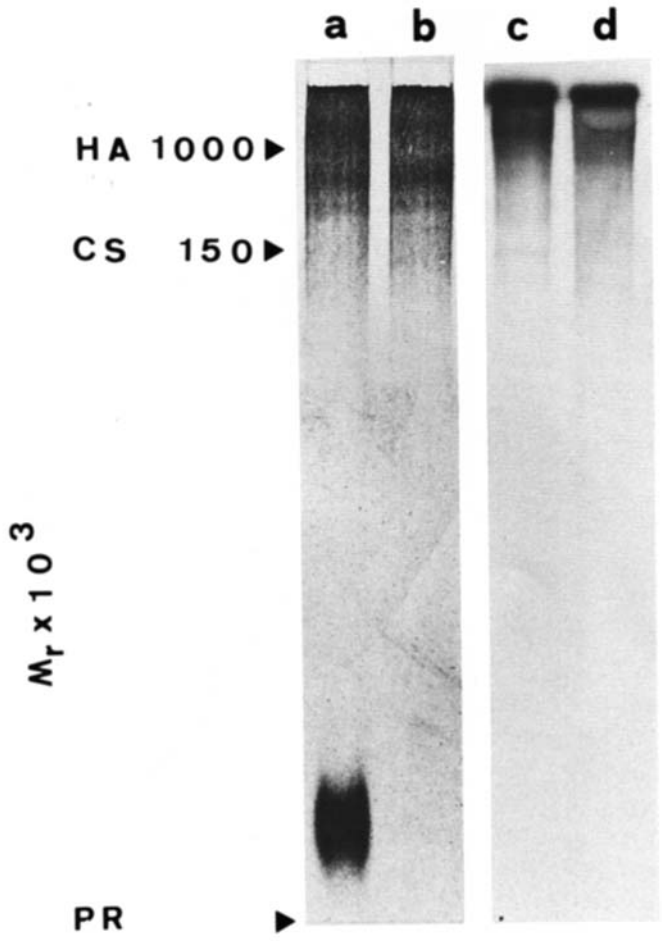


Fig. 1. Electrophoretic analysis of PNGase-treated and control MAF. Gel electrophoresis was performed on the linear 7.5–20% gradient gel as described under "Materials and Methods." Glycosaminoglycan standards were obtained from Sigma; HA is hyaluronic acid and CS is chondroitin sulfate. Alcian blue–stained gel. **a:** 5 μg of PNGase-treated MAF after 72 h digestion. **b:** 5 μg of 72 h corresponding control MAF. **c** and **d:** autoradiography of a and b.

apparent molecular weights of G-6 and MAF have been determined we calculated that one MAF molecule contained about 830 copies of the G-6 polysaccharide.

## DISCUSSION

Localization of the cell-binding site to the G-6 glycan moiety of MAF was indicated by almost complete loss of MAF binding to the homotypic cells upon the prolonged and extensive PNGase treatment which released this G-6 polysaccharide from MAF. However, since MAF dissociates into several subunits in the presence of EDTA, it was impossible to determine the association constants of the control and PNGase-treated molecules [4,5,10]. We therefore looked at the correlation between

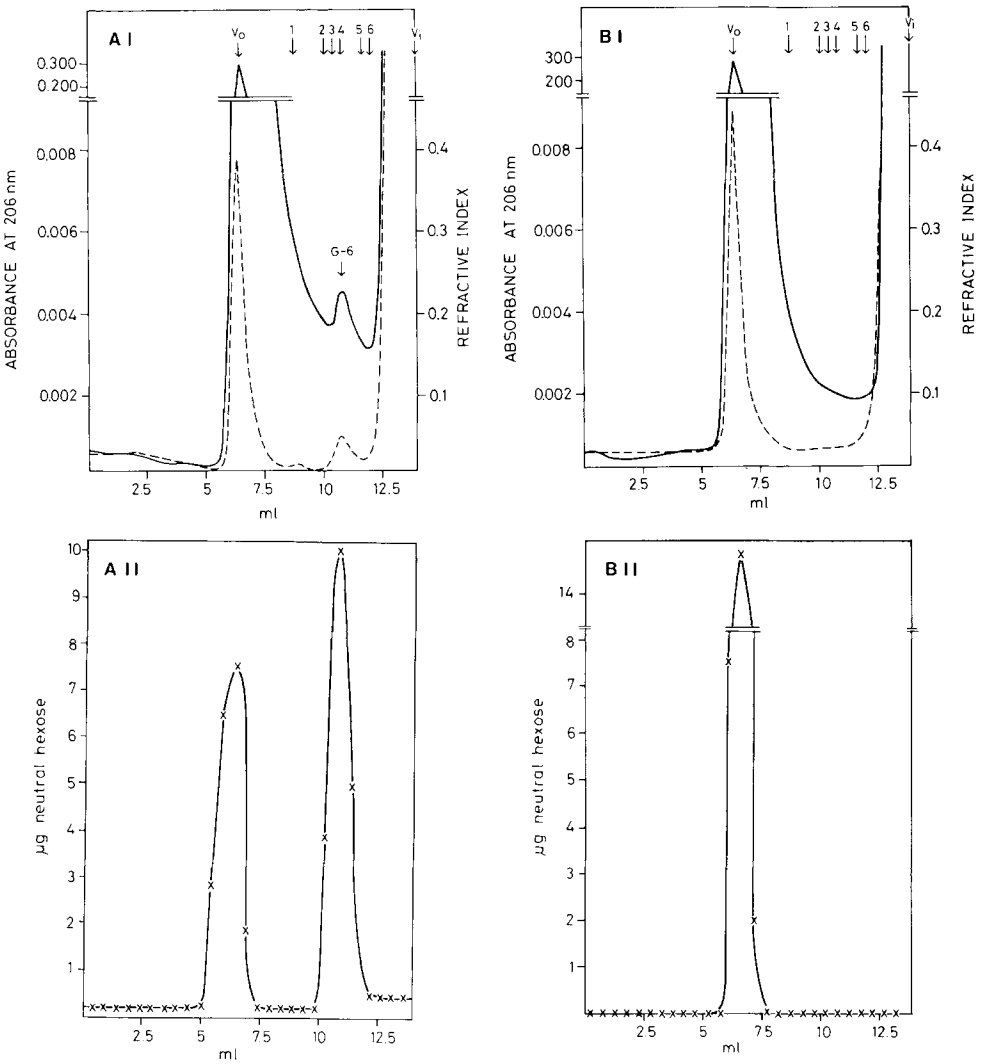


Fig. 2. Gel filtration of PNGase-treated and control MAF on a TCK G2000 SW column. Gel filtration of 6 μg MAF followed 72 h of PNGase treatment on a TCK G2000 SW column (0.8 × 30 cm) equipped with a TCK SW precolumn (0.8 × 4 cm), both obtained through LKB Sweden (**panel AI**). Gel filtration of 285 μg of MAF after 72 h PNGase digestion analyzed on the same column (**panel AII**). Identical amounts of control samples incubated for 72 h without the enzyme were analyzed on the same column (**panels BI** and **BII**). Elution was carried out in all four cases with a CSW buffered with 7 mM Tris pH 7.4 by using the flow rate of 0.5 ml/min. LKB FPLC system was used for continuous monitoring of optical density at 206 nm (—) and refractive index (- - -) (panels AI and BI). Colorimetric assay for neutral hexose (x---x) was performed on 50 μl aliquots (panels AII and BII). The following molecular weight standards were used: 1) heparin of $M_r$ = 11 × $10^3$, 2) dextran of $M_r$ = 10 × $10^3$, 3) heparin of $M_r$ = 8 × $10^3$, 4) heparin of $M_r$ = 6.3 × $10^3$, 5) heparin of $M_r$ = 4.4 × $10^3$, and 6) heparin of $M_r$ = 3.4 × $10^3$. Heparin standards are obtained from A. Lustig, University of Basel, Biocenter, Switzerland.

amount of iodinated subunits bound to cells and the quantity of the G-6 polysaccharide released enzymatically.

MAF dissociation into subunits in the presence of EDTA is completed only after several days at temperatures below 80°C [4,5,8,10]. Furthermore, MAF subunits are of lower molecular weight and thus have less repeats of cell binding site than the native MAF. Such a decrease in valency is the main cause for their lower cell-binding affinity [10,11]. It is therefore to be expected that the control samples incubated in the presence of EDTA for 24 h, which are not completely dissociated, are binding better than the almost completely dissociated samples obtained after 72 h incubation (see Table II).

Separation techniques of gel electrophoresis and FPLC gel filtration showed that the G-6 glycan is essentially pure and is represented by one type of polysaccharide chain. Thus, from the quantity of G-6 recovered after PNGase digestion of MAF, we were able to calculate that this glycan is represented by 830 N-linked polysaccharide chains per one MAF molecule. This finding raises two important questions: 1) what is the degree of carbohydrate microheterogeneity in G-6 and 2) does such microheterogeneity in the carbohydrate structure of G-6 contribute to its cell-binding function? Based on the results of polyacrylamide gel electrophoresis, microheterogeneity indeed exists in G-6. We have been unable so far to separate these forms by conventional biochemical techniques. It may be hypothesized that such subpopulations of G-6 slightly influence the binding affinity of MAF to different cell types and by this means play an important role in the control of cell-sorting processes during regeneration of tissue and in organ formation. A physiological function for the charge and size microheterogeneity of the cell-binding site may also operate in the fine regulation of some growth-related intracellular signal transduction mechanisms.

Prolonged PNGase treatment of MAF resulted in a 72% decrease in cell binding. Since under such conditions the G-6 glycan was the only component released from MAF and since about 50% of carbohydrate remained associated with MAF glycoprotein subunits, we have concluded that the loss of binding activity is not due just to release of any N-linked polysaccharides but specifically to removal of G-6. As can be seen from Figure 2, low residual binding of extensively digested MAF to the homotypic cells (5.3%) is somewhat higher than binding to heterologous cells. We can offer four possible explanations: 1) either binding is mostly unspecific (low values of bound material disable precise and reliable determination of binding specificity), or 2) MAF still contains a few more copies of G-6 which were inaccessible by PNGase owing to the steric hindrance due to close spacing of polysaccharide chains, or 3) there is a subpopulation of the same G-6 polysaccharide which is not N-linked, or 4) there is a second minor type of cell-binding site which is different than G-6.

Independent of the presence of PNGase in the binding mixture, very low binding of extensively digested MAF to homotypic cells was found (not shown). This experiment showed that small amounts of PNGase present during cell-binding assay did not effect MAF-cell association by cleaving the cell surface glycans.

In conclusion our finding of a strong correlation between the decreased level of MAF cell-binding and the amount of G-6 glycan released, together with the fact that this polysaccharide is a highly polyvalent component of MAF, strongly suggests that multiple low-affinity interactions of G-6 with cell surface receptors are involved in species-specific binding of MAF to cells. This type of MAF-cell association leads to selective cell adhesion during the reaggregation process. On the basis of these results it is

possible to propose that low-affinity polyvalent glycans may mediate specific cell-cell or cell-matrix interactions in evolutionarily more advanced organisms. Such highly multiple interactions of polysaccharides with other molecules represent an alternative, new molecular mechanism which is different from the already well-characterized lectin-carbohydrate high-affinity binding that occurs via one or only a few sites [18,19].

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## REFERENCES

1. Wilson HV: J Exp Zool 5:245–258, 1907.
2. Galtsoff PS: Biol Bull (Woods Hole) 57:250–260, 1929.
3. Humphreys T: Dev Biol 8:27–47, 1963.
4. Henkart P, Humphreys S, Humphreys T: Biochemistry 12:3045–3050, 1973.
5. Cauldwell CB, Henkart P, Humphreys T: Biochemistry 12:3051–3055, 1973.
6. Turner RS, Burger MM: Nature 244:509–510, 1973.
7. Weinbaum G, Burger MM: Nature 244:510–512, 1973.
8. Jumblatt JE, Schlup V, Burger MM: Biochemistry 19:1038–1042, 1980.
9. Varner JA, Burger MM, Kaufman JF: J Biol Chem 263:8498–8508, 1988.
10. Misevic GN, Jumblatt JE, Burger MM: J Biol Chem 257:6931–6936, 1982.
11. Misevic GN, Burger MM: J Biol Chem 261:2853–2859, 1986.
12. Misevic GN, Finne J, Burger MM: J Biol Chem 262:5870–5877, 1987.
13. Rice DJ, Humphreys T: J Biol Chem 258:6394–6399, 1983.
14. Hampson IN, Gallagher T: Biochem J 221:697–705, 1984.
15. Pelkonen S, Finne J: Methods Enzymol 179:104–110, 1989.
16. Misevic GN: Methods Enzymol 179:95–104, 1989.
17. Shields R, Burnett W: Anal Chem 32:885–886, 1960.
18. Liener IE, Sharon N, Goldstein IJ (eds): "The Lectins: Properties, Functions and Applications in Biology and Medicine." Orlando: Academic Press, 1986.
19. Lis H, Sharon N: Annu Rev Biochem 55:35–67, 1986.