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Organization and Polysaccharides of Sponge Aggregation Factor

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Aggregation factor, the macromolecular complex which mediates species-specific aggregation of dissociated sponge cells, was isolated from several species, partially characterized, and visualized by electron microscopy. All factors were large fibrous complexes with a backbone and side chains or arms. In some factors, the backbone is linear. In others it is circular and the complex appears as a sunburst with arms extending like rays from the circle. The size and location of the polysaccharide chains have been studied using purified preparations of Microciona prolifera. "Sunbursts" treated with ethylenediaminetraacetate (EDTA) for 4 weeks at 0°C dissociate into 3 protein- and polysaccharide-containing components. Sodium dodecyl sulfate does not cause the sunburst to dissociate nor does it inhibit dissociation in the presence of EDTA suggesting that dissociation is not due to hydrolytic enzymes. The dissociation products were fractionated on a 977-Å pore size micropore glass column. Fifteen percent of the material is excluded and appears in the electron microscope as the central circle of the sunburst. Digestion of the circles with 10^{-3} M dithiothreitol (DTT) and 0.5 mg/ml proteinase K for 72 h at 37°C produces 2 polysaccharide chanis of 65,000 and 6,000 daltons as fractionated and sized on a 233-Å pore size micropore glass column using Pharmacia dextrans as standards. The included fractions of the EDTA-treated material are subunits of the arms which contain 70% of the polysaccharide. A single polysaccharide of 6,000 daltons as measured on 233-Å size glass beads and Sephadex G-75 is released from these subunits by proteinase digestion. Pharmacia dextrans are used as standard on both columns. We calculate that there would be four 65,000-dalton chains and one hundred 6,000-dalton chains per circle and fifty 6,000-dalton chains per arm. The third component of the EDTA-treated preparation is partially included on the column. It appears as linear fibrils in the electron microscope and contains polydisperse polysaccharides of several-hundred-thousand daltons. It may be an impurity since there is apparently less than 1 of the large polysaccharide chains per sunburst.

Key words: aggregation factor, proteoglycans, polysaccharides, aggregation factor, glycoconjugates, glycoproteins, sponges

Sponge aggregation factor is a large glycoprotein complex which functions in re-aggregation of dissociated marine sponge cells. It is isolated from marine sponge tissue by soaking pieces of tissue in calcium- and magnesium-free sea water until the cells dissociate, thereby releasing the aggregation factor into the supernatant. When the cells are removed

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from the supernatant and returned to sea water they aggregate very slowly, but they aggregate rapidly if the material from the supernatant of dissociation is returned to the aggregating cells. This factor carries the recognition sites and the specificity for species-specific sorting that occurs when the cells from certain different species are mixed (1, 2).

Previously, we isolated and characterized the aggregation factor from Microciona parthena (2, 3). A large protein and polysaccharide complex of about 20,000,000 daltons, it consists of a central circle ~ 800 Å in diameter and 15 or 16 arms radiating outward 1,100 Å in a unique sunburst organization. Our laboratory has now succeeded in isolating the aggregation factor from Terpios zeketi, Haliclona occulata, Halichondria bowerbankii, and purifying the factor complex from Microciona prolifera. The aggregation factor of each species appears large and fibrous with a backbone and side chains and is composed of protein and polysaccharide.

Aggregation factor from each species is basically similar, yet there is species specificity in aggregation of dissociated sponge cells. Where does this specificity lie? How are the sugars and proteins arranged in each part of the complex, how do they participate in the interactions of the factor complex, and what is the basis of the specificity? This report describes our initial efforts to fragment M. prolifera aggregation factor and characterize the macromolecular subunits.

MATERIALS AND METHODS

Buffers

Ca^{2+}- and Mg^{2+}-free sea water CMF) was prepared as previously described (1). We used 0.5 N NaCl buffer. CaCMF is CMF to which 10^{-3} M $CaCl_2$ is added.

Preparation and Purification of Sponge Aggregation Factor

The aggregation factor (AF) was prepared from Microciona prolifera, Haliclona occulata, and Halichondria bowerbankii at the Marine Biological Laboratory, Woods Hole, Massachusetts by published methods (1, 2, 4). Aggregation factor from Terpios zeketi was prepared by Charles Cauldwell at the Pacific Biomedical Research Center, Honolulu, Hawaii. Microciona prolifera aggregation factor was purified (4) until the preparation was essentially only sunbursts when viewed with the electron microscope. It was dissolved at about 1,000 units of activity per ml in CaCMF and had about 1.5–5 μg protein and 0.5–2 μg polysaccharide per unit activity depending on the preparation. The preparations ranged from 60 to 80% protein and from 20 to 40% polysaccharide.

Gel Filtration and Chromatography

The 977-Å and 233-Å pore glass columns (Electro-Nucleonics, Fairfield, New Jersey) and the Sephadex G-75 (Pharmacia, Uppsala) column employed in this study were poured and eluted as recommended by the manufacturers. All columns were 48 × 0.7 cm. The 977-Å pore glass bead column equilibrated with CMF had an excluded volume of 8.33 ml, an included volume of 16.90 ml, a flow rate of 16.2 ml/h, and was used in fractionating ethylenediaminetetracetate (EDTA)-treated AF. The 233-Å pore glass bead column, equilibrated with 0.5 N NaCl, had an excluded volume of 8.16 ml, an included volume of 16.45 ml, a flow rate of 12 ml/h, and was used in fractionating the proteinase K-dithiothreitol (DTT)-, and EDTA-treated AF. The Sephadex G-75 column equilibrated with 0.5 N NaCl

had an excluded volume of 7.02 ml, an included volume of 18.36 ml, a flow rate of 12 ml/h, and was used to compare the included run of the proteinase K-, DTT-, and EDTA-treated AF from the 233-Å pore glass column. The 233-Å pore column and the Sephadex G-75 column were standardized with Pharmacia dextran standards of 10,000, 40,000 and 70,000 daltons.

EDTA Treatment

Two milliliters of purified AF was mixed with 0.5 M EDTA, pH 7, to a final concentration of 0.7 mM and left at 0°C for a 4-week period. Following this treatment the AF was run over the 977-Å pore glass column; thirty-five 0.54-ml fractions were collected and 25-μl aliquots were taken from fractions 11–35 for analysis of neutral hexoses by the phenol-sulfuric acid method (5).

Digestion With Proteinase K and DTT

The included, intermediate, and excluded peaks (1.51 ml each) of the 4-week EDTA-treated AF run over the 977-Å pore glass column were each treated with 13 mM DTT and 0.9 mg proteinase K (EM Labs, Elmsford, New York) by adding 41 μl of 0.5 M DTT, pH 7, and 91 μl of 10 mg/ml proteinase K in H_2O. These mixtures were left in a 37°C water bath for 24 h. An equal amount of proteinase K was added 2 more times at 24-h intervals. The digested fractions were then passed over a 233-Å pore glass column or over a Sephadex G-75 column. From each column, thirty-five 0.54-ml fractions were collected; 200-μl aliquots were taken from fractions 11–23 and analyzed for neutral hexoses by the phenol-sulfuric acid method (5).

Gel Electrophoresis of EDTA-Treated Aggregation Factor

One-half percent sodium dodecyl sulfate (SDS) and 10 mM DTT were added to included and excluded fractions from the aggregation factor treated with EDTA for 4 weeks and run over the 977-Å glass bead column. The eluates were treated at 100°C for 5 min and electrophoresed on 4.8% SDS-polyacrylamide gels (6).

Electron Microscopy

Positive staining. Five microliters of aggregation factor or factor fragment at 5 μg/ml in CaCMF or 10^{-3} M $CaCl_2$, with or without EDTA, was placed on a grid with a thin carbon film for about 15 sec, rinsed, and stained with freshly prepared 1% uranyl formate. Excess stain was withdrawn with filter paper.

Shadowing. Aggregation factor at 5 μg/ml in CaCMF was sprayed or dropped onto a grid or piece of mica coated with a carbon film, rinsed in distilled water or volatile buffer (0.5 M NH_4CO_3 with 0.1 M $NH_4C_2H_3O_2$) to eliminate salt, and dried. Aggregation factor in 10^{-3} M $CaCl_2$ was sprayed onto a thin carbon film on mica, not rinsed, and dried. Some preparations were spread by the aqueous basic protein film technique with the spreading solution consisting of 2.5 μg/ml aggregation factor, 0.05 mg/ml cytochrome C in 0.5 M $NH_4C_2H_3O_2$, and 1 mM EDTA, pH 7.5, with or without calcium excess to chelate the EDTA. The hypophase was 0.25 M $NH_4C_2H_3O_2$, pH 7.5. These samples were dipped into 100% ethanol and air dried. Preparations were then shadowed with C-Pt pellets at an angle of about 6–10°. Films of aggregation factor shadowed on mica were then floated onto grids.

RESULTS

Electron Microscopy

Figure 1 shows an electron micrograph of aggregation factor from Microciona prolifera applied to a grid and stained with uranyl formate without drying. The factor complex clearly consists of a circle with a number of radiating arms. This structure is very similar to the structure of the aggregation factor previously described from Microciona parthena (2). The circumference of the circle is ~ 0.4 μm depending on preparative conditions with about 16 arms or rays extending outward.

Stained preparations are of low contrast and show the aggregation factor with tangled arms; these characteristics make counting of the arms and precise measurements difficult. M. parthena was much better delineated in shadowed preparations. However, we have been unable to obtain shadowed preparations of the M. prolifera factor which are as well spread as our preparations of the M. parthena aggregation factor. Prolifera aggregation factor attached to the grids, dried, and shadowed invariably revealed collapsed structures with the circle collapsed or partially collapsed and the arms aggregating together (Fig. 2). The collapse seems to be greatly increased by drying the aggregation factor onto the substrate without support, such as that given by stain, as evidenced by the fact that aggregation factor dried on the grid before staining shows much more condensation than when stained without drying. Thus far the collapse of the circle and contraction of the arms upon drying has not been prevented by treatment of the carbon substrate by ionizing, by spreading the aggregation factor on the grids in various concentrations of volatile buffers or by chemical fixation of the aggregation factor.

Several treatments of the aggregation factor, such as incubation in ethylenediaminetetraacetate, guanidine hydrochloride, and sodium dodecyl sulfate with dithiothreitol, do

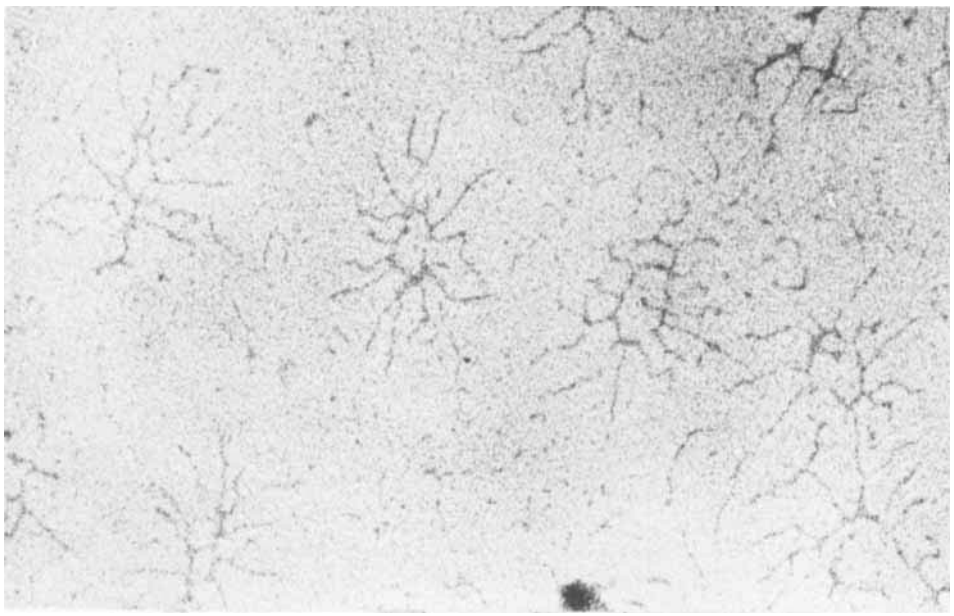

Fig. 1. Microciona prolifera aggregation factor in CaCMF positively stained with uranyl formate. The factor has a circular backbone about 0.4 μm in circumference with about 12–16 arms radiating outward giving a "sunburst" appearance. Magnification 81,000 ×.

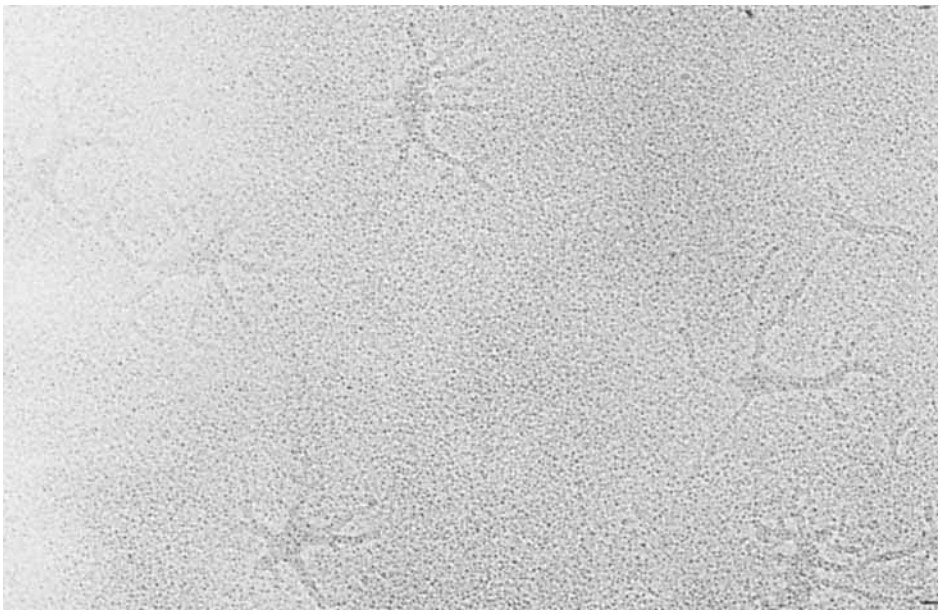

Fig. 2. Microciona prolifera aggregation factor in CaCMF rinsed in distilled water, dried, and shadowed with a C-Pt pellet. The circle and the arms collapse into solid stellate forms and more elongate forms. These condensed figures may polymerize. Magnification 81,000 ×.

seem to improve the spreading of the circular backbone in shadowed preparations. So far, however, this prevention of collapse has been associated with the removal of the arms. The nature of the reactions occurring when the sunburst "collapses" remains unknown. The collapse upon drying is real and demonstrates that the macromolecule is not rigid. Currently the method which best displays the arms of M. prolifera is a basic protein film technique. Molecules spread in this way (Fig. 3) show about 16 arms.

The circular configuration of the backbone has been observed in aggregation factors from M. prolifera (4), M. parthena (2), and Geodia cydonium (7). However, it does not seem to be a universal characteristic of aggregation factors. Halichondria bowerbankii, Terpios zeketi (Fig. 4), and Haliclona occulata (Fig. 5), extracted by methods parallel to those used for Microciona parthena (2), have a very similar size and structure of a backbone fiber with side arms. However, the backbone of the aggregation factor complexes is linear. Without further evidence, we presume that the linear backbone is the native configuration of these aggregation factors since it was extracted by rather gentle methods which did yield circular backbones from Microciona factors.

EDTA Dissociation

When calcium is removed from the active M. prolifera aggregation factor complex, activity is lost very rapidly. For example, aggregation factor treated for less than 1 min with 10^{-3} M EDTA has irreversibly lost all activity. If 2×10^{-3} M $CaCl^2$ is added, the EDTA has no effect on the aggregation factor activity. Thus the removal of calcium and not other polyvalent metal ions is the mechanism of inactivation of the aggregation factor. Although EDTA quickly inactivites aggregation factor, it has no initial obvious effect on the structure of the aggregation factor. Electron micrographs indicate that it is still a circle

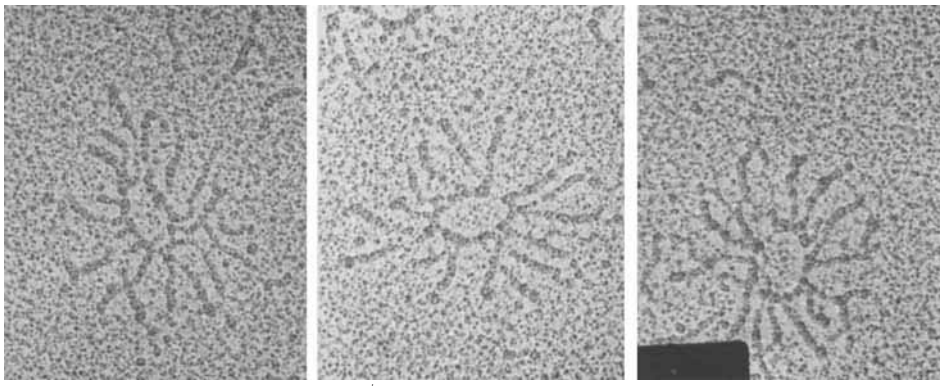

Fig. 3. Microciona prolifera aggregation factor spread with cytochrome C and shadowed display about 16 arms. Magnification 70,000 ×.

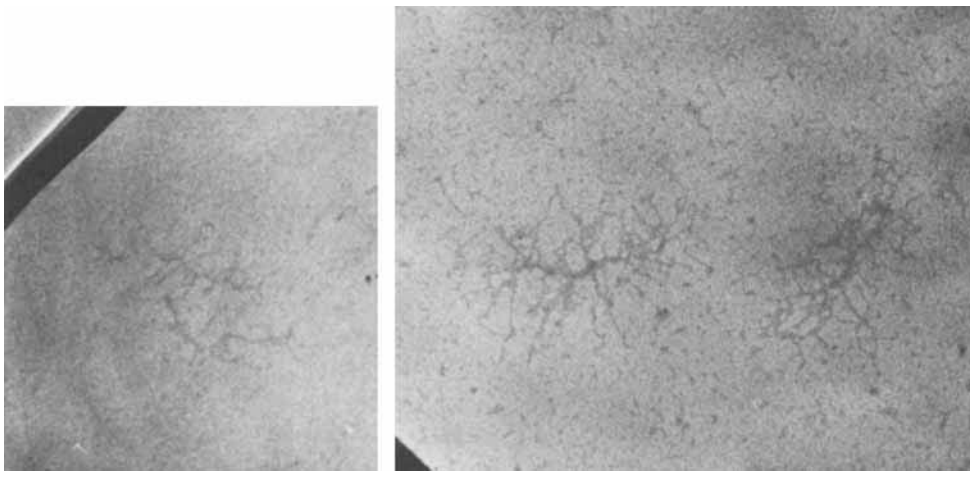

Fig. 4. Terpios zeketi aggregation factor in CaCMF positively stained with uranyl formate. The backbone is linear. Magnification 90,000 ×.

Fig. 5. Haliclona occulata aggregation factor in CaCMF positively stained with uranyl formate. The backbone is linear. Magnification 90,000 ×.

with arms and permeation chromatography shows that the aggregation factor is still excluded. However, in EDTA the aggregation factor does begin to dissociate with time. Figure 5 shows chromatography on 977-Å pore glass beads of aggregation factor treated for 2 weeks and 4 weeks at 4°C with EDTA. Active, untreated factor is completely excluded by this column, but by 2 days (not shown) about half of the protein and polysaccharide of the aggregation factor runs in the included fractions of the column. By 2 weeks (Fig. 6A) 70% of the material and by 4 weeks (Fig. 6B) 85% of the material runs in the included fractions. Most of the included material is totally included but there is

always a distinct shoulder of partially included material which apparently represents a third component.

We asked how these fractions relate to the original sunburst? Protein and polysaccharide run equally distributed across the column so we are not separating protein- or polysaccharide-rich molecules. Excluded and included fractions were examined with the electron microscope. With time, the excluded fractions appeared as circles with fewer and fewer arms and by 4 weeks it was circles devoid of arms. Such a preparation is shown in Fig. 7. The completely included fractions were very small pieces without a distinct morphology. They were not whole arms. Examination of the fractions intermediate between included and excluded showed larger pieces of linear material. It appears the arms are falling off the circles and dissociating into small subunits.

The requirement of 4 weeks for the dissociation of aggregation factor seemed curious. We wondered if it was the results of slow hydrolytic degradation of the aggregation factor by enzymes that contaminated the preparation. We had previously observed that the factor was stable in 0.5% SDS and reasoned that if SDS inhibited the EDTA effect this might indicate that the dissociation of the factor was due to an enzyme whose activity was destroyed by SDS. Figure 8 shows permeation chromatography of aggregation factor treated with SDS for 5 days and aggregation factor treated with SDS plus EDTA for 5 days. It can be seen that the aggregation factor was completely stable in SDS alone but dissociated as usual when EDTA was added to the SDS-treated aggregation factor. These results do not lend support to the idea that the complex is degraded by a hydrolytic enzyme which is denatured by SDS.

We attempted to dissociate the aggregation factor further and to measure the size of the subunits using standard SDS-polyacrylamide gel chromatography. The included fraction and the excluded fraction were each taken, treated with SDS and dithiothreitol, heated 5 min at 100°C, and electrophoresed on 4.8% polyacrylamide gels. The excluded fraction, the circle, apparently was not further degraded by treatment with SDS and dithiothreitol and failed to enter the gel. This was confirmed by passing such treated material over the 977-Å pore column. Again, the material ran as an excluded peak. The included fraction, the subunits of the arms, electrophoresed as a broad band (stained for protein and polysaccharide) ranging between 13,000 and 300,000 mol. wt. using phosphorylase A, equine heart cytochrome C, and myosin as standards. This heterodispersion is probably due to variation in length of polysaccharide chains although we have not established this point. The exact size of this subunit cannot be determined from these measurements since polysaccharide runs anomalously on acrylamide gels.

Proteinase K Digestion

Thirty percent of the material of the sunburst is polysaccharide. Polysaccharides have long been associated with cell surfaces and are believed to be intimately involved in cell surface reactions. Therefore, it seemed of special interest to analyze the polysaccharides of the factor to determine their number, size, and characteristics. To do this, we digested away the protein using proteinase K and analyzed the resultant polysaccharide chains on 233-Å pore glass bead permeation chromatography columns. When the excluded fraction of the 977-Å pore glass bead column, which contained the circles, was digested and passed over the 233-Å pore size glass bead columns 2 peaks were obtained as shown in Fig. 9A. Twenty-five percent of the material ran as an excluded peak, 50% of the material ran as an included peak, and 25% appeared as a large shoulder on the included peak. The apparent

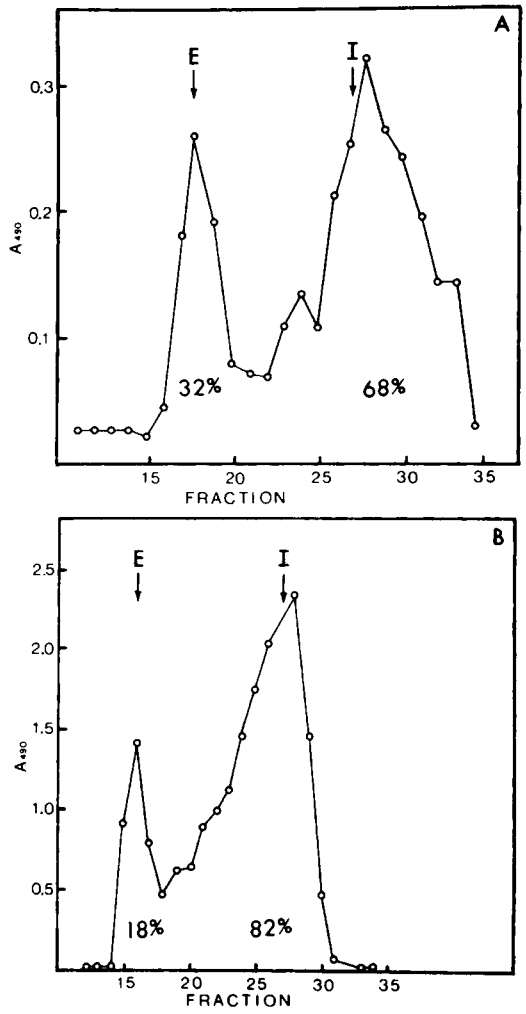

Fig. 6. Gel filtration chromatography of 2-week EDTA-treated Microciona prolifera AF (A) and 4-week EDTA-treated AF (B). A 977-Å pore glass bead column (48 × 0.7 cm) was eluted with CMF at 16.2 ml/h. Aliquots were removed for analysis for sugar (5). OD_{490}) I: included fraction. E: excluded fraction.

molecular weight of the shoulder is 65,000 and of the included peak is 6,000 when the column is standardized with Pharmacia dextrans.

Most of the mass of the sunburst appears as an included peak in the column run after EDTA dissociation and presumably represents fragments of the arms. When these are digested with proteinase K and the digest passed over a 233-Å pore size glass bead column, a single peak of polysaccharide is obtained (Fig. 9B). Its apparent molecular weight is about 6,000 when compared to dextran standards. To estimate the accuracy of this molecular weight determination, we prepared a Sephadex G-75 column and standardized it with the same dextran preparations. On this column the apparent molecular weight was also

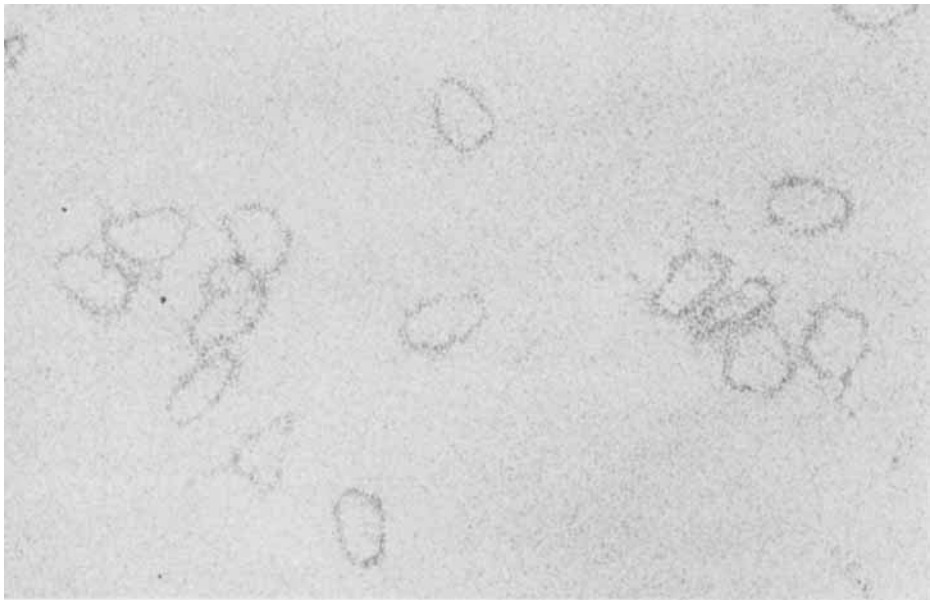

Fig. 7. The excluded peak of Microciona prolifera aggregation factor treated with EDTA for 4 weeks and run over a 977-Å glass bead column consists of circles. These circles are the backbone of the "sunbursts." The preparation was positively stained with uranyl formate. Magnification 81,000 ×.

6,000. It is difficult to judge the accuracy of either of these determinations; further analysis of the polysaccharide and comparison to other standards are required before a firm figure for the molecular weight can be achieved.

The shoulder of material that ran between the included and excluded fractions of the 977-Å pore size glass bead columns of EDTA-treated material was digested also with proteinase K and passed over the 233-Å pore size glass bead column (Fig. 9C). It ran as an excluded peak and a peak of polysaccharide equivalent to the small polysaccharide from the arms. When the excluded peak was run on larger pore size columns, it spread out and proved to be very heterogeneous and large, ranging from 100,000 to 1,000,000 daltons. The excluded peak of polysaccharide from the circles (Fig. 6A) appears to be similar to this heterodisperse polysaccharide from the intermediate shoulder. A calculation of the number of polysaccharide chains in this large heterogeneous class indicate less than 1 per 4 sunburst molecules. This led us to speculate that this polysaccharide derives from a contaminant of the aggregation factor preparation which is also released from the sunbursts in the presence of EDTA.

Other reagents were used in attempts to break the sunburst complex down into smaller subunits. When treated with 4 M guanidine hydrochloride, the reagent used to dissociate cartilage proteoglycan aggregates, the factor broke down into the same subunits as described above. Treatment of the aggregation factor with 0.1 M NaOH overnight at 0°C did not release any of the polysaccharide chains. These conditions are sufficient to hydrolyze sugar linkages to serine and thus indicate that the polysaccharide is not attached to the protein by serine linkages.

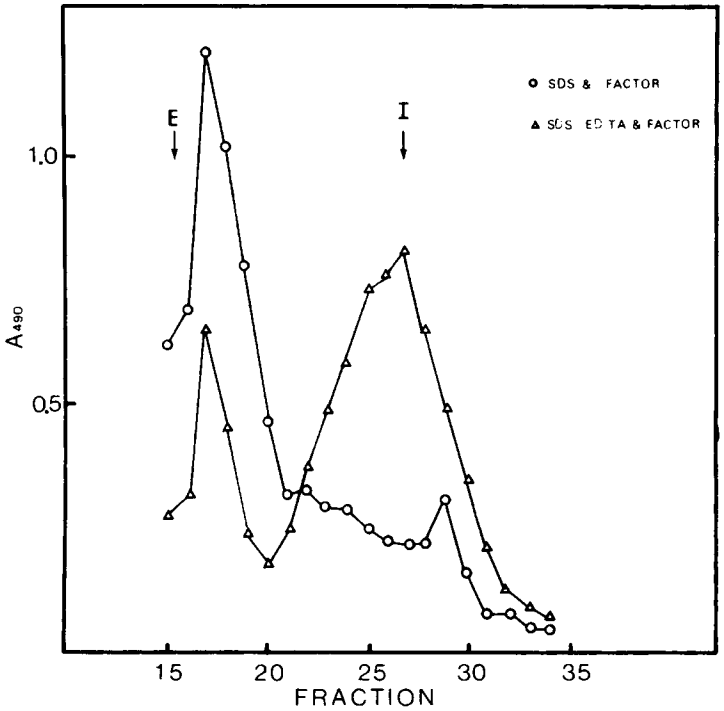

Fig. 8. Gel filtration chromatography of SDS-treated Microciona prolifera AF (○) and SDS plus EDTA-treated AF (△). A 977-Å pore glass bead column (48 × 0.7 cm) was eluted with 0.1% SDS in CMF. Fractions were analyzed for sugar (5). OD_{490}) I: included fraction. E: excluded fraction.

DISCUSSION

These results show that the aggregation factors isolated and assayed by the procedures we developed are large, fibrous macromolecular complexes with a backbone and a number of side chains. In Microciona parthena and prolifera, the backbone is a closed circle and the side chains radiate as arms from the circle producing a sunburst-like structure. However, in the other species the backbone appears linear, with arms extending from both "sides" of the backbone. All the factors we have analyzed have from 20 to 50% polysaccharide and all have been large enough to be excluded by Sepharose 2B or 3,000-Å pore size glass beads with exclusion limits of about 2×10^7 daltons.

The sunburst complex is very large, about 0.4 μm in diameter in M. prolifera when fully extended, and is much larger than the usual distance between the closely apposed membranes of adjacent cells. We have as yet been unable to localize it among aggregated cells by microscopy. The molecule could function as an essentially planar ligand between adjacent membranes, flat in the intercellular space, and binding either to receptor on apposing cell surfaces or other factor molecules. However, this planar configuration is not the only possibility. Aggregation factor molecules may be confined to specialized regions of the cell surface where membranes are not apposed or bind in a collapsed configuration.

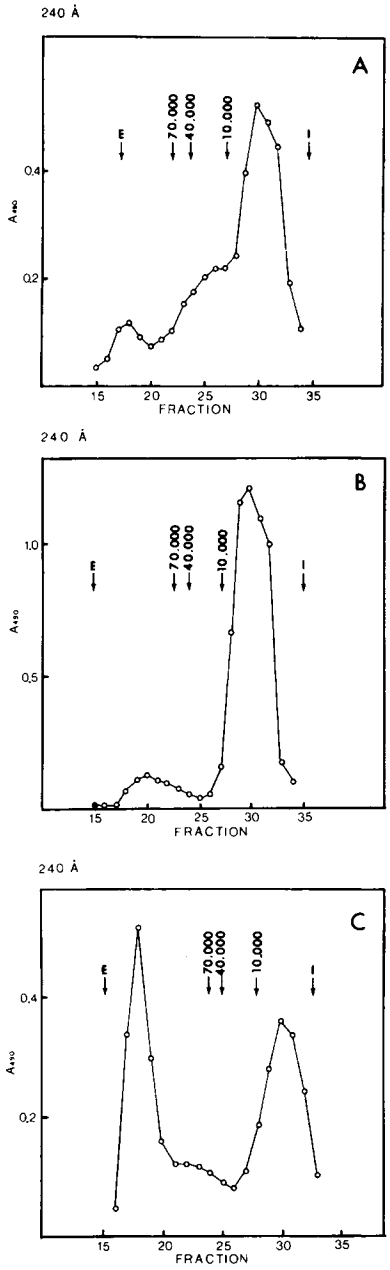

Fig. 9. Gel filtration chromatography of the excluded (A), included (B), and intermediate (C) peaks of the 4-week EDTA-treated Microciona prolifera AF from the 977-Å pore glass column, incubated in DTT and proteinase K (3 additions at 24-h intervals) at 37°C for 72 h, and run over a 233-Å pore glass bead column. The 233-Å column (48 × 0.7 cm) was eluted with 0.5 N NaCl at 12 ml/h. Fractions were analyzed for sugar (5). OD_{490}) I: included fraction. E: excluded fraction.

The development of adhesions due to apposed membranes could be secondary to the adhesion accelerated by the factor molecules.

The association of polysaccharide with cell surface reactions and the presence of polysaccharide in the factor has led to the idea that polysaccharides are intimately involved in the active sites of aggregation factors. For other such cases that are known, the specific polysaccharide of the active site inhibits the activity of the molecule. We looked for specific polysaccharide inhibition by digesting a partially purified aggregation factor preparation with pronase and testing the polysaccharides for inhibitory activity and found none (8). It will be interesting to test the individual polysaccharides of the aggregation factor for their effect on activity of intact aggregation factor.

The most analogous macromolecule and the nearest model for aggregation factor is the proteoglycan aggregate of cartilage (10). In this case, hyaluronic acid provides the backbone with proteoglycan monomer protein and link proteins attached. Each proteoglycan monomer produces a radiating side chain carrying polysaccharides of chondroitin sulfate and keratan sulfate. The sponge aggregation factor may be similar.

When treated with EDTA, the M. prolifera aggregation factor breaks down into a protein- and polysaccharide-containing circle and a series of fragments of the arms which also contain protein and polysaccharide. The organization of these fragments is far from established. However, the large polysaccharide isolated from the circle after proteinase K digestion could be equivalent to the hyaluronic acid backbone of the cartilage of the proteoglycan. We calculate that a backbone of 2,500 Å, the size we have normally seen in these aggregation factors, would have a molecular weight of 120,000–200,000 if it were a single, linear polysaccharide chain. This is larger than the apparent 65,000 daltons of the large polysaccharide on porous glass beads but it is difficult to evaluate the accuracy of this determination. If the polysaccharide were circular, this could affect the apparent molecular weight on the column.

The protein in the circular backbone may be linking proteins which attach the arms to the polysaccharide of the circular backbone. Subunits of the arms may bind end to end to produce the arms of the aggregation factor which have the smaller 6,000 dalton polysaccharide. However, the arms clearly differ in 2 respects from the cartilage proteoglycan monomers. The protein of the proteoglycan monomer of cartilage is a single polypeptide chain while the arms of the aggregation factor must have several subunits because EDTA dissociates the arms into smaller pieces. Since we do not have a molecular weight for these pieces, we cannot calculate how many subunits there might be per sunburst arm. Secondly, the polysaccharides of the aggregation factor are probably much smaller than the sulfated glycosaminoglycan side chains on the cartilage proteoglycan. The polysaccharide of the aggregation factor arms is about 6,000 while chondroitins of cartilage are about 30,000 daltons.

Sponge aggregation factor and cartilage proteoglycan may represent different evolutionary stages of the same basic type of primordial cell component. The cartilage proteoglycan is a very specialized complex developed to be a major volume occupying molecule of cartilage matrix. Sponge aggregation factor is isolated by purification procedures that give about a thousandfold purification and thus the aggregation factor is a minor component of the tissue. At present we cannot ascertain the importance of the similarities and differences between these macromolecules. It is interesting to speculate but only further analysis will tell.

ACKNOWLEDGMENTS

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