Biochem. J. (1986) 237, 343–357 (Printed in Great Britain)

Characterization of a high-M_r plasma-membrane-bound protein and assessment of its role as a constituent of hyaluronate synthase complex

Nasi MIAN
Department of Physiology, St. George's Hospital Medical School, Cranmer Terrace, Tooting, London SW17 0RE, U.K.

A high-M_r phosphoprotein (M_r 442000) was purified from Nonidet-P-40-solubilized plasma membranes of cultured human skin fibroblasts. The protein comprised one 200000-M_r subunit consisting of 116000- and 84000-M_r polypeptides and two identical 121000-M_r subunits each consisting of 66000- and 55000-M_r polypeptides. The 200000-M_r subunit and its polypeptides contained phosphotyrosine residues and were also [^{32}P]phosphorylated at these residues from [γ-^{32}P]ATP *in vitro* by an intrinsic tyrosine kinase activity of the protein molecule in response to the presence of hyaluronate precursors, UDP-glucuronic acid and UDP-*N*-acetylglucosamine. The 121000-M_r subunits and their polypeptides contained phosphoserine residues that could not be [^{32}P]phosphorylated during autophosphorylation of the protein *in vitro*. The protein molecules separated from exponential- and stationary-growth-phase cells were identical in their quaternary structure, but appeared to exist in different proportions with respect to the state of phosphorylation of their 121000-M_r subunits during different growth phases of the cell. Phosphorylation of polypeptides appeared to predispose in favour of their UDP-glucuronic acid- and UDP-*N*-acetylglucosamine-binding activities. The phosphorylated 116000- and 84000-M_r polypeptides of 200000-M_r subunits possessed a single binding site for UDP-glucuronic acid and UDP-*N*-acetylglucosamine respectively. The phosphorylated 200000-M_r subunit could also cleave the UDP moiety from UDP-glucuronic acid and UDP-*N*-acetylglucosamine precursors. The phosphorylated 121000-M_r subunit possessed two binding sites with equal affinity towards UDP-glucuronic acid and UDP-*N*-acetylglucosamine but did not possess UDP-moiety-cleavage activity. The phosphorylation of 200000-M_r subunit by an intrinsic kinase activity of the protein molecule appeared to elicit its oligosaccharide-synthesizing activity, whereas phosphorylation of 121000-M_r subunits, presumably carried out *in vivo*, abolished this activity of the protein molecule. The oligosaccharides synthesized by the protein were about M_r 5000 and about 12 disaccharide units in length. Neither nucleotide sugars nor glycosyl residues nor newly synthesized oligosaccharides were bound covalently to the protein molecule. The UDP moiety of nucleotide sugar precursors did not constitute a link between protein molecule and oligosaccharide during its synthesis. Although isolated 442000-M_r protein did not synthesize high-M_r hyaluronate *in vitro*, this protein molecule can be considered as a constituent of membrane-bound hyaluronate synthase complex because of its observed properties.

INTRODUCTION

Hyaluronate, the only non-sulphated glycosaminoglycan, has a wide distribution in both vertebrate and invertebrate connective tissues and is also present in the capsule of some bacteria such as staphylococci and group A and C streptococci. The biosynthesis of hyaluronate has been studied in some detail in streptococci (Markovitz *et al.*, 1959; Stoolmiller & Dorfman, 1969; Sugahara *et al.*, 1979), but the mechanism is imperfectly understood (Roden & Horowitz, 1978; Kleine, 1981). The mechanism of hyaluronate synthesis does not follow the same laws as that of other known glycosaminoglycans (Roden & Horowitz, 1978; Kleine, 1981). Attempts to influence hyaluronate synthesis by chemicals that affect the synthesis of other glycosaminoglycans have been unsuccessful (Hart & Lennarz, 1978; Hopwood & Dorfman, 1977; Mapleson & Buchwald, 1981). Although hyaluronate-synthesizing activity has been found to reside exclusively in the plasma membrane of the mammalian cell (Tomida *et al.*, 1975; Appel *et al.*, 1979; Prehm, 1983*a*, 1984; Mian, 1986), attempts to extract hyaluronate synthase from the plasma-membrane fractions have not been successful as yet.

As described in the preceding paper, a high-M_r protein, isolated from plasma membranes of exponentially growing cultured human skin fibroblasts, which is autophosphorylated from [γ-^{32}P]ATP in the presence of UDP-glucuronic acid and UDP-*N*-acetylglucosamine *in vitro*, exhibits an efficient nucleotide-sugar-binding activity and synthesizes oligosaccharides of about 12 repeating disaccharide units of these sugar residues (Mian, 1986). On the other hand, the corresponding protein from plasma membranes of stationary-growth-phase cells, which is also autophosphorylated and exhibits a much higher UDP-glucuronic acid- and UDP-*N*-acetylglucosamine-binding activity, does not synthesize oligosaccharides (Mian, 1986).

The unique but hyaluronate-synthesis-related activities of this plasma-membrane protein led me to characterize it in detail in order to assess its role as a component of hyaluronate synthase enzyme complex and to investigate the mechanism(s) of regulation of hyaluronate synthesis

during exponential and stationary growth phases of the cell.

MATERIALS AND METHODS

Materials

[5-^{3}H]UDP (30 Ci/mmol) and [γ-[^{35}S]thio]ATP (> 50 Ci/mmol) were obtained from Amersham International, Amersham, Bucks., U.K. Inorganic pyrophosphatase (EC 3.6.1.1) from yeast (500 units/mg), UDP-glucose dehydrogenase (EC 1.1.1.22) from bovine liver (0.6 units/mg) and yeast enzyme concentrate type II were purchased from Sigma Chemical Co., Poole, Dorset, U.K. Other materials were as described in the preceding paper (Mian, 1986).

DEAE-Sephacel, activated-thiol–Sepharose 4B and epoxy-activated Sepharose 4B were obtained from Pharmacia, Milton Keynes, Bucks., U.K. UDP-[^{14}C]-glucuronic acid and UDP-*N*-acetyl[^{3}H]glucosamine in 1:1 ratio were coupled to epoxy-activated Sepharose 4B in accordance with the manufacturer's instructions. The finally prepared resin contained about 48 μmol of UDP-[^{14}C]glucuronic acid and 55 μmol of UDP-*N*-acetyl[^{3}H]glucosamine coupled/g of conjugate.

Synthesis of [5-^{3}H]UDP-glucuronic acid and [5-^{3}H]UDP-*N*-acetylglucosamine

[5-^{3}H]UDP-glucuronic acid and [5-^{3}H]UDP-*N*-acetylglucosamine were synthesized enzymically from [5-^{3}H]UTP and glucose 1-phosphate and *N*-acetylglucosamine 1-phosphate respectively by modified procedures of Strominger *et al.* (1957) and Rao & Mendicino (1978) as described by Prehm (1983*b*). The final products were separated by t.l.c. The specific radioactivities of [5-^{3}H]UDP-glucuronic acid and [5-^{3}H]UDP-*N*-acetylglucosamine were about 1.18 and 1.33 mCi/mmol respectively.

Purification and structural analysis of high-M_r plasma-membrane protein

Human skin fibroblasts were cultured and plasma membranes were fractionated as described previously (Mian, 1986). The details of solubilization of plasma membranes and purification of high-M_r plasma-membrane protein are described in the Results section. The purified protein samples were dissociated and reduced by treatment with 6 M-urea and 100 mM-2-mercaptoethanol before their separation into individual subunits and polypeptides by gel filtration on Sepharose CL-4B. SDS/polyacrylamide-gel electrophoresis of isolated subunits and polypeptide was performed by the method of Laemmli (1970) under reducing and non-reducing conditions. The M_r values of samples on gel filtration and SDS/polyacrylamide-gel electrophoresis were estimated with reference to the standard proteins α-lactalbumin (M_r 14200), carbonic anhydrase (M_r 29000), ovalbumin (M_r 45000), bovine serum albumin (M_r 66000), rabbit muscle phosphorylase *b* (M_r 97400), *Escherichia coli* β-galactosidase (M_r 116000), rabbit muscle myosin (M_r 205000) and apoferritin monomer (M_r 450000), all obtained from Sigma Chemical Co.

Analysis of phospho amino acid residues

The protein samples were hydrolysed in 2 M-HCl at 100 °C for 6 h in sealed tubes. The hydrolysates were freeze-dried, then taken up in small volume of water and subjected to high-voltage electrophoresis at pH 1.9 (acetic acid/formic acid/water, 2:1:25, by vol.). The electrophoretograms were developed as described by Wade & Morgan (1953). Phosphoserine, phosphotyrosine and phosphothreonine were used as standard markers.

Determination of protein-bound phosphate

Protein-bound phosphate was determined as 'alkali-labile phosphate' by the following procedure. The protein sample was mixed with equal amounts of 1 M-$HClO_4$ and centrifuged at 25000 *g* for 10 min. The pellet was washed three times by suspension in 0.5 M-$HClO_4$, once in ethanol/diethyl ether (1:1, v/v) and once in chloroform/methanol (2:1, v/v). The pellet was then suspended in 5% (w/v) trichloroacetic acid, heated at 100 °C for 10 min and centrifuged. The pellet was washed once in ethanol/diethyl ether (1:1, v/v), to remove acid. The resulting pellet was then suspended (at a concentration of about 1 mg/ml) in 1 M-NaOH and heated at 100 °C for 12 min. Blanks, containing alkali but no protein, and standards, containing alkali and known quantities of KH_2PO_4, were incubated at the same time. The sample was mixed with equal volumes of 1 M-$HClO_4$ and cooled at 0 °C. The amount of phosphate was measured by the method of Shatton *et al.* (1983).

Assay of autophosphorylation activity

Protein samples were incubated under appropriate reaction conditions in the presence of [γ-^{32}P]ATP. Trichloroacetic acid-insoluble phosphorylated protein was separated from [γ-^{32}P]ATP in the reaction mixture by filtration and paper chromatography (Li & Felmly, 1973) with Whatman ET paper. The control samples were prepared with protein samples boiled at 100 °C for 2 min or by adding [γ-^{32}P]ATP after trichloroacetic acid precipitation of the protein sample.

Assay of UDP-glucuronic acid and UDP-*N*-acetylglucosamine binding to 442000-M_r protein and its subunits and polypeptides and determination of $B_{max.}$ and K_d values and number of binding sites (n)

The protein samples were incubated under appropriate conditions in the presence of UDP-[^{14}C]glucuronic acid and/or UDP-*N*-acetyl[^{3}H]glucosamine where concentrations of protein as well as of radiolabelled nucleotide sugars were varied depending on nature of the experiment. The controls were prepared by boiling protein samples for 2 min before the addition of nucleotide sugars. Portions of test and control samples were withdrawn and either centrifuged or filtered to separate protein from rest of the reaction mixture. To check the non-covalent and reversible nature of binding, protein samples were washed twice with buffer solutions free of radiolabelled nucleotide sugar substrates. The bound ligand ($[S]_b$), i.e. the amount of radioactivity bound to the protein samples, was determined by subtracting radioactivity of controls from those of test samples.

The ratios of bound to free ligand ($[S]_b/[S]_f$) and mol of ligand bound/mol of protein ($[S]_b/[S]_f \cdot [P]_t$) were calculated and plotted against $[S]_b$ and ($[S]_b/[P]_t$) as:

$$\frac{[S]_b}{[S]_f} = -\frac{1}{K_d} \cdot [S]_b + n\frac{[P]_t}{K_d} \quad (1)$$

Table 1. UDP-[^{14}C]glucuronic acid- and UDP-*N*-acetyl[^{3}H]glucosamine-binding activities of high-M_r protein (M_r 450000) separated from plasma-membrane fractions solubilized with different detergents

'*In-vitro*'-^{32}P-phosphorylated plasma-membrane fractions of exponentially growing cells were solubilized with a number of detergents as listed below. The high-M_r protein (M_r 450000) was separated by gel filtration on Sepharose CL-4B columns and nucleotide-sugar-binding assays were carried out as described in the Materials and methods section. The values given are means ± S.E.M. for three experiments.

Detergent used to solubilize plasma membranes	^{14}C radioactivity from UDP-[^{14}C]GlcA (d.p.m.)	^{3}H radioactivity from UDP-[^{3}H]GlcNAc (d.p.m.)
SDS (0.2%)	$1.62 \times 10^2 \pm 0.24 \times 10^2$	$1.49 \times 10^2 \pm 0.36 \times 10^2$
Triton X-100 (0.5%)	$1.36 \times 10^2 \pm 0.28 \times 10^2$	$1.66 \times 10^2 \pm 0.48 \times 10^2$
Sodium deoxycholate (0.5%)	$1.85 \times 10^2 \pm 0.56 \times 10^2$	$1.95 \times 10^2 \pm 0.72 \times 10^2$
Nonidet P-40 (0.5%)	$1.35 \times 10^5 \pm 0.06 \times 10^5$	$1.56 \times 10^5 \pm 0.07 \times 10^5$

$$\frac{[S]_b}{[S]_f \cdot [P]_t} = -\frac{1}{K_d} \cdot \frac{[S]_b}{[P]_t} + \frac{n}{K_d} \quad (2)$$

The Scatchard plots of $[S]_b/[S]_f - [S]_b$ and $[S]_b/[S]_f \cdot [P]_t - [S]_b/[P]_t$ were linear with a slope of $-1/K_d$. The $B_{max.}$ values and number of ligand binding sites (n) per molecule of protein were determined from the intercepts on the horizontal $[S]_b$ and $[S]_b/[P]_t$ axes of these plots.

Analysis of reaction products and assay of oligosaccharide-synthesizing activity

Depending on the nature of the experiment, reaction mixtures were mixed with saturated $(NH_4)_2SO_4$, 10% (w/v) trichloroacetic acid or 2% (w/v) SDS solutions. The supernatant and precipitated fractions were separated by filtration or centrifugation. Appropriate samples were then subjected to chromatographic analysis by ion-exchange chromatography on DEAE-Sephacel (Winterbourne & Mora, 1978), gel filtration on Sepharose CL-4B (Prehm, 1983*b*) and Bio-Gel P-6 (Mian, 1986). Synthesis of high-M_r hyaluronate was examined by paper chromatography (Tomida *et al.*, 1974; Prehm, 1983*a*) or by gel filtration (Prehm, 1983*b*). To check the authenticity of reaction products as high-M_r hyaluronate or low-M_r hyaluronate oligosaccharides, the samples were digested with hyaluronidase and/or with β-*N*-acetylglucosaminidase and β-glucuronidase (Longas & Meyer, 1981) before their chromatographic analysis. All other methods used were as described previously (Mian, 1986).

RESULTS

Extraction and purification of 442000-M_r protein

The '*in -vitro*'^{32}P-labelled plasma membranes (Mian, 1986) were solubilized with various detergents and subjected to gel filtration on Sepharose CL-4B for the separation of high-M_r plasma-membrane protein. UDP-[^{14}C]glucuronic acid- and UDP-*N*-acetyl[^{3}H]glucosamine-binding assays were used to monitor the biological activity of the protein. The high-M_r ^{32}P-labelled protein (M_r 450000) separated from plasma membranes solubilized in SDS, Triton X-100 or sodium deoxycholate appeared to have lost its nucleotide-sugar-binding activity (Table 1). Only solubilization with Nonidet P-40 was found to produce a high-M_r protein that retained efficient nucleotide-sugar-binding activity (Table 1). The high-M_r protein initially separated by gel filtration on Sepharose CL-4B (Fig. 1*a*) was rechromatographed on the same columns, where it was eluted as a single sharp peak. The elution profiles of the protein extracted from plasma membranes of exponential- and stationary-growth-phase cells were similar (results not shown).

In some experiments ^{32}P-labelled 450000-M_r protein of exponential-growth-phase cells fractionated by repeated gel filtration on Sepharose CL-4B was subjected to ion-exchange chromatography on DEAE-Sephacel equilibrated with 50 mM-Tris/HCl buffer, pH 7.1. The ^{32}P-labelled 450000-M_r protein was eluted as a single sharp peak between 0.26 M- and 0.3 M-NaCl on the application of linear gradient (0–0.4 M) of NaCl made up in the same buffer (Fig. 1*b*). Repeated gel filtration and DEAE-Sephacel chromatography increased specific ^{32}P radioactivity of finally fractionated 450000-M_r protein by about 30-fold (6.25×10^4 d.p.m./mg of protein) compared with the initial specific ^{32}P radioactivity of the 450000-M_r peak (0.21×10^4 d.p.m./mg of protein).

Since the protein exhibited efficient UDP-glucuronic acid- and UDP-*N*-acetylglucosamine-binding activity and it also contained intramolecular disulphide bonds, further purification of this protein was also performed by affinity chromatography on activated-thiol–Sepharose 4B and on affinity columns prepared by coupling [^{14}C]glucuronic acid and UDP-*N*-acetyl[^{3}H]glucosamine in equimolar ratios to epoxy-activated Sepharose 6B. Affinity chromatography on activated-thiol–Sepharose 4B with dithiothreitol as eluting agent resulted in decrease of its M_r by about 100000–120000 and a decrease in UDP-[^{14}C]glucuronic acid-binding activity from about 1.34×10^5 to 0.76×10^5 d.p.m./mg of protein. It appears that the elution step, which involves reduction of disulphide bonds, perhaps cleaved 66000- and 55000-M_r polypeptides from the protein molecule, as depicted in Fig. 4. The affinity chromatography on columns prepared by coupling UDP-glucuronic acid/UDP-*N*-acetylglucosamine on epoxy-activated Sepharose 6B was also not very effective because about 70% of biologically active ^{32}P-labelled proteins could not be adsorbed on the column. It can be assumed that the binding site(s) on the protein molecule that otherwise recognize hydroxy groups of these sugar residues failed to do so because of their coupling to the gel.

Although it can be argued that simple purification steps such as repeated filtration on Sepharose CL-4B and ion-exchange chromatography on DEAE-Sephacel may

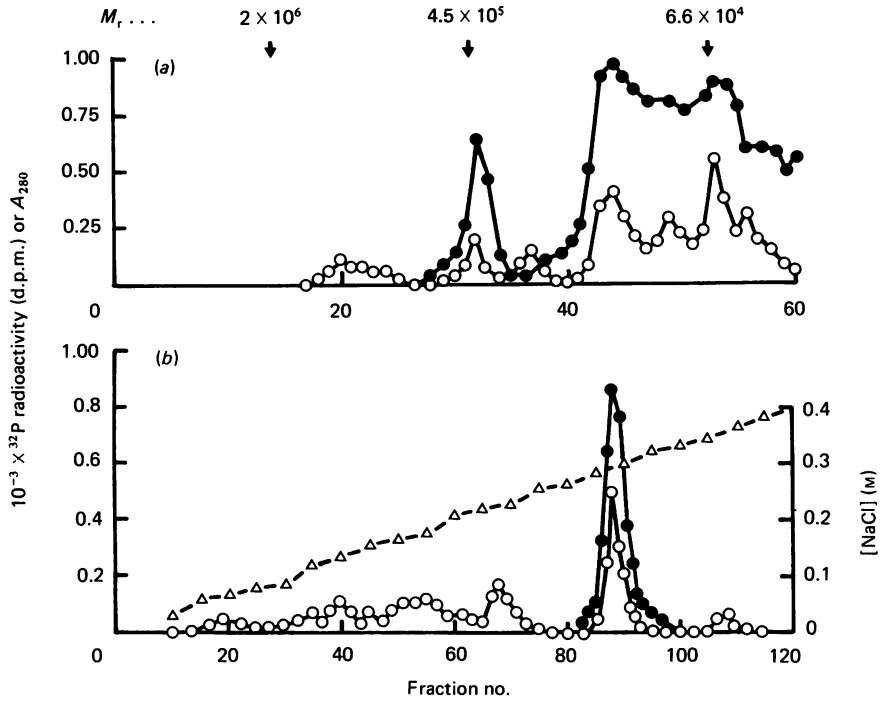

Fig. 1. Fractionation and structural analysis of 442000-M_r protein

The plasma-membrane samples of exponentially growing cells (24 h-old cell culture) were incubated for 5 min in 50 mM-Tris/HCl buffer, pH 7.1, containing 5 mM-ATP, 0.1 μCi of [γ-^{32}P]ATP, 5 mM-Mg^{2+}, 10 μM-UDP-glucuronic acid and 10 μM UDP-*N*-acetylglucosamine. The reaction mixture was centrifuged at 10000 *g* for 10 min and the resulting pellet of plasma membrane was separated and solubilized in 50 mM-Tris/HCl buffer, pH 7.1, containing 0.5% Nonidet P-40. The sample was subjected to gel filtration on a Sepharose CL-4B column, and fractions (27–35) representing high-M_r ^{32}P-labelled protein were separated (*a*). The pooled fractions were dialysed and concentrated before ion-exchange chromatography on DEAE-Sephacel (*b*). The sample was dissolved in 50 mM-Tris/HCl buffer, pH 7.1, and applied to a DEAE-Sephacel column (2.5 cm × 35 cm) equilibrated with the same buffer. The column was eluted with a linear gradient of 0–0.4 M-NaCl made up in the same buffer; 2 ml fractions were collected at a flow rate of 15 ml/h. ^{32}P-labelled 45000-M_r protein was eluted at 0.26–0.3 M-NaCl. ●, ^{32}P radioactivity; ○, A_{280}; △, concn. of NaCl.

not guarantee a high degree of purification, the homogeneity of subunits and polypeptide constituents of the protein samples as shown by gel filtration and SDS/polyacrylamide-gel electrophoresis suggests that the 450000-M_r fraction was a single protein. The other ^{32}P-labelled plasma-membrane proteins, with M_r 27000, 39000, 46000, 62000, 100000 and 200000, did not possess any UDP-[^{14}C]glucuronic acid- and UDP-*N*-acetyl[^{3}H]glucosamine-binding activities.

Another interesting feature of 450000-M_r plasma-membrane-bound protein was that it was only autophosphorylated in the presence of hyaluronate precursors. The preincubation of plasma membranes in 50 mM-Hepes buffer, pH 7.1, containing [γ-[^{35}S]thio]ATP, which caused ^{35}S-labelling of its 27000-, 39000-, 46000-, 62000-, 10000- and 210000-M_r protein components, as a result of autophosphorylation reaction, did not ^{35}S-label the 450000-M_r protein component. However, when this ^{35}S-labelled plasma-membrane sample was re-incubated with 50 mM-Hepes buffer, pH 7.1, containing [γ-^{32}P]ATP and hyaluronate precursors, only 450000-M_r protein component was ^{32}P-labelled as a result of hyaluronate-precursor-induced autophosphorylation reaction.

Structural analysis

The protein eluted from DEAE-Sephacel column was rechromatographed on Separose CL-4B (Fig. 2*a*) before its structural analysis. The protein could be dissociated into two subunits of approx. M_r 200000 and 121000 by treatment with 6 M-urea (Fig. 2*b*), which could be further cleaved by reduction with 2-mercaptoethanol into 116000- and 84000-M_r polypeptides and 66000- and 55000-M_r polypeptides respectively (Figs. 2*c* and 2*d*). The reduction of native protein with 2-mercaptoethanol alone produced polypeptides of approx. M_r 320000, 66000 and 55000. From the apparent M_r values of undissociated protein and of its subunits it can be deduced that the native protein molecule comprised one subunit of approx. M_r 200000 and two subunits of approx. M_r 121000. Since no other small polypeptide was produced during the dissociation and reduction steps, as indicated by gel filtration and SDS/polyacrylamide-gel electrophoresis of the samples (Figs. 2 and 3), it can be assumed that the M_r of the protein was about 442000. From the dissociation and reductive-cleavage data, the schematic arrangement of subunits and polypeptides within the protein molecule can be depicted as shown in Fig. 4. A comparative analysis of the protein prepared from exponential-and stationary-growth-phase cells showed no difference in its quaternary structure.

Autophosphorylation of 442000-M_r protein and its constituent subunits and polypeptides

The autophosphorylation of isolated 442000-M_r protein from [γ-^{32}P]ATP was strictly dependent on the presence of both UDP-glucuronic acid and UDP-*N*-

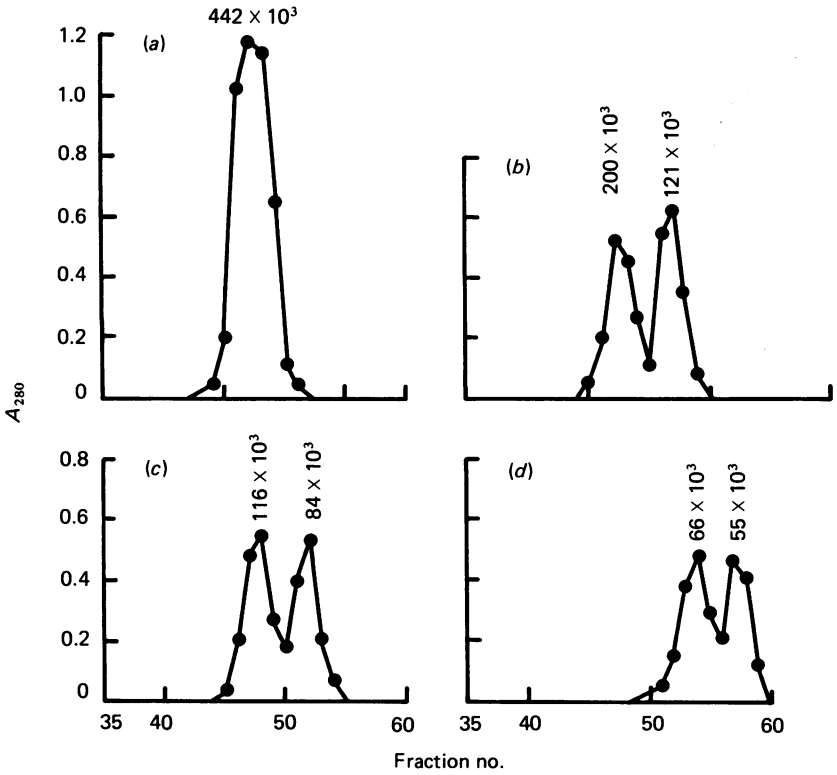

Fig. 2. Dissociation and reductive cleavage of 442000-M_r protein

The protein peak collected from DEAE-Sephacel column was dialysed against distilled water and then concentrated and subjected to a second gel filtration on Sepharose CL-4B, where the protein was eluted as a single sharp peak (*a*). The material was then dissolved in 50 mM-Tris/HCl buffer, pH 7.1, containing 6 M-urea and applied to a Sepharose CL-4B column equilibrated and eluted with the same buffer (*b*). The two peaks, fractions 40–44 and 46–49, representing 200000- and 121000-M_r subunits, were pooled separately and dialysed and concentrated. The resulting materials were dissolved in 50 mM-Tris/HCl buffer, pH 7.1, containing 100 mM-2-mercaptoethanol and applied to Sepharose CL-4B columns equilibrated and eluted with the same buffer. The 200000-M_r subunit was separated into 116000- and 84000-M_r polypeptides, fractions 44–49 and 51–54 (*c*), and the 121000-M_r subunit was separated into 66000- and 55000-M_r polypeptides, fractions 52–55 and 56–60 (*d*).

acetylglucosamine, since the omission of either nucleotide sugar or replacement by glucuronic acid or *N*-acetylglucosamine almost completely abolished autophosphorylation of the protein (Table 2). The autophosphorylation reaction was independent of bivalent cations such as Mg^{2+}, Ca^{2+} and Mn^{2+} (Table 2) and was accomplished within less than 10 s of incubation. Longer incubations did not increase the specific ^{32}P radioactivity of the protein. Preincubation of the protein with ATP for even up to 10 min in the absence of UDP-glucuronic acid and UDP-*N*-acetylglucosamine had no effect on its subsequent rapid autophosphorylation from [γ-^{32}P]ATP in the presence of these nucleotide sugar precursors. On the other hand, a 10 s preincubation of the protein with ATP in the presence of UDP-glucuronic acid and UDP-*N*-acetylglucosamine followed by incubation for even up to 10 min with [γ-^{32}P]ATP in the presence of both nucleotide sugars did not produce any detectable auto[^{32}P]phosphorylation of the protein molecule. These observations suggest that the autophosphorylatable sites were rapidly phosphorylated from unlabelled ATP during the initial 10 s incubation period. Whereas a change in pH of the reaction mixture from 7.1 to 6.5 or to 7.5 decreased ^{32}P-labelling of the protein to less than 5%, a temperature shift from 37 °C to 25 °C had no detectable effect. The addition of cyclic AMP, phospholipids or calmodulin also had no effect on the extent of ^{32}P-labelling of the protein (Table 2). However, no significant differences were observed in specific ^{32}P radioactivities and kinetics of auto[^{32}P]phosphorylation of protein samples prepared from exponential- and stationary-growth-phase cells (Table 2).

The autophosphorylation of 442000-M_r protein was also dependent on the intactness of the molecule, since isolated 200000- and 121000-M_r subunits or their constituent 116000-, 84000-, 66000- and 55000-M_r polypeptides could not be ^{32}P-labelled when incubated with [γ-^{32}P]ATP in the presence of UDP-glucuronic acid and UDP-*N*-acetylglucosamine. The protein, once auto[^{32}P]phosphorylated in the presence of hyaluronate precursors, was not dephosphorylated on the removal of hyaluronate precursors from the incubation medium or washing of the protein samples with 50 mM-Tris/HCl buffer, pH 7.1.

The analysis of isolated subunits and polypeptides revealed that only 200000-M_r subunits (11.8×10^4 d.p.m./mg) and their constituent 116000-M_r (6.8×10^4 d.p.m./mg) and 84000-M_r (4.8×10^4 d.p.m./mg) polypeptides were ^{32}P-labelled. However, no significant difference was observed between protein samples of exponential and stationary-growth-phase cells. The 121000-M_r subunits and their constituent 66000- and 55000-M_r polypeptides were not ^{32}P-labelled as a result of autophosphorylation reaction.

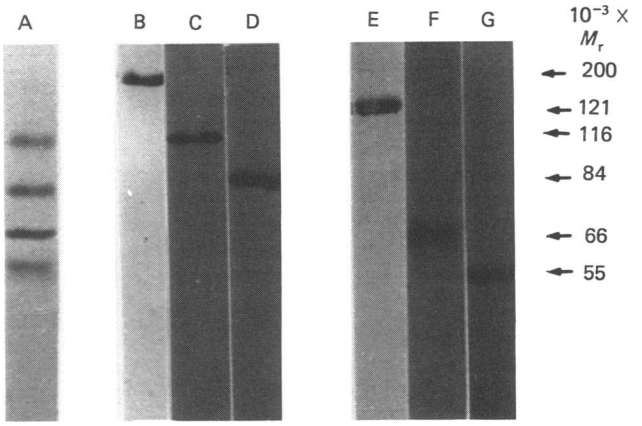

Fig. 3. SDS/polyacrylamide-gel-electrophoretic analysis of 442000-M_r protein and its constituent subunits and polypeptides

The 442000-M_r protein and its constituent subunits and polypeptides isolated by gel filtration after dissociation and reduction of the protein molecule as described in Fig. 2 were subjected to SDS/polyacrylamide-gel electrophoresis in 10% gels. Lane A shows electrophoretic separation of 116000-, 84000-, 66000- and 55000-M_r polypeptides of 442000-M_r protein under reducing conditions in the presence of 6M-urea. Lanes B, C and D show electrophoretic patterns of 200000-M_r subunit and its 116000- and 84000-M_r polypeptides under non-reducing conditions. Lanes E, F and G show electrophoretic patterns of 121000-M_r subunit and its 66000- and 55000-M_r polypeptides under non-reducing conditions.

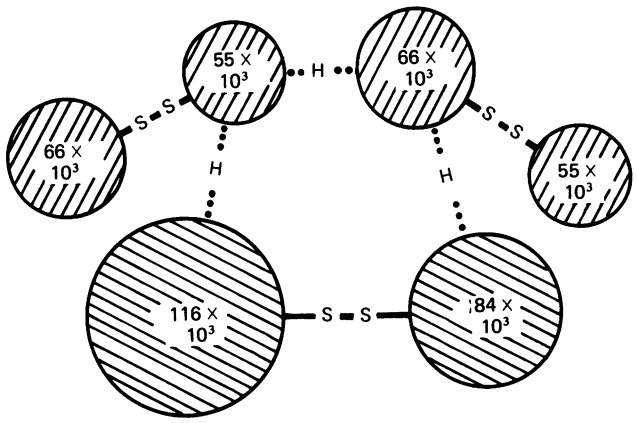

Fig. 4. Schematic arrangement of constituent subunits and polypeptides within 442000-M_r protein molecule

The data on reduction of native protein and of dissociated protein samples suggest that the constituent polypeptides within a subunit are held by disulphide bonds whereas the subunits are held by hydrogen bonds within the protein molecule as depicted.

Analysis of phospho amino acid residues and protein-bound phosphate

In protein samples of both exponential- and stationary-growth-phase cells the ^{32}P-labelled amino acid residues in 200000-M_r subunits and their constituent 116000- and 84000-M_r polypeptides were invariably tyrosine residues. The 121000-M_r subunits and their constituent polypeptides did not possess ^{32}P-labelled amino acid residues, but were found to contain non-^{32}P-labelled phosphoserine residues instead.

A quantitative analysis of alkali-labile protein-bound phosphate showed that, whereas native protein samples from exponential- and stationary-growth-phase cells contained about 2 and 4 mol of phosphate/mol of protein, the autophosphorylation *in vitro* increased their phosphate content to about 4 and 6 mol of phosphate/mol of protein respectively (Table 3). The difference in phosphate content of protein samples from exponential- and stationary-growth-cell-phase cells appeared to be due to the presence of almost double the amount of bound phosphate in 121000-M_r subunits or their polypeptides in stationary-growth-phase cells compared with that of exponential-growth-phase cells (Table 3). On the other hand, the difference between native and '*in-vitro*'-phosphorylated protein molecules was due to about a 10-fold increase in the phosphate content of 200000-M_r subunits or their polypeptides (Table 3). The data can be interpreted to mean that in the case of exponential- and stationary-growth-phase cells about 50% and almost all 442000-M_r protein molecules were phosphorylated at their 121000-M_r subunits respectively. Similarly, whereas in the case of native protein samples only 10% of the protein molecules were phosphorylated at their 200000-M_r subunits, phosphorylation *in vitro* appeared to phosphorylate all protein molecules at their 200000-M_r subunits. From these observations it can be deduced that the 442000-M_r protein molecules perhaps exist in different states with respect to phosphorylation of their constituent subunits or polypeptides during exponential and stationary growth phases of the cell.

Analysis of binding of nucleotide sugars to 442000-M_r protein and its constituent subunits and polypeptides

The UDP-glucuronic acid- and UDP-*N*-acetylglucosamine-binding activities of native protein of stationary-growth-phase cells were about 2-fold those of exponentially growing cells (Table 4). The phosphorylation *in vitro* of protein samples from exponential- and stationary-growth-phase cells increased their nucleotide-sugar-binding activity by about 67% and 44% respectively (Table 4).

The difference between exponential- and stationary-growth-phase cells appeared to be due to about a 2-fold difference in nucleotide-sugar-binding activities of 121000-M_r subunits or their constituent polypeptides (Table 4). On the other hand, the difference between native and '*in-vitro*'-phosphorylated protein samples appeared to be due to a 10-fold increase in nucleotide-sugar-binding activities of 200000-M_r subunits and their constituent polypeptides (Table 4). The data (Table 4) show the further feature that, whereas 116000- and 84000-M_r polypeptides of 200000-M_r subunits exhibited specific affinity for UDP-glucuronic acid and UDP-*N*-acetylglucosamine respectively, no such specificity of binding was observed in the case of 66000- and 55000-M_r polypeptides of 121000-M_r subunits.

The analysis of binding kinetics (Table 5) shows that the K_d values of UDP-glucuronic acid for 116000-M_r polypeptides were slightly higher than those of UDP-*N*-acetylglucosamine for 84000-M_r polypeptides. The K_d values of both nucleotide sugars for 66000- and 55000-M_r polypeptides were almost the same and were more than

Table 2. Effect of various agents on autophosphorylation of 442000-M_r protein

The controls were prepared by incubating 100–200 μg of protein in 25 μl of 50 mM-Tris/HCl buffer, pH 7.1 with 20 μl of the same buffer containing UDP-glucuronic acid and UDP-*N*-acetylglucosamine (50 μM each). The experimental samples were prepared by omitting UDP-glucuronic acid or UDP-*N*-acetylglucosamine or by adding the agents listed below. The volume of all samples was adjusted to 45 μl before the addition of 5 μl of [γ-^{32}P]ATP (0.1 μCi) solution made up in the sample buffer. The blanks were prepared by heating the protein samples at 100 °C for 2 min before the addition of other reagents. After 5 min incubation at 25 °C, 50 μl of 10% (w/v) trichloroacetic acid was added and samples were centrifuged at top speed in a Beckman Microfuge. The resulting pellets were washed twice with 200 μl of 5% trichloroacetic acid. The final pellet was dissolved in 5 ml of Ready-Solv HP/b scintillant before counting of radioactivity. The values given are means ± S.E.M. for four experiments.

Agents added or omitted	10^{-4} × Specific ^{32}P radioactivity of 442000-M_r protein (d.p.m./mg of protein)	
	Isolated from exponential-growth-phase cells (24 h-old cultures)	Isolated from stationary-growth-phase cells (98 h-old cultures)
None (control)	5.86 ± 0.32	5.62 ± 0.27
+ Mg^{2+} (5 mM)	5.72 ± 0.28	5.46 ± 0.36
+ Ca^{2+} (5 mM)	5.81 ± 0.35	5.54 ± 0.42
+ Mn^{2+} (5 mM)	5.38 ± 0.32	5.42 ± 0.25
− UDP-GlcA	0.04 ± 0.02	0.03 ± 0.01
− UDP-GlcNAc	0.06 ± 0.04	0.05 ± 0.03
− UDP-GlcA + GlcA (100 μM)	0.05 ± 0.04	0.03 ± 0.01
− UDP-GlcNAc + GlcNAc (100 μM)	0.04 ± 0.02	0.06 ± 0.02
+ Cyclic AMP (100 μM)	5.85 ± 0.35	5.72 ± 0.29
+ Lysophosphatidylcholine (4 μg/ml)	5.68 ± 0.42	5.48 ± 0.33
+ Lysophosphatidylethanolamine (4 μg/ml)	5.74 ± 0.41	5.55 ± 0.51
+ Calmodulin (100 μg/ml)	5.82 ± 0.39	5.75 ± 0.47
+ Calmodulin (100 μg/ml) + Ca^{2+} (5 mM)	5.75 ± 0.45	5.60 ± 0.39

Table 3. Quantitative analysis of phosphate covalently bound to native and '*in-vitro*'-phosphorylated 442000-M_r protein and to its constituent subunits and polypeptides

The native and '*in-vitro*'-phosphorylated 442000-M_r protein samples were fractionated into their constituent subunits and polypeptides and subjected to analysis of protein-bound phosphate as described in the Materials and methods section. The values given are means ± S.E.M. for three separate determinations.

	Protein-bound phosphate (mol/mol of protein)	
	Native protein	'*In-vitro*'-phosphorylated protein
Exponential-growth-phase cells		
442000-M_r protein	2.184 ± 0.234	3.855 ± 0.435
200000-M_r subunit	0.192 ± 0.032	1.826 ± 0.175
116000-M_r polypeptide	0.095 ± 0.036	0.942 ± 0.104
84000-M_r polypeptide	0.115 ± 0.048	0.895 ± 0.076
121000-M_r subunit	1.084 ± 0.202	1.046 ± 0.085
66000-M_r polypeptide	0.466 ± 0.035	0.476 ± 0.053
55000-M_r polypeptide	0.475 ± 0.042	0.486 ± 0.068
Stationary-growth-phase cells		
442000-M_r protein	4.235 ± 0.515	5.855 ± 0.675
200000-M_r subunit	0.216 ± 0.031	2.105 ± 0.185
116000-M_r polypeptide	0.094 ± 0.011	1.085 ± 0.094
84000-M_r polypeptide	0.089 ± 0.013	1.116 ± 0.105
121000-M_r subunit	1.895 ± 0.216	2.068 ± 0.315
66000-M_r polypeptide	1.086 ± 0.124	0.965 ± 0.175
55000-M_r polypeptide	0.978 ± 0.116	1.075 ± 0.185

Table 4. Quantitative analysis of UDP-glucuronic acid and UDP-*N*-acetylglucosamine binding to 442000-M_r protein and its isolated subunits and polypeptides

The native and '*in-vitro*'-phosphorylated 442000-M_r proteins were fractionated into constituent subunits and polypeptides as described in the Materials and methods section. Duplicate samples (50–100 μg) were incubated with equimolar concentrations of UDP-glucuronic acid and UDP-*N*-acetylglucosamine, ranging between 50 μM and 500 μM, in 50 mM-Hepes buffer, pH 7.1. The radiolabelled UDP-[^{14}C] glucuronic acid and UDP-*N*-acetyl[^{3}H]glucosamine were present in 1:100 ratio to the unlabelled nucleotide sugars. The controls were prepared by preheating protein samples at 100 °C for 2 min. After 15 min incubation at 37 °C with constant shaking, the protein was separated by filtration and its radioactivity counted. The amounts of nucleotide sugars bound (mol) per mol of protein were calculated. The values given are means ± S.E.M. for three separate experiments.

	Nucleotide sugars bound (mol/mol of protein or subunit or polypeptide molecule)			
	Native protein		'*In-vitro*'-phosphorylated protein	
	UDP-GlcA	UDP-GlcNAc	UDP-GlcA	UDP-GlcNAc
Exponential-growth-phase cells				
442000-M_r protein	1.116 ± 0.182	1.195 ± 0.175	1.972 ± 0.165	1.985 ± 0.195
200000-M_r subunit	0.096 ± 0.012	0.122 ± 0.014	1.084 ± 0.111	1.124 ± 0.131
116000-M_r polypeptide	0.108 ± 0.021	< 0.001	1.126 ± 0.118	< 0.001
84000-M_r polypeptide	< 0.001	0.105 ± 0.012	< 0.001	1.135 ± 0.150
121000-M_r subunit	0.469 ± 0.052	0.488 ± 0.061	0.554 ± 0.048	0.559 ± 0.063
66000-M_r polypeptide	0.240 ± 0.032	0.248 ± 0.040	0.234 ± 0.035	0.276 ± 0.038
55000-M_r polypeptide	0.235 ± 0.041	0.239 ± 0.031	0.227 ± 0.036	0.233 ± 0.042
Stationary-growth-phase cells				
442000-M_r protein	2.136 ± 0.255	2.178 ± 0.295	3.085 ± 0.360	3.145 ± 0.275
200000-M_r subunit	0.089 ± 0.011	0.113 ± 0.024	0.916 ± 0.104	1.108 ± 0.132
116000-M_r polypeptide	0.096 ± 0.016	< 0.001	1.012 ± 0.121	< 0.001
84000-M_r polypeptide	< 0.001	0.092 ± 0.013	< 0.001	1.106 ± 1.124
121000-M_r subunit	0.982 ± 0.108	0.994 ± 0.095	1.056 ± 0.114	1.063 ± 0.125
66000-M_r polypeptide	0.498 ± 0.056	0.553 ± 0.062	0.559 ± 0.071	0.503 ± 0.061
55000-M_r polypeptide	0.509 ± 0.065	0.464 ± 0.057	0.556 ± 0.071	0.538 ± 0.068

Table 5. Comparison of K_d values of UDP-glucuronic acid and UDP-*N*-acetylglucosamine for isolated polypeptides of 442000-M_r protein

The 116000-, 84000-, 66000- and 55000-M_r polypeptides were isolated from 442000-M_r protein samples as described in the Materials and methods section. The polypeptide samples, in duplicate, were incubated with nucleotide sugars as described in Table 4 legend. The amounts of protein-bound and free nucleotide sugars, after 15 min incubation, were determined after filtration of the samples. The $[S]_b/[S]_f$ ratios were plotted against $[S]_b$. The Scatchard plots were linear with a slope of $-1/K_d$. The K_d values given are means ± S.E.M. for three separate experiments.

	K_d			
	Native protein		'*In-vitro*'-phosphorylated protein	
	UDP-GlcA	UDP-GlcNAc	UDP-GlcA	UDP-GlcNAc
Exponential-growth-phase cells				
116000-M_r polypeptide	1.23 ± 0.11	—	1.12 ± 0.09	—
84000-M_r polypeptide	—	0.87 ± 0.09	—	0.85 ± 0.07
66000-M_r polypeptide	6.85 ± 0.46	6.57 ± 0.68	6.75 ± 0.57	6.48 ± 0.69
55000-M_r polypeptide	6.75 ± 0.58	6.87 ± 0.72	6.65 ± 0.79	6.57 ± 0.82
Stationary-growth-phase cells				
116000-M_r polypeptide	1.36 ± 0.15	—	1.22 ± 0.16	—
84000-M_r polypeptide	—	0.85 ± 0.11	—	0.81 ± 0.09
66000-M_r polypeptide	6.25 ± 0.49	6.85 ± 0.64	6.46 ± 0.71	6.39 ± 0.81
55000-M_r polypeptide	6.75 ± 0.62	6.45 ± 0.58	6.52 ± 0.64	6.67 ± 0.56

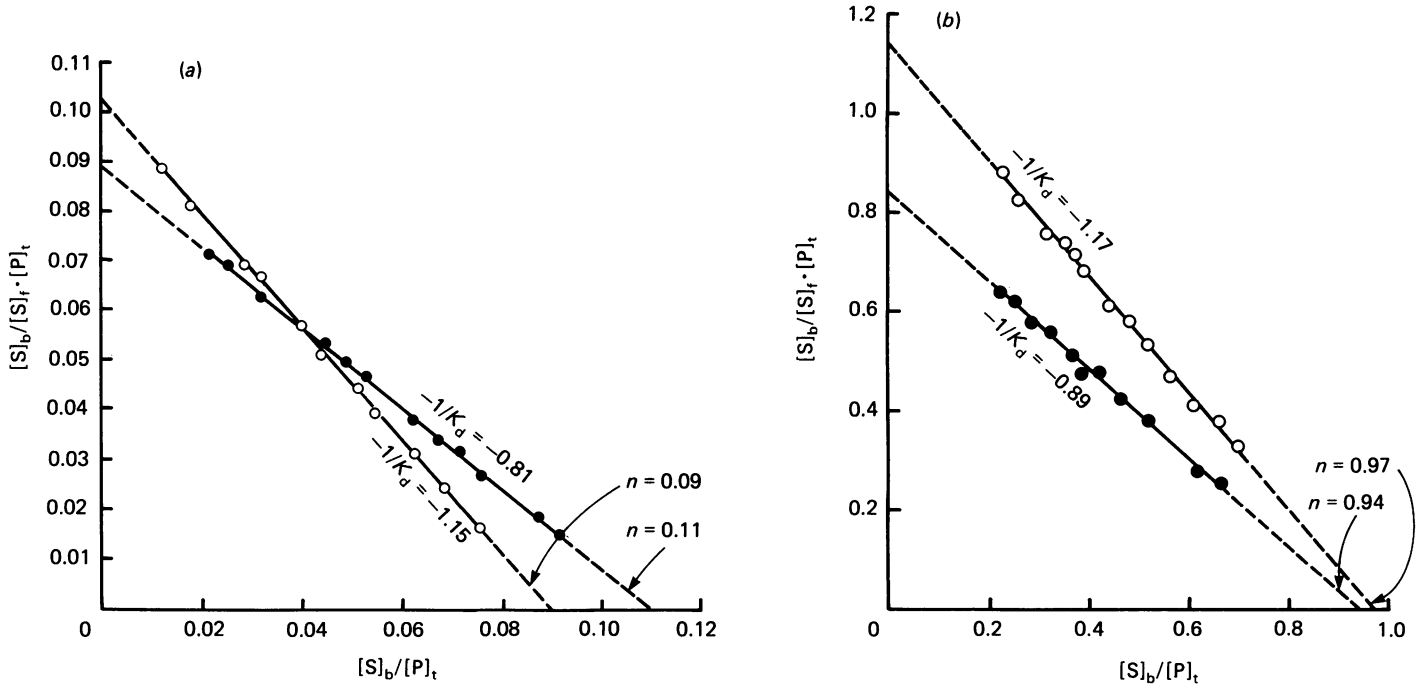

Fig. 5. Analysis of number of UDP-glucuronic acid-binding sites in 116000-M_r polypeptides and of UDP-*N*-acetylglucosamine-binding sites in 84000-M_r polypeptides isolated from native and '*in-vitro*'-phosphorylated 442000-M_r protein samples prepared from exponentially growing cells

The 116000- and 84000-M_r polypeptides (approx. 100 μg) were incubated with UDP-[^{14}C]glucuronic acid and UDP-*N*-acetyl[^{3}H]glucosamine respectively as described in Table 4 legend. After 15 min incubation at 37 °C with constant shaking, the polypeptides were separated by filtration and their radioactivities counted. The $[S]_b/[S]_f \cdot [P]_t$ and $[S]_b/[P]_t$ ratios were calculated and Scatchard plots drawn. The intercept on the horizontal $[S]_b/[P]_t$ axis gives the number of nucleotide-sugar-binding sites per polypeptide molecule. The values plotted are the averages for three determinations. (*a*) and (*b*) represent polypeptides isolated from native and '*in-vitro*'-phosphorylated 442000-M_r protein separated from plasma membranes of exponentially growing cells. ●, UDP-glucuronic acid binding to 116000-M_r polypeptides; ○, UDP-*N*-acetylgalactosamine binding to 84000-M_r polypeptides.

Table 6. Analysis of number of UDP-glucuronic acid- and UDP-*N*-acetylglucosamine-binding sites (*n* values) per polypeptide molecule

The isolated polypeptides were incubated with nucleotide sugars and the amounts of protein-bound and free nucleotide sugars were determined as described in Table 5 legend. The $[S]_b/[S]_f \cdot [P]_t$ ratios were plotted against $[S]_b/[P]_t$. The Scatchard plots were linear with a slope of $-1/K_d$. In each case the number of binding sites (*n* value) was determined from the intercept on the horizontal $[S]_b/[P]_t$ axis of the plot. The values given are means ± S.E.M. for three experiments.

	n			
	Native protein		'*In-vitro*'-phosphorylated protein	
	UDP-GlcA	UDP-GlcNAc	UDP-GlcA	UDP-GlcNAc
Exponential-growth-phase cells				
116000-M_r polypeptide	0.11 ± 0.03	—	0.94 ± 0.06	—
84000-M_r polypeptide	—	0.09 ± 0.02	—	0.97 ± 0.05
66000-M_r polypeptide	0.47 ± 0.08	0.52 ± 0.06	0.52 ± 0.03	0.55 ± 0.06
55000-M_r polypeptide	0.53 ± 0.06	0.48 ± 0.07	0.49 ± 0.05	0.56 ± 0.04
Stationary-growth-phase cells				
116000-M_r polypeptide	0.09 ± 0.03	—	0.96 ± 0.11	—
84000-M_r polypeptide	—	0.12 ± 0.04	—	1.02 ± 0.09
66000-M_r polypeptide	1.06 ± 0.08	0.93 ± 0.11	0.98 ± 0.06	1.04 ± 0.12
55000-M_r polypeptide	0.95 ± 0.07	1.04 ± 0.08	0.96 ± 0.08	1.08 ± 0.09

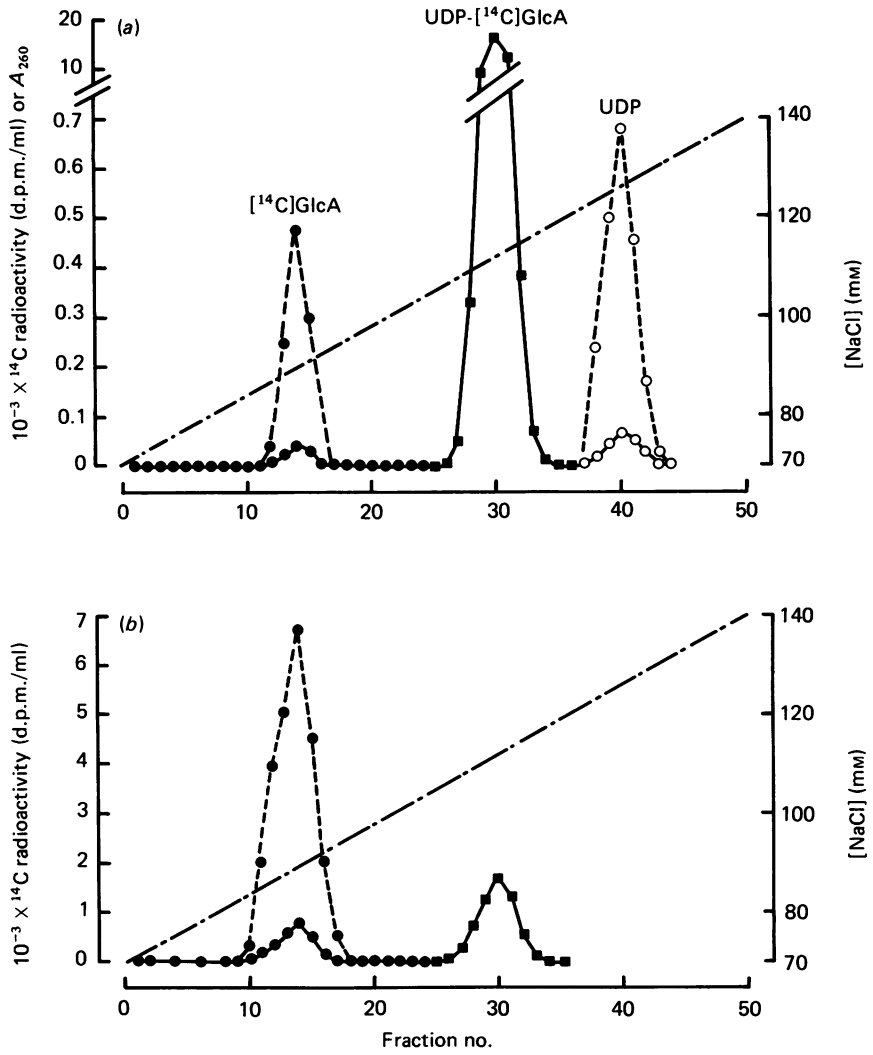

Fig. 6. DEAE-Sephacel ion-exchange chromatography of reaction mixture containing 200000-M_r subunit and UDP-[^{14}C]glucuronic acid and UDP-*N*-acetylglucosamine

The native and phosphorylated 200000-M_r subunits (approx. 100 μg) were incubated in 20 mM-Tris/HCl buffer, pH 7.1, containing 10 μM-UDP-[^{14}C]glucuronic acid and UDP-*N*-acetylglucosamine for 10 min at 37 °C with constant shaking. The protein was separated by filtration. (*a*) shows the analysis of reaction products in the filtrate fraction on DEAE-Sephacel ion-exchange chromatography, indicating the UDP moiety after cleavage from nucleotide sugars was liberated into medium and did not bind to the protein molecule. (*b*) shows the analysis of supernatant obtained after precipitation of the protein fraction with saturated $(NH_4)_2SO_4$, indicating that [^{14}C]glucuronic acid moiety was held non-covalently bound to the protein molecule. ^{14}C radioactivity: ●--●, phosphorylated subunits; ●—●, native subunits. UDP (A_{260}): ○--○, phosphorylated subunits; ○—○, native subunits. —·—, Concn. of NaCl.

5 times those for 116000- and 84000-M_r polypeptides (Table 5). The polypeptides isolated from native and '*in-vitro*'-phosphorylated protein samples of exponential- and stationary-growth-phase cells showed no significant difference with respect to their K_d values of nucleotide sugars (Table 5).

Analysis of the number of binding sites per polypeptide molecule (Figs. 5*a* and 5*b* and Table 6) shows that the 116000- and 84000-M_r polypeptides isolated from '*in-vitro*'-phosphorylated protein of exponential- and stationary-growth-phase cells possess a single binding site (*n* values ranged between 0.94 to 1.02) for UDP-glucuronic acid and UDP-*N*-acetylglucosamine respectively. The *n* values for corresponding polypeptides of native protein are about 0.1, indicating that only 10% of these polypeptide molecules were present in a form that could bind to the nucleotide sugars (Table 6). Similarly both 66000- and 55000-M_r polypeptides of native and '*in-vitro*'-phosphorylated protein of stationary-growth-phase cells appeared to possess a single and common binding site for the appropriate nucleotide sugar (*n* values ranging between 0.93 to 1.08). The *n* values for the corresponding polypeptides of exponential-growth-phase cells are between 0.47 to 0.56, indicating that about 50% of these polypeptide molecules were present in a form capable of linking with the nucleotide sugars.

A close relationship between the data on protein-bound phosphate and nucleotide sugars bound per mol of protein molecule or its subunit or polypeptides (Tables 3 and 4) clearly indicates that phosphorylation of these molecules was an essential prerequisite for the induction of their nucleotide-sugar-binding activity. The similarity

in K_d values of nucleotide sugars for corresponding polypeptides and the existence of a single binding site per polypeptide molecule also suggest that the observed differences in nucleotide-sugar-binding activities of native and '*in-vitro*'- phosphorylated protein samples of exponential-and stationary-growth-phase cells were due to differences in the proportion of protein molecules with respect to the state of phosphorylation of their constituent polypeptides.

Mechanism of binding of UDP-[^{14}C]glucuronic acid and UDP-*N*-acetyl[^{3}H]glucosamine to 442000-M_r protein and its constituent polypeptides and subunits

The binding of nucleotide sugars to isolated phosphorylated polypeptides was independent of the presence of ATP, Ca^{2+} and Mg^{2+}. It was a non-covalent and reversible reaction since repeated washing of the mixture with 50 mM-Na_2HPO_4/NaH_2PO_4 buffer, pH 7.1, or precipitation with 5% (w/v) trichloroacetic acid or saturated $(NH_4)_2SO_4$ completely removed all UDP-[^{14}C]glucuronic acid and UDP-*N*-acetyl[^{3}H]glucosamine as intact molecules.

Similar experiments on 200000- and 121000-M_r subunits of protein samples of exponentially growing cells showed that binding of nucleotide sugars was independent of the presence of ATP, Mg^{2+} and Ca^{2+}. Chromatographic analysis of reaction mixture, however, revealed that a fraction of nucleotide sugar molecules was hydrolysed into UDP and sugar residue moieties during incubation with phosphorylated 200000-M_r subunits (Fig. 6*a*). Whereas the UDP moiety cleaved during this reaction was liberated into the medium, the sugar residue moiety was held non-covalently bound to the 200000-M_r subunit molecule (Fig. 6*b*). The UDP-moiety-cleavage reaction and non-covalent binding of sugar residues to the subunit molecule were independent of the presence of ATP, Mg^{2+} and Ca^{2+}, but were severely affected by a change in pH from pH 7.1 to 7.5 or 6.5 and almost abolished when either of the nucleotide sugars was omitted from the incubation mixture (Table 7). The binding of both

Table 7. UDP-moiety cleavage and non-covalent binding of sugar residues to 200000-M_r subunits under different reaction conditions

The samples of 200000-M_r subunits (50–100 μg) were incubated with equimolar concentrations of UDP-glucuronic acid and UDP-*N*-acetylglucosamine (50 μM) in 50 mM-Hepes bufer, pH 7.1. The radiolabelled UDP-[^{14}C]glucuronic acid and UDP-*N*-acetyl[^{3}H]glucosamine or [^{3}H]UDP-glucuronic acid and [^{3}H]UDP-*N*-acetylglucosamine were present in 1:100 ratio to the unlabelled nucleotide sugars. In each set of incubation mixture only one nucleotide sugar was added in radioactively labelled form whereas the other nucleotide sugar was unlabelled. The test samples were prepared by adding or omitting agents listed below or by altering the pH of the incubation mixture. The blanks were prepared by preheating the subunit samples at 100 °C for 2 min. After 5 min incubation at 37 °C, the supernatants and protein samples were separated by filtration. The radioactivities of one set of filter discs were counted for the determination of protein-bound radioactivity whereas the other set was washed twice with 50 mM-Hepes buffer, pH 7.1, and filtrate collected for the determination of non-covalently bound radioactivity to these subunit samples. The supernatants of the sample containing unlabelled UDP-nucleotide sugars were chromatographed on DEAE-Sephacel as described in the Materials and methods section and the absorbance at 260 nm of fractions representing UDP peak maxima was measured. In experiments where [^{3}H]UDP-labelled UDP-nucleotide sugars were used, the amounts of ^{3}H radioactivity in the supernatant and protein fraction were taken to be [^{3}H]UDP released after its cleavage from the nucleotide sugars and that bound to the subunit molecule. The values given are means ± S.E.M. for three experiments.

	Amount of UDP released (arbitrary absorbance units)		10^{-5} × Amount of sugar residues bound to the subunits (d.p.m./mg)		10^{-5} × Amount of sugar residues released from the subunits (d.p.m./mg)	
Agents added or omitted	UDP-[^{14}C]GlcA	UDP-[^{3}H]GlcNAc	UDP-[^{14}C]GlcA	UDP-[^{3}H]GlcNAc	UDP-[^{14}C]GlcA	UDP-[^{3}H]GlcNAc
None (control)	0.17 ± 0.04	0.19 ± 0.04	0.92 ± 0.16	1.16 ± 0.17	0.85 ± 0.08	1.02 ± 0.12
+ ATP (5 mM)	0.18 ± 0.04	0.18 ± 0.05	0.84 ± 0.16	1.12 ± 0.18	0.94 ± 0.09	1.06 ± 0.09
+ Mg^{2+} (5 mM)	0.17 ± 0.03	0.17 ± 0.04	0.95 ± 0.15	1.18 ± 0.16	0.88 ± 0.07	1.08 ± 0.11
+ Ca^{2+} (5 mM)	0.19 ± 0.05	0.19 ± 0.04	0.85 ± 0.17	1.04 ± 0.14	0.84 ± 0.08	1.04 + 0.12
− UDP-GlcA	0	0	< 0.01	< 0.01	< 0.01	< 0.01
− UDP-GlcNAc	0	0	< 0.01	< 0.01	< 0.01	< 0.01
At pH 6.5	0.04 ± 0.02	0.03 ± 0.01	0.18 ± 0.02	0.23 ± 0.06	0.16 ± 0.03	0.25 ± 0.06
At pH 7.5	0.04 ± 0.02	0.05 ± 0.03	0.15 ± 0.01	0.18 ± 0.05	0.09 ± 0.02	0.14 ± 0.04

	10^{-4} × Amount of UDP released (d.p.m./mg)		10^{-4} × Amount of sugar residues bound to the subunits (d.p.m./mg)		10^{-4} × Amount of sugar residues released from the subunits (d.p.m./mg	
	[^{3}H]UDP-GlcA	[^{3}H]UDP-GlcNAc	[^{3}H]UDP-GlcA	[^{3}H]UDP-GlcNAc	[^{3}H]UDP-GlcA	[^{3}H]UDP-GlcNAc
None (control)	0.56 ± 0.04	0.62 ± 0.06	< 0.001	< 0.001	< 0.001	< 0.001
+ ATP (5 mM)	0.57 ± 0.05	0.58 ± 0.07	< 0.001	< 0.001	< 0.001	< 0.001
+ Mg^{2+} (5 mM)	0.62 ± 0.07	0.65 ± 0.08	< 0.001	< 0.001	< 0.001	< 0.001
+ Ca^{2+} (5 mM)	0.55 ± 0.04	0.59 ± 0.06	< 0.001	< 0.001	< 0.001	< 0.001
− UDP-GlcA	< 0.01	< 0.01	< 0.001	< 0.001	< 0.001	< 0.001
− UDP-GlcNAc	< 0.01	< 0.01	< 0.001	< 0.001	< 0.001	< 0.001

Table 8. UDP-moiety cleavage and non-covalent binding of sugar residues to 442000-M_r protein in the absence of Mg^{2+} in the reaction mixture

The 442000-M_r protein samples (100 μg) were incubated and processed as described in the Table 7 legend. The values given are means ±S.E.M. for three separate experiments.

	10^{-4} × Amount of [^{3}H]UDP released (d.p.m./mg)		10^{-5} Amount of sugar residues bound to the protein (d.p.m./mg)		10^{-5} × Amount of sugar residues released from the protein (d.p.m./mg)	
	[^{3}H]UDP-GlcA	[^{3}H]UDP-GlcNAc	UDP-[^{14}C]GlcA	UDP-[^{3}H]GlcNAc	UDP-[^{14}C]GlcA	UDP-[^{3}H]GlcNAc
Exponential-growth-phase cells						
Native protein	0.03±0.01	0.04±0.02	0.11±0.03	0.13±0.04	0.09±0.03	0.12±0.04
'*In-vitro*'-phosphorylated protein	0.29±0.03	0.34±0.04	0.95±0.10	1.22±0.09	0.88±0.07	1.08±0.09
Stationary-growth-phase cells						
Native protein	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
'*In-vitro*'-phosphorylated protein	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01

UDP-glucuronic acid and UDP-*N*-acetylglucosamine together to 200000-M_r subunit appears to produce some conformational changes that induce in it the ability to hydrolyse α-glycosidic linkage between glycosyl and UDP moieties of the substrate molecules. The ability of phosphorylated 200000-M_r subunits to cleave UDP moiety from nucleotide sugars was further confirmed by using [^{3}H]UDP-glucuronic acid and [^{3}H]UDP-*N*-acetylglucosamine precursors. The analysis (Table 7) shows that hardly any [^{3}H]UDP was found bound to the 200000-M_r subunits after this reaction. The UDP-moiety-cleavage reaction showed a linear increase with increasing concentration of 200000-M_r subunits (tested up to 100 μg/incubation) and with time up to 1 min of incubation period with no further progress with increasing incubation period. These observations suggest that, perhaps, the catalytic sites are rendered unavailable for further reaction because of the sugar residues being held non-covalently bound to these sites. The 200000-M_r subunits of protein from stationary-growth-phase cells showed no difference in their ability to cleave the UDP moiety and to hold sugar residues non-covalently (results not shown).

The 121000-M_r subunits, which exhibited efficient nucleotide-sugar-binding activity (see Table 4), did not hydrolyse the UDP moiety from the substrate molecules. The binding of nucleotide sugars to 121000-M_r subunits was non-covalent and reversible.

The native and '*in-vitro*'-phosphorylated 442000-M_r protein samples of exponential-growth-phase cells also cleaved the UDP moiety from nucleotide sugars. This intermediate reaction step between binding of nucleotide sugars and their polymerization into oligosaccharides was observed when Mg^{2+} was omitted from the reaction mixture. The relative amounts of [^{3}H]UDP released from [^{3}H]UDP-glucuronic acid and [^{3}H]UDP-*N*-acetylglucosamine and of [^{14}C]glucuronic acid and *N*-acetyl[^{3}H]-glucosamine bound non-covalently to the protein from UDP-[^{14}C]glucuronic acid and UDP-*N*-acetyl[^{3}H]glucosamine precursors after incubation with '*in-vitro*'-phosphorylated protein were considerably higher than those produced by the native protein (Table 8). The native and '*in-vitro*'-phosphorylated 442000-M_r protein samples of stationary-growth-phase cells did not cleave α-glycosidic bonds of nucleotide sugar substrates being non-covalently held by them (Table 8).

Glucuronosyl- and *N*-acetylglucosaminosyl-residue-polymerization or oligosaccharide-synthesizing activity of 442000-M_r protein

The glycosyl-residue-polymerization or oligosaccharide-synthesizing activity was expressed only by 442000-M_r plasma-membrane protein of exponentially growing cells in the presence of Mg^{2+} in the reaction mixture. The activity of '*in-vitro*'-autophosphorylated protein was about 10 times that of native protein. Whereas the '*in-vitro*'-autophosphorylated protein incorporated $5.74 \times 10^4 \pm 0.34 \times 10^4$ and $5.91 \times 10^4 \pm 0.62 \times 10^4$ d.p.m. from UDP-[^{14}C]glucuronic acid and UDP-*N*-acetyl[^{3}H]glucosamine respectively into oligosaccharides per mg of protein ($n = 5$), the native protein samples incorporated about $0.58 \times 10^4 \pm 0.07 \times 10^4$ and $0.62 \times 10^4 \pm 0.05 \times 10^4$ d.p.m. respectively into oligosaccharides per mg of protein ($n = 5$). On the other hand, both native and '*in-vitro*'-phosphorylated protein of stationary-growth-phase cells showed no oligosaccharide-synthesizing activity.

The largest oligosaccharide synthesized by isolated 442000-M_r protein was about M_r 5000 and about 12 disaccharide units in length (Fig. 7*a*). The synthesis of smaller oligosaccharides ranging between tetrasaccharides and dodecasaccharides (approx. M_r 800–5000) was not normally observed unless reaction was stopped within less than 10 s (Fig. 7*a*). From the data on [^{14}C]glucuronic acid- and *N*-acetyl[^{3}H]glucosamine-incorporation, the complete digestion of oligosaccharides into monosaccharides by the combined action of β-glucuronidase and β-*N*-acetylglucosaminidase and fragmentation of oligosaccharides into hexasaccharides (M_r approx. 2500) by hyaluronidase (Fig. 7*b*) it can be ascertained that oligosaccharides synthesized by the protein comprised repeating disaccharide units of glucuronic acid and *N*-acetylglucosamine. Incubation of protein samples for a longer period (15 min), which increased the overall production of radiolabelled oligosaccharides, did not increase their size or M_r (Fig. 7*c*). The synthesis of

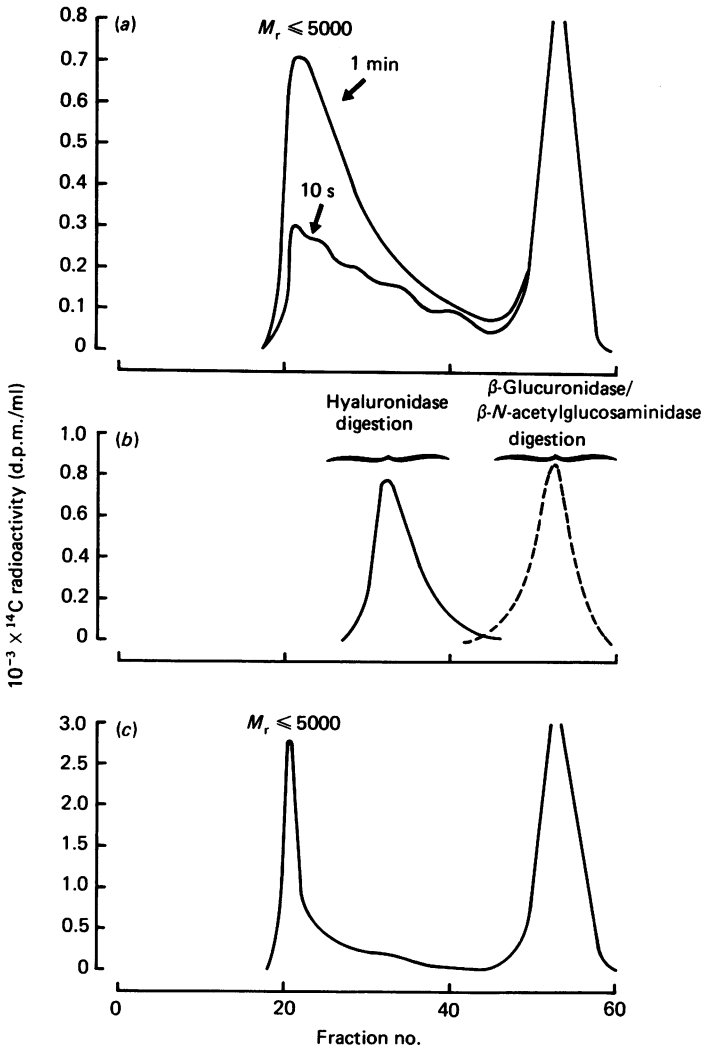

Fig. 7. Gel-filtration analysis of oligosaccharides synthesized by 442000-M_r protein isolated from plasma membranes of exponentially growing cells

The 442000-M_r protein samples (100–200 μg) were incubated in 50 mM-Tris/HCl buffer, pH 7.1, containing 5 mM-ATP, 0.1 mM-Mg^{2+}, 10 μM-UDP-[^{14}C]glucuronic acid and UDP-*N*-acetylglucosamine at 37 °C for different intervals. The samples were then heated at 100 °C for 2 min and subjected to gel filtration on Bio-Gel P-6 columns. (*a*) shows oligosaccharides of M_r < 5000, eluted near the excluded volume of the column, being synthesized during 10 s and 1 min incubation periods. (*b*) shows the hyaluronidase-digested (——) and β-glucuronidase-and-β-*N*-acetylglucosaminidase-digested (– – – –) products of oligosaccharide fractions collected from the previous column (*a*). (*c*) shows the elution pattern of oligosaccharides synthesized during a 15 min incubation period. Unincorporated UDP-[^{14}C]glucuronic acid was eluted between column fractions 45 and 60.

oligosaccharides of approx. M_r 5000 within 10–12 s indicates that the rate of polymerization of glycosyl residues was about 1 disaccharide unit/s. The radiolabelled oligosaccharides were not covalently linked to the protein molecule during their synthesis, since on termination of the reaction with 0.1% (w/v) SDS or 5% (w/v) trichloroacetic acid no radioactivity was found bound with the protein molecules.

The oligosaccharides did not carry a UDP moiety either during their synthesis on 442000-M_r protein molecules or after their release from the protein. Analysis of the reaction products of incubation mixtures containing [^{3}H]UDP-glucuronic acid and UDP-*N*-acetyl[^{3}H]glucosamine showed that, whereas $5.62 \times 10^4 \pm 0.7 \times 10^4$ d.p.m. of ^{3}H radioactivity from UDP-*N*-acetyl[^{3}H]glucosamine was incorporated into oligosaccharides, no ^{3}H radioactivity was found bound to the protein. Similarly, when [^{3}H]UDP-*N*-acetylglucosamine and UDP-[^{14}C]glucuronic acid were used as precursors, only ^{14}C radioactivity ($5.58 \times 10^4 \pm 0.69 \times 10^4$ d.p.m./mg of protein) was incorporated into oligosaccharides and no ^{14}C or ^{3}H radioactivity was found bound to the protein.

The oligosaccharide-synthesis reaction was absolutely dependent on the presence of Mg^{2+}. The concentration of Mg^{2+} (0.1 mM) required for the optimal reaction carried out by the isolated 442000-M_r protein was much lower than the amount (5 mM) required by the intact plasma membranes (Mian, 1986). Other bivalent cations such as Ca^{2+} or Mn^{2+} could not replace Mg^{2+}. Once the protein molecule has been initially autophosphorylated, the presence or absence of ATP had no effect on oligosaccharide-synthesis reaction (Table 9). Both GTP and UTP appeared to have no inhibitory or stimulatory effect on this reaction (Table 9). On the other hand, glucuronic acid, *N*-acetylglucosamine and UDP at 5 mM concentration were found to inhibit this reaction by about 15–25% (Table 9).

The rate of oligosaccharide synthesis reaction was dependent on concentration of nucleotide sugar substrates at 1:1 ratio in the incubation mixture. The optimal pH of the reaction was 7.1, and even a small change (0.1–0.2 pH unit) on either side lowered the rate of reaction by about 80%. Other agents such as cyclic AMP, calmodulin and phospholipids had no effect on the oligosaccharide-synthesis reaction (Table 9).

Various attempts to induce a 'high-M_r-hyaluronate-chain'-synthesizing activity in isolated 442000-M_r protein molecules turned out to be futile. The addition of Ca^{2+}, calmodulin, cyclic AMP and phospholipids in reaction mixture did not promote the production of oligosaccharides larger than M_r 5000 (results not shown). Similarly the addition of hyaluronate of approx. M_r 400000 (Sigma Chemical Co.) or of its hyaluronidase-produced fragments also failed to improve the size of newly-synthesized oligosaccharide molecules.

The isolated 442000-M_r protein did not bind to either high-M_r ^{14}C-labelled hyaluronate ($M_r > 2 \times 10^6$) or low-M_r hyaluronate fragments produced by digestion with hyaluronidase, since precipitation of the mixture with 5% (w/v) trichloroacetic acid or with saturated $(NH_4)_2SO_4$ by the method of Underhill *et al.* (1983) showed no detectable ^{14}C radioactivity being co-precipitated with the protein.

DISCUSSION

A high-M_r protein (approx. M_r 442000), separated from Nonidet-P-40-solubilized plasma membranes, was of some interest in that it was autophosphorylated from [γ-^{32}P]ATP in the presence of UDP-glucuronic acid and UDP-*N*-acetylglucosamine (hyaluronate precursors) and possessed efficient nucleotide-sugar-binding activity. Depending on the source of plasma membranes, whether prepared from exponential- or stationary-growth-phase

Table 9. Effect of various agents on glycosyl-residue-polymerization activity of '*in-vitro*'-phosphorylated 442000-M_r protein of exponential-growth-phase cells

The '*in-vitro*'-phosphorylated 442000-M_r protein was incubated with UDP-glucuronic acid and UDP-*N*-acetylglucosamine (100 μM each) in 50 mM-Hepes buffer, pH 7.1, containing 0.25 mM-Mg^{2+}. The ratio of labelled to non-radiolabelled nucleotide sugars was 1:100 in the mixture. After 15 min incubation at 37 °C, SDS was added to give a final concentration of 0.2% (w/v) and samples were heated at 100 °C for 2 min. The samples were then chromatographed on Bio-Gel P-6, and the excluded radioactivity peak was separated, which represents oligosaccharides being polymerized by the protein. The values given are means ± S.E.M. for three experiments.

Agents added or omitted	10^{-4} × Radioactivity incorporated into oligosaccharides synthesized by 1 mg of protein (d.p.m.)	
	^{14}C from UDP-[^{14}C]GlcA	^{3}H from UDP-[^{3}H]GlcNAc
None (control)	5.38 ± 0.42	5.75 ± 0.36
−Mg^{2+}	< 0.01	< 0.01
−UDP-GlcA	< 0.01	< 0.01
−UDP-GlcNAc	< 0.01	< 0.01
+GlcA (100 μM)	4.15 ± 0.36	4.88 ± 0.43
+GlcNAc (100 μM)	4.32 ± 0.32	4.63 ± 0.48
+UDP (5 mM)	3.95 ± 0.34	4.37 ± 0.45
+ATP (5 mM)	5.12 ± 0.42	5.70 ± 0.53
+GTP (5 mM)	5.25 ± 0.45	5.45 ± 0.59
+UTP (5 mM)	4.96 ± 0.46	5.38 ± 0.51
+Calmodulin (100 μg/ml)	5.20 ± 0.52	5.56 ± 0.48
+Cyclic AMP (100 μM)	4.90 ± 0.51	5.48 ± 0.55
+Lysophosphatidylcholine (4 mg/ml)	5.15 ± 0.47	5.78 ± 0.59
+Lysophosphatidylethanolamine (4 mg/ml)	5.25 ± 0.46	5.40 ± 0.48
+Ca^{2+} (5 mM)	5.06 ± 0.48	5.48 ± 0.52
+Mn^{2+} (5 mM)	5.16 ± 0.53	5.22 ± 0.57
At pH 6.9	1.08 ± 0.13	1.27 ± 0.14
At pH 7.3	1.12 ± 0.16	0.98 ± 0.12

cultured human skin fibroblasts, the protein also exhibited hyaluronate-oligosaccharide (approx. M_r 5000)-synthesizing activity. Whereas purification of the protein by affinity chromatography on activated-thiol–Sepharose 4B or epoxy-activated Sepharose 6B coupled to nucleotide sugars did not produce encouraging results, repeated gel filtration on Sepharose CL-4B and ion-exchange chromatography on DEAE-Sephacel enriched the protein by about 30-fold. The protein appeared to belong to the unusual category of proteins possessing a quaternary structure with an odd number of three subunits per oligomer (Klotz *et al.*, 1975). The oligomer consisted of one 200000-M_r subunit and two identical 121000-M_r subunits held together by hydrogen bonds, which in turn consisted of 116000- and 84000-M_r polypeptides and 66000- and 55000-M_r polypeptides respectively, held together by disulphide bonds.

The protein molecule possessed endogenous tyrosine kinase activity that was elicited only in the presence of hyaluronate precursors and depended on the conformation of the molecule. The treatment with SDS as reported in the present paper and reconstitution of the protein molecule after its dissociation and reduction (N. Mian, unpublished work) completely destroyed its endogenous tyrosine kinase activity. The tyrosine kinase activity of the protein molecule was restricted to phosphorylation of its own 200000-M_r subunit constituent, since immobilized 442000-M_r protein on Sepharose 4B (N. Mian, unpublished work), which was itself [^{32}P]phosphorylated under the appropriate reaction conditions, did not phosphorylate soluble 442000-M_r protein or its isolated 200000-M_r subunit in the reaction mixture. The autophosphorylation of 442000-M_r protein was irreversible, since the removal of nucleotide sugar precursors did not result in dephosphorylation of the molecule. Although a specific protein tyrosine-phosphatase has not been found as yet, substrate-induced endogenous phosphorylation of a protein molecule with an extremely short half-life (2.1 h) (N. Mian, unpublished work) that predisposes in favour of its substrate-binding activity suggests that the existence of a specific phosphatase system that would reverse this process may not be of great advantage to the cell.

The 442000-M_r protein molecule was also interesting in the sense that its two identical 1210000-M_r subunits were phosphorylated and dephosphorylated at serine residues by a Ca^{2+}-dependent cytoplasmic kinase/phosphate system (N. Mian, unpublished work). Although phosphorylation of these subunits also predisposed in favour of their nucleotide-sugar-binding activity like that of endogenous phosphorylation of 200000-M_r subunit which resulted in stimulation of hyaluronate-oligosaccharide-synthesizing activity of the molecule, the phosphorylation of 121000-M_r subunits was found to have an opposite effect. The lack of hyaluronate-oligosaccharide-synthesizing activity in 442000-M_r protein samples of stationary-growth-phase cells was found to be due to the presence of almost all protein molecules in their phosphorylated state with respect to 121000-M_r subunits. On the other hand, the protein samples of exponential-growth-phase cells that synthesized hyaluronate oligosaccharides were present in equilibrium

between the phosphorylated and the non-phosphorylated state with respect to phosphorylation of their 121000-M_r subunits. From these observations it can be concluded that substrate-dependent endogenous phosphorylation of 200000-M_r subunits and Ca^{2+}-dependent exogenous phosphorylation of 121000-M_r subunits of the protein molecule were clearly implicated in regulation of its hyaluronate-oligosaccharide-synthesizing activity.

The exact mechanism of hyaluronate-oligosaccharide synthesis by 442000-M_r plasma-membrane protein is not clearly understood as yet. However, the availability of a single specific binding site for UDP-glucuronic acid and UDP-*N*-acetylglucosamine in phosphorylated 200000-M_r subunit and its ability to cleave the UDP moiety from the precursors suggest that perhaps this subunit of the protein molecule plays a central role in the 'ordered' polymerization of glucuronic acid and *N*-acetylglucosamine residues into oligosaccharide structures. The abrogation of this activity of the protein molecule as a result of exogenous phosphorylation of its 121000-M_r subunits may be due to overcrowding of the molecule with non-covalently bound nucleotide sugars, which, perhaps, creates spatial disorder and hinders cleavage of UDP moieties and 'ordered' polymerization of glucuronic acid and *N*-acetylglucosamine residues otherwise carried out by the 200000-M_r subunit constituent of the protein molecule.

The isolated 442000-M_r protein could synthesize only a low-M_r hyaluronate oligosaccharide (M_r approx. 5000) consisting of about 12 repeating disaccharide units of glucoronic acid and *N*-acetylglucosamine residues. Various attempts to elicit a high-M_r-hyaluronate-synthesizing activity in the isolated protein were, however, unsuccessful. It can be argued that either 442000-M_r protein and its reaction product, i.e. low-M_r oligosaccharides of glucuronic acid and *N*-acetylglucosamine residues, were completely unrelated to hyaluronate synthase and its reaction product, i.e. high-M_r hyaluronate (M_r $10^6 - 10^7$) or that this protein was a part of the membrane-bound hyaluronate synthase complex and because of its dissociation from the rest of the complex it was left with a rather diminished functional activity. The answer to these questions awaits the successful separation of intact hyaluronate synthase complex. Meanwhile it can be speculated that the reason that this protein produces only oligosaccharides could be that these non-covalently held oligosaccharides, perhaps, 'fall off' from the protein molecule in the absence of surrounding membrane components that otherwise support these structures while they are being elongated at their growing end. Another possibility could be that hyaluronate synthase complex possesses additional oligosaccharide polymerase activity that keeps polymerizing the newly synthesized oligosaccharide units into a high-M_r hyaluronate chain.

The present work clearly demonstrates that throughout the synthesis reaction, from binding of nucleotide sugar substrates to polymerization of their sugar residues into an oligosaccharide structure, there was no evidence of covalent interactions between the protein and substrate molecules or finally synthesized product. These observations also rule out the possibility of glycosylation of protein molecule itself during the course of oligosaccharide synthesis. The present work also provides clear evidence that UDP moiety of the incoming sugar residue was not involved as a link between protein molecule and the oligosaccharide during its synthesis as suggested by Prehm (1983*b*). On the contrary, the UDP moieties were found to be cleaved off from nucleotide sugar substrates before polymerization of their glycosyl residues.

I thank Dr. P. W. Kent, Nuffield Department of Clinical Biochemistry, Radcliffe Infirmary, Oxford, for invaluable discussions and Professor J. G. Widdicombe, Department of Physiology, St. George's Hospital Medical School, for his support and encouragement. Thanks are also due to Kathryn Marks and Mark Camp for their technical assistance and Miss Adele de Souza and Marion Astudillo for preparation of the manuscript. The work was supported by the Cystic Fibrosis Research Trust, U.K.

REFERENCES

Appel, A., Horwitz, A. L. & Dorfman, A. (1979) J. Biol. Chem. **254**, 12199–12203
Hart, G. W. & Lennarz, W. J. (1978) J. Biol. Chem. **253**, 5795–5801
Hopwood, J. J. & Dorfman, A. (1977) J. Biol. Chem. **252**, 4777–4785
Kleine, T. O. (1981) Int. Rev. Connect. Tissue Res. **9**, 27–98
Klotz, I. M., Darnell, D. W. & Langerman, N. R. (1975) in Proteins, 3rd edn. (Neurath, H., Hill, R. L. & Boeder, C. L. eds.), vol. 1, pp. 336–338, Academic Press, New York and London
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Li, H. C. & Felmly, D. A. (1973) Anal. Biochem. **52**, 300–304
Longas, M. O. & Meyer, K. (1981) Biochem. J. **197**, 275–282
Mapleson, J. L. & Buchwald, M. (1981) J. Cell. Physiol. **109**, 215–222
Markovitz, A., Cifonelli, J. A. & Dorfman, A. (1959) J. Biol. Chem. **234**, 2343–2350
Mian, N. (1986) Biochem. J. **237**, 333–342
Prehm, P. (1983*a*) Biochem. J. **211**, 181–189
Prehm, P. (1983*b*) Biochem. J. **211**, 191–198
Prehm, P. (1984) Biochem. J. **220**, 597–600
Rao, A. K. & Mendicino, J. (1978) Anal. Biochem. **91**, 490–495
Roden, L. & Horowitz, M. I. (1978) in The Glycoconjugates (Horowitz, M. I. & Pigman, W., eds.), vol. 2, pp. 3–71, Academic Press, New York
Shatton, J. B., Ward, C., Williams, A. & Weinhouse, S. (1983) Anal. Biochem. **130**, 114–119
Stoolmiller, A. C. & Dorfman, A. (1969) J. Biol. Chem. **244**, 236–246
Strominger, J. L., Maxwell, E. S., Axelrod, J. & Kalckar, H. M. (1957) J. Biol. Chem. **224**, 79–90
Sugahara, K., Schwartz, N. B. & Dorfman, A. (1979) J. Biol. Chem. **254**, 6252–6261
Tomida, M., Koyama, H. & Ono, T. (1974) Biochim. Biophys. Acta **338**, 352–363
Tomida, M., Koyama, H. & Ono, T. (1975) J. Cell. Physiol. **86**, 121–130
Underhill, C. B., Chi-Rosso, G. & Toole, B. P. (1983) J. Biol. Chem. **258**, 8086–8091
Wade, H. E. & Morgan, D. M. (1953) Nature (London) **171**, 529–530
Winterbourne, D. J. & Mora, P. T. (1978) J. Biol. Chem. **253**, 5109–5120

Received 9 September 1985/20 January 1986; accepted 12 March 1986