Journal of Cell Science 108, 3119-3126 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995



# Cell adhesion in sponges: potentiation by a cell surface 68 kDa proteoglycan-binding protein

**Judith A. Varner**

327 Via Andalusia, Encinitas, CA 92024, USA

## SUMMARY

**Constitutive, stable intercellular adhesion is one of the distinguishing properties of metazoans, of which the sponges (Phylum Porifera) are the most primitive representatives. In sponges, intercellular adhesion is mediated by the large proteoglycan-like cell agglutinating molecule 'aggregation factor', which binds to cell surfaces via an oligosaccharide moiety. Previous studies indicated that this aggregation factor binds to two proteins associated with the surface of sponge cells. One of these, a 68 kDa peripheral membrane protein, was isolated by affinity chromatography on aggregation factor conjugated to Sepharose. This monomeric 68 kDa glycoprotein plays a key role in sponge cell adhesion since it potently inhibits the binding of aggregation factor to cell surfaces and completely prevents aggregation factor-mediated cell adhesion. The 68 kDa aggregation factor ligand binds with high affinity to both aggregation factor ($K_D$ = $2\times10^{-9}$ M) and cell surfaces ($K_D$ = $6\times10^{-8}$ M) providing evidence that it serves as an intramolecular bridge between the aggregation factor molecule and a cell surface receptor. Therefore, this early metazoan protein may represent one of the earliest extracellular matrix adhesion proteins to have arisen in the course of metazoan evolution.**

Key words: cell adhesion, proteoglycan, sponge

## INTRODUCTION

Rapid advances in mammalian cell and molecular biology have helped to elucidate some of the mechanisms of intercellular adhesion (Albelda and Buck, 1990; Ruoslahti, 1991; Hynes, 1992; Cheresh, 1993). Cell-to-cell and cell-to-substrate adhesion proteins play critical roles in numerous normal or pathological processes, including development (Hynes and Lander, 1992; Kintner, 1992), inflammation (Hemler, 1990; Kassner, 1992; Springer, 1990), cellular proliferation (Varner et al., 1995; Meredith et al., 1993; Montgomery et al., 1994), angiogenesis (Brooks et al., 1994a,b; Bischoff, 1995), tumorigenicity (Varner et al., 1995; Giancotti and Ruoslahti, 1990; Felding-Habermann et al., 1992), and metastases (Zetter, 1990; Albelda et al., 1990). These adhesion proteins can be classified into several groups that are based upon both DNA sequence and functional similarities, which include the evolutionarily conserved integrin (Ruoslahti, 1991; Hynes, 1992; Cheresh, 1993), cadherin (Takeichi, 1991; Kemler et al., 1989), selectin (Lasky et al., 1994) and immunoglobulin (Edelman, 1988) superfamilies.

Although much is now known about mammalian adhesion molecules, nonmammalian and invertebrate cell adhesion molecules remain less well characterized. An understanding of more primitive cell adhesion molecules is important to permit additional insight into the processes of evolution and into the basic mechanisms of cell adhesion in lower and higher organisms. Nonmammalian animal models of cell adhesion include slug formation in the slime mold, *Dictyostelium discoideum* (Springer et al., 1984; Barondes et al., 1985; Gabius et al., 1985) and embryogenesis in echinoderm (Adelson et al., 1992; McClay, 1991; Hardin and McClay, 1990), amphibian (Boucat et al., 1984; Lee et al., 1984; DeSimone, 1994; Lallier et al., 1994) and avian embryos (Bronner-Fraser et al., 1992; Lallier and Bronner-Fraser, 1993).

Studies of intercellular adhesion began, however, with a still simpler model, as early as 1907, when Wilson (1907, 1910) demonstrated that sponge cells would regroup and reform intact sponges from a single cell suspension. Since Wilson's pioneering work, the molecular mechanisms of this process have only been partially clarified. Several researchers have demonstrated that the reaggregation of sponge cells is mediated by divalent cation dependent proteoglycan-like 'aggregation factors' (Humphreys, 1963; Misevic et al., 1982; Müller et al., 1982). Aggregation factors are very large ($2\times10^7$ Da) stable proteoglycan-like linear or circular lampbrush structures that are over 50% carbohydrate by weight (Henkart et al., 1973) and that bind to cell surfaces with high affinity (Misevic et al., 1982; Misevic and Burger, 1986). Aggregation factors interact in a homotypic, divalent cation- and oligosaccharide-dependent manner with other aggregation factors (Jumblatt et al., 1980; Misevic et al., 1982; Misevic and Burger, 1993) but bind to the cell surface in a divalent cation independent manner (Jumblatt et al., 1980; Misevic et al., 1982). A repeated oligosaccharide moiety has been implicated in the aggregation factor cell binding site (Misevic and Burger, 1990).

Putative cell surface ligands for the aggregation factor have been identified using an aggregation factor as a probe in a novel western blotting assay (Varner et al., 1988). These two aggregation factor binding proteins from the sponge *Microciona prolifera*, of molecular masses 210 and 68 kDa, were identified and partially characterized as two distinct peripheral membrane/extracellular matrix proteins by incubating nitrocellulose transfers of non-reducing SDS-polyacrylamide gels containing sponge cell or membrane extracts with iodinated aggregation factor (Varner et al., 1988). These two proteins were characterized as peripheral membrane proteins in that they could be extracted from sponge cell membranes with high pH, low salt or 6 M urea, that they remained soluble in the absence of detergent, that they did not incorporate into liposomes and that they partitioned into the aqueous phase of a Triton X-114 phase shift experiment (Varner et al., 1988).

In order to examine further the biological and biochemical properties of one of these early metazoan adhesion proteins, the 68 kDa aggregation factor ligand was purified to homogeneity. This novel cell adhesion protein potently inhibits the binding of aggregation factor to the cell surface as well as aggregation factor mediated cell adhesion. The 68 kDa protein is a high affinity ligand for aggregation factor, exhibiting a $K_D$ of $2\times10^{-9}$ M in Scatchard analysis (Scatchard, 1959). It also displays a high affinity for the cell surface. This monomeric glycoprotein may be among the most evolutionarily primitive adhesion proteins described to date.

## MATERIALS AND METHODS

Sponges were obtained from the supply department of the Marine Biological Laboratory in Woods Hole, Massachusetts, and maintained in artificial sea water at 15°C. Nitrocellulose was obtained from Schleicher and Schuell (Keene, NH). Iodobeads were from Pierce Chemical Company (Rockford, IL). Sepharose 4B and CNBr sepharose were obtained from Pharmacia LKB (Piscataway, NJ). X-AR film was obtained from Kodak (Rochester, NY). $Na^{125}I$ was from Amersham (Arlington Heights, IL). Pre-stained molecular mass markers were from Gibco/Bethesda Research Labs (Gaithersburg, MD). Peptide-*N*-glycosidase was from Boehringer Mannheim Biochemicals (Indianapolis, IN). All other chemicals were reagent grade.

### Preparation of cells and membranes

Sponges were cut into 5 mm pieces and cells were squeezed through nylon mesh into $Ca^{2+}$ and $Mg^{2+}$ free sea water (CMFSW; Humphreys, 1963). They were washed at a final concentration of $4\times10^7$ cells/ml for 2-4 hours at 4°C on a rotary shaker and twice by centrifugation at 1,000 rpm. They were frozen at –80°C or were fixed as described (Misevic et al., 1982) and stored at 4°C in normal sea water (MBLSW; Humphreys, 1963). Membranes were prepared by a slight modification of the method of Maeda et al. (1983). A 40 g sample of cells ($10^{11}$) was twice thawed and frozen in 400 ml of 2 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM $NaHCO_3$, 1 mM $MgCl_2$, 30 mM NaCl and 1 mM dithiothreitol (DTT) and subsequently homogenized with 10 strokes of a Potter-Elvejhem homogenizer. Aliquots (30 ml) were layered on 10 ml 50% sucrose cushions in the above buffer and centrifuged at 25,000 rpm in an SW27 rotor for 90 minutes at 4°C. Membranes were removed from the interface between the sucrose cushion and sample, pelleted at 100,000 ***g*** for 90 minutes and resuspended in 20 ml CMFSW, 50 mM Tris-HCl, pH 7.8.

### Preparation of aggregation factor affinity columns

A 3 mg sample of CsCl purified aggregation factor (Misevic et al., 1982) in 0.5 M NaCl, 0.1 M $NaHCO_3$, pH 9, was coupled to 3 ml of activated CNBr-Sepharose overnight at 4°C, with rotation. Unoccupied sites were then blocked by washing in 0.2 M glycine in 0.5 M NaCl, 0.1 M $NaHCO_3$, pH 9, for 2 hours at room temperature. The resin was washed three times by alternating high (0.1 M $NaHCO_3$, pH 9) and low (0.1 M Na acetate, pH 4) pH buffers. The resin was stored at 4°C in 0.5 M NaCl, 50 mM Tris-HCl, pH 7.8.

### Small scale affinity chromatography

Aliquots of $5\times10^7$ sponge cells were solubilized for 30 minutes on ice in 0.25% octylpolyoxyethylene, 0.5 M NaCl, 0.05 M Tris-HCl, pH 8, and centrifuged for 10 minutes at 10,000 rpm in a microcentrifuge at 4°C to remove insoluble material. The lysates were incubated with 100 μl of packed aggregation factor Sepharose (at 2 mg/ml) or unconjugated Sepharose for 2 hours at 4°C. Unbound lysates were removed after centrifugation at 10,000 rpm for 5 minutes and resins were washed four times in lysis buffer. Resins were boiled 3 minutes in SDS sample buffer, as were samples of lysates. All samples were separated by 7.5% SDS-polyacrylamide gel electrophoresis and analyzed using the previously described western blotting assay.

### Preparative affinity chromatography

Sponge membranes were extracted on ice with 0.25% octylpolyoxyethylene, 0.5 M NaCl, 0.05 M Tris-HCl, pH 8. Insoluble proteins were removed by centrifugation at 10,000 rpm for 30 minutes. The extracts were dialyzed overnight versus two changes of 0.5 M NaCl, 0.05 M Tris-HCl, pH 8.0, to remove detergent and then were centrifuged to remove insoluble proteins. The extracts were then applied to a 3 ml aggregation factor Sepharose column and incubated for 30 minutes at 4°C. The column was then washed consecutively with 20 ml of ice-cold buffer (0.5 M NaCl, 0.05 M Tris-HCl, pH 8.0), 10 ml of ice-cold buffer adjusted to 1 M NaCl, 20 ml of ice-cold buffer at 0.5 M NaCl and 20 ml of ice-cold buffer at 2 M NaCl. Column fractions were stored at 4°C. Fractions were monitored for protein by absorbance at 280 nm. Pooled protein peaks fractions were dialyzed versus 0.5 M NaCl, 50 mM Tris-HCl, pH 8.0, before further analysis.

### Inhibition of aggregation and binding assays

A 200 μl (5 μg) sample of affinity purified protein was serially diluted twofold (in triplicate) in 12 wells of a 24-well tissue culture plate. Four micrograms of aggregation factor was added to each well. Thirty minutes later 200 μl of fixed *M. prolifera* cells ($4\times10^7$) were added. After shaking for 20 minutes at room temperature, each well was compared to controls in which 12 μg of aggregation factor had been serially diluted in order to assign an inhibition titer (specific activity equals that number of μg of inhibitor needed to inhibit 1 μg of aggregation factor-mediated aggregation). Bovine serum albumin (at the same concentrations) was also serially diluted and assayed for inhibition as for the 68 kDa protein.

Aggregation factor-cell binding inhibition assays were performed in triplicate as follows: purified protein was serially diluted twofold in 100 μl of 0.5 M NaCl, 0.05 M Tris-HCl, pH 8, in a 96-well microtiter plate. Then 50 μl of fixed sponge cells ($2\times10^5$ cells) and 10 μl of iodinated aggregation factor ($1\times10^5$ cpm or 0.1 mg) were added for 8 hours at 4°C. Cell-bound aggregation factor was separated from free by centrifugation at 1,000 rpm for 5 minutes after three washes of cells with 200 μl 0.5 M NaCl, 50 mM Tris-HCl, pH 7.8, 2 mM $CaCl_2$, and bound cpm determined by gamma counting.

### SDS-polyacrylamide gel electrophoresis and western blots

Protein samples solubilized in nonreducing buffer (2% SDS, 100 mM Tris-HCl, pH 8.3, 10% glycerol, 0.01% Bromphenol Blue) or in reducing buffer (as above, with 5% 2-mercaptoethanol) were electrophoresed on 7.5% SDS-polyacrylamide gels prepared by the method of Laemmli (1970). Gels were either stained with Coomassie Brilliant Blue or silver, or were transferred to nitrocellulose in 25 mM Tris, 192 mM glycine at 1 A for 1.5 hours at 4°C (Towbin et al., 1979).

Blots were blocked for 8 hours at 4°C with 3% BSA, 0.1% NP40, 0.5 M NaCl, 50 mM Tris-HCl, pH 7.8, at room temperature and incubated overnight in iodinated aggregation factor ($10^6$ cpm/ml). Blots were washed with 5 changes of 0.5 M NaCl, 50 mM Tris-HCl, pH 7.8, at room temperature and autoradiographed for 2 days.

### Peptide-*N*-glycosidase digestion

An aliquot of $4\times10^7$ sponge cells was solubilized in 100 µl 1% NP40, 0.5 M NaCl, 0.05 M Tris-HCl, pH 8, on ice for 30 minutes. Detergent insoluble residues were removed by centrifugation at 10,000 rpm in a microcentrifuge for 10 minutes at 4°C. The lysates were acetone precipitated and resuspended in 40 µl of 0.5% SDS, 100 mM Tris-HCl, pH 8.8, 10 mM EDTA, and boiled for 3 minutes. Samples were adjusted to 1% NP40 by addition of 5 µl of 10% NP40 and divided into two samples of equal volume. A 5 µl (0.25 U) aliquot of peptide-*N*-glycosidase was added to one sample and a 5 µl aliquot of glycerol was added to the other. Samples were incubated at 37°C overnight. Then 25 µl of 50% glycerol, 10% SDS, 0.1% Bromphenol Blue were added to each sample; the samples were boiled for 3 minutes and electrophoresed on 7.5% SDS-polyacrylamide gels, transferred to nitrocellulose and assayed by the aggregation factor western blotting assay.

### Iodination of proteins

Aggregation factor was iodinated by incubating 10 µg of CsCl purified proteoglycan (100 µl) with 1 mCi of $Na^{125}I$ and one iodobead for 30 minutes at room temperature. Free iodine was separated from bound by gel filtration on a 1 ml Sephadex G50 column in a Pasteur pipette. A specific activity of $10^6$ cpm/µg was obtained. Affinity purified 68 kDa protein was iodinated by combining 5 µg (200 ml) of 68 kDa protein, 1 mCi $Na^{125}I$ and one iodobead for 15 minutes at room temperature. Protein was separated from free iodine in a one ml column of Sephadex G50 in a Pasteur pipette. A specific activity of $1\times10^6$ cpm/µg was obtained.

### Gel filtration

Pooled and concentrated affinity column eluate fractions containing the 68 kDa protein were applied to a 210 ml Sepharose 4B gel filtration column (1.5 cm × 120 cm). A single peak eluted with 0.5 M NaCl, 50 mM Tris-HCl, pH 7.8, in 3 ml fractions. Elution was monitored for unlabelled protein by absorbance at 280 nm. The column was standardized with Blue Dextran ($V_e$ = 70 ml), BSA (68 kDa, $V_e$ =185 ml) and Phenol Red ($V_e$ = 210 ml).

### Determination of affinity of 68 kDa protein for aggregation factor

In six separate determinations, triplicate serial dilutions of iodinated, purified 68 kDa protein (12.5-400 ng) were prepared in the presence and absence of a 100-fold excess of unlabelled 68 kDa protein in a final volume of 100 µl of 0.1% NP40, 0.5 M NaCl, 50 mM Tris-HCl, pH 7.8, 2 mM $CaCl_2$ in 1 ml microfuge tubes. Five micrograms of aggregation factor was added to each tube and the tubes were incubated at 4°C for 12 hours, which was the incubation time required to achieve equilibrium. Bound receptor was separated from unbound by centrifugation for 30 minutes in a Beckman airfuge at 20 psig (100,000 ***g*** at 4°C). Pellets of aggregation factor and bound receptor were rinsed three times with buffer and both bound and free radiolabelled 68 kDa protein were counted. The amount of bound 68 kDa protein was calculated and plotted versus total. Affinity binding constants were determined by Scatchard analysis (Scatchard, 1959).

### Determination of affinity of 68 kDa protein for cell surfaces

Serial dilutions of iodinated 68 kDa protein ranging from 0.1 to 1.0 µg in 100 µl were incubated in the presence and absence of excess unlabelled 68 kDa protein with aliquots of low salt washed *M. prolifera* cells. Bound protein was separated from free by pelleting the cells by centrifugation for 15 minutes at 4°C. Bound and unbound protein were quantified by γ counting and bound plotted versus total. Affinity constants were determined as described above.

## RESULTS

Previous studies into the nature of cell adhesion in sponges indicated that sponge cell adhesion is mediated by a high affinity $2\times10^7$ Da proteoglycan-like aggregation factor that binds cell surfaces through an oligosaccharide moiety (Misevic et al., 1990). Attempts to identify the cell surface receptors for aggregation factor using a novel western blotting assay indicated that two peripheral membrane protein ligands for the aggregation factor, of molecular masses 68 and 210 kDa, could be extracted from cell surfaces using either detergent or non-detergent extraction procedures (Varner et al., 1988). An optimal extraction procedure consisted of an octylpolyoxyethylene extract of sponge membranes followed by dialysis and centrifugation to remove integral membrane proteins (Varner et al., 1988). In an attempt to purify these proteins to homogeneity and to characterize their biological and biochemical properties, purified sponge membranes were extracted with this nonionic detergent and the extracts were dialyzed to remove detergent. Both the 68 kDa and the 210 kDa aggregation factor ligands were present in significant quantities in the dialyzed octylpolyoxyethylene extracts of sponge membranes. Their presence was detected by the binding of radioiodinated aggregation factor to western blots of nonreducing SDS-polyacrylamide gels containing samples of the extracts (Fig. 1).

### Isolation of 68 kDa ligand by affinity chromatography

Affinity chromatography is a powerful means of rapidly and selectively purifying proteins and has been used to purify a variety of antigens. In order to determine if affinity chromatography on aggregation factor conjugated to Sepharose could effi-

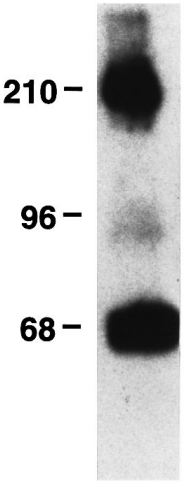


**Fig. 1.** Identification of aggregation factor ligands by western blotting of sponge membrane extracts. An aliquot of an octylpolyoxyethylene extract of purified sponge membranes was electrophoresed on a 7.5% nonreducing SDS-polyacrylamide gel, transferred to nitrocellulose by western blotting and incubated with iodinated aggregation factor after washing to remove unbound aggregation factor. The blot was autoradiographed on Kodak X-AR film. Molecular mass markers indicated are 210, 96, and 68 kDa.

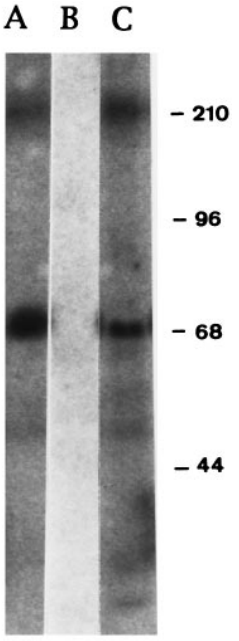


**Fig. 2.** Small scale affinity chromatography. Octylpolyoxyethylene extracts of *M. prolifera* membranes (A) were incubated with 100 µl aliquots of either unconjugated Sepharose (B) or MAF coupled to Sepharose at 1 mg/ml (C) at 4°C. Unbound fractions of lysates were removed by centrifugation, resins were washed four times in buffer (500 mM NaCl, 50 mM Tris-HCl, pH 8), and boiled 3 minutes in SDS nonreducing sample buffer. Samples were electrophoresed on 7.5% SDS-polyacrylamide gels. Gels were blotted onto nitrocellulose and assayed for MAF binding proteins as described in Materials and Methods. Molecular mass markers are 210, 96, and 68 kDa.

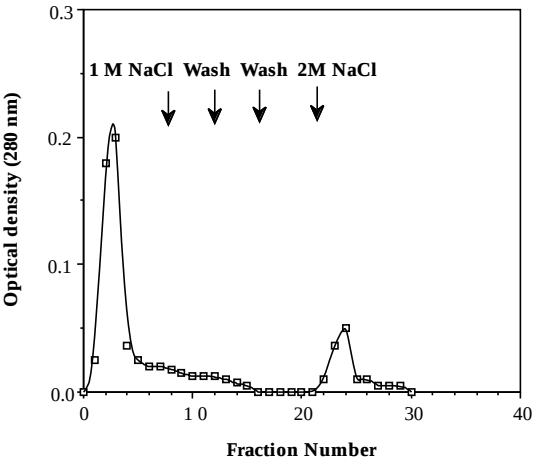


**Fig. 3.** Preparative aggregation factor affinity chromatography. Extracts from sponge membranes were incubated with a 3 ml MAF column for 30 minutes. The unbound material was allowed to pass through the column and the column was washed in 20 ml of 0.5 M NaCl, 0.05 M Tris-HCl, pH 8. Bound protein was eluted with 1 M NaCl, 0.05 M Tris-HCl, pH 8, and further eluted with 2 M NaCl, 50 mM Tris-HCl, pH 8, after washing the column twice with application buffer (WASH). Optical density readings at 280 nm for each fraction collected were plotted versus fraction number.

ciently extract the 68 and 210 kDa protein ligands from the sponge membrane extracts, small aliquots of either aggregation factor-Sepharose or unconjugated Sepharose were incubated for 2 hours with the sponge extracts. After the resins were washed several times to remove unbound proteins, bound proteins were extracted by boiling the resins in SDS sample buffer and were analyzed by incubation of western blots in radioiodinated aggregation factor. Both the 68 and 210 kDa proteins were present in the lysate that was incubated with the resins (Fig. 2A) but neither bound to the unconjugated Sepharose (Fig. 2B). Neither protein was present in the aggregation factor that was coupled to the resin, as determined by analyzing resins that were boiled directly in SDS without exposure to sponge membrane extracts by the aggregation factor western blotting assay (not shown). In contrast, both proteins bound to the aggregation factor Sepharose resin (Fig. 2C), suggesting that affinity chromatography could be a useful strategy to purify these proteins.

Larger scale affinity chromatography of sponge membrane lysates on aggregation factor Sepharose was performed to isolate useful quantities of the aggregation factor ligands. The dialyzed, octylpolyoxyethylene membrane extract was applied in batches to a 3 ml affinity column containing 1 mg/ml aggregation factor. When the affinity column was washed successively with buffered 0.5 M NaCl, 1 M NaCl, 0.5 M NaCl (wash) or 2 M NaCl, a single protein peak eluted from the column after application of the 2 M NaCl buffer (Fig. 3). Samples of column fractions, including unbound as well as eluted material, were analyzed by silver stained non-reducing SDS-polyacrylamide gels and by western blotting. Whereas the unbound material consisted of numerous proteins as observed on silver stained gels of the unbound material (Fig. 4A), the eluted protein peak consisted of two protein bands of approximately 68 kDa molecular mass (Fig. 4B). When blotted onto nitrocellulose and incubated in radioiodinated aggregation factor, the affinity column eluate was shown to contain only a single 68 kDa and not the 210 kDa aggregation factor binding ligand (Fig. 4C). Western blot analysis of the unbound material indicated that the 68 kDa aggregation factor binding activity had been effectively removed from it (not shown). The 210 kDa protein present in extracts bound weakly to the column but was eluted from it during the initial 0.5 M NaCl washes; it thus had to be purified by alternate means (J. A. Varner, unpublished). Eluted protein peaks from successive column runs were pooled, concentrated and dialyzed prior to further analysis.

## Purified 68 kDa aggregation factor ligand is a monomer

In order to purify and analyze the affinity column eluate further, it was chromatographed on a previously standardized Sepharose 4B sizing column that was equilibrated with 0.5 M NaCl, 50 mM Tris-HCl, pH 7.8. A single protein peak, corresponding to a native molecular mass of approximately 70 kDa, was eluted from the column, at an elution volume of 185 ml (not shown). When a sample of the sizing column peak was iodinated and subsequently analyzed on non-reducing SDS-polyacrylamide gels by Coomassie Blue staining and by autoradiography, the peak fraction appeared to contain only a single 68 kDa protein band (Fig. 5, $^{125}$I, CBB). Analysis by the aggregation factor western blotting assay of a sample of the peak fraction indicated that the 68 kDa protein that eluted from the sizing gel column retains the aggregation factor binding activity of the 68 kDa protein from the original extract (Fig. 5,

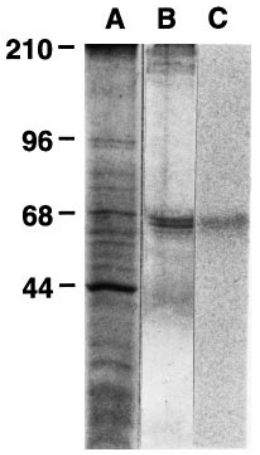


**Fig. 4.** Gel electrophoretic analysis of affinity chromatography fractions. Aliquots (100 µl) of the flow through (A) and 2 M NaCl eluted fractions (B) collected from aggregation factor affinity column chromatography were acetone precipitated, solubilized in 100 µl SDS nonreducing sample buffer and electrophoresed on a 10% SDS-polyacrylamide gel. The gel was silver stained. A parallel sample of the affinity column eluate was electrophoresed on the same gel; the gel was transferred to nitrocellulose and incubated in iodinated aggregation factor (C) as described in Materials and Methods. Molecular mass markers indicated are 210, 96, 68 and 44 kDa.

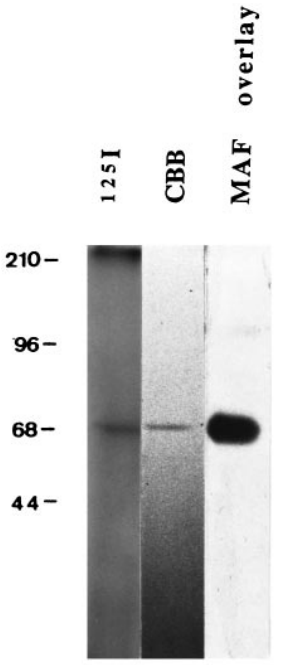


**Fig. 5.** Analysis of gel filtration chromatography purified 68 kDa protein. Iodinated 68 kDa protein was electrophoresed on a 3-20% gradient gel under nonreducing conditions. The material was analyzed by either autoradiography ($^{125}$I) or Coomassie Blue staining (CBB). A similar sample of unlabelled 68 kDa protein was electrophoresed on a nonreducing polyacrylamide gel and was analyzed by the aggregation factor western blotting assay (MAF overlay). Molecular mass markers indicated by arrows include 210, 96, 68, and 44 kDa.

MAF overlay). Thus, the material eluted from the affinity column appeared to consist of a single protein species, on the basis of gel filtration chromatography, SDS gel analysis of iodinated and Coomassie Blue stained material and analysis by the aggregation factor western blotting assay. The 68 kDa aggregation factor binding protein was thus efficiently purified using an aggregation factor affinity column.

### Intrachain disulfide bonds affect 68 kDa ligand function

The purified 68 kDa aggregation factor ligand was characterized further to examine the role of intrachain disulfide bonding in its function. Identical samples of the 68 kDa protein were electrophoresed on SDS-polyacrylamide gels in the presence and absence of beta-mercaptoethanol. Under non-reducing conditions, the aggregation factor ligand electrophoreses at an apparent molecular mass of 68 kDa (Fig. 6, NR). Under reducing conditions, however, it electrophoreses at an apparent molecular mass of 76 kDa (Fig. 6, R). Since this protein exhibits significant differences in mobility on SDS-polyacrylamide gels depending on its oxidation/reduction state, it contains a significant degree of disulfide bond-determined secondary structure. Reduction significantly inhibits the ability of the 68 kDa protein to bind aggregation factor in the western blotting assay (Varner et al., 1988). Thus it is probable that the binding site on the 68 kDa protein is composed of a disulfide bond determined, non-linear structural unit.

### The 68 kDa protein is a glycoprotein

To determine if the 68 kDa protein is a glycoprotein and if glycans play a role in its function, the protein was subjected to deglycosylation analysis. Peptide-*N*-glycosidase digestion of sponge cell lysates, followed by analysis with the western blotting assay, indicates that the 68 kDa protein is glycosylated. The migration of the 68 kDa aggregation factor ligand on non-reducing SDS gels (Fig. 7, –) increases upon deglycosylation by peptide-*N*-glycosidase (Fig. 7, +), as evaluated by the aggregation factor blotting assay. Because the protein still is recognized by iodinated aggregation factor on the western blot after deglycosylation, it is possible to conclude that the glycan moieties on the 68 kDa protein that can be removed with peptide-*N*-glycosidase do not contribute significantly to the aggregation factor-68 kDa ligand interaction. The appearance of two bands on the blot after deglycosylation suggests that the protein contains at least one glycan; the upper band may represent an incompletely deglycosylated species. Thus, these analyses suggest that the 68 kDa protein is a glycoprotein, but that its glycans do not impact its affinity for the aggregation factor.

### The 68 kDa protein inhibits cell adhesion and aggregation factor-cell binding

In order to determine if the 68 kDa aggregation factor ligand plays a significant role in aggregation factor-mediated cell adhesion, the 68 kDa protein purified by aggregation factor affinity chromatography was tested for its ability to inhibit the binding of iodinated aggregation factor to cells and to inhibit aggregation factor-mediated cell aggregation. When 68 kDa protein was preincubated for 30 minutes with aggregation factor before addition of cells (to allow saturation of 68 kDa protein binding sites on the proteoglycan), as little as 0.6 µg of the purified protein was required to inhibit 50% of aggregation

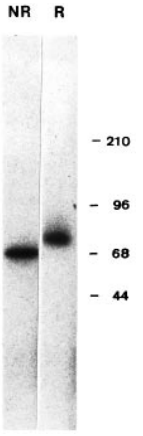


**Fig. 6.** Reduction/nonreduction analysis of iodinated purified 68 kDa protein. Two micrograms of iodinated 68 kDa protein was electrophoresed on a 3-20% gradient gel in nonreducing (NR) and reducing (R) conditions. The material was analyzed by autoradiography. Molecular mass markers indicated by arrows include 210, 96, 68, and 44 kDa.

factor-mediated cell aggregation (Fig. 8A). Control substances, including additional purified sponge proteins, sponge extracts from which the 68 kDa and 210 kDa aggregation factor ligands were removed by anion exchange chromatography (Varner et al., 1988) and proteins such as albumin, fibronectin, and gelatin, were unable to inhibit the aggregation of sponge cells (not shown).

Besides potently inhibiting cell adhesion mediated by aggregation factor, the 68 kDa protein prevents the aggregation factor from binding to the cell surface. Although the 68 kDa protein does not completely inhibit binding of aggregation factor to the cell surface, only 0.5 μg was required to inhibit 50% of the aggregation factor from binding to the cell surface (Fig. 8B). The 68 kDa protein inhibition of aggregation factor binding to the cell surface and of cell adhesion indicates that it plays a major role in aggregation factor-mediated cell adhesion. Because another aggregation factor binding protein has been identified (the 210 kDa protein), it is not unexpected that the 68 kDa protein does not completely inhibit aggregation factor binding to the cell.

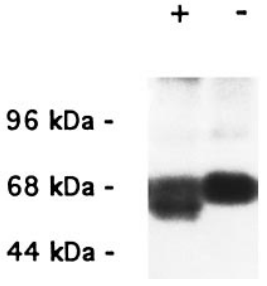


**Fig. 7.** Peptide-*N*-glycosidase treatment of the 68 kDa protein. NP40 extracts of sponge cells were incubated in the presence (+) or absence (–) of peptide-*N*-glycosidase at 37°C overnight, were electrophoresed on 7.5% SDS-polyacrylamide gels and were analyzed by the aggregation factor western blotting assay. Molecular mass markers indicated are 210, 96, 68 and 44 kDa.

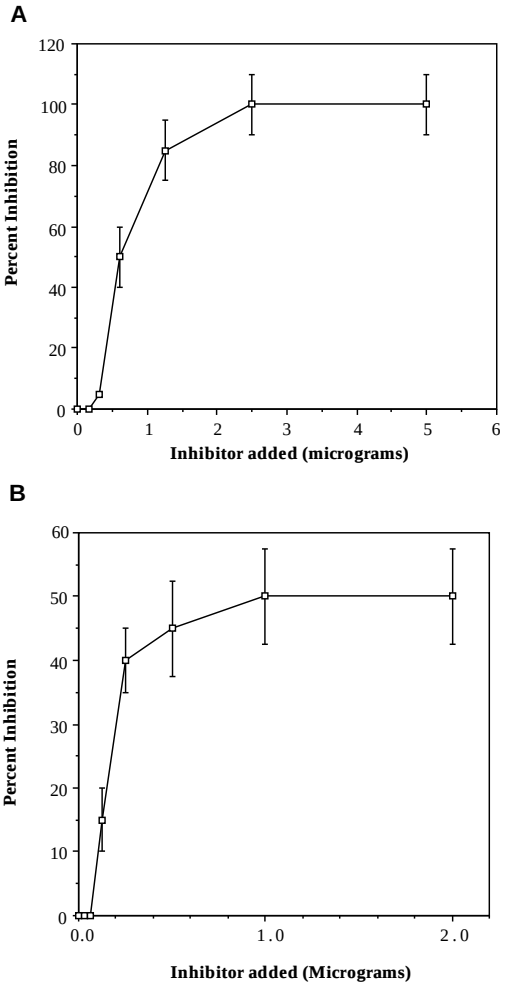


**Fig. 8.** Inhibition of aggregation and $^{125}$I aggregation factor-cell binding. (A) Twofold serial dilutions of MAF affinity column purified 68 kDa protein (0.15-5 μg) were incubated with 4 μg of MAF under inhibition assay conditions described in Materials and Methods. The titer of aggregation was determined for each dilution and inhibition calculated as percentage decrease from the standard 4 μg aggregation titer. Per cent inhibition is plotted here versus μg protein added. (B) $^{125}$I-MAF was incubated with twofold serial dilutions of purified 68 kDa protein for 30 minutes and cells added for standard cell binding assays. Per cent inhibition was calculated by determining counts of MAF bound and dividing by counts bound under the same conditions but in the absence of added 68 kDa protein.

## The 68 kDa protein is a high affinity ligand for aggregation factor

The purified 68 kDa protein inhibits aggregation factor binding to the cell and aggregation factor-mediated cell adhesion by directly binding to the aggregation factor in solution. Scatchard analysis (Scatchard, 1959) of radioiodinated 68 kDa protein binding to the aggregation factor indicates that the 68 kDa protein has a high affinity ($K_D = 2\times10^{-9}$) for the aggregation factor (Fig. 9A).

The 68 kDa protein is a peripheral membrane protein on the basis of its solubility in the absence of detergent (Varner et al., 1988) and its monomeric state in these buffers. Thus, it is likely that there is an integral membrane protein receptor for it. In fact, the 68 kDa protein binds to cell surfaces (which had been

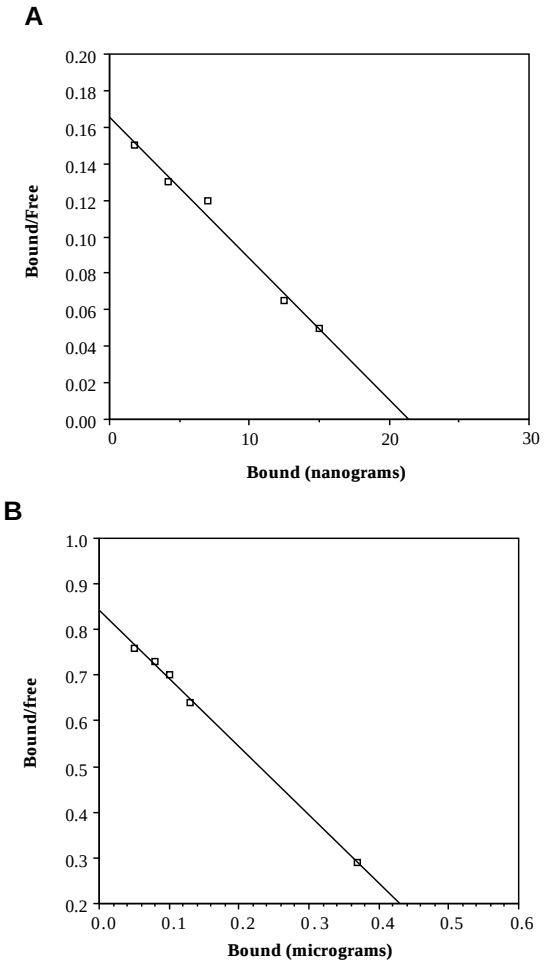


**Fig. 9.** Scatchard analysis of 68 kDa protein binding to aggregation factor and cells. (A) Serial dilutions of iodinated 68 kDa protein (12.5-400 ng) were incubated as described in Materials and Methods with aliquots of MAF in a total volume of 100 μl for 12 hours at 4°C. Bound ng were divided by free (Bound/free) and plotted versus bound ng per μg aggregation factor. (B) Dilutions of iodinated 68 kDa protein (0.1 to 1.0 μg) were incubated with $10^6$ washed sponge cells as described in Materials and Methods. Bound μg were divided by free (Bound/free) and plotted versus bound μg.

washed with low salt to remove the majority of the bound 68 kDa protein; Varner et al., 1988) with a dissociation constant of $6.7\times10^{-8}$ M, as determined by Scatchard analysis (Fig. 9B).

## DISCUSSION

Cell adhesion molecules play essential roles in a number of normal and pathological events (Hynes, 1992). Integrins (Cheresh, 1993), cadherins (Takeichi, 1991), selectins (Lasky et al., 1994) and immunoglobulin family molecules (Edelman, 1988) are among the cell adhesion molecules that have been extensively studied in the last decade. Although much is known about adhesion in higher species, less is understood about the molecular events that govern the process of cellular adhesion in nonmammalian and invertebrate species. To further the understanding of the origins of cell adhesion, two sponge adhesion proteins, ligands for the proteoglycan-like aggregation factor (of molecular masses 210 and 68 kDa) were identified and characterized as peripheral membrane proteins using 'aggregation factor' as a probe on western blots of sponge cell extracts (Varner et al., 1988).

To analyze in detail the structure-function relationships of these proteins, one of them was purified to homogeneity using affinity chromatography. Although both the 210 and the 68 kDa proteins bound to aggregation factor conjugated to Sepharose in a small scale experiment, only the 68 kDa aggregation factor ligand was efficiently purified by aggregation factor-Sepharose affinity chromatography on a larger scale. It eluted with the application of a high salt buffer, whereas the 210 kDa protein eluted during initial washing steps, suggesting that the 68 kDa protein has a higher affinity for aggregation factor than the 210 kDa protein. The 68 kDa protein that eluted from the affinity column chromatographed as a monomer in gel filtration chromatography and electrophoresed as a single discrete band when analyzed by Coomassie blue staining, autoradiography and western blotting. In material eluted from the affinity column, occasionally a 68 kDa doublet was observed which disappeared in further purification steps. This doublet was likely the product of slight proteolysis in occasional preparations or due to minor carbohydrate heterogeneities.

Previous studies have indicated that the 68 kDa protein-aggregation factor interaction is reduction sensitive (Varner et al., 1988). This sensitivity, together with the change in electrophoretic migration of the protein on polyacrylamide gels before and after reduction, suggests that the 68 kDa protein contains a significant degree of disulfide dependent secondary structure in the binding site. The binding to aggregation factor is not, however, dependent on the presence of Peptide-*N*-glycosidase sensitive glycans, as their removal by peptide-*N*-glycosidase does not alter aggregation factor binding.

Several lines of evidence demonstrate that the 68 kDa protein is a major sponge adhesion protein. First, the 68 kDa protein is one of only two proteins that bind the aggregation factor (Varner et al., 1988). Second, it binds tightly to aggregation factor, as demonstrated by aggregation factor-Sepharose affinity chromatography, eluting only with high molarity saline. Third, the purified 68 kDa molecule displays a high affinity for native aggregation factor ($K_D = 2\times10^{-9}$ M) as well as for cell surfaces. Fourth, it completely inhibits the aggregation of sponge cells and significantly prevents the binding of aggregation factor to sponge cell surfaces. These results suggest that it is the major cell surface component responsible for effecting the binding of aggregation factor to cells and aggregation factor-mediated cell adhesion.

It is likely that there is an integral membrane protein receptor for the 68 kDa protein, based upon the affinity of the purified 68 kDa protein for sponge cell surfaces. Indeed, other extracellular matrix molecules such as fibronectin, vitronectin, collagen, and laminin bind to integral membrane protein receptors (integrins) as well to extracellular matrix proteoglycans and are responsible for mediating cell-substrate adhesion in many species (Hynes, 1992). Like these vertebrate matrix proteins, the 68 kDa aggregation factor ligand binds to other matrix molecules and to the cell surface, while playing a role in cell adhesion. Thus, this early metazoan extracellular matrix adhesion protein may be related to vertebrate extracellular matrix proteins.

Continued studies, including sequence analysis and identification of an integral membrane receptor will enable further understanding of the role of this protein in the evolution of cellular adhesion.

## REFERENCES


**Albelda, S. and Buck, C.** (1990). Integrins and other cell adhesion molecules *FASEB J*. **4**, 2868-2880.

**Albelda, S., Mette, B. A., Elder, D. E., Stewart, R., Damjanovich, L., Herlyn, M. and Buck, C.** (1990). Integrin distribution in malignant melanoma: Association of the beta 3 subunit with tumor progression. *Cancer Res*. **50**, 6757-6764.

**Adelson, D. L., Alliegro, M. C. and McClay, D. R.** (1992). On the ultrastructure of hyalin, a cell adhesion protein of the sea urchin embryo extracellular matrix. *J. Cell Biol.* **116**, 1283-1289.

**Barondes, S. H., Haywood-Reid, P. L. and Cooper, D. N.** (1985). Discoidin I, an endogenous lectin, is externalized from Dictyostelium discoideum in multilamellar bodies. *J. Cell Biol*. **100**, 1825-33.

**Bischoff, J.** (1995). Approaches to studying cell adhesion molecules in angiogenesis. *Trends Cell Biol.* **5**, 60-74.

**Boucat, J.-C., Darribiere, T., Poole, T. J., Aoyama, H. M., Yamada, K. and Theiry, J. P.** (1984). Biologically active synthetic peptides as probes of embryonic development: A competitive inhibitor of fibronectin function inhibits gastrulation in amphibian embryos and neural crest cell migration in avian embryos. *J. Cell Biol*. **99**, 1822-1829.

**Bronner-Fraser, M., Artinger, M., Muschler, J. and Horwitz, A. F.** (1992). Developmentally regulated expression of alpha 6 integrin in avian embryos. *Development* **115**, 197-211.

**Brooks, P. C., Montgomery, A. M. P., Rosenfeld, M., Reisfeld, R. A., Hu, T., Klier, G. and Cheresh, D. A.** (1994a). Integrin αvβ3 antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. *Cell* **79**, 1157-1164.

**Brooks, P. C., Clark, R. F. and Cheresh, D. A.** (1994b). Requirement of vascular integrin αvβ3 for angiogenesis. *Science* **264**, 569-571.

**Cheresh, D. A.** (1993). Integrins: Structure, function and biological properties. *Advan. Mol. Cell. Biol.* **6**, 225-252.

**DeSimone, D.** (1994). Integrin function in early vertebrate development: perspectives from studies in amphibian embryos. In *Integrins: The Biological Problems* (ed. Y. Takada), pp. 217-234. Academic Press, San Diego, CA.

**Edelman, G. M.** (1988). Morphoregulatory molecules. *Biochemistry* **27**, 3533-43.

**Felding-Habermann, B., Mueller, B., Romerdahl, C. and Cheresh, D. A.** (1992). Involvement of integrin αv gene expression in human melanoma tumorigenicity. *J. Clin. Invest.* **89**, 2018-2022.

**Gabius, H. J., Springer, W. R. and Barondes, S. H.** (1985). Receptor for the cell binding site of discoidin I. *Cell* **42**, 449-56.

**Giancotti, F. G. and Ruoslahti, E.** (1990). Elevated levels of the α5β1 receptor suppresses the transformed phenotype of Chinese hamster ovary cells. *Cell* **60**, 848-859.

**Hardin, J. and McClay, D. R.** (1990). Target recognition by the archenteron during sea urchin gastrulation. *Dev. Biol*. **142**, 86-102.

**Hemler, M. E.** (1990). VLA proteins in the integrin family: Structures, functions, and their role on leukocytes. *Annu. Rev. Immunol*. **8**, 365-400.

**Henkart, P., Humphreys, S. and Humphreys, T.** (1973). Characterization of sponge aggregation factor: a unique proteoglycan complex. *Biochemistry* **12**, 3045-3050.

**Humphreys, T.** (1963). Chemical and in vitro reconstitution of sponge cell adhesion. I. Isolation and functional demonstration of the components involved. *Dev. Biol*. **8**, 27-47.

**Hynes, R. O.** (1992). Integrins: versatility, modulation and signalling in cell adhesion. *Cell* **69**, 11-25.

**Hynes, R. O. and Lander, A. D.** (1992). Contact and adhesive specificities in the associations, migrations, and targeting of cells and axons. *Cell* **68**, 303-322.

**Jumblatt, J., Schlup, V. and Burger, M. M.** (1980). Cell-cell recognition: Specific binding of *Microciona* sponge aggregation factor to homotypic cells and the role of calcium ions. *Biochemistry* **12**, 3045-3050.

**Kassner, P. D., Teixido, J., Chan, B. M. C., Parker, C. M. and Hemler, M. E.** (1992). Analyses of VLA-4 Structure and Function. In *Mechanisms of Lymphocyte Activation and Immune Regulation IV: Cellular Communications* (ed. S. Gupta and T. A. Waldmann), pp. 163-170. Plenum Press, NY, NY.

**Kemler, R., Ozawa, M. and Ringwald, M.** (1989). Calcium-dependent cell adhesion molecules. *Curr. Opin. Cell Biol*. **1**, 892-897.

**Kintner, C.** (1992). Regulation of embryonic cell adhesion by the cadherin cytoplasmic domain. *Cell* **69**, 225-236.

**Laemmli, U. K.** (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

**Lasky, L., Presta, L. G. and Erbe, D. V.** (1994). Structure-function aspects of selectin-carbohydrate interactions. In *Cellular Adhesion: Molecular Definition to Therapeutic Potential* (ed. B. Metcalf, B. Dalton and G. Poste), pp. 37-53. Plenum Press, New York, New York.

**Lallier, T. and Bronner-Fraser, M.** (1993). Inhibition of neural crest cell attachment by integrin antisense oligonucleotides. *Science* **259**, 692-695.

**Lallier, T., Hens, M. and DeSimone, D.** (1994). Integrins in Development. In *Integrins: Molecular and Biological Responses to the Extracellular Matrix* (ed. D. Cheresh and R. Mecham), pp. 111-140. Academic Press, San Diego, CA.

**Lee, G., Hynes, R. O. and Kirschner, M.** (1984). Temporal and spatial regulation of fibronectin in early Xenopus development. *Cell* **36**, 729-740.

**McClay, D. R.** (1991). Gastrulation. *Curr. Opin. Genet. Dev*. **1**, 191-5.

**Maeda, T., Balakrishnan, K. and Mehdi, S. Q.** (1983). A simple and rapid method for the preparation of plasma membranes. *Biochem. Biophys. Acta* **731**, 115-120.

**Meredith, J. E., Fazeli, B. and Schwartz, M. A.** (1993). The extracellular matrix as a cell survival factor. *Mol. Biol. Cell* **4**, 953-961.

**Misevic, G., Jumblatt, J. and Burger, M. M.** (1982). Cell binding fragments from a sponge proteoglycan-like aggregation factor. *J. Biol. Chem*. **257**, 6931-6936.

**Misevic, G. and Burger, M. M.** (1986). Reconstitution of high cell binding affinity of a marine sponge aggregation factor by cross-linking of small low affinity fragments into a large polyvalent polymer. *J. Biol. Chem.* **261**, 2853-2859.

**Misevic, G. and Burger, M. M.** (1990). The species-specific cell-binding site of the aggregation factor from the sponge *Microciona prolifera* is a highly repetitive novel glycan containing glucuronic acid, fucose and mannose. *J. Biol. Chem*. **265**, 20577-20584.

**Misevic, G. and Burger, M. M.** (1993). Carbohydrate-carbohydrate interactions of a novel acidic glycan can mediate sponge cell adhesion. *J. Biol. Chem.* **268**, 4922-4929.

**Montgomery, A. M. P., Reisfeld, R. A. and Cheresh, D. A.** (1994). Integrin αvβ3 rescues melanoma cells from apoptosis in a three-dimensional dermal collagen. *Proc. Nat. Acad. Sci. USA* **91**, 8856-8860.

**Müller, W. E. G., Conrad, G., Pondeljack, V., Steffan, R. and Zahn, R.** (1982). Electron microscopical characterization of sponge aggregation factors. *Tissue & Cell* **14**, 219-233.

**Ruoslahti, E.** (1991). Integrins. *J. Clin. Invest*. **87**, 1-5.

**Scatchard, C.** (1959). *Ann. NY Acad. Sci.* **51**, 660-672.

**Springer, T. A.** (1990). Adhesion receptors of the immune system. *Nature* **346**, 425-434.

**Springer, W. R. D., Cooper, W. W. and Barondes, S.** (1984). Discoidin I is implicated in cell-substratum attachment and ordered cell migration of *Dictyostelium discoideum* and resembles fibronectin. *Cell* **39**, 557-564.

**Takeichi, M.** (1991). Cadherin cell adhesion receptors as a morphogenetic regulator. *Science* **251**, 1451-1455.

**Towbin, H., Staehlin, T. and Gordan, J.** (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Nat. Acad. Sci. USA* **76**, 4350-4354.

**Varner, J., Burger, M. M. and Kaufman, J.** (1988). Two cell surface proteins bind the sponge *Microciona prolifera* aggregation factor. *J. Biol. Chem*. **263**, 8498-8508.

**Varner, J. A., Emerson, D. A. and Juliano, R. L.** (1995). Integrin α5β1 negatively regulates cell growth: Reversal by attachment to fibronectin. *Mol. Biol. Cell* **6**,

**Wilson, H. V.** (1907). On some phenomena of coalescence and regeneration in sponges. *J. Exp. Zool*. **5**, 245.

**Wilson, H. V.** (1910). *Bull. Bur. Fisheries* **30**, 1.

**Zetter, B. R.** (1990). The cellular basis of site-specific tumor metastasis. *New. Engl. J. Med*. **322**, 605-612.


(Received 7 March 1995 - Accepted 22 June 1995)