Isolation of a novel cell-attachment and spreading-promoting protein from human serum

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A protein with potent cell-attachment and spreading-promoting activity was isolated from fibronectin-free human serum. The purification steps included affinity chromatography on heparin-agarose and preparative isoelectric focusing. The purified protein was homogeneous as judged from dodecyl sulphate/polyacrylamide-gel electrophoresis. It had an isoelectric point of 5.0 and an M_r of 52 000. The protein promoted the spreading of Chinese-hamster ovary cells to plastic in a manner similar to that observed with fibronectin.

The growth of adherent cells *in vitro* is dependent on a supply of growth factors as well as of specific protein factors that enable the cells to adhere and spread on solid surfaces. Both types of factors are provided by serum added to the cell-culture media. A number of serum proteins capable of mediating cell attachment and spreading on various substrates have already been described. Fibronectin, a dimeric protein with a subunit M_r of 220 000, is the best-characterized protein in this respect (Hynes & Yamada, 1982). Chondronectin, an adhesive factor for chondrocytes, has an M_r of 180 000 and comprises subunits with an M_r of 70 000 (Hewitt *et al.*, 1982). Epibolin, a single-chain protein (M_r 65 000) supporting spreading of epithelial cells, has recently been isolated from plasma (Stenn, 1981). Additional adhesive factors having M_r values in the range 62 000–75 000 have been isolated from serum by several laboratories (Whateley & Knox, 1980; Barnes & Silnutzer, 1983; Hayman *et al.*, 1983). It has also been found that collagens (Kleinman *et al.*, 1978) and laminin, a high- M_r protein of basement membranes (Timpl *et al.*, 1979; Carlsson *et al.*, 1981) are involved in the attachment of many cells *in vitro*.

It was suggested fairly recently that cell-surface glycosaminoglycans or proteoglycans may have an important role in cell adhesion (Rollins *et al.*,

1982). It is thought that cell-adhesion-mediating proteins bind simultaneously to cell-surface glycosaminoglycans and to the substratum. It is known that many of the recognized cell-adhesion-mediating proteins bind to glycosaminoglycans (Sakashita *et al.*, 1980; Kleinman *et al.*, 1981; Hynes & Yamada, 1982; Barnes & Silnutzer, 1983). We have isolated an attachment and spreading factor for CHO cells from human serum, taking advantage of its affinity to heparin. This factor has an M_r of 52 000 and an isoelectric point of 5.0.

Materials and methods

Cells

CHO cells obtained from the American Type Culture Collection (A.T.C.C.) were routinely cultured in Ham's F-12 medium supplemented with 10% (v/v) human serum. Cells were grown on Petri dishes (90 mm diameter) at 37°C in an air/CO₂ (19:1) atmosphere, and they were passaged by using 0.05% (w/v) trypsin/0.02% (w/v) EDTA in Dulbecco's phosphate-buffered saline for cell detachment. In the attachment experiments, the cells were washed with phosphate-buffered saline and seeded in serum-free F-12 medium supplemented with bovine insulin (2.5 µg/ml; Sigma, St. Louis, MO, U.S.A.) and human transferrin (5 µg/ml; Sigma; approx. 98% pure).

Determination of attachment and spreading activity

Plastic cell-culture dishes (15 mm diameter; Nunclon delta SI; Nunc, Roskilde, Denmark)

Abbreviations used: CHO cells, Chinese-hamster ovary cells; PMSF, phenylmethanesulphonyl fluoride; SDS, sodium dodecyl sulphate.

were coated with the proteins to be studied by incubating the dishes with the protein solution (20 µg/ml in phosphate-buffered saline unless indicated otherwise) for 2 h at room temperature. Solutions of the purified 52000- M_r protein were prepared by diluting fractions from isoelectric focusing with phosphate-buffered saline to an appropriate protein concentration. Dishes incubated with carrier ampholytes did not support attachment of CHO cells in control experiments. After the incubation, the dishes were washed with phosphate-buffered saline and the cells were added in serum-free medium at a density of 10^5 cells/dish. The dishes were routinely incubated for 16 h, examined under the microscope, and washed three times with phosphate-buffered saline. Cells attached to the dishes were then detached with trypsin/EDTA, and counted in a cell counter (Coulter Counter Industrial D; Coulter Electronics, Harpenden, Herts., U.K.). The measurements were made in duplicate. Dishes coated with bovine serum albumin (fraction V; Sigma) were used as negative controls. Attachment to albumin-coated dishes varied between 5 and 10%. Dishes coated with purified human plasma fibronectin (Vuento & Vaheri, 1979) were used as positive controls. Plating efficiency on fibronectin-coated dishes was 80–90%.

Preparation of fibronectin-free serum

Pooled human serum was prepared from blood samples obtained with informed consent from healthy volunteers. The samples were allowed to clot for 2 h at room temperature and further kept at +4°C for 16 h. Serum samples were centrifuged (10000g, 20 min) and pooled. PMSF and EDTA were added to pooled serum to a concentration of 1 mM. The samples were stored at -20°C until used. Fibronectin was removed from serum by affinity chromatography on gelatin-agarose (Vuento & Vaheri, 1979) followed by affinity chromatography on a column of anti-fibronectin antibody coupled to agarose. Briefly, serum (50 ml) was run through a column (2.6 cm × 4 cm; 20 ml) of gelatin-Sepharose prepared as described by Vuento & Vaheri (1979) and equilibrated with 50 mM-Tris/HCl, pH 7.5, containing 1 mM-EDTA and 1 mM-PMSF.

The column was eluted with the equilibrating buffer until no protein was eluted from the column. The combined eluates, containing serum proteins except fibronectin, were run through a second column (1 cm × 6 cm; 5 ml) of anti-fibronectin antibody coupled to Sepharose. This affinity gel contained 1 mg of immunoglobulin fraction from a specific high-titre rabbit antiserum (Vuento *et al.*, 1980)/ml of gel. The column was equilibrated with the above buffer, and eluted as above. The absence

of fibronectin in the combined eluates was confirmed with Ouchterlony immunodiffusion analysis with specific anti-fibronectin antibody (Vuento *et al.*, 1980) and with immunoblotting analysis (Towbin *et al.*, 1979). In the immunoblotting analysis, the samples were fractionated by polyacrylamide-gel electrophoresis in the presence of SDS, and then transferred electrophoretically from polyacrylamide gel to a nitrocellulose membrane. Immunoreactive fibronectin was detected on the nitrocellulose membrane after reaction with anti-fibronectin antibodies by using peroxidase-conjugated antibodies against rabbit immunoglobulins. Purified fibronectin was used as a control. In these studies, no fibronectin could be detected in fibronectin-depleted serum, nor could fibronectin be detected in the eluate from heparin-Sepharose chromatography (results not shown).

Affinity chromatography on heparin-agarose

Fibronectin-free serum (50 ml) was applied on a column (2.5 cm × 8 cm; 40 ml) of heparin-Sepharose (Pharmacia, Uppsala, Sweden) equilibrated with 10 mM-Tris/HCl, pH 7.5, containing 150 mM-NaCl and 1 mM-EDTA. The column was washed with 1000 ml of the equilibrating buffer and eluted with a linear gradient of NaCl (0.15–0.5 M; 500 ml), followed by batchwise elution with 1.5 M-NaCl and finally with 1.5 M-NaCl/8 M-urea. The eluted fractions were pooled as indicated below, concentrated by ultrafiltration with Amicon (Lexington, MA, U.S.A.) PM 10 membranes, and subjected to isoelectric focusing.

Isoelectric focusing

Isoelectric focusing was performed on a 110 ml focusing column (LKB, Bromma, Sweden). Ampholine carriers (LKB) with a pH range 3.5–10 were used. Gradients were stabilized with sucrose (5–53%, w/v) containing 4 M-urea and 0.01% Nonidet P40. The presence of urea and detergent was necessary to prevent aggregation of samples during the focusing experiments. A focusing time of 48 h with a voltage of 900 V was routinely used. Gradients run without protein samples were used as controls in the cell-attachment experiments.

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was carried out using the method of Laemmli (1970) with vertical gel slabs (7%, w/v, acrylamide). M_r markers were obtained from Sigma. They included bovine erythrocyte carbonic anhydrase (M_r

29000), chicken egg albumin (M_r 45000), bovine albumin (M_r 66000), rabbit muscle phosphorylase *a* (M_r 97000) and rabbit muscle myosin (M_r 205000). The gels were silver-stained with a commercial reagent kit (Bio-Rad, Richmond, CA, U.S.A.).

Determination of protein concentration

Determination of protein concentration was carried out with a commercial dye reagent (Bio-Rad), the method of Bradford (1976) being used. Bovine serum albumin (Sigma) was used as a standard.

Results

We have previously found that serum depleted of fibronectin was able to support the attachment and spreading of CHO cells on plastic dishes. The attachment- and spreading-promoting activity was adsorbed to heparin-Sepharose and eluted with NaCl as a heterogeneous group of proteins differing in M_r and in isoelectric point (M. Vuento, M. Korkolainen, P. Kuusela, I. Eronen & E. Hölttä, unpublished work). One of these protein factors

bound to heparin-Sepharose relatively strongly, which made possible its effective purification.

Elution of the heparin-Sepharose column with an NaCl gradient (0.15–0.5M) is shown in Fig. 1. Much of the protein bound to the column from fibronectin-free serum was eluted in the early fractions of the gradient (0.15–0.3M-NaCl). A considerable portion of the bound protein resisted elution with 0.5M-NaCl, but was eluted with 1.5M-NaCl (Fig. 1). A further portion of the bound proteins could only be eluted with buffer containing 1.5M-NaCl/8M-urea (Fig. 1). Cell-attachment-promoting activity was found in all fractions eluted with the NaCl gradient, as well as in fractions eluted with 1.5M-NaCl and with 1.5M-NaCl/8M-urea (Fig. 1). However, the fractions eluted with 0.2–0.25M-NaCl were relatively inefficient in promoting cell attachment (Fig. 1).

The eluted fractions were divided into pools as follows: fractions 17–25, pool 1; fractions 26–40, pool 2; fractions 44–50, pool 3; fractions 59–66, pool 4. The isoelectric-focusing pattern given by pool 3 is shown in Fig. 2. The protein material focused as a single peak at pH 5.0. The protein peak coincided with a peak of cell-attachment-promoting activity (Fig. 2). However, some cell-attachment-promoting activity was also found in

Fig. 1. Elution of cell-attachment-promoting activity from heparin-Sepharose

A 50 ml sample of human serum, depleted of fibronectin by specific affinity gels, was passed through a column (40 ml) of heparin-Sepharose, equilibrated with 10 mM-Tris/HCl (pH 7.5)/150 mM-NaCl/1 mM-EDTA. After an extensive washing, the column was eluted with a gradient (0.15–0.5M, 500 ml) of NaCl (shown by the broken line), followed by stepwise elutions with 1.5M-NaCl and 1.5M-NaCl/8M-urea (starting points of stepwise elutions are shown by arrows 1 and 2 respectively). Cell-attachment-promoting activity in the eluted fractions (□) was determined by incubating plastic cell-culture dishes with eluted proteins at a concentration of 20 µg/ml. Attachment of CHO cells to the dishes was determined as described in the Materials and methods section. The attachment is expressed as a percentage of the attachment observed in positive-control dishes coated with purified fibronectin. Protein concentration is shown as A_{280} (O).

Fig. 2. Isoelectric focusing of proteins eluted with 1.5M-NaCl from heparin-Sepharose
 ●, A_{280} ; □, attachment activity; ○, pH gradient. For experimental details, see the Materials and methods section.
 The attachment activity is expressed as in Fig. 1.

fractions with basic pH values (Fig. 2). Fractions from control gradients had no cell-attachment-promoting activity (results not shown). A similar isoelectric-focusing pattern was obtained with pool 2. However, pools 1 and 4 gave more complex focusing patterns, with several protein peaks.

When the protein peaks (pI 5.0) from focused pools 2 and 3 were analysed by SDS/polyacrylamide-gel electrophoresis under non-reducing conditions, a single polypeptide band corresponding to M_r 52000 was observed (Fig. 3). After treatment with the reducing agent β -mercaptoethanol, the protein again migrated as a single polypeptide band, but now at a distinctly lower migration rate corresponding to an apparent M_r of 70000. Attempts to determine the M_r of the purified protein by gel filtration under non-dissociating conditions were unsuccessful, owing to an aggregation of the protein in the columns.

The 52000- M_r polypeptide was the only protein component present in the protein peak (pI 5.0) of focused pools 2 and 3. It was also found, together with other polypeptide bands, in the pI 5.0 fraction of focused pool 1, but not in the corresponding fraction of focused pool 4. The yield of the purified 52000- M_r protein recovered after isoelectric focusing of pools 2 and 3 was 2 mg from 50 ml of serum.

When used to coat plastic cell-culture dishes, the purified protein was active in promoting attachment of CHO cells at concentrations of 1 μ g/ml and

above (Fig. 4). CHO cells showed a rapid spreading on dishes coated with the purified protein. The kinetics of spreading were similar to those observed with purified fibronectin (Fig. 5). The spread cells had a fibroblast-like morphology similar to that of cells spreading on fibronectin-coated dishes (Fig. 5). In contrast, CHO cells did not attach nor spread on albumin-coated dishes (Fig. 5).

Discussion

We have presented a simple and efficient method for obtaining, in a highly purified state, a cell-attachment- and spreading-promoting protein from the heparin-binding fraction of fibronectin-free human serum. This protein was the major component eluted from heparin-Sepharose with 0.3–1.5M-NaCl, and it could be purified from the eluate to apparent homogeneity by preparative isoelectric focusing. The protein had a pI of 5.0 and an M_r (from SDS/polyacrylamide-gel electrophoresis under non-reducing conditions) of 52000. Under reducing conditions, the protein had a migration rate corresponding to a significantly higher M_r of 70000. The reason for this anomalous behaviour on reduction is not known, but it may depend on changes in molecular shape that could lead to changes in frictional properties.

The size of the purified protein distinguishes it

Fig. 3. SDS/polyacrylamide-gel electrophoresis of the purified protein

Protein from the pI 5.0 peak (Fig. 2) was analysed by SDS/polyacrylamide-gel electrophoresis under non-reducing conditions. The gel is shown with the anode at the bottom, and its upper and lower ends are indicated by arrows. The gel was stained with silver stain. Migration positions of M_r markers are shown on the right.

from previously characterized adhesive proteins such as laminin, fibronectin and chondronectin. Furthermore, fibronectin was completely removed from serum before purification of the 52000- M_r protein. The relationship between the purified protein and the previously known serum spreading factors (M_r 62000–75000) is less clear. However, the human serum spreading factor isolated by Barnes & Silnutzer (1983) has been shown to comprise two polypeptides with M_r values of 65000–70000 (SF 65) and 75000–78000 (SF 75). The M_r values for these polypeptides are significantly higher than that obtained in the present study, which makes it unlikely that we have isolated the

Fig. 4. Attachment of CHO cells to plastic dishes coated with the purified 52000- M_r protein

Cell-culture dishes were coated with purified 52000- M_r protein (●), fibronectin (○) or with albumin (□) by incubation of the dishes with protein solutions (conc. 0.1–100 µg/ml). For details of the cell-attachment assay, see the Materials and methods section.

same protein as Barnes & Silnutzer (1983). Furthermore, the serum spreading factor has been shown to bind to heparin–Sephacrose only weakly, whereas the protein isolated here bound very strongly to heparin. Another adhesive serum protein called vitronectin has been characterized by Hayman *et al.* (1983). Like serum spreading factor, vitronectin comprises polypeptides with M_r values of 65000 and 75000. It is unlikely that we are dealing with vitronectin, since this protein has been shown to migrate at similar rates on SDS/polyacrylamide-gel electrophoresis under both non-reducing and reducing conditions (Hayman *et al.*, 1983). A cell spreading factor with M_r 62000 has been isolated from foetal-calf serum by Whateley & Knox (1980). This protein was isolated from a material different from our starting material. It also has a different M_r .

It is a striking feature of most cell-adhesion-promoting proteins that they bind to heparin. This binding activity may be related to a role of cell-surface glycosaminoglycans in cell adhesion (Rollins *et al.*, 1982), and it suggests that adhesive proteins may belong to a protein family sharing common structural characteristics. Further structural characterization of the 52000- M_r protein isolated in the present study should provide information on the mutual relationships between the adhesive serum proteins.

Fig. 5. *Spreading of CHO cells on plastic dishes coated with the purified 52000-M_r protein*
 Cell-culture dishes were coated with the purified 52000-M_r protein (100 µg/ml) (a-d). The photographs show cell morphology after 2 h (a), 4 h (b), 6 h (c) and 24 h (d) of incubation. Photographs of CHO cells on fibronectin-coated (e-g) or on albumin-coated (h) dishes are shown for comparison. The incubation times were 4 h (e), 6 h (f) or 24 h (g, h). Magnification 340 ×.

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