The hyaluronate synthase from a eukaryotic cell line

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The hyaluronate synthase complex was identified in plasma membranes from B6 cells. It contained two subunits of molecular masses 52 kDa and 60 kDa which bound the precursor UDP-GlcA in digitonin solution and partitioned into the aqueous phase, together with nascent hyaluronate upon Triton X-114 phase separation. The 52 kDa protein cross-reacted with poly-

and monoclonal antibodies raised against the streptococcal hyaluronate synthase and the 60 kDa protein was recognized by monoclonal antibodies raised against a hyaluronate receptor. The 52 kDa protein was purified to homogeneity by affinity chromatography with monoclonal anti-hyaluronate synthase.

INTRODUCTION

Several attempts have been made to isolate the hyaluronate synthase from a eukaryotic cell source. Mian (1986b) reported the isolation of an enzymically active complex of several subunit proteins from detergent-solubilized plasma membranes. Ng and Schwartz (1989) described an extensive purification scheme and isolated a 60 kDa protein. We used an indirect approach for the identification and isolation of a eukaryotic hyaluronate synthase. Enzyme kinetic analysis of the synthase indicated that the active enzyme was present in plasma membranes at very low abundance (Prehm, 1983). Furthermore we were unable to maintain enzymic activity after disintegration of membranes with detergents (Prehm, 1983, 1989). But we could identify the hyaluronate synthase in streptococcal protoplast membranes and solubilize the active enzyme with cholate (Prehm and Mausolf 1986). The inactive synthase could be enriched from protoblast membranes by extraction using Triton X-114 phase separation into a detergent and an aqueous phase containing hyaluronate together with the synthase. A 52 kDa protein could be affinity-labelled with periodate-oxidized UDP-GlcA or UDP-GlcNAc. Antibodies against this protein inhibited synthase activity. These antibodies recognized a protein in eukaryotic membranes and stained the cell surface of fibroblasts (Prehm, 1989). Here we describe the properties and isolation of the hyaluronate synthase from B6 cells, a hybrid mouse/hamster cell line.

MATERIALS AND METHODS

Monoclonal antibodies raised against the p60 hyaluronate-binding protein isolated from mouse 3T3 fibroblasts have been described (Turley et al., 1987, 1991; Hardwick et al., 1992). Mouse monoclonal antibodies raised against human p85 hyaluronate-binding protein CD44 were from Dianova, Hamburg, Germany. Radioactive chemicals were from Amersham International, protein-A-Sepharose, Sephacryl S-300 and CNBr-activated Sepharose CL4B were from Pharmacia and all other chemicals were from Sigma Chemical Co.

General methods

Determination of the hyaluronate synthase activity (Prehm 1983a), Western blotting (Tsang et al., 1983), and isolation of plasma membranes (Brunnette and Till, 1971) have been de-

scribed. Proteins were separated after reduction with mercaptoethanol on 10% (w/v) polyacrylamide gels. Purified hyaluronate synthase was loaded on to a gradient of 5–25% (w/v) sucrose in PBS, containing 0.1% digitonin and centrifuged for 24 h at 250 000 *g* at 4 °C.

Cell culture

B6 cells, a hybrid cell line derived from mouse mammary carcinoma and a Chinese hamster lung cell line which produced large amounts of hyaluronate (Koyama et al., 1970; Koyama and Ono, 1970) were grown in Dulbecco's modified Eagle's medium supplemented with streptomycin/penicillin (100 units of each/ml), kanamycin (100 units/ml) and 5% (v/v) foetal calf serum.

Extraction of hyaluronate-binding proteins from plasma membranes

Plasma membranes from B6 cells (10 mg) were suspended in 10 ml of 2 M NaCl/10 mM Tris/malonate, pH 7.0, and sedimented at 40 000 *g* for 10 min. The membranes were resuspended in 10 ml of 10 mM Tris/malonate (pH 7.0)/10 mM MgCl₂ containing 0.3 mg of UDP-GlcA and 3 mg of UDP-GlcNAc and incubated for 30 min at 37 °C to load the membranes with nascent hyaluronate. The solution was cooled to 0 °C, 1 ml of 10% (v/v) Triton X-114 was added and the solution was homogenized in a Potter–Elvehjem homogenizer. Undissolved material was sedimented at 40 000 *g* for 10 min and the supernatant was rapidly heated to 37 °C in a 60 °C water-bath. The exact temperature was controlled by a thermometer in the solution. The turbid solution was immediately centrifuged for 5 min at 2800 *g*, the upper aqueous phase was collected and 0.1 ml of 10% (w/v) cetylpyridinium chloride was added. The solution was incubated for 15 min at 37 °C and the precipitate was removed by centrifugation at 40 000 *g* for 10 min. Proteins in the supernatant were precipitated by the method of Wessel and Flügge (1984) and analysed by gel electrophoresis on 10% (w/v) polyacrylamide.

Surface labelling of intact cells

B6 cells were suspended in 2 ml of Dulbecco's medium, 5% (v/v) foetal calf serum at 4 × 10⁴ cells/ml and incubated over-

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night at 37 °C. The cultures were supplemented with an additional 0.2 ml of foetal calf serum and incubation was continued for 6 h. The cells were washed twice with cold PBS and surface-iodinated by incubation at 0 °C with 0.5 ml of PBS containing 0.2 μ M H_2O_2 , 10 μ g of lactoperoxidase and 0.6 mCi of [125 I]iodine for 15 min. The cells were washed twice with cold PBS and then incubated at 0 °C with 0.5 ml of PBS containing 20 μ l of rabbit pre-immune serum, 20 μ l of polyclonal anti-hyaluronate synthase, 20 μ l of anti-CD44 or 20 μ l of anti-hyaluronate receptor for 1 h. The solution was removed, the cells were washed twice with cold PBS and suspended in 0.5 ml of cold PBS containing 0.5 % Triton X-114. The suspension was centrifuged for 1 min at 10000 *g* and the supernatant was brought to 37 °C in a water-bath. The turbid solution was centrifuged for 1 min at 10000 *g* and the upper aqueous phase was removed from the lower detergent phase. The detergent phase was diluted with 0.5 ml of cold PBS. A suspension of protein A-Sepharose (1:1, 50 μ l) in PBS was added to both phases and the suspensions were rotated at 4 °C for 1 h. Protein A-Sepharose was removed by centrifugation at 1000 *g* for 30 s, washed twice with PBS, boiled in disintegration buffer and the supernatant was applied to a 10 % SDS/polyacrylamide gel. Radioactive bands were visualized by autoradiography.

Detergent extraction

Crude membranes were prepared as described (Brunette and Till, 1971) and resuspended to a final concentration of 5 mg/ml protein in PBS containing 1 % (w/v) digitonin, 1 μ g of DNase, 1 μ g of RNAase, 0.1 % aprotinin, and 10 % (w/v) glycerol. After 1 h at 4 °C the extract was subjected to ultracentrifugation at 100000 *g* for 30 min. The supernatant was subjected to affinity chromatography.

Affinity chromatography by polyclonal anti-hyaluronate synthase

Polyclonal rabbit antibodies were purified on protein A-Sepharose CL4B and coupled with CNBr-activated Sepharose 4B. The supernatant of digitonin extracts was incubated with 2 ml of affinity matrix for 1 h at 4 °C, unbound proteins were removed by washing the matrix with 10 ml of extraction buffer and bound antigen was eluted with 0.1 M sodium acetate, pH 2.5, containing 0.1 % digitonin. The pH value of the eluate was adjusted to 7.4 by adding 1 M $NaHCO_3$.

Production and characterization of monoclonal anti-hyaluronate synthase

Monoclonal antibodies were prepared against the streptococcal hyaluronate synthase by injecting mice with 125 μ g of synthase in 100 μ l of PBS four times over a 6-week period. Four days before cell fusion, the mice were primed using 100 μ g of synthase. The spleen was then removed and hybridomas were prepared by fusing spleen cells with X63-Ag8 myeloma cells using 50 % (w/v) polyethylene glycol. Cells were plated on to peritoneal macrophage feeder layers in HAT medium. Positive colonies were cloned three to four times by limiting dilution, and synthase binding was assessed with an e.l.i.s.a. (Engvall, 1980). Clones were injected into mice and ascites obtained were again tested for a positive reaction to the synthase by e.l.i.s.a.

E.I.I.s.a. on intact B6 cells

The reaction of the monoclonal antibodies to the cell surface of intact B6 cells was measured by the procedure of Heusser et al. (1981) with the following modification. B6 cells were washed

with PBS, resuspended in Dulbecco's medium containing 5 % (v/v) foetal calf serum, and seeded at a density of 10^6 cells/ml into poly-L-lysine-coated microtiter plates (NUNC-Immuno Plate Maxisorp). After 6 h at 37 °C cells were sedimented by centrifugation for 5 min at 1000 *g* on to the bottom of the microtiter plates, medium was removed and cells were fixed with PBS containing 0.25 % glutaraldehyde for 5 min. Additional steps were done as described (Heusser et al., 1981).

Affinity chromatography by monoclonal anti-hyaluronate synthase

The IgM antibody was purified from ascites by gel filtration on Sephacryl S-300 and 1 mg was biotinylated as described (Harlow and Lane, 1988). The biotinylated antibody was incubated with 5 mg of a Triton X-100 extract of B6 membranes at 0 °C for 24 h. The solution was shaken at 4 °C with 500 μ l of a suspension of avidin-agarose (1:1, 38.4 units/ml) for 1 h. The agarose beads were separated by centrifugation at 16000 *g* for 5 min and washed 10 times with PBS containing 0.1 % Emulphogen. Bound proteins were eluted four times with 500 μ l of 100 mM glycine/HCl, pH 2.5. The eluted proteins were precipitated by the method of Wessel and Flügge (1984) and analysed by SDS/PAGE.

RESULTS

Characterization of the eukaryotic hyaluronate synthase

B6 cells were used for isolation of the hyaluronate synthase, because they produced large amounts of hyaluronate in suspension culture (Prehm, 1984). The cells were grown to the stationary phase and supplemented with an additional 5 % (v/v) foetal calf serum to stimulate the induction of the synthase. Plasma membranes were prepared, extracted with Triton X-100 and proteins were separated on a polyacrylamide gel. Western blotting with antibodies raised against the bacterial synthase stained a protein at 52 kDa (Figure 1).

The rabbit antiserum raised against the bacterial synthase was

Figure 1 Western blot of B6 membrane extract with anti-hyaluronate synthase

Membrane proteins from B6 cells were separated on a 10 % (w/v) polyacrylamide gel, transferred to nitrocellulose and stained with antibodies against the bacterial hyaluronate synthase.

Figure 2 Affinity purification of B6 membrane extract on immobilized anti-hyaluronate synthase

Membrane proteins from B6 cells were extracted with digitonin and adsorbed to anti-hyaluronate synthase coupled to Sepharose CL 4B. The adsorbed proteins were eluted and separated on a 10% (w/v) polyacrylamide gel. Lane A, digitonin extract; lane B, unadsorbed proteins; lane C, adsorbed proteins.

Figure 3 Determination of molecular mass by sucrose-velocity centrifugation

Purified hyaluronate synthase was subjected to sucrose-velocity centrifugation as described in the Materials and methods section. Fractions were collected and tested for binding of UDP-[¹⁴C]GlcA. Reference proteins [(1) apoferritin, 400 kDa; (2) BSA, 67 kDa; (3) ovalbumin, 45 kDa; (4) carbonic anhydrase, 29 kDa] were centrifuged on parallel gradients.

used to purify the B6-cell synthase using affinity chromatography. Specific antibodies were isolated from the antiserum by affinity chromatography on the bacterial synthase coupled to Sepharose. A digitonin extract of B6 membranes was loaded on to the

affinity-purified antibodies coupled to Sepharose. Bound proteins were desorbed by decreasing the pH to 2.5 and analysed by SDS/PAGE. Two proteins were separated with molecular masses of 60 kDa and 52 kDa (Figure 2). The antibodies thus recognized again the 52 kDa protein which appeared to be linked to the 60 kDa protein. This preparation was tested for synthase activity by incubation with the nucleotide sugars UDP-GlcNAc and UDP-[¹⁴C]GlcA as described earlier (Prehm 1983). There was no time-dependent increase of radioactivity into labelled hyaluronate, but the preparation bound 85000 d.p.m. UDP-[¹⁴C]GlcA (specific activity 0.3 mCi/mmol) per μ g of protein, corresponding to 2 mol of UDP-[¹⁴C]GlcA/mol of 60 kDa and 52 kDa complex. In a similar experiment the complex bound 1 mol of UDP-[³H]GlcNAc. This result indicated that the isolated complex had lost the enzyme activity of hyaluronate-chain elongation, but retained the property of nucleotide-sugar binding. From 5 mg of plasma membranes 48 μ g of purified protein complex was recovered from the affinity column.

The molecular mass of the complex was determined by sucrose-velocity centrifugation of a digitonin extract from plasma membranes (Figure 3). The fractions were incubated with radioactive substrate for hyaluronate synthesis. Incorporation of radioactivity occurred at a molecular mass of 110 kDa, but again the fraction did not synthesize hyaluronate. Since this was the only fraction which was labelled, we assume that the radioactive [¹⁴C]UDP-GlcA precursor was adsorbed by the hyaluronate synthase complex. Thus the complex is a dimer consisting of two subunits, the 52 kDa and 60 kDa proteins.

Extraction of the synthase from plasma membranes

The bacterial synthase had been enriched from protoplast membranes by extraction with Triton X-114. Membrane proteins passed into the detergent phase, except for the synthase which remained in the aqueous phase, because it was bound to nascent hyaluronate. We utilized this property in order to extract proteins from B6 plasma membranes. The proteins in the aqueous phase were analysed by PAGE. Figure 4 shows prominent bands at 110 kDa, 85 kDa, 60 kDa and 52 kDa, indicating that these membrane proteins have an affinity for hyaluronate. Also this preparation did not have any synthase activity.

Identification of the subunit proteins in the hyaluronate synthase complex

The Triton X-114 extraction procedure would not only enrich the synthase, but also cell-surface receptors for hyaluronate. Two different receptors have been described on fibroblasts, the CD44 protein (Miyake et al., 1990) and a 60 kDa receptor (Turley et al., 1987). Monoclonal antibodies are available against the receptors, and they were used to identify the proteins in the Triton X-114 extract. In Western blots only the monoclonal antibody raised against CD44 reacted with an 85 kDa protein from B6 plasma membranes, but the monoclonal antibody raised against the 60 kDa receptor failed to detect an antigen (results not shown). Therefore proteins were immunoprecipitated from cells which had been radiolabelled with [¹²⁵I]iodine and lactoperoxidase on the cell surface. The antibodies against the bacterial synthase and against the 60 kDa receptor both precipitated two proteins of molecular mass 52 kDa and 60 kDa. Only background staining was observed with rabbit pre-immune serum and anti-CD44 (Figure 5). No proteins were precipitated from the detergent phase (results not shown). This result indicated that the cells contained a protein complex on the cell surface consisting of two subunits with molecular masses of 52 kDa and 60 kDa,

Figure 4 Triton X-114 extraction of B6 membranes

Membranes from B6 cells were incubated with substrate for hyaluronate synthesis and extracted with Triton X-114. The extract was subjected to phase separation and the proteins in the aqueous phase were analysed by PAGE.

Figure 5 Analysis of cell-surface-labelled proteins

Cells were surface-labelled with ^{125}I and lactoperoxidase and incubated with antibodies: lane A, pre-immune serum; lane B, anti-hyaluronate synthase; lane C, anti-CD44; and lane D, anti-hyaluronate receptor p60. Unadsorbed antibodies were washed off and the cells were extracted with Triton X-114. The extract was subjected to phase separation and the aqueous phase was analysed by PAGE.

which passed, together with hyaluronate, into the aqueous phase of the Triton X-114 extract. These proteins were recognized by antibodies raised against the bacterial synthase as well as by antibodies against the 60 kDa receptor. In addition the result showed

Figure 6 E.I.I.s.a. of monoclonal antibodies on intact cells

The binding of monoclonal antibodies raised against the streptococcal hyaluronate synthase to the surface of B6 cells was measured as described in the Materials and methods section.

Figure 7 Affinity purification of the synthase by monoclonal anti-hyaluronate synthase

Membranes from B6 cells were extracted with Emulphogen. The extract was incubated with biotinylated monoclonal anti-hyaluronate synthase. The antibodies were adsorbed to avidin-agarose, bound antigens were eluted and analysed by PAGE as described in the Materials and methods section.

that the 52 kDa synthase was labelled and recognized by the antibodies on intact cells and must therefore contain an extracellular domain.

Preparative isolation of the hyaluronate synthase

In preliminary experiments we found that extensive chromatographic procedures were inconvenient for the purification of

larger amounts of the synthase, because of massive losses during each purification step. Affinity purification on immobilized antibodies promised to be an applicable approach; however, the polyspecific antiserum raised against the bacterial synthase contained only limited amounts of cross-reactive antibodies against the B6-cell synthase. Therefore we produced a monoclonal antibody against the bacterial synthase. The hybridoma clones were screened for their reactivity towards B6 plasma membranes with an e.l.i.s.a. We isolated a clone producing a monoclonal IgM antibody which recognized B6 membranes in dot blots. The antibody stained the cell surface of intact B6 cells, as determined by an e.l.i.s.a. reaction (Figure 6). The antibody was purified by gel filtration, biotinylated and adsorbed to a Triton X-114 extract of B6 membranes. The extract was passed over an avidin-agarose column, bound proteins were eluted with an acidic buffer, and analysed by PAGE. Figure 7 shows that the monoclonal antibody purified a single protein with a molecular mass of 52 kDa. This preparation was unable to bind the nucleotide sugars UDP-[¹⁴C]GlcA or UDP-[³H]GlcNAc. We succeeded in purifying about 50 µg of p52 from 10 mg of plasma membranes.

DISCUSSION

The identification of the synthase from a eukaryotic cell line had to rely on indirect methods, because the enzymic activity was lost upon dissolution of plasma membranes. Several criteria indicated that a 52 kDa protein was the synthase. This protein cross-reacted with polyclonal and monoclonal antibodies raised against the streptococcal synthase, it partitioned into the aqueous phase together with nascent hyaluronate on Triton X-114 separation, and it adsorbed equimolar amounts of the precursor nucleotide sugars. However, it did not incorporate the sugar into hyaluronate. Since Mian (1986a,b) and Ng and Schwartz (1989) did not assay hyaluronate chain elongation in a time-dependent manner, they might have erroneously concluded that their solubilized synthase was enzymically active.

A complex of 52 kDa and 60 kDa proteins was isolated by affinity chromatography from digitonin-extracted membranes. This complex bound UDP-[¹⁴C]GlcA and UDP-[³H]GlcNAc and had an apparent molecular mass of 110 kDa as determined by sucrose-velocity centrifugation.

When membranes were extracted with Triton X-100 instead of digitonin and subjected to affinity chromatography only the 52 kDa subunit was isolated, indicating that the 52 kDa and 60 kDa subunits were separated by stronger detergents. It appears that the separation of the 52 kDa and 60 kDa subunits depended

on the type of detergent and the duration of exposure. Extraction with digitonin or Triton X-114 within several hours left the complex intact, whereas extraction and purification with Triton X-100 dissociated the subunits. The synthase complex was recovered from B6 plasma membranes in high yields (1% of membrane proteins).

Both subunits appeared to have extracellular domains, because they could be iodinated on intact cells. The radiolabelled complex was precipitated by antibodies raised against the streptococcal synthase, as well as by a monoclonal antibody against a hyaluronate receptor. Therefore the hyaluronate synthase complex consisted of two subunits containing the synthase and the receptor.

The close association of the synthase and the receptor is significant regarding their functions. The synthase was required for fibroblast detachment during mitosis (Brecht et al., 1986) and the receptor was involved in fibroblast migration (Turley et al., 1987, 1991). The receptor was localized in the leading lamella and the retraction process of migrating fibroblasts (Turley and Torrance, 1984). It is therefore likely that the complex is responsible for cell detachment in mitosis as well as in migration.

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