Characterization of a Major Fibroblast Cell Surface Glycoprotein†

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ABSTRACT: We have isolated and partially characterized CSP, the major glycoprotein on the surface of chick embryo fibroblasts. Isolated CSP exists in an insoluble form in isotonic solutions at pH 7 even in the presence of nonionic detergents or 0.1 M ethylenediaminetetraacetate (EDTA), or at high ionic strength. CSP is soluble at high pH (pH ≥11) or low pH (pH ≤3), and in 8 M urea or 6 M guanidine-HCl. When nonreduced CSP is chromatographed on Sepharose CL 4B at pH 11 and analyzed by electrophoresis in sodium dodecyl sulfate ($NaDodSO_4$) gels, it consists of a major species of disulfide-linked multimer that is probably a dimer, plus lesser amounts of monomer and larger complexes. Upon reduction with or without alkylation all these species of CSP migrate as the monomer. The Stokes radius of the CSP monomer estimated by gel filtration is 110 Å, whether or not it is reduced and alkylated. Prior to disulfide reduction, the CSP of chick fibroblast cell homogenates also migrates as multimers when analyzed on $NaDodSO_4$ gels, and as the monomer after reduction. The amino acid composition of CSP has been determined and indicates that CSP does not have a preponderance of any particular class of amino acid, and has only 20% hydrophobic residues. The amino terminus is blocked and is not removed by pyroglutamate aminopeptidase. CSP is a glycoprotein containing 5–6% carbohydrate, which consists of *N*-acetylglucosamine, mannose, galactose, sialic acid, and glucose.

A glycoprotein of high molecular weight has been identified on the surface of a variety of fibroblastic cells using procedures that label cell surface proteins by radioactive compounds or by specific antibodies (Hynes, 1973, 1976; Gahmberg and Hakomori, 1973; Wickus et al., 1974; Stone et al., 1974; Vaheri and Ruoslahti, 1974; Hogg, 1974; Yamada and Pastan, 1976a). This LETS[1] (large, external, transformation sensitive) protein is often decreased after establishment of a permanent cell line (Yamada et al., 1977), and is further decreased after transformation. Absence of this glycoprotein from the surface of a series of cell lines generally correlates with tumorigenicity (Chen et al., 1976; Gallimore et al., 1977).

We have isolated CSP, the LETS protein of chick fibroblasts, and have established that it is an adhesive protein (Yamada and Weston, 1974; Yamada and Pastan, 1976a; Yamada et al., 1975, 1976b). Reconstituting this protein on the surface of transformed cells from several species results in morphologic reversion toward a more normal phenotype, apparently due to increased cell adhesion (Yamada et al., 1976a,b; Ali et al., 1977). Recently, the decrease in CSP after transformation of chick fibroblasts was shown to result from decreased biosynthesis, as well as increased turnover (Olden and Yamada, 1977b). The five- to six-fold decrease in biosynthesis is apparently due to decreased quantities of translatable CSP messenger RNA (Adams et al., 1977).

In this paper we describe studies on the solubility properties, composition, and structure of CSP. These studies were performed to elucidate the mechanism by which CSP increases cellular adhesion and helps to maintain normal cell behavior.

Materials and Methods

Materials. Cross-linked Sepharose 4B (CL 4B) and Blue Dextran 2000 were purchased from Pharmacia; phenylmethanesulfonyl fluoride, Triton X-100, and sodium deoxycholate were from Sigma; pyroglutamate aminopeptidase (EC 3.4.11.8) was from Boehringer Mannheim; rabbit and bovine γ-globulin and fibrinogen were from Miles; ovalbumin and cytochrome *c* were from Calbiochem; electrophoresis supplies were from Bio-Rad; 3× crystallized trypsin was from Worthington; and dithiothreitol, ammonium sulfate (Ultra Pure), and urea (Ultra Pure) were from Schwarz/Mann. Bovine thyroglobulin was a gift from Drs. Alan Burkhardt and Jacob Robbins (National Institutes of Health), and protease-free collagenase was a gift from Dr. Beverly Peterkofsky (National Institutes of Health).

Solubility Studies. Roller bottles (690 cm^2) of confluent chick embryo fibroblasts were cultured in modified GM medium as described previously (Vogt, 1969; Olden and Yamada, 1977b). Cells in 100 mL of GM were incubated with 1 μCi/mL L-[^{14}C]leucine (New England Nuclear, 320 mCi/mmol) for 24 h, and CSP was extracted using 1 M urea, fractionated with ammonium sulfate, and stored in 10 mM Caps (cyclohexylaminopropanesulfonic acid) buffer at pH 11 at 2.5–3 mg/mL as described previously (Yamada et al., 1975, 1976b). This procedure extracts approximately 50% of the CSP from heavily confluent roller bottle cultures of chick fibroblasts.

Aliquots of CSP containing 0.5 mg (35 000 dpm) were incubated in 1.5-mL conical polypropylene centrifuge tubes (Beckman) as indicated in the text in a final volume of 1.0 mL at 0 °C. After 18 h, the samples were centrifuged at 16 000*g* for 20 min. The supernatants were carefully aspirated and counted in Aquasol (New England Nuclear) in a scintillation spectrometer. The pellets were dissolved in 0.2 mL of 1 N NaOH at room temperature for 1 h, neutralized with 0.1 mL of 2 N HCl, and counted in Aquasol. To correct for quenching, disintegrations per minute were determined by a channels ratio method, and the fraction of total counts per tube remaining in the supernatant was calculated after subtracting the baseline 700 dpm found to remain in the tube after aspiration of uncentrifuged samples.

Column Chromatography. Confluent cultures of chick embryo fibroblasts (1.5 × 10^7 cells per 100 mm dish) were iodinated with carrier-free Na^{125}I (New England Nuclear, 400 μCi/mL final concentration) using lactoperoxidase and glucose

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[1] Abbreviations used are: LETS, large, external, transformation sensitive; Caps, cyclohexylaminopropanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetate; CIg, cold insoluble globulin.

oxidase (Hynes, 1973). CSP was extracted (Yamada et al., 1975), and the extract was pooled with unlabeled CSP isolated from roller bottles. The combined CSP was precipitated by adding ammonium sulfate crystals to a final concentration of 70% saturation at 4 °C. After centrifugation, the CSP was resuspended in buffer A (0.15 M NaCl, 1 mM $CaCl_2$, and 10 mM Caps, pH 11.0). The pH was immediately readjusted to pH 11.0 with 5 N NaOH, and the CSP was dialyzed against buffer A (3 × 4 L for 18–24 h).

In a representative experiment, 11 mg of CSP in 5.7 mL of buffer A was applied to a 2.5 × 100 cm column of cross-linked Sepharose 4B preequilibrated in buffer A, and chromatographed by upward flow in buffer A at 18 mL/h at 4 °C; 8-mL fractions were collected. Recovery of protein as determined by the procedure of Lowry et al. (1951) using bovine serum albumin standards was 100%, and recovery of ^{125}I as determined with a γ counter was 94%. Parallel CSP preparations were reduced and alkylated either by reduction at pH 11.0 with 0.2 M β-mercaptoethanol, then alkylation at pH 8.5 with 0.25 M iodoacetic acid, or by reduction with a 50-fold molar excess of dithiothreitol (usually 0.1 M) to CSP cysteine in 6 M guanidine for 4 h at pH 8.5 and alkylation with iodoacetamide (Haeberli et al., 1975).

Polyacrylamide Gel Electrophoresis. For $NaDodSO_4$ gel electrophoresis, CSP fractions were dialyzed 18 h against deionized water, lyophilized, and resuspended in 2% $NaDodSO_4$, 10 mM sodium phosphate (pH 7.0), and 2 mM phenylmethanesulfonyl fluoride, with or without 0.1 M dithiothreitol as indicated in the text. After heating at 100 °C for 3 min the samples were applied to 5% slab polyacrylamide–$NaDodSO_4$ gels. Electrophoresis and staining with 0.25% Coomassie blue in 50% trichloroacetic acid were performed as described (Studier, 1973; Yamada and Weston, 1974).

Electrophoresis was also performed in slab polyacrylamide gels containing 8 M urea without $NaDodSO_4$ at acid or alkaline pH as described by Shuster (1971). CSP samples were heated to 100 °C for 2 min in the corresponding gel buffer containing 8 M urea with or without 0.1 M dithiothreitol and incubated for 30 min at 37 °C. Omitting the heating step resulted in poorer resolution of protein bands. The pH 8.9 alkaline gels utilized a discontinuous buffer system with a pH 6.8 stacking gel and pH 8.3 electrode buffer, and the pH 4.3 acid gel used a continuous buffer system (Shuster, 1971).

Amino Acid and End Group Analyses. Amino acid analyses were performed on native or performic acid oxidized CSP as described by Spackman et al. (1958). For performic acid oxidation, CSP samples were dissolved in 300 μL of performic acid prepared by mixing 9.5 mL of formic acid (88%) and 1.0 mL of hydrogen peroxide (30%) at 0 °C (Schlesinger et al., 1974). After oxidation for 2 h at 0 °C, the samples were diluted with distilled water and lyophilized. After drying, dilution and lyophilization were repeated to remove traces of acid.

Samples of CSP for amino acid analysis were hydrolyzed in 6 N HCl containing 0.05% (v/v) β-mercaptoethanol at 110 °C in vacuo for 24 h. Amino acids were identified and quantitated on a Beckman Model 121M amino acid analyzer.

Tryptophan content was determined spectrophotometrically by titration of tryptophan residues with *N*-bromosuccinimide (Witkop, 1959; Scoffone and Fontana, 1970). CSP (2.2 mg) was dialyzed against 4 L of water for 6 h and lyophilized. The dried protein was dissolved in 6 M guanidine-HCl and the pH was lowered to 4.4. The change in absorbance at 280 nm was measured after successive additions of *N*-bromosuccinimide.

Automated sequence analyses were performed on native or pyroglutamate aminopeptidase treated CSP by the method of Edman and Begg (1967) using a double cleavage Quadrol program. Coupling with phenyl isothiocyanate was performed twice before initiating automated sequencing. Phenylthiohydantoin (Pth) amino acid derivatives were identified by gas–liquid (Pisano and Bronzert, 1969) and thin-layer chromatography (Edman, 1970; Schlesinger et al., 1975).

CSP was digested with pyroglutamate aminopeptidase using a modification of the method of Doolittle (1972). Native CSP (28 nmol) in 2 mL of 0.1 M NH_4HCO_3 (pH 8.4) containing 2 μL of β-mercaptoethanol and 20 μL of a solution of 0.05 M ethylenediaminetetraacetate (EDTA) was incubated with 0.2 mg of pyroglutamate aminopeptidase for 24 h at 37 °C and then dialyzed against water (3 × 4 L for 24 h) and lyophilized. The dried protein was dissolved in 0.05 M NH_4OH and applied to the spinning cup of the sequencer. This batch of pyroglutamate aminopeptidase readily removed the pyrrolidonecarboxylic acid group of a human salivary phosphoprotein (Schlesinger et al., 1977) under identical conditions.

Carbohydrate Analyses. The carbohydrate composition of the major CSP multimer was analyzed by Dr. John Codington (Carbohydrate Research, Massachusetts General Hospital) by the method of Chambers and Clamp (1971) as modified by Reinhold (1972). Two separate 1.0-mg preparations of the CSP dimer were dialyzed against water (3 × 4 L for 72 h) and lyophilized. Following methanolysis in 1.0 N HCl in methanol for 20 h at 80 °C, acetylation, and de-O-acetylation, the Me_3Si (trimethylsilyl) derivatives of CSP were identified and quantitated by gas–liquid chromatography.

Sialic acid was hydrolyzed from CSP using either 0.1 N H_2SO_4 for 1 h at 80 °C (Warren, 1963) or boiling in 1 N HCl for 90 s at 100 °C (Gottlieb et al., 1974), and was measured by the thiobarbituric acid method (Warren, 1963). Internal controls of *N*-acetylneuraminic acid were included in parallel tubes with or without CSP and subjected to the same conditions of hydrolysis.

Results

Solubility. CSP exists as an insoluble aggregate on the cell surface. Cells release CSP when exposed to 1 M urea; this released CSP can be concentrated with ammonium sulfate and resolubilized at pH 11 in Caps buffer (Yamada et al., 1976b). When CSP isolated in this manner is incubated at pH 7 under a variety of conditions of salt, chelating agents, or nonionic detergents, it is poorly soluble (Table I). In other experiments, omitting the ammonium sulfate precipitation step or attempting to resuspend the ammonium sulfate precipitated CSP in 1 M urea also does not result in soluble CSP (data not shown). The only agents found to completely solubilize CSP are 8 M urea, 6 M guanidine-HCl, and 0.1% $NaDodSO_4$ (Table I). These denaturing agents are toxic to cells, and would interfere with studies of CSP's biological activity.

The solubility of CSP is dependent on pH (Figure 1). It is soluble at pH 11 and above. It is least soluble at physiological pH, and again becomes soluble as the pH is lowered to 2, although a small fraction of CSP remains insoluble at low pH. These findings have permitted studies of CSP under nondenaturing conditions. We have performed all structural studies on CSP at pH 11.0 using buffer A, since after neutralization the CSP remained active and could be utilized in a series of biological studies described elsewhere (see Discussion).

Gel Chromatography. After molecular sizing on a column of cross-linked Sepharose 4B in buffer A at pH 11, unreduced CSP is separated into at least three components: material in the void volume (A), a major protein peak (B), and a smaller third peak (C) (Figure 2). The profile of ^{125}I labeling follows the profile of total protein. Six other CSP preparations show

TABLE I: Solubility of CSP.[a]

Treatment	Fraction in supernatant
0.15 M NaCl, 50 mM sodium phosphate, pH 7.0 (PBS)[b]	0.39
0.15 M NaCl, 50 mM Tris-HCl, pH 7.0	0.33
3 M KCl, PBS	0.52
0.1 M EDTA, PBS	0.39
2% Triton X-100, PBS	0.43
1% Nonidet P40, PBS	0.30
5% Nonidet P40, PBS	0.26
2% deoxycholate, PBS	0.75
6 M guanidine-HCl, PBS	1.00
8 M urea, PBS	0.90
8 M urea, 2% Triton X-100, PBS	0.78
0.1% $NaDodSO_4$, PBS	0.91
Buffer A (0.15 M NaCl, 1 mM $CaCl_2$, 10 mM Caps, pH 11.0)	1.00

[a] CSP was labeled by incubating cells in 1 μCi/mL [^{14}C]leucine for 24 h, then isolated as described under Materials and Methods. Labeled CSP was incubated at 0.5 mg/mL for 18 h at 0 °C under the conditions indicated and then centrifuged at 16 000g for 20 min. The fraction of total counts remaining in the supernatant was determined as described under Materials and Methods. Values are the means of duplicate samples. [b] Phosphate-buffered saline.

TABLE II: Stokes Radius of CSP by Gel Filtration.[a]

	Stokes radius (Å)	No. of expts
CSP monomer	112 ± 2	5
Reduced/alkylated CSP	111 ± 2	7

[a] Stokes radii were obtained from 12 experiments in which unreduced or reduced and alkylated CSP were examined by gel filtration on cross-linked Sepharose 4B at pH 11.0. Standards included bovine fibrinogen (108 Å), bovine thyroglobulin (85 Å), bovine γ-globulin (51-53 Å), and ovalbumin (27-30 Å) (values for Stokes radii obtained from Tanford et al., 1974; Andrews, 1970; and Doolittle, 1973). Values indicate mean ± standard error.

a similar pattern when chromatographed on Sepharose CL 4B, although in two preparations, the shoulder between peaks A and B is more pronounced, suggesting the existence of another species of multimer. After reduction and alkylation, the material in both peaks A and B is shifted to a peak which comigrates with the original peak C. Peak A is not eliminated completely unless the reduction and alkylation is performed in 6 M guanidine-HCl.

The materials in peaks A and B have very large apparent Stokes radii and do not fit on our standard curve. The Stokes radius of the material in peak C is approximately 110 Å (Table II). Reduced and alkylated CSP also has a Stokes radius of 110 Å. The elution volumes of the standard marker proteins are inversely proportional to Stokes radius even at pH 11, with the exception of the fibrinogen marker which is slightly retarded (data not shown); fibrinogen and other large, asymmetric molecules have been reported to be anomalously retarded in their elution from molecular sizing columns (Nozaki et al., 1976). Fractions comprising the major peak (B) were pooled and used for subsequent biochemical analysis.

Electrophoresis. On 4 or 5% polyacrylamide-$NaDodSO_4$ gels, unreduced CSP is resolved into three protein size classes (Figure 3A). Material is found on the top of the gel, indicating a size excluded by this porosity of gel, in a region of apparent molecular weight of 300 000, and in a region of mol wt of

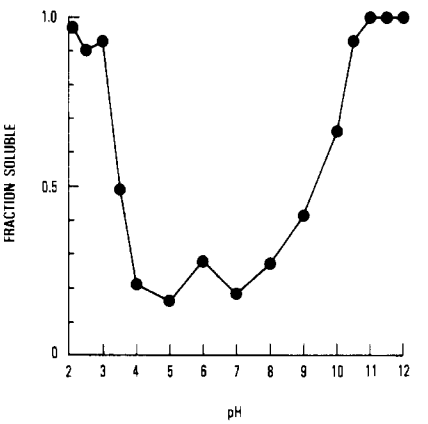

FIGURE 1: Solubility of CSP as a function of pH. ^{14}C-Labeled CSP was incubated in 50 mM sodium phosphate, 100 mM NaCl at a concentration of 0.5 mg/mL at each indicated pH. After 18 h at 0 °C, samples were centrifuged and the percent of the radioactivity remaining in the supernatant was calculated as described under Materials and Methods. Points indicate means of duplicate samples.

220 000. In the absence of molecular weight standards larger than 240 000, the estimated size of the larger band is solely for purposes of identification. After reduction by 0.1 M dithiothreitol or reduction and alkylation (Haeberli et al., 1975), all of the isolated CSP migrates as a single band of apparent mol wt 220 000 as reported previously (Figure 3A,C: Yamada and Weston, 1974; Yamada et al., 1975). We have been unable to detect any low molecular weight subunits (>5000 apparent molecular weight) by electrophoresis in 10 or 15% polyacrylamide-$NaDodSO_4$ gels with or without a stacking gel (Yamada and Weston, 1974; Studier, 1973); under these conditions we readily detect the light chains of rabbit skeletal muscle myosin (mol wt 16 000-20 000).

To determine the components of the three major peaks resolved by gel filtration (Figure 2), we analyzed each fraction from the Sepharose column on $NaDodSO_4$ gels (Figure 3B). The protein peaks separated by molecular sieve chromatography correspond to the material on the top of the gel (peak A, extending into peak B), the 300 000 molecular weight band (peak B), and the apparent monomer band (peak C). In the small peak of low molecular weight material, no discrete protein bands were detected. After reduction with dithiothreitol, the CSP in all fractions migrated as the 220 000 monomer (Figure 3C).

In approximately one-quarter of the CSP preparations, the major multimer appears to migrate in $NaDodSO_4$ gels as a closely spaced doublet. This heterogeneity disappears after reduction of the samples to the monomer, which always migrated as a single band regardless of the gel system utilized. The gel systems employed to analyze reduced CSP included our standard buffer system with 4, 5, 10, and 15% polyacrylamide gels (Yamada and Weston, 1974); this same system with 8 M urea included in all buffers with 5% gels; the Laemmli discontinuous buffer system with stacking and resolving gels of 3%/5%, 3%/6%, 4%/7.5%, or 5%/15% (Studier, 1973); or an alkaline, continuous buffer system with a 5% polyacrylamide gel consisting of 0.1% $NaDodSO_4$, 0.1 M sodium phosphate, pH 11.0 (samples were heated in 2% $NaDodSO_4$ at 100 °C for 3 min in pH 11 buffer with or without 8 M urea).

Similar evidence for interchain disulfide linkages in isolated CSP is also found after electrophoresis in polyacrylamide gels containing 8 M urea without $NaDodSO_4$ at alkaline or acid pH (Figure 4). In 5% polyacrylamide-urea gels at pH 8.9, unreduced CSP is separated into three components. A portion

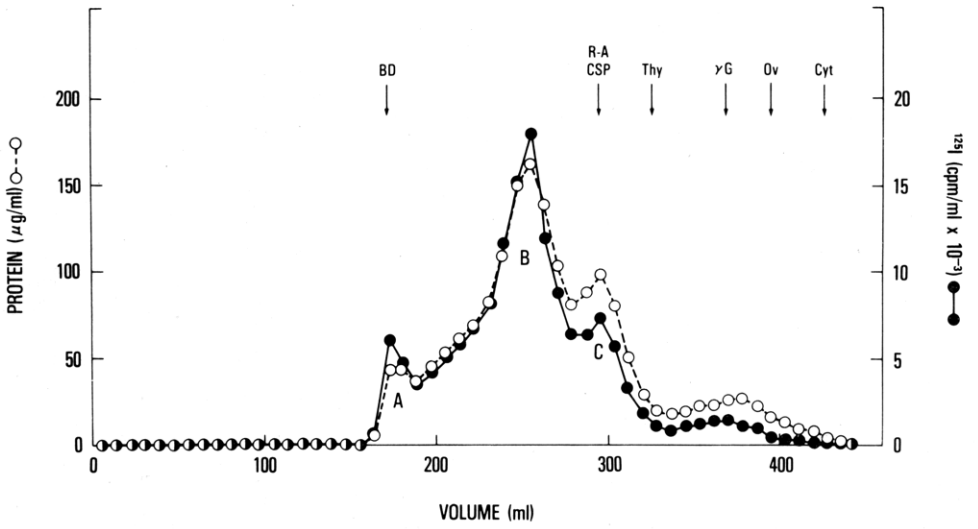

FIGURE 2: Gel filtration of unreduced CSP. CSP was isolated from chick fibroblast cultures iodinated with ^{125}I or from unlabeled roller bottle cultures, pooled, and concentrated using 70% ammonium sulfate. After dialysis against column buffer, 8 mL containing 11 mg of protein was applied to a 2.5 × 100 cm column of Sepharose CL 4B and chromatographed at pH 11.0 in buffer A using upward flow: (○) protein; (●) ^{125}I counts/minute. Markers included Blue Dextran (BD), bovine thyroglobulin (Thy), rabbit γ-globulin (γG), ovalbumin (Ov), and cytochrome *c* (Cyt).

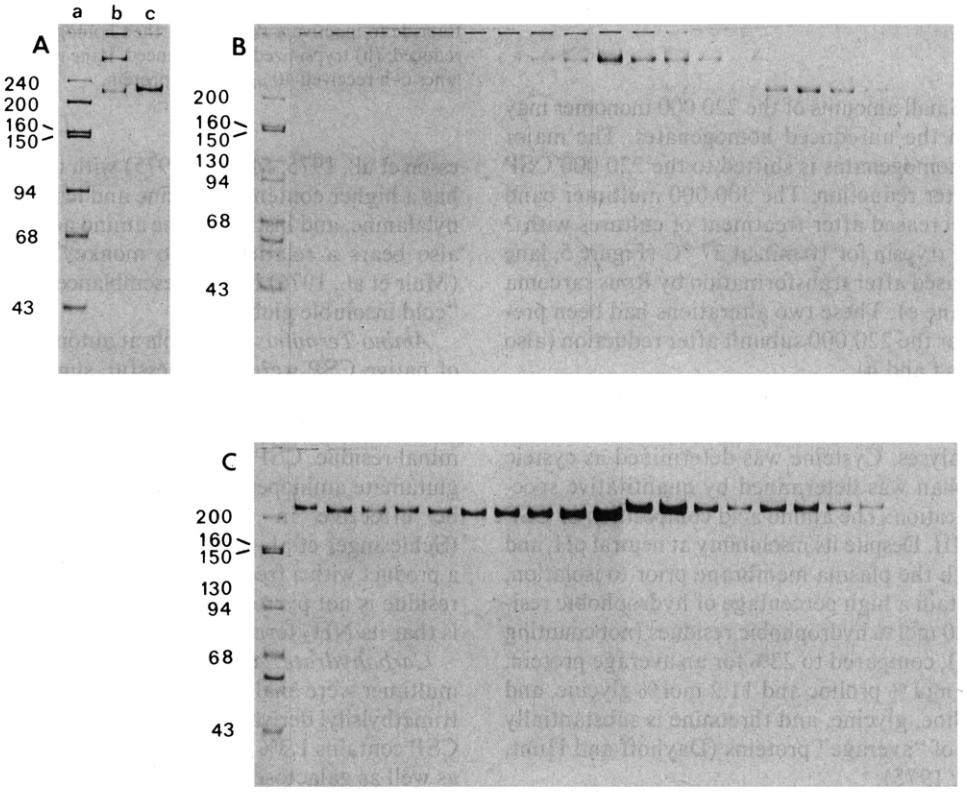

FIGURE 3: $NaDodSO_4$ gel electrophoresis of unreduced and reduced CSP. (Panel A) (a) Protein standards, indicating molecular weight × 10^3. (b) Isolated unfractionated CSP (2 μg) electrophoresed on 5% polyacrylamide–$NaDodSO_4$ gel without reduction. (c) CSP after reduction with 0.1 M dithiothreitol. (Panel B) CSP fractionated by gel filtration electrophoresed without reduction; 0.1-mL fractions from the CL-Sepharose 4B column of Figure 2 were dialyzed, lyophilized and resuspended in 2% $NaDodSO_4$ electrophoresis buffer, and boiled, and half of each sample was electrophoresed. This panel is a composite of two gels. Protein standards are to the left. (Panel C) The other half of each fraction in $NaDodSO_4$ of Figure 3B was combined with 0.1 M dithiothreitol, boiled, and electrophoresed.

of the CSP is excluded from the gel, and two bands corresponding to monomer and dimer enter the gel (Figure 4a). After reduction with 0.1 M dithiothreitol, CSP migrates as the monomer band (Figure 4b). At pH 4.3 in 5% polyacrylamide–urea gels, CSP does not migrate toward the anode, but does migrate slowly toward the cathode (Figures 4c–f). A similar pattern of bands appears to be present in unreduced samples, and reduction results in the single monomer band (Figures 4c,d).

We also investigated whether the pattern of interchain disulfide linkages found in CSP isolated by urea treatment and ammonium sulfate precipitation was similar to the protein pattern found originally on cells. These studies were necessary to rule out the possibilities that the disulfide linkages were an artifact of disulfide exchange, or that the CSP we isolated was not representative of total CSP. A similar protein pattern is found in unreduced homogenates of cultures of chick fibroblasts examined on $NaDodSO_4$–polyacrylamide gels (Figure

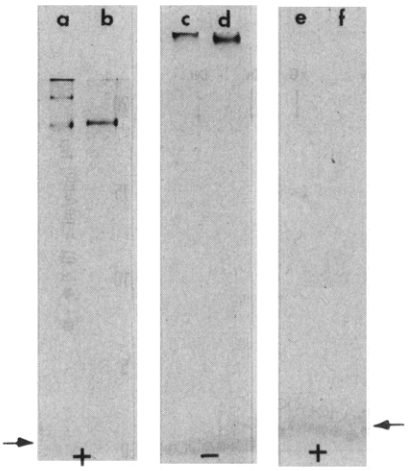

FIGURE 4: Urea–polyacrylamide gels at alkaline vs. acid pH. CSP in 8 M urea was electrophoresed in 5% polyacrylamide slab gels (1 mm thick) at pH 8.9 or 4.3. (a) CSP on alkaline gel without reduction. (b) CSP on alkaline gel after reduction. (c) CSP on acid gel without reduction. (d) CSP on acid gel after reduction. (e) CSP on acid gel without reduction, opposite polarity. (f) CSP on acid gel after reduction, opposite polarity. Each lane received 4 μg of CSP. Arrows indicate bromophenol blue markers. The acid gels were electrophoresed in parallel for the same length of time.

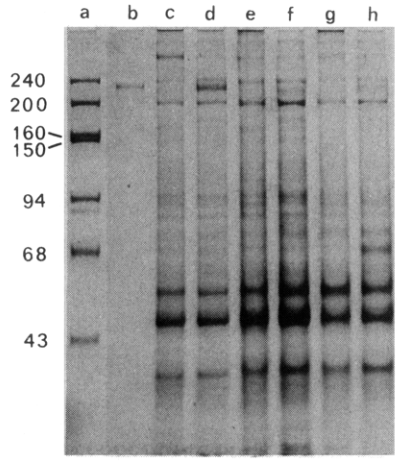

FIGURE 5: $NaDodSO_4$ gel electrophoresis of homogenates of chick embryo fibroblasts. Confluent cultures were homogenized in $NaDodSO_4$ and electrophoresed as indicated under Materials and Methods: (a) protein standards; (b) reduced CSP standard; (c) chick fibroblast homogenate, not reduced; (d) chick fibroblast homogenate reduced with 0.1 M dithiothreitol; (e) chick fibroblasts transformed by the Schmidt-Ruppin strain of Rous sarcoma virus, not reduced; (f) transformed CEF, reduced; (g) chick fibroblasts pretreated with 2 μg/mL crystalline trypsin for 10 min at 37 °C, washed with PBS containing 2 mM phenylmethanesulfonyl fluoride to inactivate the trypsin, then homogenized in $NaDodSO_4$; not reduced; (h) trypsinized CEF, reduced. Lane b received 1 μg of CSP, and lanes c–h received 40 μg of total protein.

5, lanes c and d). Small amounts of the 220 000 monomer may also be present in the unreduced homogenates. The major 300 000 band of homogenates is shifted to the 220 000 CSP monomer band after reduction. The 300 000 multimer band is substantially decreased after treatment of cultures with 2 μg/mL crystalline trypsin for 10 min at 37 °C (Figure 5, lane g). It is also decreased after transformation by Rous sarcoma virus (Figure 5, lane e). These two alterations had been previously reported for the 220 000 subunit after reduction (also see Figure 5, lanes f and h).

Amino Acid Composition. Two separate preparations of the major CSP multimer (peak B) were hydrolyzed and subjected to amino acid analyses. Cysteine was determined as cysteic acid, and tryptophan was determined by quantitative spectrophotometric titration. The amino acid composition of CSP is shown in Table III. Despite its insolubility at neutral pH, and its association with the plasma membrane prior to isolation, CSP does not contain a high percentage of hydrophobic residues. It contains 20 mol % hydrophobic residues (not counting glycine or proline), compared to 23% for an average protein. CSP contains 8.1 mol % proline and 11.2 mol % glycine, and its content of proline, glycine, and threonine is substantially greater than that of "average" proteins (Dayhoff and Hunt, 1972; Jukes et al., 1975).

CSP does not contain hydroxyproline. However, since cells were not grown in the presence of ascorbate, this finding does not unequivocally rule out a similarity to procollagen. We therefore digested CSP with a high enzyme-to-substrate ratio (1:20, w/w) of protease-free bacterial collagenase (provided by Dr. Beverly Peterkofsky) according to Peterkofsky and Diegelmann (1971). Incubation with collagenase for 1.5 h at 37 °C has no effect on either the amount or the electrophoretic mobility of CSP as analyzed on 5% polyacrylamide–$NaDodSO_4$ gels (data not shown). The same batch of collagenase completely digested collagen under the same conditions.

A comparison of the amino acid compositions of CSP, the plasma glycoprotein "cold insoluble globulin", α1 collagen, microfibrillar protein, and the "average" protein is shown in Table III. The amino acid composition of CSP is similar to that of human cold insoluble globulin isolated from plasma (Mosesson et al., 1975; Mosher, 1975) with the exceptions that CSP has a higher content of glycine and less cysteine, valine, phenylalanine, and histidine. The amino acid composition of CSP also bears a relationship to monkey microfibrillar protein (Muir et al., 1976) but the resemblance is not as strong as with "cold insoluble globulin".

Amino Terminus. Attempts at automated sequence analysis of native CSP were unsuccessful, suggesting that CSP possessed a blocked residue in the NH_2-terminal position. To determine whether CSP possesses a pyroglutamyl NH_2-terminal residue, CSP was digested with a preparation of pyroglutamate aminopeptidase that had previously been shown to be effective in removing pyrrolidonecarboxylic acid (Schlesinger et al., 1977). This treatment failed to generate a product with a free NH_2-terminal residue indicating that the residue is not pyrrolidonecarboxylic acid; a likely possibility is that its NH_2 terminus is acetylated.

Carbohydrate Analysis. Two preparations of the CSP multimer were analyzed for carbohydrate after methanolysis, trimethylsilyl derivatization, and gas–liquid chromatography. CSP contains 1.3% mannose and 1.6% *N*-acetylglucosamine, as well as galactose and glucose (Table IV). No *N*-acetylgalactosamine or fucose was detected. Separate samples were assayed for *N*-acetylneuraminic acid by the thiobarbituric acid assay (Warren, 1963); CSP contains 0.7% or 5 mol of sialic acid per mol of CSP monomer (Table IV).

Phosphate. The phosphate content of two separate preparations of CSP multimer was determined as described by Ames (1966) after ashing in the presence of magnesium nitrate. CSP contains 0.8 mol of phosphate per mol of CSP monomer. The phosphate is completely extracted from CSP by chloroform–methanol (Folch et al., 1957), indicating that the phosphate is not covalently attached to CSP; it may therefore represent residual phospholipid.

Discussion

CSP, the major cell surface protein of chick embryo fibroblasts, constitutes 3% of total cell protein (Yamada and

TABLE III: Amino Acid Composition of CSP Compared to Other Proteins (Residues per 1000).[a]

	CSP	CIg	CIg	Microfibrillar protein	α1 collagen	Av protein
Asp	94.1	92.5	95	87.6	41	104
Thr	100.3	96.7	108	75.3	18	63
Ser	74.0	67.9	80	101.0	27	76
Glu	117.5	116.1	115	130.0	78	94
Pro	76.9	76.1	82	69.9	123	48
Cys	18.4	26.3	25	10.2	0	28
Gly	106.2	79.7	86	131.0	329	77
Ala	47.5	42.9	42	65.5	127	84
Val	65.6	80.6	82	61.9	13	69
Met	12.7	11.2	8	10.7	9	17
Ile	37.5	44.0	45	35.6	7	48
Leu	55.4	57.1	54	57.9	20	75
Tyr	36.6	45.4	43	33.0	2	36
Phe	18.8	27.1	24	27.0	12	36
Trp	36.0	27.7			0	13
Lys	34.1	36.5	36	37.0	29	69
His	15.6	20.7	23	22.4	2	22
Arg	53.4	51.6	54	44.1	51	41
Hyp	0			0	107	

[a] Sources of data: human plasma cold insoluble globulin (CIg)—Mosesson et al., 1975; Mosher, 1975; monkey microfibrillar protein—Muir et al., 1976; chick α1 collagen—Miller, 1969; "average protein" value is mean of values in Dayhoff and Hunt, 1972, and Jukes et al., 1975.

Weston, 1974). We had previously isolated CSP from chick cells, and shown that it is an adhesive protein which would agglutinate cells and increase cell to substratum adhesion (Yamada et al., 1975, 1976b). After reconstitution on the surface of transformed cells, CSP restores or partially restores one class of transformation-induced phenotypic alterations which include altered morphology, alignment, and surface microvilli and ruffles. Metabolic changes which include growth control, nutrient transport, and cyclic AMP levels were not affected (Yamada et al., 1976a; Yamada and Pastan, 1976b; Ali et al., 1977).

We have now investigated the solubility properties, subunit organization, and composition of isolated CSP. It should be noted that it is conceivable that some of the CSP on fibroblasts may not be identical with the CSP we have characterized, since the isolation procedure recovers approximately half of the CSP originally present on chick fibroblasts.

After isolation, CSP at a concentration of 0.5 mg/mL did not remain soluble when transferred from pH 11 to 0.15 M sodium chloride solutions buffered by phosphate or Tris at pH 7. High salt, EDTA, and detergents other than $NaDodSO_4$ were also unable to maintain CSP solubility. CSP was soluble in 8 M urea and 6 M guanidine-HCl. These results are complementary to those of Hynes et al. (1976) who were unable to solubilize iodinated LETS protein from hamster Nil cells by extracting with various solutions other than strong denaturing agents. The inability to solubilize CSP in all but strong denaturing conditions seriously hampered previous attempts to further purify and to characterize the biological activities and structure of this protein.

We found that CSP would remain soluble at alkaline pH (and less reproducibly at acidic pH). We chose to use pH 11 for the studies discussed below, since the CSP solutions could be neutralized and used immediately in biological experiments (Yamada et al., 1976a,b).

We find that purified CSP, after chromatography on cross-linked Sepharose, is separated into three peaks, which on $NaDodSO_4$ gels are found to represent a monomer of apparent molecular weight 220 000, plus multimers. Similar results have been obtained after sedimentation on sucrose gradients (unpublished data). Reduction with or without alkylation results in migration of all the CSP as the monomer by chromatography and by gel electrophoresis. The patterns of migration of the major multimer on molecular sizing columns and on polyacrylamide gels and the absence of detectable subunits smaller than the 220 000 dalton monomer after reduction of disulfide bonds strongly suggest that this predominant species of CSP is a disulfide-linked dimer.

TABLE IV: Carbohydrate Composition of CSP.

	%	mol/mol of CSP monomer
N-Acetylglucosamine	1.63	16
Mannose	1.33	16
Glucose	1.11	14
Galactose	0.78	10
Sialic acid	0.67	4.8
Xylose	0.03	0.4
N-Acetylgalactosamine	N.D.[a]	0
	5.55	61

[a] None detected.

The major CSP multimer (dimer) could also be identified by protein staining in homogenates of chick embryo fibroblasts, not treated with reducing agents. Only minimal amounts of monomer were seen. The major multimer migrated as the monomer after reduction, and as previously reported for reduced samples (Hynes, 1973; Gahmberg and Hakomori, 1973; Wickus et al., 1974; Stone et al., 1974; Vaheri and Ruoslahti, 1974; Hogg, 1974; Yamada and Weston, 1974), was decreased after trypsinization or transformation. These results indicate that the multimeric form of CSP is the principal state of CSP in chick fibroblasts.

In an analysis of unreduced, iodinated proteins of Nil hamster cells on $NaDodSO_4$ gels, Hynes and Destree (1977) and Keski-Oja et al. (1977) reported that LETS proteins existed in disulfide-linked aggregates that were probably dimers and larger aggregates. Our data permit us to conclude that such aggregates do not include significant amounts of disulfide linkages to other proteins of the cell surface, and that this pattern found with the isolated, purified protein is represen-

tative of the state of CSP in homogenates as visualized by protein staining.

Interchain disulfide linkages may play an important role by cross-linking CSP into multivalent adhesive complexes that can form a bridge between cells, or bind cells to the substratum. In preliminary experiments, reduction of the disulfide bonds of CSP using dithiothreitol destroyed the ability of CSP to agglutinate formalinized sheep erythrocytes (unpublished results).

The large apparent Stokes radius of the CSP monomer (110 Å) indicates that CSP is an elongated, asymmetric molecule. This configuration may help account for its tendency to form aggregates and adhesive interactions. The comigration of the CSP monomer and of CSP that had been reduced and alkylated suggests that CSP may not contain intrachain disulfide bonds that substantially alter the elongated shape of the molecule.

The amino acid composition of CSP is not unusual. It rules out the possibility that CSP could be a procollagen (Robbins et al., 1974) and the possibility that it is adhesive because it is a simple polyelectrolyte. CSP's content of 20% nonpolar residues is even lower than the 23% average of many other proteins (Dayhoff and Hunt, 1972; Jukes et al., 1975), and is consistent with CSP's resistance to solubilization by nonionic detergents that solubilize many hydrophobic membrane proteins.

CSP is a glycoprotein, with a sugar composition that suggests that it possesses several asparagine-linked oligosaccharides per molecule (Kornfeld and Kornfeld, 1976). Since oligosaccharides linked to serine can be removed by prolonged incubation at pH 12, it is conceivable that our pH 11 conditions could have resulted in loss of particularly sensitive oligosaccharides of this type. Glucose was detected in similar amounts in two separate preparations of the CSP multimer. It should be of interest to determine its structural significance, since glucose is generally found only in collagen-like proteins or in precursor oligosaccharides (Spiro et al., 1976; Robbins et al., 1977). It is likely that the glucose or mannose residues account for the ability of CSP to bind concanavalin A (Olden and Yamada, 1977a; Burridge, 1976). The presence of 5 mol of sialic acid per mol of CSP suggests that CSP contributes significantly to the surface charge of cells, particularly since it is present in such large quantities on the cell surface.

The amino acid composition of CSP resembles that of two other proteins thought to be similar to LETS proteins: the human plasma protein "cold insoluble globulin" (CIg), and to a lesser extent, the presumptive connective tissue protein subunit of microfibrils from cultured smooth muscle cells (Mosesson et al., 1975; Mosher, 1975; Muir et al., 1976). In addition, the overall carbohydrate compositions of CSP and CIg are similar (Mosesson et al., 1975), although the specific sugar composition of CIg is not yet available. However, the blocked NH_2 terminus of CSP is not removed by pyroglutamate aminopeptidase, suggesting that CSP probably possesses an acetylated amino-terminal residue rather than the terminal pyrrolidonecarboxylic acid reported for CIg (Mosesson et al., 1975). Moreover, although they are immunologically cross-reactive (Ruoslahti and Vaheri, 1975; Chen et al., 1976), CIg and human LETS protein reportedly do not comigrate in $NaDodSO_4$ gels (Keski-Oja et al., 1977). Whether these proteins are products of the same gene, but processed differently after transcription, or products of separate genes remains to be determined.

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Effect of Nucleotide Binding on the Proximity of the Essential Sulfhydryl Groups of Myosin. Chemical Probing of Movement of Residues during Conformational Transitions†

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ABSTRACT: The reaction of myosin with three bifunctional sulfhydryl reagents of differing cross-linking span is reported. In the absence of nucleotide only *p-N,N'*-phenylenedimaleimide with a cross-linking span of 12–14 Å can bridge between the two essential sulfhydryls of myosin. The other two reagents, 2,4-dinitro-1,5-difluorobenzene and 4,4'-difluoro-3,3'-dinitrodiphenyl sulfone with cross-linking spans of 3–5 and 7–10 Å, respectively, react under identical conditions with the SH_1 sulfhydryl but do not bridge to the SH_2 group. In the presence of MgADP, both *p-N,N'*-phenylenedimaleimide and 4,4'-difluoro-3,3'-dinitrodiphenyl sulfone bridge across the SH_1 and SH_2 groups indicating a closer proximity of these two sulfhydryls in the presence of bound nucleotide. These results are discussed in relation to the conformational change induced in myosin by binding of the nucleotide.

It is now well established that the binding and hydrolysis of nucleotides by myosin causes conformational perturbations of the protein which can be readily monitored by a variety of steady-state techniques such as UV[1] difference spectroscopy (Morita, 1967), fluorescence (Werber et al., 1972), ESR spectroscopy (Seidel and Gergely, 1973), and circular dichroism (Murphy, 1974). The detection of these conformational changes provided new impetus for attempts to link the

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[1] Abbreviations used: MalNEt, *N*-ethylmaleimide; pPDM, *p-N,N'*-phenylenedimaleimide; FDNB, fluorodinitrobenzene; F_2DNB, 2,4-dinitro-1,5-difluorobenzene; F_2DPS, 4,4'-difluoro-3,3'-dinitrodiphenyl sulfone; ADP, adenosine diphosphate; UV, ultraviolet; ESR, electron spin resonance.