Expression and Function of a Putative Cell Surface Receptor for Fibronectin in Hamster and Human Cell Lines

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Abstract. We have previously reported the use of monoclonal antibodies to identify a 140-kD cell surface glycoprotein in mammalian cells that is specifically involved in fibronectin-mediated cell adhesion. We now report the purification of this molecule using immunoaffinity chromatography and the subsequent generation of polyclonal antibodies that selectively immunoprecipitate 140-kD putative fibronectin receptor glycoprotein (gp140) extracted from rodent or human cells; these antibodies also specifically block fibronectin-mediated cell adhesion but not adhesion mediated by other factors in serum. Expression of gp140-like molecules was detected on the surfaces of several adherent human cell lines (HDF, WISH, and EFC) but not on erythrocytes; however, gp140 was also detected on a nonadherent human lymphoid line (DAUDI). Analysis of gp140 on nonreducing SDS gels revealed two closely migrating bands. Protease digestion and peptide mapping suggests that the two bands are closely related polypeptides.

THE adhesion of cells to the extracellular matrix is a complex, multistage process. An early stage presumably involves interaction between adhesive protein ligands in the matrix such as fibronectin (Fn),[1] vitronectin, or laminin, and their receptors on the cell surface (4, 22, 32, 33). Later stages include cellular metabolic activities and the recruitment of cytoskeletal elements involved in morphological changes and cell spreading (9, 13). Fn, a major adhesion ligand for mesenchymal cells, is a complex multidomain protein (16). Recently most of the adhesion promoting activity of Fn has been shown to reside in a tetrapeptide segment that is part of one domain of the molecule (25); however the overall conformation of the Fn molecule may also be important in modulating the adhesion activity of this protein (29–31).

Establishment of the identity of the surface receptor for Fn has been elusive, probably because of the relatively low affinity of soluble Fn for its receptor (2, 22). Investigators have suggested several molecular classes, including glycolipids (8) and proteoglycans (21), as candidates. Recently, however, attention has mainly focused on cell surface glycoproteins as the receptors involved in the adhesion promoting functions of Fn (19, 24, 28). Thus our laboratory has recently described two monoclonal antibodies (designated PB1 and PB2) that can selectively inhibit Fn-mediated adhesion in hamster fibroblastic cells (Chinese hamster ovary [CHO] line) without affecting adhesion processes mediated by laminin, vitronectin, or the complex mixture of adhesion factors in serum (6). Both monoclonals immunoprecipitate a 140-kD glycoprotein from cells labeled biosynthetically ([^{3}H]-glucosamine, [^{35}S]methionine), or with ^{125}I using surface radioiodination. Ruoslahti's laboratory has also recently described a 140-kD protein isolated from human osteosarcoma cells that binds to Fn affinity columns and is specifically eluted by a hexapeptide with adhesion promoting activity (26). The 140-kD components described above are thus likely candidates for the mammalian cell Fn receptor. The 140-kD component may also be a member of a group of adhesion-related glycoproteins previously described in mammalian cells (19, 11). In avian cells, adhesion has been associated with a complex composed of three distinct cell surface glycoproteins with molecular masses of 155–160, 135, and 110–120 kD (7, 18, 23). Thus, it is becoming evident that the cellular receptors for Fn in a variety of cells, are likely to comprise one or more cell surface glycoproteins.

We wished to explore the expression and function of the 140-kD putative Fn receptor glycoprotein (gp140) in adhesive and nonadhesive mammalian cells. However, the monoclonal antibodies PB1 and PB2 were so selective that they recognized the gp140 molecule in CHO cells but not in human or murine cell lines. For this reason we elected to use the PB1 monoclonal to purify gp140 to apparent homogeneity and subsequently raised a polyclonal antibody to this component. In this report we demonstrate that the polyclonal anti-gp140 antibody specifically immunoprecipitates gp140 in CHO cells. Furthermore, the polyclonal antibody, like the PB1 and PB2 monoclonals, selectively blocks Fn-dependent cell adhesion, but not other adhesion processes. Polyclonal

1. *Abbreviations used in this paper*: CHO, Chinese hamster ovary; DPBS, Dulbecco's PBS; Fn, fibronectin; gp140, 140-kD putative Fn receptor glycoprotein; L band, lower band; NGIgG, nonimmune goat IgG; NP-40, Nonidet P-40; U band, upper band.

anti-hamster gp140 recognizes and immunoprecipitates cross-reactive glycoproteins of similar molecular mass in a number of human cell lines. This antibody affects the adhesion and morphology of cells expressing gp140-like molecules on their surfaces including fibroblasts, amnion cells, and glioma cells. Thus evidence suggests that cell surface molecules similar to CHO cell gp140 play a role in the Fn-mediated adhesion processes of a number of cell types.

Materials and Methods

Cell Culture

CHO cells were grown and maintained in suspension culture as previously described (12). Human diploid fibroblasts (HDF) (a gift of Dr. C. Moore, Shriner's Children Hospital, Houston, TX) were grown in Eagle's minimum essential alpha medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Hazleton Research Products, Inc., Denver, PA) and 1% antibiotics (Gibco). The human cell lines, EFC (glioblastoma), WISH (amnion), and DAUDI (lymphoblastoid), were provided by Dr. P. J. Kelleher (M.D. Anderson Hospital, Houston, TX). Suspension cells (CHO and DAUDI) were harvested by centrifugation when the cell density attained 8×10^5 cells/ml and washed three times with Dulbecco's PBS (DPBS). Subconfluent monolayer cultures (HDF, WISH, and EFC) were washed three times with DPBS and the cells removed by treatment with 5 mM EDTA in DPBS and scraping.

Protein Determination

Protein concentrations were determined using the BCA protein assay reagent according to the manufacturer's directions (Pierce Chemical Co., Rockford, IL). BSA was used as the standard.

Radiolabeling

CHO cells were metabolically labeled with 4 μCi/ml [^{3}H]leucine overnight in suspension culture. Cells ($2-3 \times 10^7$) were surface-labeled with 1 mCi [Na]^{125}I using the Iodogen^R surface-labeling technique (17).

Preparation of Affinity Columns

Monoclonal antibody PB1 was purified from ascites fluid as described previously (6), and covalently coupled to Affi-gel 10 (Bio-Rad Laboratories, Richmond, CA) as recommended by the manufacturer. The resulting gel contained ~15–20 mg/ml covalently coupled monoclonal antibody. Goat anti-gp140 and preimmune goat IgG were covalently coupled to Affi-gel 10 with ~25 mg antibody/ml of Affi-gel for each.

Isolation of gp140

Approximately 2×10^{10} CHO cells were harvested by centrifugation at 750 *g* for 10 min and were washed twice with ice cold DPBS. The cell pellet (5×10^7 cells/ml) was extracted with 20 mM Tris HCl (pH 8.3), 100 mM NaCl, 1% Nonidet P-40 (NP-40), 0.1% NaN_3, 2 mM phenylmethylsulfonyl floride, and 1 mM EDTA (lysis buffer) for 60 min at 4°C with occasional vortexing. The cell extract was centrifuged for 1 h at 42,400 *g* (JA-21, JA-20 rotor; Beckman Instruments Inc., Fullerton, CA) and for 45 min at 150,000 *g* (L5-40, 70.1 Ti rotor; Beckman Instruments Inc.). The supernatant was first applied to a Sepharose Cl-4B (10 ml bed volume; Pharmacia Fine Chemicals Inc., Piscataway, NJ) column previously equilibrated in lysis buffer to remove any nonspecific binding material from the extract. The flow through from the Sepharose column was pumped onto a prewashed immunoaffinity column. The column consisted of 70 mg of PB1 monoclonal covalently coupled to 5 ml Affi-gel 10 according to the manufacturer's directions. The sample was passed twice over the PB1 immunoaffinity column. The column was washed with 20 bed volumes of TNEN buffer (20 mM Tris HCl [pH 8.2], 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) (5) and eluted with 0.2 N acetic acid, (pH 2.5), 100 mM NaCl, and 1% NP-40. 1-ml fractions were collected and neutralized to pH 7.3 with 0.233 ml 1 M Tris.

Production of Polyclonal Anti-gp140 Antibodies

Antiserum against gp140 was produced in a 1-yr-old male goat by three injections at 2-wk intervals of 100 μg gp140 mixed 1:1 with complete Freund's adjuvant. Half of the gp140 (50 μg) was in its native state while 50 μg had been blotted onto nitrocellulose and dissolved in dimethyl sulfoxide. Serum was collected 1 wk after the third immunization and tested for anti-CHO binding in an enzyme-linked immunosorbant assay using anti-goat-horseradish peroxidase conjugate (Cappel Laboratories, Cochranville, PA) with *O*-phenylenediamine (Sigma Chemical Co., St. Louis, MO) as the substrate. The IgG fraction of the antiserum was obtained by two ammonium sulfate fractionations, (30 and 45%). The final pellet was dissolved in a minimum volume of DPBS and dialyzed against four changes of DPBS at 100 times the volume of the dissolved pellet.

Immunoprecipitations

^{125}I surface-labeled cells were extracted with lysis buffer (1×10^7 cells/ml) for 60 min at 4°C and were subsequently centrifuged at 12,500 *g* in an Eppendorf microfuge for 15 min. The supernatant was passed over a 0.5-ml column of Sepharose Cl-4B pre-equilibrated in a lysis buffer. The flow through was collected and the protein concentration of each sample was determined by the Pierce BCA Protein assay. The specific activity was determined by 10% TCA precipitation of a 10-μl aliquot with 20 μg bovine albumin providing carrier protein. The pellet was washed twice with 10% TCA and counted in a gamma counter. The protein concentration of each sample was adjusted to 360 μg/ml and 1.2 ml added to 50 μl Affi-gel 10 coupled to goat-anti gp140 (1.2 mg) or 50 μl Affi-gel 10 coupled to preimmune goat (1.2 mg). In addition, some samples of CHO cells were also immunoprecipitated with PB1 monoclonal coupled to Affi-gel. The immunoprecipitations were carried out at 4°C for 4 h on a rotating platform. The samples were washed four times with TNEN buffer and solubilized by boiling with 100 μl Laemmli sampler buffer with 2-mercaptoethanol (20).

Electrophoresis

SDS PAGE was carried out essentially according to the method of Laemmli (20) except that both the stacking and separating gels contained 0.3% linear polyacrylamide (Polysciences Inc., Warrington, PA) (13).

Adhesion Blocking Assays

The wells of 24 well tissue culture plates were coated with the appropriate ligand (Fn, 100 μg/ml or Fn-depleted serum, 20%) according to published procedures (12, 13). Dilutions of goat anti-gp140 or preimmune goat IgG (NGIgG) were made in ice cold Eagle's essential minimum alpha medium with 0.1% BSA and 1% antibiotics (adhesion medium) and were added to the ligand-coated wells. $1-2 \times 10^5$ washed cells were pipetted into each well and the plate incubated at 4°C for 60 min to allow antibody binding. The plates were then transferred to 37°C and incubated for a period of time allowing control cells to attach and spread. For CHO cells, the time required was 60 min, whereas the other cell lines required 3 h. In some experiments monolayers were formed followed by the addition of the appropriate antibody dilutions. The plates were then incubated at 37°C for 18 h and results recorded photographically. All experiments were done in triplicate.

Determination of the Number of gp140-Binding Sites

Suspension culture CHO cells were washed with PBS and fixed with 3% formaldehyde for 10 min at room temperature. The cells were washed with PBS and with Eagle's essential minimum alpha medium and resuspended at 5×10^7 cells in PBS plus 0.2% BSA. PB1 monoclonal antibody was radiolabeled using Protag-125^{TM} (J.T. Chemical Baker Co., Phillipsburg, NJ) according to the manufacturer's protocol. Dilutions of radiolabeled antibody were made in PBS with 0.2% BSA and 1 mg/ml mouse gamma globulin or with PBS 0.2% BSA and 80 μg/ml cold PB1 monoclonal antibody. 100 μl of cell suspension (5×10^6 cells) was added to each tube and the binding carried out at 4°C for 2 h. After the incubation, the cells were spun down and a 100-μl aliquot of the supernatant retained for determining free labeled antibody. The cells were washed four times and then counted in a gamma counter.

Immunoblotting

Samples of adhesion-related proteins and antibodies to these proteins were compared by immunoblot techniques. 100 μl of appropriate dilutions of an-

tigen were loaded into each well of a micro-filtration apparatus (BioRad Laboratories) with a nitrocellulose membrane and allowed to bind during gravity filtration. The membrane was air dried and stored until use. The membrane was blocked with 3% gelatin in Tris-buffered saline (pH 7.5) (20 mM Tris, and 500 mM NaCl) for 30–60 min and then incubated with an appropriate dilution of primary antibody in 1% gelatin in Tris-buffered saline for 2 h at 25°C with gentle agitation. The membrane was washed with two changes of 0.05% Tween 20 in Tris-buffered saline followed by incubation with horse radish peroxidase-coupled second antibody (BioRad Laboratories) (1/1000 in 1% gelatin in TBS), and washed and developed in horse radish peroxidase color development solution. The antigens and antibodies used were (*a*) normal goat IgG; (*b*) goat anti-hamster gpl40 IgG; (*c*) affinity-purified goat anti-hamster gpl40 IgG; (*d*) affinity-purified rabbit anti-human Fn receptor IgG (19) obtained from E. Ruoslahti, La Jolla Cancer Research Center Foundation, La Jolla, CA; (*e*) normal rabbit IgG; (*f*) rabbit anti-CSAT antigen serum; (*g*) normal rabbit serum; and (*h*) CSAT antigen (23). Items *f-h* were obtained from C. Buck (Wistar Institute of Anatomy and Biology, Philadelphia, PA).

Peptide Mapping

Two-dimensional peptide maps of radioiodinated upper (U) and lower (L) gpl40 bands were prepared by the method of Elder et al. (10). Briefly, purified gpl40 was resolved on a 6% nonreducing gel. Gel regions containing the resolved U and L bands were identified by Coomassie Blue staining. Slices containing the separated U and L bands were ^{125}I-labeled in situ using chloramine T as described (10). After removal of excess isotope, the proteins were digested overnight at 37°C with 50 μg/ml each of trypsin and chymotrypsin in 50 mM NH_4CO_3 buffer (pH 8). The supernatants were lyophilized, rehydrated in appropriate buffer, and then spotted onto cellulose-coated thin layer chromatography plates and resolved by high voltage electrophoresis in the first dimension and thin layer chromatography in the second dimension. The electrophoresis buffer was acetic acid/formic acid/water (15:5:80), whereas the chromatography solvent was butanol/pyridine/acetic acid/water (33:25:5:20). The dried thin layer chromatography plates were autoradiographed on Kodak XRP film to visualize the ^{125}I peptides generated. Approximately equal cpm were spotted on to the plates for analysis of U or L bands.

Results

gpl40 in CHO Cells

As shown previously, monoclonal antibodies PB1 and PB2 specifically inhibit Fn-mediated adhesion in CHO cells (6); these antibodies selectively precipitate a 140-kD cell surface glycoprotein from the cells. This result is confirmed in Fig. 1 where lanes *b* and *c* show the immunoprecipitation pattern on SDS PAGE of PB1 and PB2 respectively, while lane *d* shows the immunoprecipitation pattern for 3F9, an unrelated monoclonal that does not block adhesion and that precipitates a cell surface component with a molecular mass of 265 kD. The band precipitated by PB1 and PB2 is one member of a complex of ^{125}I-surface labeled proteins in the 130–150-kD range (lane *e*) (13).

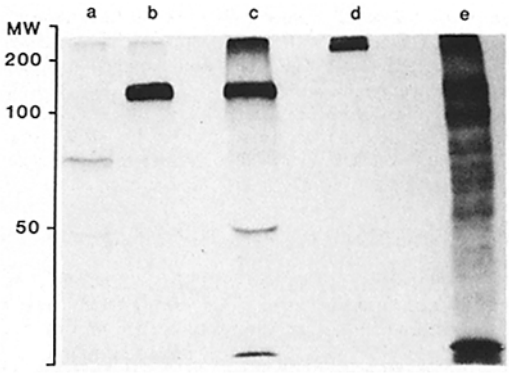

Figure 1. Immunoprecipitation of cell surface proteins by adhesion blocking monoclonals. ^{125}I surface-labeled cells were solubilized in 1% NP-40 plus 1 mM phenylmethylsulfonyl fluoride in PBS and lysates subjected to immunoprecipitation using monoclonal antibodies coupled to Protein A Sepharose beads (Pharmacia Fine Chemicals) via rabbit anti-mouse IgG (Miles Laboratories, Inc., Elkhart, IN). Approximately 1 × 10^7 cell equivalents were immunoprecipitated and 1–2 × 10^6 cell equivalents were run in each lane. Lane *a*, Control, no monoclonal antibody; lane *b*, PB1 monoclonal antibody; lane *c*, PB2 monoclonal antibody; lane *d*, 3F9 monoclonal antibody; lane *e*, total ^{125}I-labeled cell surface proteins. 7.5% SDS PAGE run under reducing conditions.

The gpl40 antigen is moderately abundant on the cell surface. Thus binding assays with ^{125}I-labeled PB1 shows that saturation occurs at a concentration of ~10 nM PB1 with 0.7 pmol/10^6 cells specifically bound (Fig. 2 *A*). A Scatchard analysis of the data indicates a single PB1 binding site present at ~1 × 10^5 copies per cell and having an affinity of 5 × 10^8 M (Fig. 2 *B*). It is not clear if the apparent deviation from linearity at low PB1 concentration represents positive cooperativity, or simply reflects errors in the data due to low counts. The number of PB1 binding sites agrees well with previous estimates of the numbers of cell surface binding sites for Fn (1, 3, 22).

gpl40 was purified from NP-40 lysates of CHO suspension cells using PB1 coupled to Affi-gel 10 as affinity column (see Materials and Methods). Judged by silver staining after SDS PAGE, gpl40 appears to be homogeneous (Fig. 3). Approximately 600 μg purified gpl40 was obtained from 2 × 10^{10}

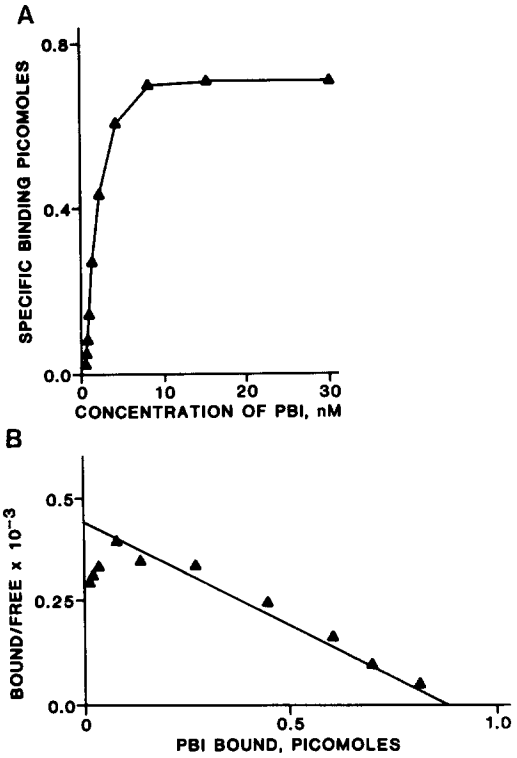

Figure 2. Binding of PB1 monoclonal antibody to CHO cells. ^{125}I PB1 binding to fixed CHO suspension cells was measured as described in Materials and Methods. Specific binding was calculated by the difference in the cpm bound in the absence and presence of 500 nM "cold" PB1. Points represent means of triplicate determinations differing by <5%. (*A*) Specific binding in picomoles/10^6 cells. (*B*) Scatchard plot.

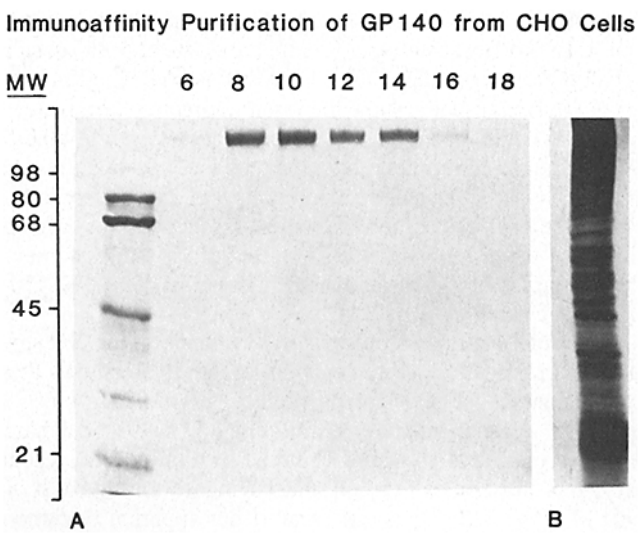

Figure 3. Immunoaffinity purification of gpl40. A PB1 monoclonal antibody affinity column was used to purify gpl40 from CHO cells as described in Materials and Methods. The samples were analyzed by 7.5% SDS PAGE under reducing conditions, and detected by silver staining. (*A*) Fractions eluted from the PB1 affinity column with 0.2 N acetic acid. Fractions 8–14 were retained for polyclonal antibody production. (*B*) The original CHO cell lysate.

cells, an amount representing 0.03% total cell protein. If one assumes one PB1 binding site per gpl40 molecule expressed at the cell surface, then the surface pool of gpl40 would represent 0.002% total cell protein. This suggests the existence of a large intracellular pool of gpl40 that is reactive with PB1 in cell lysates but not in intact cells. The purified gpl40 was used to immunize a goat, and antibodies were obtained as described in Materials and Methods.

Characterization of Polyclonal Anti-gpl40

NP-40 lysates of radioiodinated CHO cells were subjected to immunoprecipitation using goat anti–gpl40 IgG, preimmune goat IgG, or PB1 all covalently bound to Affi-gel 10. Fig. 4 shows the results. The typical pattern of ^{125}I surface-labeled cells is shown in Fig. 4, lane *d*. The predominant band precipitated by goat anti-gpl40 has an apparent molecular mass of 140,000 D (Fig. 4, lane *a*) and aligns with the immunoprecipitate of PB1 (Fig. 4, lane *c*). Thus, it appears that the goat anti-gpl40 immunoprecipitates the same or similar species of surface protein as PB1. Fig. 4, lane *b* is the immunoprecipitation pattern of preimmune goat IgG, a control for nonspecific binding. There is some nonspecific binding of the 140,000-D component as well as a band migrating with the molecular mass of 40–50 kD. However, the predominant band specifically immunoprecipitated by both goat anti-gpl40 and PB1 is a 140,000-D component.

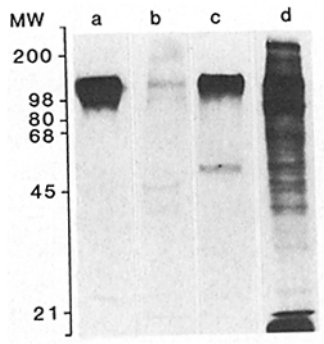

Figure 4. Immunoprecipitations of CHO cell surface proteins by PB1 or goat anti-gpl40. NP-40 extracts of ^{125}I surface-labeled CHO cells (~2 × 10^7 cells/sample) were immunoprecipitated with goat anti-gpl40 or NGIgG bound to Affigel-10; alternatively PB1 monoclonal antibody coupled to Affigel-10 was used. Approximately 3 × 10^6 cell equivalents were loaded on each lane. Samples were analyzed by SDS PAGE (7.5%) and detected by autoradiography. (*a*) goat anti-gpl40; (*b*) NGIgG; (*c*) PB1 monoclonal; (*d*) whole cells.

Because the goat anti–gpl40 polyclonal antibody precipitates a protein similar to that precipitated by PB1, our next question was whether goat anti-gpl40 was also specific in blocking CHO cell adhesion to Fn. The data Fig. 5 presents indicates that the polyclonal antibody is as functionally specific as the PB1 monoclonal. Thus goat anti-gpl40 blocks the adhesion of CHO cells to Fn-coated substrata but does not perturb the adhesion of the cells to Fn-depleted serum-coated substrata. Such substrata should contain serum spreading factors but not Fn (4). Approximately 1 mg/ml goat anti-gpl40 was required to block 50% of CHO cell adhesion to Fn; this amount has no effect on adhesion of the cells to serum-coated substrates. Thus goat anti-gpl40, like PB1 and PB2, specifically inhibits Fn-mediated adhesion, but not other adhesion processes.

Expression and Function of gpl40-Related Protein in Human Cells

A number of human cell types were screened for surface expression of molecules immunologically related to CHO gpl40 using the goat anti-gpl40 in immunoprecipitation of detergent extracts of ^{125}I surface-labeled cells. As Fig. 6 shows, the polyclonal antibody specifically precipitated surface-labeled components comigrating with CHO cell gpl40 from human diploid fibroblasts (HDF), human am-

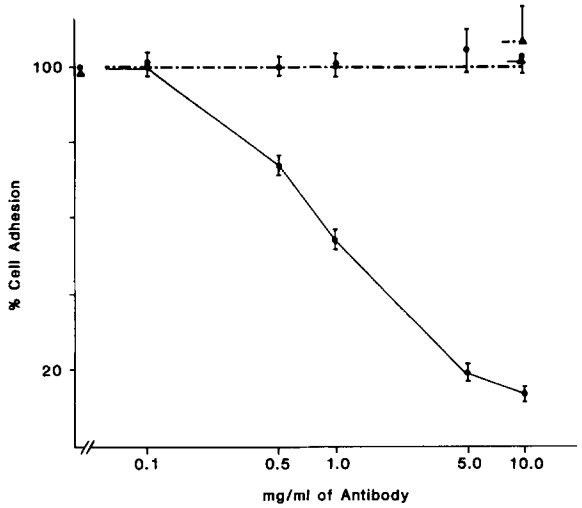

Figure 5. Effect of goat anti-gpl40 on adhesion of CHO cells to Fn-coated or to serum-coated substrata. Tissue culture plates (24-well; Costar, Data Processing Corp., Cambridge, MA) were coated either with bovine Fn (100 μg/ml) or Fn-depleted bovine serum (20%) in Eagle's essential minimum alpha medium. The wells were then co-coated with 1% bovine albumin. Each well was filled with 1 ml of adhesion medium containing various dilutions of goat anti-gpl40 or of NGIgG. 2 × 10^5 CHO cells were added and incubated for 1 h at 4°C. The cells were then warmed to 37°C and allowed 1 h to adhere. The fraction of cells adhering was determined as described in Materials and Methods. (●——●), goat anti-gpl40 on a Fn substratum; (●– –●), goat anti-gpl40 on a Fn free serum substratum; (▲), NGIgG on a Fn substratum.

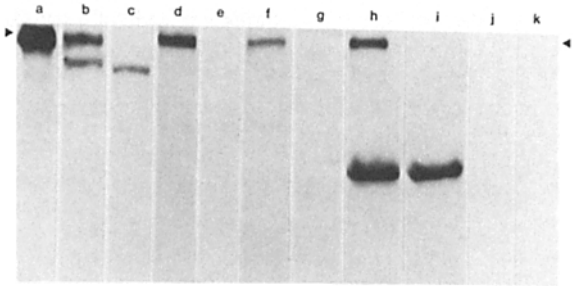

Figure 6. Immunoprecipitation of gpl40 from various cell lines. 2–3 × 10^7 cells were surface-labeled with 1 mCi ^{125}I via the Iodogen surface-labeling procedure and solubilized with 1% NP-40. The protein concentration was adjusted to 360 μg/ml and immunoprecipitated with goat anti-gpl40 or NGIgG covalently coupled to Affigel 10. Samples were solubilized in 125 μl sample buffer. 50 μl were applied to each lane of a 7.5% SDS polyacrylamide gel. Labeled proteins were visualized by autoradiography. Lane *a*, CHO cells: lanes *b* and *c*, EFC cells; lanes *d* and *e*, WISH cells; lanes *f* and *g*, HDF cells; lanes *h* and *i*, DAUDI cells; lanes *j* and *k*, human erythrocytes. Lanes *a*, *b*, *d*, *f*, *h*, and *j*, goat anti-gpl40. Lanes *c*, *e*, *g*, *i*, and *k*, NGIgG.

nion cells (WISH), human glioblastoma cells (EFC), and human lymphoblastoid cells (DAUDI). No specific immunoprecipitate was observed with extracts of human red blood cells. In the case of EFCs and DAUDI cells, ^{125}I components of lower molecular weight were found in the immunoprecipitates of both the specific and preimmune IgG. This suggests that these components are precipitation artifacts.

Monolayers of CHO, HDF, WISH, and EFC cells on Fn substrata were treated with polyclonal anti-CHO gpl40. As Fig. 7 shows, CHO cells were completely rounded and detached by 10 mg/ml antibody. The other cell types were not completely prevented from adhesion by anti-gpl40, but all displayed distinct morphological alterations. Thus, in WISH monolayers the lateral cell-to-cell contact seemed perturbed and the cells formed "islands" or patches, rather than an even monolayer. For EFC cells, the extension of long neurite-like processes seemed to be perturbed and the cells had a generally rounded appearance. For HDF cells, the usual parallel orientation of these fibroblasts seemed perturbed. In no case, however, was the adhesion or spreading of the various human cell lines on Fn completely blocked by anti-gpl40. On substrata coated with Fn-depleted serum, neither CHO cells nor the various human cell lines were affected by 10 mg/ml anti-gpl40 antibody (data not shown).

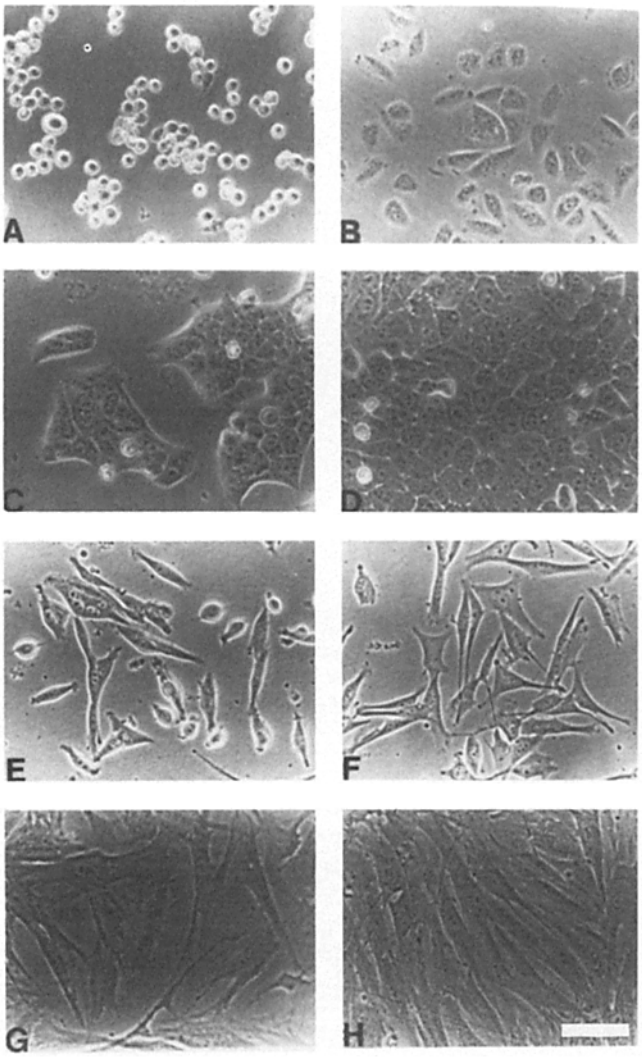

Figure 7. Effects of anti-gpl40 on the morphology of human and hamster cell lines. Tissue culture plates were treated with 100 μg/ml bovine Fn and then co-coated with 1% bovine albumin. Various cell lines in adhesion medium were allowed 3 h at 37°C to adhere. The cells were then treated with 10 mg/ml of goat anti-gpl40 or NGIgG in adhesion buffer for 18 h at 37°C. Cell morphology was examined and photographed using a Nikon Diaphot inverted phase microscope. (*A* and *B*) CHO cells; (*C* and *D*) WISH cells; (*E* and *F*) EFC cells; (*G* and *H*) HDF cells; (*A*, *C*, *E*, and *G*) goat anti-gpl40; (*B*, *D*, *F*, and *H*) NGIgG. Bar, 15 μm.

Peptide Mapping

Avian proteins involved in cell–matrix adhesion seem to be composed of a complex of three polypeptides (7, 18). These seem to be more clearly manifest when the proteins are run on gels under nonreducing conditions. For this reason we elected to examine the migration of our affinity-purified hamster gpl40 under nonreducing conditions on 6% SDS PAGE. As seen in Fig 8 *A*, the gpl40 component can be resolved into two bands. In contrast to the case of avian adhesion proteins, no third band was detected under a variety of gel analysis conditions. The two bands resolved on nonreducing gels seem to be closely related polypeptides. Two-dimensional maps of iodopeptides from U and L bands were very similar (Fig. 8, *b* and *c*), although the L band map showed three spots (marked *A*, *B*, and *C*,) not found in the U map. Another four spots (crosshatched in Fig. 8 *C*) were more intense in the U band map than the L band map. These results suggest that there is a high degree of homology between U and L bands of gpl40, although some minor differences are clearly present. At present it is not clear if the U and L bands represent distinct polypeptides or if the observed small differences in peptide maps are due to proteolysis or to different posttranslational modifications. Interestingly, lower band consistently incorporated more cpm/mg protein using chloramine T radioiodination, which suggests a greater abundance of tyrosine, histidine, and phenylalanine residues in this component (28).

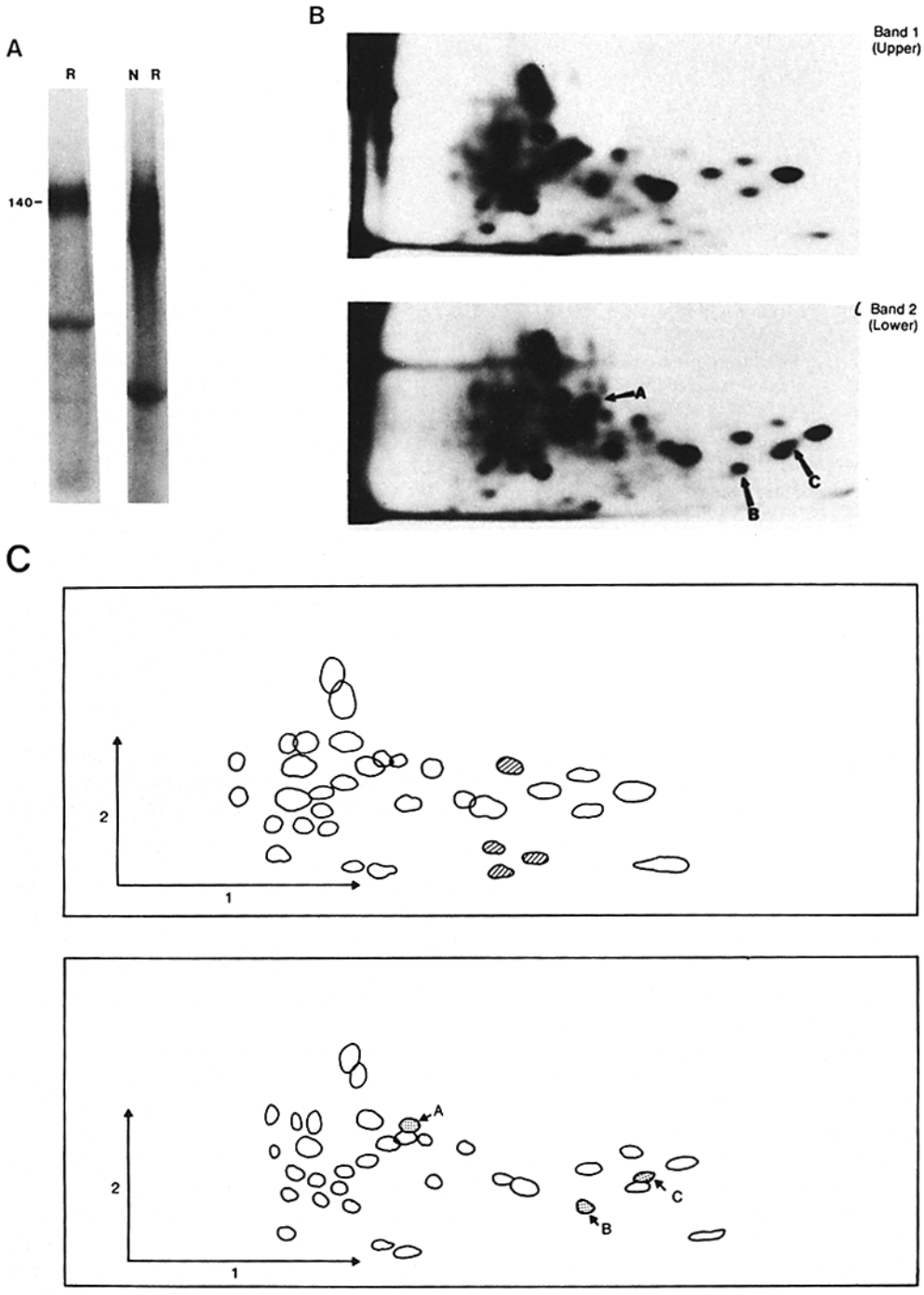

Figure 8. Peptide map of gpl40. (*A*) Purified gpl40 was iodinated using Iodogen[R] (Pierce Chemical Co.) with a resultant sp act of 32 μCi/μg. 20 ng of gpl40 having 1.4 × 10^6 cpm were electrophoresed on 6% SDS polyacrylamide gels with 0.2% linear polyacrylamide under reducing or nonreducing conditions. Under nonreducing conditions, gpl40 separates into two distinct bands (U and L) that could be subsequently cut out of the gel. (*B*) For two-dimensional peptide maps areas containing nonradiolabeled U or L bands were identified by Coomassie Blue staining of the gel and were sliced from the gel and ^{125}I labeled in situ using chloramine T reagent as described (28). Iodopeptides were generated by digestion with trypsin and chymotrypsin (50 μg/ml each) overnight at 37°C. The iodopeptides were analyzed by high voltage electrophoresis and thin layer chromatography and detected by autoradiography (10). (*A*) Affinity-purified ^{125}I gpl40 under reducing (R) and nonreducing (NR) conditions. (*B*) Two-dimensional maps of ^{125}I peptides from U and L bands of gpl40. The peptides that seem distinct between the two bands are indicated by A, B, and C in L. Vertical direction, thin layer chromatography. Horizontal direction, high voltage electrophoresis. (*C*) Line drawings of peptide maps. Spots that seem distinct between the two bands are marked by A, B, and C in L. Spots that have a different intensity in U than in L are cross hatched.

Relation of Hamster gpl40 to Other Adhesion Proteins

We have compared the immunological cross reactivity of hamster gpl40 with the recently described human gpl40 Fn receptor (26) and with the avian adhesion protein complex known as CSAT (15) or JG22 (7). As seen in Fig. 9 *A*, an affinity-purified rabbit antibody against human gpl40 reacts with hamster gpl40, as does affinity-purified goat anti-hamster gpl40. Both antibodies can detect 20 ng of hamster gpl40 at an IgG level of 1 μg/ml. This indicates strong cross reactivity for the hamster and human gpl40 components. In Fig. 9 *B*, the cross reactivity of gpl40 and CSAT antigen are compared. Interestingly, rabbit anti-CSAT antiserum (1:20 dilution), which detects CSAT antigen at levels of 0.2–1.0 ng, can also readily detect hamster gpl40 at levels of 1–5 ng. However, goat anti-hamster gpl40 IgG (100 μg/ml), which detects gpl40 at 1–5 ng, does not react with CSAT antigen at levels of up to 500 ng. Thus epitopes cross reactive with those in CSAT as well as unique epitopes are found on gpl40.

Discussion

Recent evidence from our laboratory (6) and from others (26) strongly suggests that cell surface glycoproteins of ~140 kD are critically involved in the Fn-mediated adhesion pro-

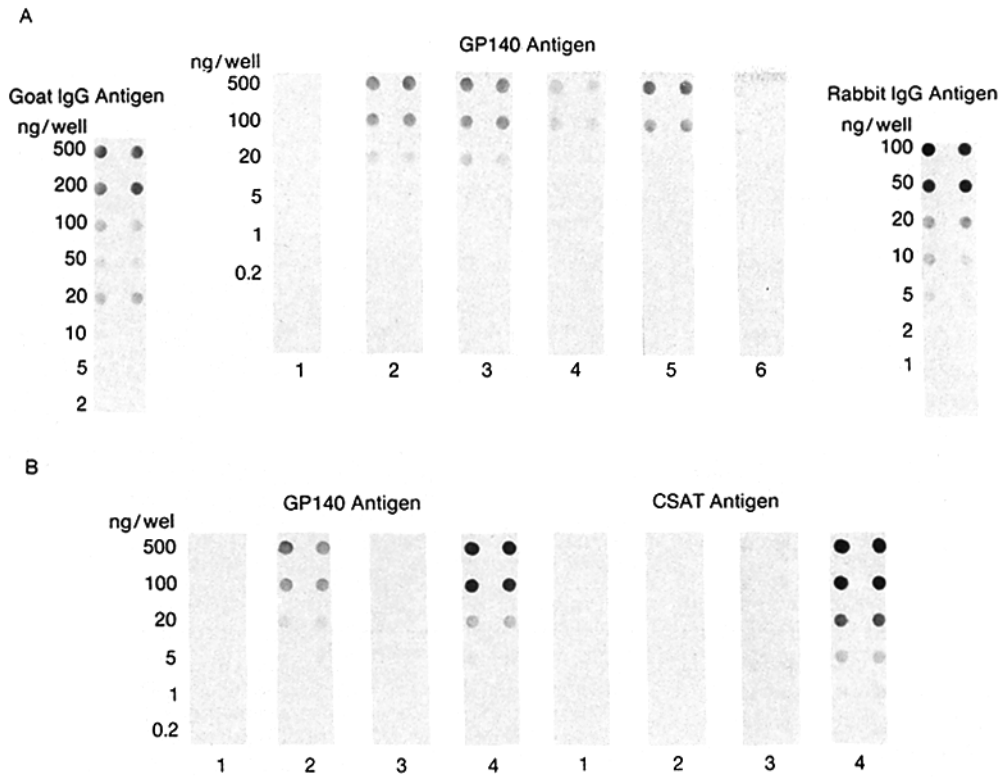

Figure 9. Immunoblot comparison of gpl40 to other adhesion-related proteins. Hamster gpl40 antigen and chick CSAT antigen blotted on nitrocellulose sheets were reacted with goat anti-hamster gpl40 IgG, rabbit anti-human 140 kD Fn receptor IgG (26), or rabbit anti-CSAT antisera (23), along with appropriate control antibodies. Antibody-antigen binding was detected using a peroxidase-linked second antibody. Goat IgG and rabbit IgG dotted into nitrocellulose served as quantitation controls for detection by anti-goat peroxidase conjugate or anti-rabbit peroxidase conjugate respectively. (*A*) Reactivity of hamster gpl40 antigen with anti-hamster gpl40 or anti-human gpl40 antibodies. Primary antibodies. Lane *1*, normal goat IgG 100 μg/ml; lane *2*, goat anti-hamster gpl40 100 μg/ml; lane *3*, affinity purified goat anti hamster gpl40 10 μg/ml; lane *4*, affinity purified goat anti hamster gpl40 1 μg/ml; lane *5*, affinity purified rabbit anti human gpl40 1 μg/ml; lane *6*, normal rabbit IgG 1 μg/ml. (*B*) Reactivity of hamster gpl40 or CSAT antigen. *Left* lanes *1–4*, gpl40; *Right* lanes *1–4*, CSAT. Lane *1*, normal goat IgG 100 μg/ml; lane *2*, goat anti-hamster gpl40 100 μg/ml; lane *3*, normal rabbit serum 1:20 dilution; lane *4*, rabbit anti-CSAT serum 1:20 dilution.

cess in some mammalian cell types. We have used PB1, a monoclonal antibody that recognizes gpl40, a putative Fn receptor in CHO cells, to purify this molecule to apparent homogeneity by SDS PAGE and to generate a polyclonal antibody to it. The polyclonal goat anti-gpl40 precipitates the same protein as PB1 from lysates of surface-labeled CHO cells and does not precipitate other components. Moreover, like PB1, the goat anti-gpl40 selectively blocks Fn-mediated adhesion but not other adhesion processes in CHO cells. Thus the goat anti-gpl40 is effectively monospecific in reacting with gpl40 on the CHO cell surface. We have not at this point investigated the reactivity of anti-gpl40 with lysates of metabolically labeled cells. Presumably the polyclonal antibody would recognize intracellular precursor forms of gpl40 as well as the fully processed molecule expressed at the cell surface.

We have used the goat anti-gpl40 to probe the surface expression and function of gpl40-related molecules in human cell types. Thus the goat anti-gpl40 immunoprecipitates surface-labeled components of ~140 kD from several adherent human cell lines including HDF, WISH, and EFC. Treatment of these cells with anti-gpl40 induces changes in cell spreading and morphology. This suggests that gpl40-related molecules are expressed and function in Fn-mediated adhesion in a number of adherent cell types. By contrast, erythrocytes, a totally nonadherent cell, seem completely devoid of gpl40-related cell surface proteins. One should note, however, that DAUDI cells, a lymphoblastoid line which, in our hands, proved to be poorly adherent to Fn substrata, also clearly expresses cell surface gpl40. Thus one cannot make a simple correlation between expression of gpl40-related molecules and adhesion to Fn. As we have shown elsewhere, cells have several ways of modifying their adhesive behavior in addition to modulation of surface receptor expression, for example, by modification of cAMP-protein kinase systems (9).

At this point it is not clear why anti-gpl40 only partially affects the adhesion and morphology of the human cell types while fully blocking CHO cell adhesion. One possibility is that most of the antibodies in the anti-hamster gpl40 IgG fraction do not react with the site of the human gpl40 type molecule which functions in adhesion. Another possibility is that the human cell lines, but not CHO cells, can condition substrata by secretion of other (nonfibronectin) adhesion factors during the rather protracted course (18 h) of the experiment. Consistent with this is our observation that anti-gpl40 seems to strongly retard early stages of adhesion, but eventually this block is partially overcome (data not shown). In any case it seems clear that perturbation of gpl40-like molecules can result in alteration in cell morphology.

An important issue is whether or not gpl40 is actually a receptor for Fn. Certainly formal pharmacological criteria for receptor status have not been met for this molecule. These criteria would include demonstration of specific, competable ligand binding to gpl40, and correlation of receptor occupancy and biological function. Nonetheless, the evidence implicating gpl40 as a Fn receptor is at least strongly suggestive. Thus, (*a*) monoclonal antibodies to hamster gpl40 specifically block Fn-mediated hamster cell adhesion but not adhesion mediated by other ligands (18); (*b*) human

gp140 binds specifically to Fn columns (26); (*c*) antibodies to human gp140 strongly cross react with hamster gp140 (this paper); and (*d*) polyclonal antibodies raised against hamster gp140 but cross reactive with human gp140 perturb morphology in human cells adhering to Fn (this paper). All of these observations would suggest that hamster gp140 is critically and specifically involved in Fn-mediated adhesion, is immunologically closely related to the human 140-kD Fn-binding protein, and thus is a good candidate as a Fn receptor. One should keep in mind, however, that the identification of one receptor does not rule out the possible existence of other cell surface binding sites for Fn; in fact some lines of evidence strongly support additional sites (8, 21, 28).

Another issue is the possible relationship between recently isolated mammalian gp140 adhesion molecules (6, 26) and previously described adhesion molecules in mammalian and avian cells. It seems very likely that the hamster cell gp140 described here is a member of a complex of partially purified adhesion-related cell surface glycoproteins described previously (11, 19, 24). However, there seem to be some sharp distinctions as well as some similarities between the 140-kD mammalian Fn receptor protein described here and previously described adhesion-related proteins (CSAT and JG22) in avian cells (7, 23). First, the avian adhesion proteins seem to be comprised of three quite distinct polypeptides in the 110–160-kD range, with rather unique one- and two-dimensional peptide maps (14, 18). In contrast, the mammalian gp140 adhesion protein seems to be comprised of two closely related polypeptides, based on one- and two-dimensional peptide mapping. Second, antibodies to the avian proteins disrupt the adhesion of avian cells to complex, serum-coated substrata (23) where a number of adhesion promoting ligands such as vitronectin and other "spreading factors" are present in addition to Fn (4); this suggests that the avian proteins have receptor functions for more than one adhesion ligand. By contrast, both monoclonal and polyclonal antibodies to mammalian gp140 disrupt only Fn-mediated adhesion processes and not adhesion mediated by vitronectin, laminin, or serum spreading factors (6, also this paper). Third, there is direct evidence that mammalian gp140 specifically binds to Fn but not to other adhesion ligands (26, 27). By contrast, the avian adhesion complex (CSAT and JG22) binds not only to Fn (2) but also to laminin (15). Finally, although anti-CSAT antibody cross reacts with gp140 the converse is not true (Fig. 9); therefore gp140 must have epitopes not found on the CSAT complex. Thus, the mammalian and avian adhesion glycoproteins seem to be biochemically, immunologically, and functionally distinct, despite a similarity in apparent molecular weight and a degree of immunlogical cross reactivity.

In summary, present findings suggest that a 140-kD surface glycoprotein found in both hamster and human cells is critically and specifically involved in Fn-mediated cell adhesion. The availability of monoclonal and polyclonal antibodies to the 140-kD component opens the path to rapid, large scale purification and analysis of biochemical and functional characteristics of this important molecule.

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