

# The major cell surface glycoprotein of chick embryo fibroblasts is an agglutinin

(hemagglutination/cell adhesion/proteolysis/divalent cations/transformation)

KENNETH M. YAMADA, SUSAN S. YAMADA, AND IRA PASTAN

Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014

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**ABSTRACT** A major cell surface protein, CSP, of chick embryo fibroblasts has been shown to constitute up to 3% of total cell protein, and to be decreased after viral transformation. Its role in normal cell behavior is not known. We have isolated CSP from chick embryo fibroblasts by extraction with 1 M urea and find that these preparations of CSP agglutinate formalinized sheep erythrocytes at protein concentrations of under 2  $\mu\text{g}/\text{ml}$ . In extracts of chick embryo cells, the quantity of such hemagglutinating activity parallels that of CSP determined by electrophoresis, and both are substantially decreased in chick cells transformed by the Bryan high-titer strain of Rous sarcoma virus. Both CSP and hemagglutinating activity are progressively adsorbed onto erythrocytes and can be released by 1 M urea. An antiserum to purified CSP specifically blocks the agglutination. The agglutinating activity is destroyed by boiling or treatment with proteases. The agglutination reaction is inhibited by the chelating agents EDTA and EGTA [ethyleneglycol-bis( $\beta$ -aminoethyl ether) $N,N'$ -tetraacetic acid]. Agglutination is also inhibited to a lesser degree by amino sugars and other amines, increased osmolarity, and urea. Other monosaccharides, hyaluronidase, DNase, and RNase have little or no effect on the agglutination reaction. This demonstration that CSP has an agglutinating activity that is sensitive to proteases and that requires divalent cations suggests that this molecule may play a role in cell adhesion.

A high-molecular-weight protein is present on the surface of cultured cells and is reportedly decreased after transformation (see refs. 1 and 2). We have isolated such a cell surface protein (CSP) from chick embryo cells and found that it is a major cell protein constituting up to 3% of the total protein of these cells (3). The function of CSP is unknown, but the low level present in virally transformed cells suggests that its absence may be responsible for some of the altered surface properties of transformed cells.

One important function of the cell surface is that of cell-cell adhesion. Rosen *et al.* (4) have shown that formalinized sheep erythrocytes can be used to detect and isolate substances that are apparently responsible for the increased adhesiveness of aggregating slime mold cells. To investigate the possibility that CSP has such adhesive properties, we have examined its ability to bind to and agglutinate cells. Using a simple hemagglutination assay, we find that CSP agglutinates sheep erythrocytes and have characterized this biological activity of CSP in detail.

## MATERIALS AND METHODS

**Preparation of Cell Surface Protein.** We modified the previously described extraction procedure (3) to increase the

Abbreviation: EGTA, ethyleneglycol-bis( $\beta$ -aminoethyl ether) $N,N'$ -tetraacetic acid.

yield and purity of CSP. Monolayers of chick embryo fibroblasts were plated in disposable 690  $\text{cm}^2$  roller bottles (Bellco) and fed daily. After reaching confluence, monolayers were washed at 37° with 50 ml of Hanks' balanced salts solution four times, then rinsed 60 min with 25 ml of serum-free medium (Diploid Growth Medium or Dulbecco's modified Eagle's medium) containing 2 mM phenylmethylsulfonyl fluoride. After another rinse with salts solution, the monolayers were extracted for 2 hr with 25 ml of serum-free medium containing phenylmethylsulfonyl fluoride plus 1.0 M urea (Schwarz/Mann, ultrapure grade). After centrifugation at 25,000  $\times g$  for 15 min, the preparations were dialyzed for 20 hr against three changes of 40 volumes each of serum-free medium or calcium-magnesium free Dulbecco's phosphate-buffered saline at 4° with vigorous stirring. CSP was prepared weekly and stored at 4°.

**Hemagglutination Assay.** Formalinized sheep erythrocytes were prepared according to Butler (5), using calcium-magnesium free phosphate/saline (pH 7.4). Hemagglutinating activity was routinely assayed at room temperature using methods modified from those of Rosen *et al.* (4). A sample of 0.025 ml was serially 2-fold diluted in calcium-magnesium free phosphate/saline in Linbro "U"-shaped wells. Then 0.025 ml of a 2.5% suspension of formalinized red cells was added to each well. After sealing, the plate was agitated for 5 sec, again 3 min later, and then kept at room temperature for 16 hr. Although the assay could be read after 2 hr, all readings were made from photographs taken after 16 hr. Identical agglutinin activities were obtained if the assay was performed at 4°. Agglutinin patterns were similar to those of classical antibody hemagglutination assays (e.g., refs. 6 and 7). For inhibitor studies, volumes of material added to the wells were adjusted to give a final erythrocyte concentration of 1.25% and a hemagglutination activity of 4-8 units per the final assay volume of 0.05 ml.

**Electrophoresis.** Extracts were prepared for electrophoresis in 5% polyacrylamide sodium dodecyl sulfate gels as described (3). Protein was estimated relative to bovine serum albumin standards by densitometry at 550 nm of gels stained with Coomassie blue. Absorbance was linearly proportional to CSP (3) or albumin concentration up to 8  $\mu\text{g}$  per gel.

**Erythrocyte Binding and Release of CSP.** Formalinized sheep erythrocytes were pretreated with 1 M urea in calcium-magnesium free phosphate/saline containing 1 mM phenylmethylsulfonyl fluoride for 2 hr, then washed five times by centrifugation and resuspension in phosphate/saline. Packed erythrocytes were resuspended in 9 volumes of a CSP preparation in phosphate/saline, and maintained in

suspension by rotary shaking at 100 rpm at room temperature. In the parallel control, an equal volume of phosphate/saline was substituted for the erythrocytes. At intervals, 0.5 ml of each mixture was centrifuged in a Beckman Microfuge (approximately  $10,000 \times g$ ) for 15 sec. The supernatants were removed, recentrifuged, and stored on ice. The pellets were vigorously resuspended in phosphate/saline with a pasteur pipet, centrifuged, and washed twice more. The pellets were then resuspended in phosphate/saline containing 1 M urea and incubated 30 min at  $23^\circ$  with occasional agitation. After centrifugation, all the supernatants were assayed for hemagglutinating activity. Other aliquots were dialyzed against water, lyophilized, and analyzed by sodium dodecyl sulfate gel electrophoresis for CSP. Because we knew that high concentrations of urea would inhibit agglutination (see *Results*), we extracted the erythrocytes in urea using half the original volume (0.25 ml) to increase the concentration of agglutinin 2-fold. As a result, the serial dilution assay could be performed with the end-point in less than the lowest known inhibitory concentration of urea (200 mM). To correct for the 50% reduction in volume, we have divided the activity by two, and electrophoresed only half the volume of extract.

**Materials.** All media were obtained from the NIH Media Unit. Human umbilical cord hyaluronic acid (Grade I) and pig skin chondroitin sulfate were from Sigma; bovine vitreous humor hyaluronic acid was from Worthington; heparin was from Hynson, Westcott, and Dunning. All enzymes were the purest grade available from Worthington: trypsin, 206 U/mg;  $\alpha$ -chymotrypsin, 50 U/mg; deoxyribonuclease I, 2450 U/mg; ribonuclease A, 5139 U/mg; and hyaluronidase, 3000 U/mg.

## RESULTS

### Hemagglutination by CSP extracts

Previously we had prepared CSP by extracting cells with 0.2 M urea (3). We were able to increase the yield of CSP approximately 8-fold by increasing the concentration of urea to 1 M and including the protease inhibitor phenylmethylsulfonyl fluoride during extraction. Purity of CSP, as determined by Coomassie blue staining of gels, rose from 20% to at least 60–75% (Fig. 1). Such preparations contained an average of 55  $\mu\text{g/ml}$  of protein by the Lowry *et al.* method (8) with a bovine serum albumin standard and 5  $\mu\text{g/ml}$  of carbohydrate by the phenol sulfuric acid method (9) with a galactose standard.

CSP, prepared as described in *Materials and Methods* agglutinated formalinized sheep erythrocytes (Fig. 2) with activities of 4–16 units, where a unit is the reciprocal of the

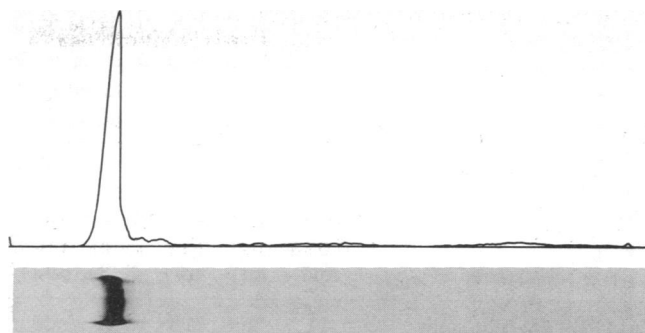


FIG. 1. Electrophoresis of isolated CSP. CSP was prepared as described in *Materials and Methods*. A sodium dodecyl sulfate 5% polyacrylamide gel was stained with Coomassie blue, and a densitometer tracing was made recording absorbance at 550 nm. Origin is at left.

dilution giving half-maximal agglutination. The final concentration of protein in the assay mixture at this end-point dilution was less than 2  $\mu\text{g/ml}$ . By phase microscopy, unagglutinated controls consisted of single erythrocytes, whereas assays containing CSP consisted of clumps of erythrocytes, with occasional single cells.

The amount of hemagglutinating activity detected in different preparations of CSP was roughly proportional to the CSP content of extracts, as determined by gel electrophoresis (Table 1). In the standard extraction procedure, the first 60-min rinse with serum-free medium contained little CSP or agglutinating activity. Shortening the extraction in urea to 15 min yielded reduced quantities of both CSP and hemagglutinating activity. Since virus-transformed chick fibroblasts contain substantially reduced quantities of CSP (refs. 10 and 11; unpublished results), we compared yields of extractable CSP from cultures transformed by the Bryan high-titer strain of Rous sarcoma virus. We found little CSP or hemagglutinating activity in such urea extracts (Table 1). The lower specific activity could be due to an inhibitor of the agglutinin in these transformed cells.

CSP preparations gradually lost agglutinating activity on storage at  $4^\circ$  at a rate of roughly 10–20% per day. The loss of activity was up to 2-fold faster in calcium-magnesium free phosphate/saline compared to phosphate/saline or serum-free medium. Consequently, we prepared fresh CSP each week and used CSP in media without  $\text{Ca}^{++}$  only for the studies with chelating agents.

Hyaluronic acid has been reported to increase the aggregation of dissociated cells from several cell lines (12), suggesting that the agglutinating activity of CSP preparations might be due to contaminating glycosaminoglycans. However, CSP preparations contained only 5  $\mu\text{g/ml}$  of sugar

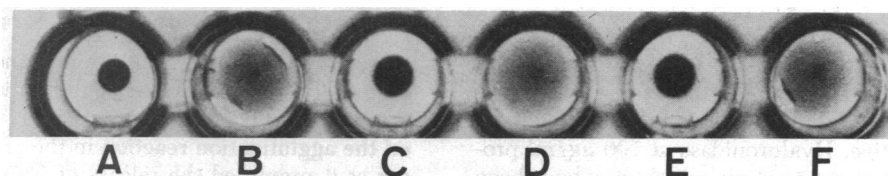


FIG. 2. Hemagglutination of formalinized sheep erythrocytes by CSP and its immunological inhibition. (A) Control, showing button of unagglutinated cells. (B) Dialyzed CSP extract, showing carpet of agglutinated cells covering the bottom of microtiter well. (C) CSP with anti-CSP gamma globulin diluted  $\frac{1}{100}$ . Antiserum prepared against electrophoretically purified CSP was precipitated three times with 40% saturated ammonium sulfate, dissolved in the original volume of calcium-magnesium free phosphate/saline, and then incubated 30 min at room temperature with CSP prior to the addition of erythrocytes. (D) CSP with anti-CSP gamma globulin pre-absorbed with CSP and incubated as in (C). Absorbed sera were prepared by incubating 300  $\mu\text{g/ml}$  of CSP or bovine serum albumin with  $\frac{1}{10}$  volume of anti-CSP gamma globulin for 16 hr at  $4^\circ$ , then centrifuging at  $10,000 \times g$  for 10 min. (E) CSP with anti-CSP pre-absorbed with bovine serum albumin. (F) CSP with  $\frac{1}{100}$  diluted gamma globulin fraction of an antiserum prepared against electrophoretically purified platelet actin.

Table 1. Comparison of hemagglutinating activity and CSP content of extracts of chick embryo fibroblasts

Extracts	Activ-ity		Total protein (μg/ml)	Specific activity	
	(units/0.025 ml)	(μg/ml)		(units/μg of total protein)	(units/μg of total protein)
(a) CEF, 60-min rinse	<1	2.4	13	<17	<3
(b) CEF, 15-min urea	3	7.5	10	16	12
(c) CEF, 120-min urea	8	20.0	31	16	10
(d) BH-CEF, 60 min rinse	0	0.1	8	—	—
(e) BH-CEF, 120-min urea	<1	4.9	21	<8	<2

Cultures of nontransformed chick embryo fibroblasts (CEF) or cells transformed by Bryan high-titer Rous sarcoma virus (BH-CEF) containing equal amounts of protein were extracted for CSP as described in *Materials and Methods*. The 60-min rinses in serum-free medium (a and d) were saved and directly analyzed by the hemagglutination assay. The subsequent urea extractions were performed for 15 min (b) or the usual 120 min (c and e). These urea extracts were dialyzed 20 hr against three changes of serum-free medium to remove urea, then assayed for activity. An aliquot of each sample was also dialyzed against deionized water and assayed for total protein by the Lowry *et al.* method and for CSP by densitometry of polyacrylamide gels.

by the phenol-sulfuric acid method (9), and hemagglutination assays performed in the presence of hyaluronic acid, chondroitin sulfate, and heparin at concentrations of 1, 10, 100, and 1000 μg/ml did not mimic the agglutination.

#### Binding to erythrocytes

If CSP is responsible for the hemagglutinating activity, both CSP and hemagglutinating activity should be removed from the medium by the added erythrocytes, and both activities might be recovered if the erythrocytes were then extracted in urea, as for the original isolation of CSP. We found that a 10% suspension of formalinized erythrocytes progressively depleted CSP (identified electrophoretically) and hemagglutinating activity, and that incubation of such erythrocytes in 1 M urea released both CSP and agglutinating activity (Table 2). The low specific activity of CSP remaining unabsorbed to erythrocytes after 20 min of incubation, and the high specific activity of the material released by urea from the erythrocytes, suggest that a portion of the CSP is not active as an agglutinin. This may be explained by our finding that CSP preparations gradually lose hemagglutinating activity during dialysis and storage.

#### Agglutinin enzyme and temperature sensitivity

CSP is known to be rapidly hydrolyzed by low concentrations of trypsin (1, 3). We found that the agglutinin activity was also inactivated by treatment with low quantities of proteases prior to the assay (Table 3). Deoxyribonuclease and ribonuclease were inactive. Hyaluronidase at 100 μg/ml produced a small amount of inactivation, which may have been due to a contaminating protease. Agglutinin activity was also destroyed by incubation for 2 min at 100°.

#### Immunological inhibition of agglutination

We have prepared goat antiserum to CSP isolated by gel electrophoresis (in preparation). The antibody blocked agglutination at dilutions as low as 1:200 (Fig. 2). The inhi-

Table 2. Binding and release of hemagglutinating activity and CSP

Time (min)	CSP remaining in supernatant			CSP eluted by urea		
	Activ-ity*	Pro-tein†	S.A.‡	Activ-ity	Protein	S.A.
0	8	4.2	15§	0	0.1	—§
0.5	4	3.0	11	3	1.0	24
5	2	1.3	12	4	0.6	53
10	1.5	1.5	8	4	0.7	46
20	1	0.8	10	5	0.9	44

A CSP preparation was incubated with sheep erythrocytes as described in *Materials and Methods*. At the times indicated, aliquots were removed and centrifuged. The supernatants were then assayed for hemagglutinating activity and CSP (determined electrophoretically). The pellets were washed three times and then extracted for 30 min with 1 M urea. After the erythrocytes were sedimented, these supernatants were also assayed for hemagglutinating activity and CSP.

\* Hemagglutinating activity in units/0.025-ml aliquot.

† Protein (μg) in the CSP peak of a 0.2-ml aliquot by electrophoresis and densitometry relative to bovine serum albumin standards.

‡ Specific activity (units/μg of CSP).

§ The zero time point was determined prior to the addition of erythrocytes. CSP preparations incubated without erythrocytes maintained the same activity and protein values at each of the later time points.

tion of agglutination did not occur if the antibody to CSP was pre-absorbed with CSP, but not with equal quantities of bovine serum albumin. A control immune antiserum prepared against electrophoretically purified platelet actin did not block agglutination (Fig. 2).

#### Divalent cation requirement

Activities of CSP preparations dialyzed against calcium-magnesium free phosphate/saline were the same whether the hemagglutination assay was performed with or without added calcium. However, agglutinin activity was markedly inhibited by the addition of micromolar concentrations of the chelating agents EDTA or EGTA [ethyleneglycol-bis(β-aminoethyl ether)*N,N'*-tetraacetic acid] (Table 4). The inhibition of agglutination by EGTA was overcome by adding divalent cations to the reaction mixture: the concentration of cation necessary to permit half-maximal agglutination in the presence of 20 μM EGTA were 5 μM for Ca<sup>++</sup> and Mn<sup>++</sup>, but over 1000 μM for Mg<sup>++</sup>, which is consistent with the known stability constants of EGTA and each of these cations (13). These findings indicate that a divalent cation such as Ca<sup>++</sup> or Mn<sup>++</sup> is required for the agglutination reaction.

#### Sensitivity to salts, urea, and sugars

Increasing the salt concentration in the assay mixture above the usual 0.15 M inhibited agglutination, although maximal inhibition did not occur until 0.35 M (Table 4). Urea inhibited the agglutination reaction in the same concentration ranges as it promoted the release of CSP from cell monolayers (Table 4). Hemagglutination was maximally inhibited by 1 M urea, the concentration that was maximally effective for releasing CSP from chick cells.

Many agglutinins, including lectins, are inhibited by specific monosaccharides. We therefore tested a wide variety of monosaccharides; none of these proved to be a specific inhibitor of CSP-mediated agglutination (Table 4). However,

Table 3. Enzymatic inactivation of hemagglutinating activity

Enzyme	Concentration ( $\mu\text{g/ml}$ )	Decrease of activity (%)
Trypsin	0.1	0
	0.5	35
	1	50
	2	100
Chymotrypsin	0.5	0
	1	70
	2	100
Deoxyribonuclease	100	0
Ribonuclease A	100	0
Hyaluronidase	20	0
	50	15
	100	20

CSP preparations in Diploid Growth Medium were incubated at the indicated final concentrations of enzyme at 37° for 20 min. The mixtures were then chilled on ice, then assayed for hemagglutinating activity along with parallel incubated controls without enzyme. The action of trypsin was terminated by the addition of soybean trypsin inhibitor at 200  $\mu\text{g/ml}$  prior to assay. With chymotrypsin, no inhibition of agglutination was noted if added to assay without preincubation with CSP.

the three amino sugars tested, glucosamine, galactosamine, and mannosamine, inhibited agglutination half-maximally at 30 mM, while all their *N*-acetyl derivatives were inactive up to 100 mM. The amino sugars had been obtained in the HCl form, and the solutions were neutralized to pH 7.4 with NaOH prior to testing. By conductivity measurements, 0.20 M stocks of these sugars contained the equivalent of 0.10–0.11 M NaCl above the conductivity of calcium-magnesium free phosphate/saline alone. This quantity of NaCl would not be sufficient to account for all the inhibition seen by the amino sugars (e.g., a 30 mM sugar mixture would contain only 15–17 mM extra NaCl). We tested other amines, including tris (hydroxy methyl) amino methane, arginine, lysine, histidine, and glutamine. They all proved to be inhibitors of hemagglutination by CSP (Table 4). It is of interest that amines have also been found to inhibit the aggregation of embryonic liver and neural retinal cells (14, 15).

## DISCUSSION

How the cell surface influences cell behavior could be effectively analyzed if each of the surface components could be isolated and characterized. One major cell surface glycoprotein, CSP, has been isolated from chick embryo fibroblasts by urea extraction (3). Isolated CSP has an apparent molecular weight of 220,000 on sodium dodecyl sulfate polyacrylamide gels, and is markedly trypsin-sensitive (3).

We show here that extracts containing CSP as the major protein component agglutinate formalinized sheep erythrocytes. We have investigated whether it is CSP itself or a contaminant present in the extract which is the agglutinin. The following data strongly suggest that CSP is the active substance: On sodium dodecyl sulfate polyacrylamide gels, CSP accounts for up to 80% of the protein in extracts. The minor contaminating peaks vary in intensity in different preparations, and can be virtually absent from highly active preparations (see Fig. 1).

Both agglutinating activity and CSP are nondialyzable, trypsin-sensitive, poorly extracted from cells by serum-free medium, readily extracted by urea, progressively adsorbed

Table 4. Inhibitors of hemagglutinating activity

	Concentration to inhibit agglutination (mM)		
	Minimum	Half-maximal	Maximal
EDTA	0.007	0.010	$\geq 0.015$
EGTA	0.007	0.008	$\geq 0.015$
NaCl	10	50	200
KCl	20–30	50	200
Urea	200	250	1000
D-Glucosamine	5–10	30	100
D-Galactosamine	10	30	100
D-Mannosamine	5	30	100
<i>N</i> -Acetyl neuraminic acid	6–12	100	>100
D-Glucuronic acid	60	100	>100
D-Gluconic acid	20	100	>100
Tris-HCl	30	80–100	>100
L-Arginine	10	40	>100
L-Lysine	6	30	>100
L-Histidine	6	40	>100
L-Glutamine	10	100	>100
L-Glutamic acid	$\geq 100$	—	—
Glycine	>100	—	—

Inhibitors were added to calcium-magnesium free phosphate/saline and adjusted to pH 7.4 with HCl or NaOH and assayed in duplicate in at least two dilution series as indicated in *Materials and Methods*. Minimum inhibitory concentration is the lowest that results in a ring pattern in the well (see ref. 7). Half-maximal inhibition is the concentration at which the diameter of settled erythrocytes is half the difference between the diameters of fully agglutinated and nonagglutinated wells (wells with CSP without inhibitor and wells without CSP). Maximal inhibition is the concentration that results in the nonagglutinated pattern (a compact button of erythrocytes at the bottom of the well). All reagents were tested for artifactual hemagglutination patterns without CSP; all were negative at the highest concentration indicated. The following sugars did not inhibit agglutination even minimally at 100 mM: D-glucose, D-galactose, D-mannose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, and *N*-acetyl-D-mannosamine; also L-fucose,  $\alpha$ -methyl-D-glucoside,  $\alpha$ -methyl-D-mannoside, D-xylose, L-rhamnose, L-arabinose, 3-*O*-methylglucose, maltose, lactose, and sucrose.

onto formalinized red cells, and released by urea. Moreover, both are present in only low quantities in urea extracts of transformed chick fibroblasts. Finally, antibodies prepared against electrophoretically purified CSP will inhibit the hemagglutination reaction.

Trypsin and agents that chelate divalent cations are routinely used to dissociate cells. The marked sensitivity of agglutination by CSP to trypsin and to EDTA and EGTA suggests that CSP may play a role in cell-cell or cell-substrate adhesion. Besides binding to erythrocytes, CSP can also be reabsorbed back onto the surface of chick fibroblasts (2). Preliminary experiments using rotation-mediated assays for cell aggregation indicate that CSP preparations can adsorb to and rapidly agglutinate several other cell types, including mechanically dissociated 24-hr chick embryo cells and NRK (Normal Rat Kidney) cells transformed by murine sarcoma virus. We have not yet investigated the specificity of agglutination by CSP to establish whether it is tissue-specific, as is the 50,000-dalton cell-aggregating factor of neural retinal cells (16), or whether it is a more general type of agglutinin.

Our finding that CSP is an agglutinin provides the first demonstration of a biological activity for this major cell sur-

face component. The hemagglutination assay provides a rapid, simple biological assay for CSP which complements the measurement of denatured CSP by electrophoresis in polyacrylamide gels. Our information should facilitate further purification of CSP for biochemical studies and quantitation of CSP in various tissues *in vivo*, as well as providing an approach to investigating whether the reported decreases in cell-cell adhesion of tumor cells *in vivo* (17) is related to decreased production of CSP or related cell surface molecules.

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