The Cell Substrate Attachment (CSAT) Antigen Has Properties of a Receptor for Laminin and Fibronectin

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ABSTRACT The cell substrate attachment (CSAT) antigen is an integral membrane glycoprotein complex that participates in the adhesion of cells to extracellular molecules. The CSAT monoclonal antibody, directed against this complex, inhibited adhesion of cardiac and tendon fibroblasts and skeletal myoblasts to both laminin and fibronectin, thus implicating the CSAT antigen in adhesion to these extracellular molecules.

Equilibrium gel filtration was used to explore the hypothesis that the CSAT antigen functions as a cell surface receptor for both laminin and fibronectin. In this technique, designed for rapidly exchanging equilibria, the gel filtration column is pre-equilibrated with extracellular ligand to ensure receptor occupancy during its journey through the column. Both laminin and fibronectin formed complexes with the CSAT antigen. The association with laminin was inhibited by the CSAT monoclonal antibody; the associations with both fibronectin and laminin were inhibited by synthetic peptides containing the fibronectin cell-binding sequence. Estimates of the dissociation constants by equilibrium gel filtration agree well with those available from other measurements. This suggests that these associations are biologically significant. SDS PAGE showed that all three glycoproteins comprising the CSAT antigen were present in the antigen–ligand complexes. Gel filtration and velocity sedimentation were used to show that the three bands comprise an oligomeric complex, which provides an explanation for their functional association. The inhibition of adhesion by the CSAT monoclonal antibody and the association of the purified antigen with extracellular ligands are interpreted as strongly implicating the CSAT antigen as a receptor for both fibronectin and laminin and perhaps for other extracellular molecules as well.

The interaction of cells with components of the extracellular matrix plays a major role in determining cell morphology, cell migration, and tissue maintenance (1). Putative sites of cell–matrix contact have been identified and studied using various microscopic techniques. In fibroblasts they occur in regions where the cellular cytoskeleton and associated components interact with the cell surface (2–4). Integral membrane proteins are hypothesized to be present in these sites and to serve as transmembrane links connecting cytoskeletal and the extracellular matrix components. These transmembrane proteins would then serve as dual receptors for cytoskeletal and extracellular matrix components.

We have identified a candidate for such a cell surface molecule using an adhesion and morphology perturbing cell substrate attachment (CSAT)[1] monoclonal antibody (5). This monoclonal antibody alters the adhesion of several different cell types in culture. The nature and degree of perturbation by the CSAT monoclonal antibody is characteristic for different cell types (6). The antigen to which the antibody is directed has been purified by immunoaffinity chromatography and partially characterized (7). It is a complex of three distinct integral membrane glycoproteins that migrate in the molec-

[1] *Abbreviations used in this paper:* CMF-PBS, calcium- and magnesium-free phosphate-buffered saline; CSAT, cell substrate attachment; DME, Dulbecco's modified Eagle's medium; RCF, relative centrifugal force; TNC, 0.01 M Tris-acetic acid, 0.5% Nonidet P-40, 0.5 mM $CaCl_2$, 0.15 M NaCl, pH 8.0.

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ular weight range of 140,000 on SDS PAGE. The antigen on fibroblasts has been localized using immunofluorescence to regions along portions of stress fibers and in regions surrounding focal contacts as identified by vinculin staining (8). Similar observations and conclusions have been made using another monoclonal antibody, JG-22, which was isolated independently and is directed against the same protein complex (9–12).

In this paper, we explore further the role of the CSAT antigen in adhesion to extracellular matrix molecules by addressing three issues: (*a*) the extracellular matrix molecules with which adhesions involving the CSAT antigen interact; (*b*) the role of the CSAT antigen in adhesion to these extracellular matrix molecules; and (*c*) the origin of the characteristic responses of different cells to the CSAT monoclonal antibody. We have used three cell types in these studies–tendon fibroblasts, skeletal myoblasts, and cardiac fibroblasts. Cell cultures of the first two cell types are responsive to the CSAT monoclonal antibody, whereas cultures of the cardiac fibroblasts are not (6). The adhesion to two well characterized and readily available extracellular molecules, fibronectin and laminin, were studied (13–17). We report that the CSAT antigen participates in adhesion to both fibronectin and laminin. We also report that the purified antigen is an oligomeric complex that binds to both of these extracellular molecules. These data along with previous observations provides very strong evidence that the CSAT antigen functions as a receptor for both fibronectin and laminin and perhaps for other extracellular matrix molecules as well. They are also consistent with the suggestion that the antigen functions as an extracellular matrix–cytoskeleton transmembrane link.

MATERIALS AND METHODS

Avian Cell Cultures: Skeletal myoblasts were explanted, dissociated, and cultured as described previously (6). The dissociated pectoral muscle cells were plated onto Falcon tissue culture dishes (Falcon Labware, Oxnard, CA) coated with 0.1% gelatin (BBL Microbiology Systems, Cockeysville, MD) at a density of 3.5–4.5 × 10^5 cells/35-mm dish. The cells were grown in calcium-free Dulbecco's modified Eagle's medium (DME) containing 10% horse serum, 2% chick embryo extract or 40 μg/ml conalbumin (Sigma Chemical Co., St. Louis, MO), and 0.2–0.3 mM EGTA to prevent fusion.

Tendon fibroblasts were obtained from 15–18-d embryos as described previously (6). The isolated tendons were incubated for 40 min at 37°C in 1 mg/ml collagenase (CLS II, Worthington Biochemicals, Freehold, NJ) and 0.25% trypsin (Gibco, Grand Island, NY) in calcium- and magnesium-free phosphate-buffered saline (CMF-PBS) followed by pipetting. The dissociated cells were washed and plated onto 100-mm Falcon tissue culture dishes (Falcon Labware) at a density of 2–5 × 10^5 cells/plate. The cells were passaged at least two times, seeded in tissue culture dishes at a density of 2–5 × 10^5 cells/plate, and grown in DME containing 10% fetal calf serum. Cells passaged more than 10 times were discarded.

Cardiac fibroblasts were isolated from 14-d embryo ventricles after dissociation by 2.5 mg/ml collagenase and pipetting (6). The cells were plated onto Falcon tissue culture dishes (3 ventricles per 100-mm dish) in DME containing 10% fetal calf serum. After 1 h the nonadherent cells were decanted and fresh medium added. The cells were passaged at least three times before use, seeded at a density of 2–5 × 10^5 cells/100-mm plate, and grown in DME containing 10% fetal calf serum. Cells passaged more than 10 times were discarded.

Cell Substratum Adhesion Assays: 2-d cultures prepared as described above at a density of 75–85% of confluency were washed twice with CMF-PBS containing 0.02% EDTA and incubated for 5 min (tendon fibroblasts and skeletal myoblasts) or 30 min (cardiac fibroblasts) in 0.02% EDTA in CMF-HEPES Hanks' buffered balanced salt solution. The rounded cells were harvested by gentle pipetting using a fire polished Pasteur pipette and centrifuged at 160 *g* for 3–4 min. The cells were resuspended into DME containing 2% bovine serum albumin (BSA; Sigma Chemical Co.) and counted. Viability was estimated using 0.4% trypan blue. 1.25 × 10^5 cells/well were added to each laminin or fibronectin-coated Linbro tissue culture or nontissue culture multiwell plate (No. 76-000-04 or 76-000-05, Flow Laboratories, McLean, VA) and the final volume brought to 0.5 ml. The CSAT monoclonal antibody was added either at the time of plating or after cell adhesion and spreading (30–180 min). For experiments using fibronectin peptides, spot cultures of 10 μl containing 3–6 × 10^5 cells/ml were plated onto multiwells coated as above. These were gently flooded with 0.5 ml of medium for optical observation.

For trypsinization experiments cultures were incubated with 25 μg/ml cycloheximide (Sigma Chemical Co.) for 2 h before harvest; cycloheximide was present in all subsequent solutions. (This procedure was also used for cycloheximide-treated cells.) The cells were washed two times in HEPES Hanks' buffer, and 4 ml of trypsin (type III; Sigma Chemical Co.) at a concentration of 1 mg/ml in HEPES Hanks' was added. The cultures were incubated for 30 min at 37°C. The cells were then harvested by pipetting, 8 mg soybean trypsin inhibitor (type IIs, Sigma Chemical Co.) added, and the cells centrifuged for 3–4 min at 180 *g*. The cells were washed in DME containing soybean trypsin inhibitor and finally resuspended in DME containing 2% BSA for assays described above.

A more quantitative assay of cell substratum adhesion, based on that described by McClay et al. (18), was also used. 1.8 ml of a cell suspension containing 9 × 10^4 cells/ml in DME with 2% BSA and 20 mM HEPES were aliquoted into Linbro tissue culture vinyl or nontissue culture multiwells (No. 76-000-04 and No. 76-000-05, Flow Laboratories). The wells were sealed with plastic cell cover sheets (No. 76-401-05, Flow Laboratories) and centrifuged at 66 *g* for 10 min at 4°C. The cells were then incubated for 30 min (tendon fibroblasts and skeletal myoblasts) or 45 min (cardiac fibroblasts) at 37°C. The wells were inverted and centrifuged again at 13 *g* or 66 *g* or just allowed to sit inverted (1 *g*) for 10 min at 4°C. The cover sheets were cut open with a scalpel, and the supernatant gently decanted. The wells were then placed on a warm plate at 37°C, and the adherent cells were removed by trypsinization and then counted. The data are expressed as a percent of the total number of cells initially added to the well.

Wells were coated either with laminin, derived from an Englebreth Holm Swarm (EHS) sarcoma (Bethesda Research Laboratories, Gaithersberg, MD; Collaborative Research, Waltham MA; or a gift from Hynda Kleinman), human plasma fibronectin (Collaborative Research; Bethesda Research Laboratories; or a gift from Jeremy Paul and Richard Hynes), poly-L-lysine (22,000 mol wt; Sigma Chemical Co.), type I collagen (Collaborative Research), type IV collagen (a gift of Hynda Kleinman), or vitronectin (Calbiochem-Behring Corp., La Jolla, CA). 10 μl from a stock solution of laminin or fibronectin at a concentration of 1 mg/ml in CAPS buffer (cyclohexylaminopropane sulfonic acid, pH 11; Sigma Chemical Co.) was added to each well and diluted to a total volume of 0.5 ml with CMF-PBS. In later experiments the laminin stock was in 10 mM Tris-HCl buffer, pH 7.2. The wells were incubated for 2 h, or overnight at 37°C, aspirated, and washed twice with CMF-PBS. Multiwells were coated with other substrates by adding appropriate stock solutions to the wells, incubating for 2–4 h at 37°C, and rinsing with medium or PBS. The poly-L-lysine was used at a concentration of 18 μg/well from a stock solution at 35 μg/ml in borate buffer pH 8.0; types I and IV collagen were used at a concentration of 12 μg/well from a stock solution at 0.5 mg/ml in 0.1 N acetic acid; and vitronectin was used at a concentration of 2.5–10 μg/well from a stock solution at 1 mg/ml in PBS. The collagen solutions were first spread evenly and then allowed to dry on the wells.

Antigen Isolation: [^{35}S]Methionine labeling of cell cultures and the subsequent CSAT antigen purification and analysis by SDS PAGE were performed as described previously except that the acetone precipitation step was omitted (5–7). Unlabeled antigen was prepared as described for the labeled antigen from decapitated and eviscerated chick embryos.

Equilibrium Gel Filtration Assay for Macromolecular Associations: Gel filtration was routinely performed on an Ultrogel AcA22 (LKB, Gaithersburg, MD) column with a bed volume of 0.2 × 30 cm. 60-μl fractions were collected at a flow rate of 0.7 ml/h. The void and included volumes of this column were 0.54 and 1.5 ml, respectively. For fractionation of larger volumes a 1 × 12-cm bed volume was used which had void and included volumes of 3.5 and 13 ml, respectively. 1-ml fractions were collected at a flow rate of 5 ml/h. Ultrogel AcA22 has a linear fractionation range of 100,000–1,200,000 D.

When interactions between either fibronectin or laminin with the antigen were studied, a void volume equivalent of ligand in 0.01 M Tris-HAc, 0.5% Nonidet P-40, 0.15 M NaCl, 0.5 mM $CaCl_2$ at pH 8 (TNC) was first run into the column. The mixture of ^{35}S-labeled antigen and ligand was then added to the column followed by a void volume equivalent of ligand in TNC. Two specific activities of antigen were used: 2.2 × 10^5 mCi/mmol and 43 mCi/mmol. The column was eluted further with TNC alone. Thus throughout its journey in the column the antigen saw a roughly constant, high concentration of ligand. This was done to ensure continued receptor occupancy in the case of rapid ligand–receptor equilibria. In concept, this technique is reminiscent of that described by Hummel and Dryer (19). However there are significant and noteworthy differences.

Antigen affinities to the CSAT antibody, laminin, or fibronectin were estimated using linearizations of the following expression:

$$K_d = \frac{L\,(1-\theta)}{\theta},$$

where θ is the fraction of occupied receptors, L is the ligand concentration, and K_d is the dissociation constant of the antigen–ligand complex.

The synthetic tetramer (arg-gly-asp-ser), hexamer (gly-arg-gly-glu-ser-pro), and decamer (arg-gly-asp-ser-pro-ala-ser-ser-lys-pro) were obtained from Peninsula Laboratories, Inc., Belmont, CA; the synthetic nonamer (arg-lys-lys-thr-gly-gln-glu-ala-cys) was a gift from Jeremy Paul and Richard Hynes. The affinities of the nonamer and tetramer for the antigen were estimated from their inhibition of fibronectin or laminin binding using the following expression:

$$K_i = \frac{K_d \theta I}{L - \theta L - \theta K_d},$$

where I is the peptide concentration, and K_i is the inhibition (dissociation) constant for the peptide–antigen complex.

For antigen and laminin binding, the fractional occupancy was estimated by integrating the areas under the peaks in the elution profiles representing the antigen and antigen–antibody complexes, respectively. The fractional occupancy for the fibronectin interaction was estimated by decomposing the peak in the elution profile into components arising from the antigen–fibronectin complex (peak shoulder) and the antigen alone and by estimating the fractional peak shift. The binding constants derived from the fibronectin data, due to their complex nature, provide only an order of magnitude estimate. The inhibition constants for the peptides were determined by measuring the fraction of occupied receptors in the presence of a constant 400 μg/ml of laminin or fibronectin and varying concentrations of the peptide. The estimates of receptor–ligand equilibria provided by this method should only be considered as first approximations. Possible receptor and ligand aggregation, micellar interactions of the antigen, and details of the column elution phenomena were ignored in making them.

Determination of Hydrodynamic Parameters: Sedimentation of the CSAT antigen in sucrose gradients were performed and analyzed as described elsewhere (20–22). Linear density gradients from 5–20% sucrose were poured into 5-ml cellulose nitrate ultracentrifuge tubes. The sucrose solutions were buffered in TNC (without NaCl) at pH 8. The gradients were overlaid with 100 μl of TNC containing labeled antigen and calibrating enzymes (β-galactosidase 15.93 S, 25 μg/ml; catalase 11.3 S, 100 μg/ml; fumarase 9.09 S, 50 μg/ml; lactic dehydrogenase 6.95 S, 30 μg/ml; and cytochrome c 1.71 S, 2 mg/ml) (Sigma Chemical Co.). The tubes were spun in a Beckman SW 60 rotor (Beckman Instruments, Inc., Palo Alto, CA) at 45,000 *g* for 9 h at 4°C. Fractions containing roughly 150 μl were collected. The calibrating enzymes were assayed spectrophotometrically. The sedimentation coefficient for the antigen was determined graphically by comparing it with that of calibrating enzymes or by use of the sedimentation equation.

The Stokes radius for the antigen was determined by comparing its elution profile from an Ultrogel AcA22 (LKB) column with that of calibrating enzymes: thyroglobin (8.5 nm), ferritin (6.1 nm), catalase (5.22 nm), and aldolase (4.81 nm) (High Molecular Weight Standard Kit, Pharmacia Fine Chemicals, Piscataway, NJ). The labeled antigen and calibrating enzymes were run in TNC buffer (without NaCl) at a flow rate of 5 ml/h. (The presence or absence of NaCl did not affect the elution profiles.)

The molecular weight and frictional coefficient for the detergent–antigen complex was calculated from the above gel filtration and sedimentation data as described by Clarke (20). The detergent contribution to the molecular weight was estimated using a value of 0.948 g/ml and 0.735 g/ml for the partial molar volumes of the detergent (23) and antigen (20), respectively.

RESULTS

Effect of CSAT Monoclonal Antibody on Spread Cells Adhering to Defined Substrata

When chick embryo tendon or cardiac fibroblasts from cell cultures were removed with EDTA and added to tissue culture dishes coated with either fibronectin or laminin, the majority of cells spread and assumed fibroblast-like morphologies. On fibronectin 90% of the tendon fibroblasts were spread after 30 min, whereas the cardiac fibroblasts plated with a lower efficiency and required slightly longer times, 40–60 min, to spread. The rate of adhesion of both types of fibroblasts to laminin was slower than to fibronectin by 30–60 min; however, by 2 h nearly all of the attached cells had spread and assumed a fibroblast-like morphology on either substratum. The adhesion of skeletal myoblasts to these substrata also was investigated. On the former substratum most of the cells assumed a bipolar morphology, whereas on the latter they are more fibroblast-like (Fig. 1). Comparable effects for all cell types and substrata were obtained using either tissue culture or nontissue culture wells. The nontissue culture wells did not promote any cell attachment in the absence of added substrate material. Uncoated tissue culture wells support only a modest

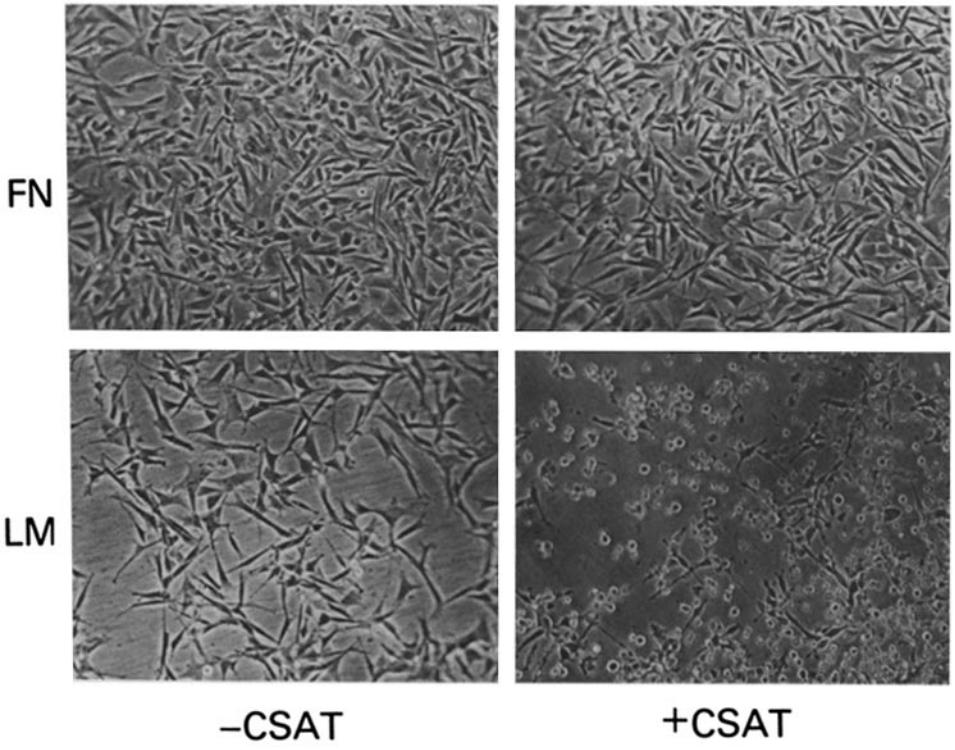

FIGURE 1 Effect of CSAT monoclonal antibody on adhesion of cardiac fibroblasts to laminin and fibronectin. Cardiac fibroblasts, pretreated for 2 h with 25 μg/ml cycloheximide, were plated in cycloheximide containing serum-free DME onto wells (200 mm^2) coated with either fibronectin or laminin at a density of 1.25×10^5 cells/well. The cells were allowed to adhere and spread for 2 h. The CSAT monoclonal antibody was then added at a concentration of 40 μg/ml. The cultures were photographed 2 h after antibody addition. Bar, 40 μm.

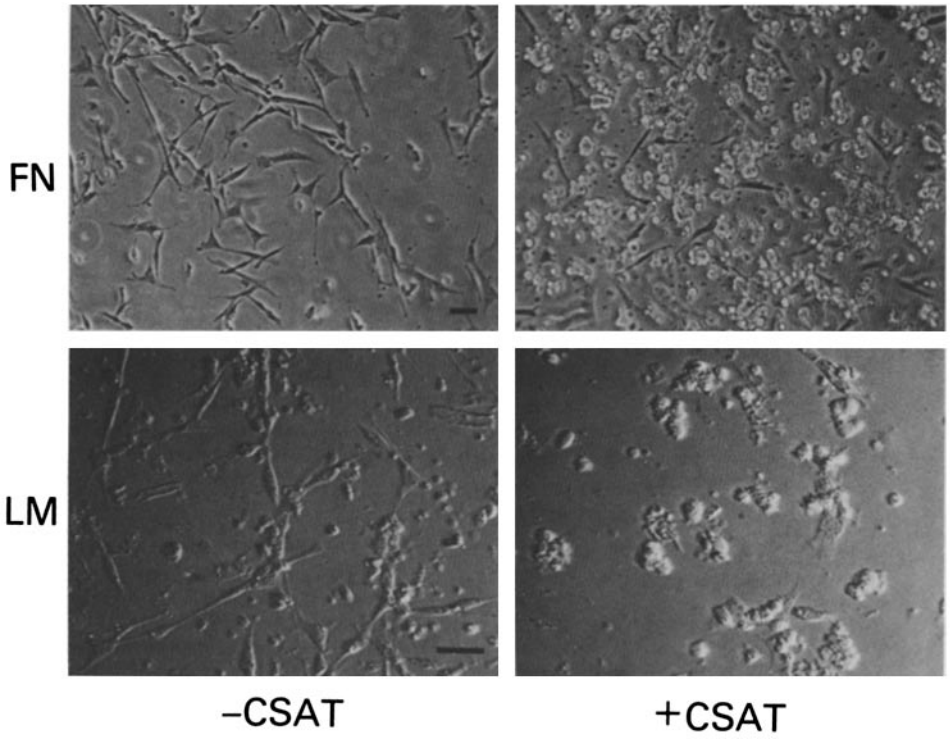

FIGURE 2 Effect of CSAT monoclonal antibody on adhesion of skeletal myoblasts to laminin and fibronectin. Skeletal myoblasts were plated on laminin or fibronectin-coated dishes (35 mm) in DME containing 10% horse serum at a density of 3.5×10^5 cells/dish. The CSAT monoclonal antibody (20 μg/ml) was added at the time of plating. The cultures were observed 48 h after plating. Bar, 40 μm.

attachment under the conditions of these assays. The time course of this adhesion is considerably slower (two to three times) than on wells coated with either fibronectin or laminin. Finally when the CSAT monoclonal antibody itself was used to coat the wells, cells adhered and spread. This was unique among several antibodies tested and suggests that the CSAT antibody can serve as an adhesion-promoting ligand.

Addition of the CSAT monoclonal antibody to tendon fibroblasts, allowed first to adhere and spread on laminin for 1–2 h, induced the rounding of nearly all the cells (Table I). In contrast, the CSAT monoclonal antibody added to tendon fibroblasts after prior plating on fibronectin caused a retraction of their edges and rounding of only 20–30% of the cells. The response of the skeletal myoblasts to the CSAT monoclonal antibody was analogous to that of the tendon fibroblasts. Cardiac fibroblasts, on the other hand, when plated and allowed to spread on either substratum, were not detectably affected by the CSAT monoclonal antibody. Cardiac fibroblasts, first preincubated with cycloheximide (25 μg/ml for 2 h), plated onto a laminin coated dish, and allowed to adhere and spread for 2 h, could now be rounded and detached by addition of the CSAT monoclonal antibody (Fig. 2). This suggests that cardiac fibroblasts synthesize molecules that mediate adhesions in which the CSAT antigen does participate. Tendon and cardiac fibroblasts, allowed to adhere and spread on poly-L-lysine for 2 h, were not detached by the antibody lending specificity to our results.

TABLE I. *Summary of Effects of CSAT Monoclonal Antibody on Cell Adhesion and Morphology on Either Fibronectin or Laminin*

	Laminin		Fibronectin	
Cell type	Initial adhesion	Morphology	Initial adhesion	Morphology
Cardiac fibroblast	+	−	−	−
Cyclohex-cardiac fibroblast	+	+	−	−
Tryp-cardiac fibroblast	NA	NA	+	−
Tendon fibroblast	+	+	+	p
Cyclohex-tendon fibroblast	+	+	+	p
Tryp-tendon fibroblast	NA	NA	+	p
Skeletal myoblast	+	+	+	p

Morphology, CSAT monoclonal antibody added after cells have adhered and spread for 30–180 min. Initial adhesion, CSAT monoclonal antibody added at the time of plating. +, rounded and detached cells when added to spread cells and inhibits initial adhesion when added at the time of plating. −, no detectable effect. p, partial effect; caused a retraction of edges and rounding of only some cells when added to spread cells and inhibited adhesion of only some cells when added at the time of plating. NA, no adhesion; the cells do not adhere under these conditions. Cyclohex-cardiac and cyclohex-tendon fibroblast, tendon and cardiac fibroblasts preincubated with 25 μg/ml cycloheximide for 2 h before harvesting. Tryp-cardiac and tryp-tendon fibroblast, tendon and cardiac fibroblasts preincubated for 2 h with 25 μg/ml cycloheximide and then treated with 1 mg/ml trypsin for 20–30 min. Cells were plated onto wells in DME containing the CSAT monoclonal antibody and incubated for 30–180 min (initial adhesion). The cultures were scored visually for the relative fraction of adherent cells. For some experiments the cells were allowed to first adhere and spread for 30–180 min (morphology) before additions. These cultures were generally scored 60 min subsequently.

Effect of the CSAT Monoclonal Antibody on Initial Adhesive Events

The observations just presented address the effects of the CSAT monoclonal antibody on the adhesion and morphology of cells that had already adhered and spread on defined substrata. We have also studied the effects of the antibody on initial adhesive events that occur before extensive organization of adhesion plaques and the cytoskeletal apparatus. We have developed a quantitative assay, based on that described by McClay et al. (18), to measure the relative adhesive strength of cells to defined substrata. In this assay cells are centrifuged onto the bottom of a well, allowed to incubate, and then the wells are inverted, and the weakly adherent cells removed by centrifugation. The key variables are the choice of molecules used to coat the wells, the incubation time and temperature,

and the relative centrifugal force (RCF) used to detach the weakly adherent cells.

The fraction of cells remaining adherent was measured after different incubation times using an RCF of 66 *g*, the highest force assayed. The adhesion of tendon and cardiac fibroblasts reached a plateau after ~30–45 min and 45–60 min, respectively, at which time 50–80% of the cells had adhered. For both cell types the kinetics of adhesion to either fibronectin or laminin were similar; however, fewer cells adhered to laminin than to a fibronectin-coated substratum. In general fewer cardiac fibroblasts adhered than did tendon fibroblasts. This likely reflects the lower plating efficiency and viability of the cardiac fibroblasts.

The fraction of cells remaining adherent after centrifugation at three different RCFs, 1 *g* (1.2×10^{-7} dynes), 13 *g* (1.6×10^{-6} dynes), and 66 *g* (8×10^{-6} dynes), is presented in Fig. 3. Adhesion of both tendon and cardiac fibroblasts to fibronectin showed a steady decrease in the fraction of adhering cells with increasing RCF. The shapes of the curves for laminin, in contrast, showed little variation with *g*-force between 1–13 *g* after which the fraction of cells remaining adherent decreases.

The effect of the CSAT monoclonal antibody on the adhesion of both cell types to wells coated with either laminin or fibronectin was studied using this assay (Figs. 3 and 4). The presence of the CSAT monoclonal antibody at the time of plating inhibited the adhesion of the tendon fibroblasts to both substrata. Adhesion to laminin was inhibited essentially completely (>10-fold) at all RCFs assayed including 1 *g*, whereas adhesion to fibronectin was inhibited only partially (2–3-fold) at the same RCFs. In contrast to the effects of the CSAT monoclonal antibody on the adhesion of tendon fibroblasts, the antibody inhibited only minimally (<25%) the adhesion of the cardiac fibroblasts to fibronectin (Fig. 4). This small difference, though within experimental error, was seen in all experimental pairs. As described for the adhesion of tendon fibroblasts to laminin, the CSAT monoclonal antibody inhibited essentially completely adhesion of cardiac fibroblasts to laminin. When CSAT inhibition experiments were done using uncoated wells not treated for tissue culture, the fraction of cells remaining on the dish remained at the null value seen on these wells without antibody.

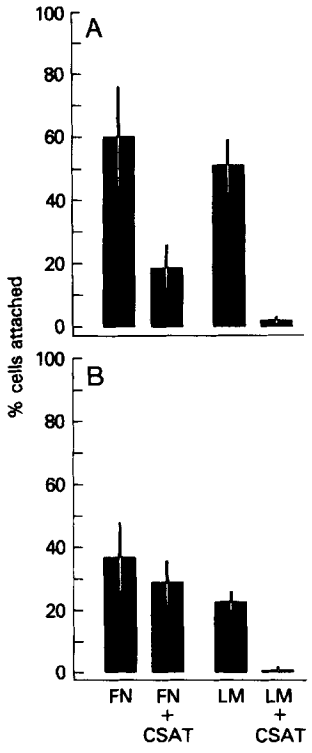

FIGURE 3 Initial adhesion of tendon (*A*) and cardiac (*B*) fibroblasts to fibronectin and laminin substrata. 48-h cultures were harvested with EDTA and centrifuged onto multiwells coated with either laminin or fibronectin at a concentration of 6 $\mu g/cm^2$. The wells were incubated for 30 min (tendon fibroblasts) or 45 min (cardiac fibroblasts) at 37°C, the wells were inverted, the weakly adherent cells were sedimented at 66 *g*, and the remaining cells were counted. The percentage of cells attached are the number of cells remaining adherent divided by the number of cells added to the wells, times 100. CSAT monoclonal antibody was added at a concentration of 20 $\mu g/ml$ at the time of plating. *FN*, fibronectin-coated wells; *LM*, laminin-coated wells.

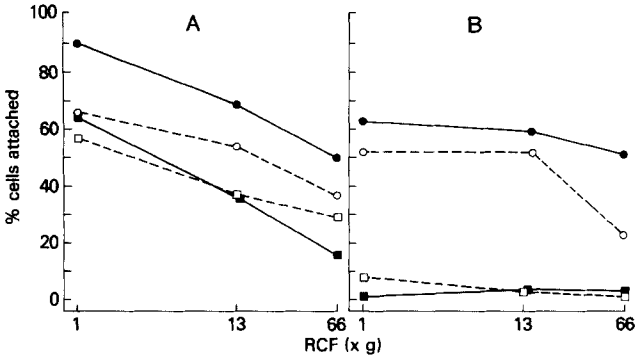

FIGURE 4 Relative adhesive strength of tendon and cardiac fibroblasts plated on fibronectin (*A*) and laminin (*B*). Adhesion was assayed as described in the legend to Fig. 3 except that the weakly adherent cells were sedimented at either 1 *g*, 13 *g*, or 66 *g*. The data are the averages of at least two separate determinations. The error bars are omitted for clarity. (●) Tendon fibroblasts; (■) tendon fibroblasts with CSAT monoclonal antibody; (○) cardiac fibroblasts; (□) cardiac fibroblasts with CSAT monoclonal antibody.

The response to the CSAT monoclonal antibody and the force-adhesion data for tendon and cardiac fibroblasts binding to laminin are readily interpreted as arising from an essentially homogeneous population of cells possessing a single (or at least a very dominant), CSAT monoclonal antibody sensitive adhesion mechanism for laminin. The adhesion to fibronectin appears more complex. The fractional inhibitions of tendon fibroblast adhesion, at all RCFs, by the CSAT monoclonal antibody suggests heterogeneity in the cell population. Adhesive heterogeneities among cells in a population could arise from different numbers of receptors per cell, variations in their interactions, or intrinsic heterogeneity of the cell types in the population. In any case our observation that the initial adhesion on fibronectin of some cells is inhibited by the CSAT monoclonal antibody, e.g., the tendon fibroblasts, while the adhesion of others is not, e.g., the cardiac fibroblasts, suggests the presence of CSAT sensitive and insensitive adhesion mechanisms to fibronectin.

Additional experiments were performed to characterize further the adhesion of these cells. In one the cardiac and tendon fibroblasts were pre-incubated for 3–5 h with cycloheximide to inhibit protein synthesis; in another, cytochalasin b was used to inhibit cell spreading and cytoskeletal organization. With either treatment, the effects of the CSAT monoclonal antibody on the adhesion of the two cell types to laminin and fibronectin remained similar to those just described for cells without these treatments. In contrast, pre-incubating the fibroblasts with trypsin altered some adhesive properties. This was explored by plating cells, first incubated with cycloheximide and subsequently digested with 1 mg/ml trypsin in HEPES-Hanks buffer for 20–30 min (24), onto coated wells, incubating the wells for 30–120 min, gently pipetting to remove the nonadherent cells, and scoring the fraction of cells remaining on the dish.[2] The adhesion of trypsin-treated car-

[2] Skeletal myoblasts were unique in that the CSAT monoclonal antibody delayed their attachment to both laminin and fibronectin for long periods of time (at least 48 h) even in serum-supplemented cultures.

diac fibroblasts to fibronectin was inhibited by the CSAT monoclonal antibody (Table I). In the absence of antibody most of the trypsin-treated cells attach and spread after an hour in culture. In the presence of antibody, only ~40% attach. The CSAT monoclonal antibody did not detectably affect trypsin-treated cells that had been allowed to first adhere and spread. Neither tendon nor cardiac fibroblast adhered to laminin after the cycloheximide plus trypsin treatments.

The adhesion of these cells to some other substrata was also investigated using this assay. The initial adhesion of cardiac and tendon fibroblasts to poly-L-lysine was not inhibited by the CSAT monoclonal antibody, whereas adhesion of tendon fibroblasts to both type I and type IV collagen was inhibited completely by the antibody. The antibody partially inhibited adhesion to vitronectin.

Interactions between the Purified CSAT Antigen and Extracellular Ligands

The above observations implicate the CSAT antigen in adhesion to both laminin and fibronectin. We have explored the possibility that it functions as a receptor for these substrates by assaying for interactions between the purified antigen and these putative ligands. Most assays for associations of integral membrane components with cytoskeletal or extracellular components rely on relatively high affinities. Raising the effective valency by presenting the protein in liposomes or coupled to an inert support is one way to circumvent this problem and assay for weak or rapidly exchanging interactions. Another approach is to use separation techniques that can be performed in the continued presence of high concentrations of one of the constituents. This ensures receptor occupancy even in the presence of rapid equilibria. We have chosen this latter approach using a gel filtration column (Ultrogel AcA22) that is preloaded with ligand at the concentration to be assayed. Thus in this experiment the antigen, as it passes through the column, is exposed to a roughly uniform ligand concentration. This equilibrium gel filtration method also provides estimates of antigen–ligand dissociation constants from measurements of the fraction of antigen in the ligand receptor complex (slow exchange) or the fractional shift of the peak representing the complex (fast exchange).

Fig. 5*A* shows the elution profile of detergent-solubilized antigen. As a positive control, the elution profile of antigen in the presence of the CSAT monoclonal antibody is also shown. The presence of the antibody produced a shift in the elution profile of the antigen toward the void volume. This shift is expected from an antigen–antibody complex which would have a larger effective Stokes radius than that of the antigen alone. The shift did not require pre-equilibration of the column with antibody. The elution profiles were studied over an antibody concentration range of 0.1–20 μg/ml. A K_d of 6×10^{-9} M (Table II) was estimated from linearizations of the binding data. This affinity compared well with that measured for CSAT monoclonal antibody binding to whole cells (6×10^{-9} M) (5).

Fig. 5*B* shows the effect of pre-equilibrating the column with laminin on the elution profile of the CSAT antigen. An additional peak that migrates in the void volume appears at a laminin concentration of 50 μg/ml.[3] The presence of this extra peak reveals an association between the antigen and laminin. The fraction of antigen migrating in this region increased as the laminin concentration increased and was linear, over the concentration range studied, i.e., 25–400 μg/ml, on Eadie-Hofstee or double reciprocal plots (25). The K_d extrapolated from these representations was 2×10^{-6} M. An analysis of the material in the void volume peak using SDS PAGE showed the same three bands that are present in the original antigen (Fig. 6). Prior incubation of the antigen with CSAT antibody blocked the formation of the extra peak in the void volume (Fig. 5*C*). The CSAT monoclonal antibody concentration required for this effect was the same as that required to form the antigen–antibody complex as shown in Fig. 5*A*.

An analogous series of measurements were made using a column preloaded with fibronectin rather than laminin. At fibronectin concentrations above 50 μg/ml, the elution profile of the antigen appeared shifted toward higher excluded volume, and a prominent shoulder appeared on the void volume side of the peak (Fig. 7*A*). The presence of this shifted peak and shoulder reveals an interaction between the antigen and fibronectin. The magnitude of this effect increased over the limited fibronectin concentration range studied—100–400 μg/ml. These data, due to the overlap between the antigen–fibronectin complex and the original antigen, provided only a very rough estimate of 10^{-6} M for the K_d of this interaction. SDS PAGE of the material in the shoulder shows the same three bands as in the native antigen. The position of this shoulder was very similar to that of the antigen–antibody complex. This complicates demonstrating that the CSAT antibody inhibits this interaction. We have taken another approach to ascertaining the significance of this interaction by using a tetrapeptide (arg-gly-asp-ser) reported to represent the cell binding domain of fibronectin (26, 27). This peptide, when included with the fibronectin in the usual protocol, inhibited formation of the shoulder assigned to the fibronectin–antigen complex (Fig. 7*B*). The inhibition occurred in the concentration range of 10–100 μg/ml. The inhibition data were used to estimate a K_i of 10^{-3}–10^{-4} M for the tetrapeptide inhibition of the fibronectin–antigen interaction. A decapeptide (arg-gly-asp-ser-pro-ala-ser-ser-lys-pro) containing the cell binding peptide sequence also inhibited the formation of the complex (not shown). These same peptides were used in the laminin experiments and were found to inhibit the interaction of laminin with the CSAT antigen as well (Fig. 5*D*). When the column was preloaded with both fibronectin and laminin, the elution profile showed both the shoulder characteristic of the fibronectin–antigen interaction and a peak in the void volume characteristic of the laminin–antigen interaction. This result along with the inhibition of the antigen interactions with both fibronectin and laminin by the cell binding tetrapeptide from fibronectin suggest that laminin and fibronectin are binding to neighboring or sterically related sites.

These interactions appear to be both meaningful and significant. An unrelated antibody that inhibits adhesion to substrata other than fibronectin and laminin (unpublished

[3] The peak at the void volume was not apparent unless the column was pre-loaded with laminin; when the peak fractions were isolated and rerun without preloading the column, the complex was no longer present. The presence of a resolvable peak for the antigen–ligand complex suggests that the exchange rate of the antigen–ligand complex is slow on the time scale of the gel filtration experiment. Whereas this may be the intrinsic nature of the interaction, it may also arise from antigen binding to a laminin aggregate whose formation is concentration dependent.

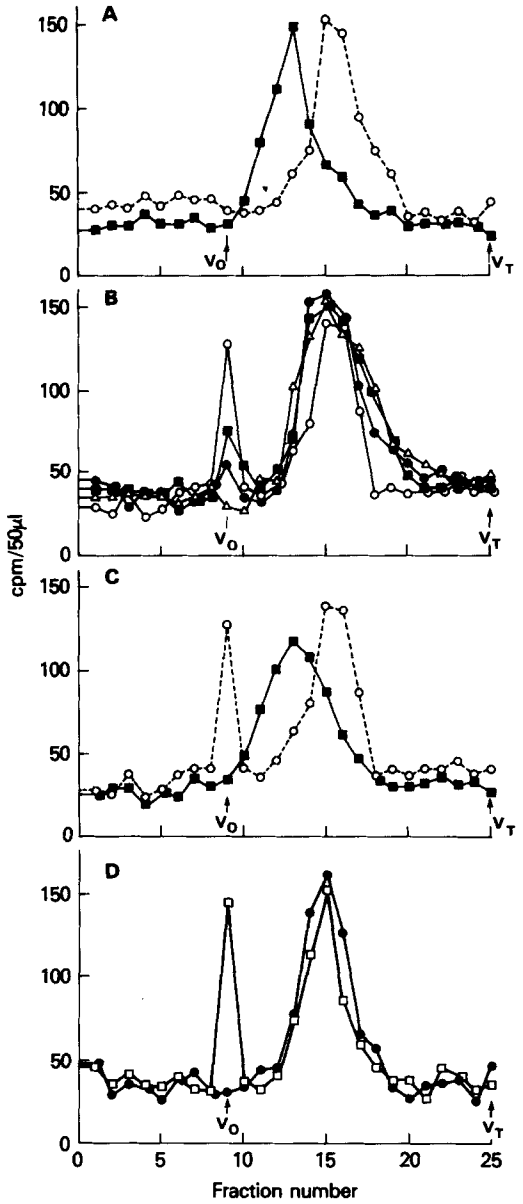

FIGURE 5 Equilibrium gel filtration elution profile of CSAT antigen from columns pre-equilibrated with the CSAT monoclonal antibody (*A*), laminin (*B*), CSAT monoclonal antibody and laminin (*C*), or fibronectin peptide analogue and laminin (*D*). Gel filtration was performed using Ultrogel AcA22 with TNC buffer containing 0.1% Nonidet P-40. The column was first preloaded with the desired ligand, and ^{35}S-labeled CSAT antigen (with cold ligand) was then run through the column. 60-μl fractions were collected and the labeled antigen counted. V_o is the void volume, and V_T is the total included volume. (*A*) (○) antigen alone; (■) antigen passed through column pre-equilibrated with 10 μg/ml CSAT antibody. (*B*) CSAT antigen passed through column pre-equilibrated with: (△) 25 μg/ml laminin, (●) 100 μg/ml laminin, (■) 200 μg/ml laminin; (○) 400 μg/ml laminin. (*C*) (○) CSAT antigen passed through column pre-equilibrated with 400 μg/ml laminin; (■) CSAT antigen first preincubated with 20 μg/ml CSAT monoclonal antibody and then passed through column pre-equilibrated with 400 μg/ml laminin. (*D*) CSAT antigen passed through the column pre-equilibrated with: (●) 400 μg/ml laminin and 1 mg/ml cell binding tetrapeptide; (□) 400 μg/ml laminin and 1 mg/ml nonapeptide.

observations) does not inhibit formation of the ligand–antigen complexes. Two synthetic peptides that do not inhibit cell adhesion, a nonapeptide (arg-lys-lys-thr-gly-gln-glu-ala-cys)

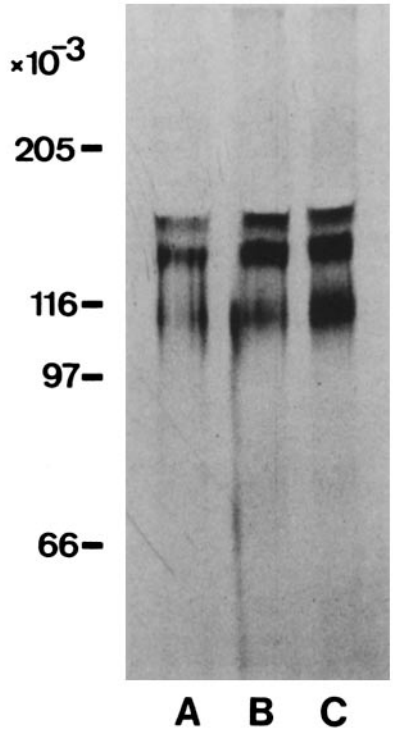

FIGURE 6 SDS PAGE of CSAT antigen and CSAT antigen–laminin complexes. Gel filtration was performed as described in Fig. 5 (with the column pre-equilibrated with 400 μg/ml laminin). The void volume fraction corresponding to the antigen–laminin complex (fraction 9) (*A*), the fractions corresponding to free antigen (fractions 14–16), and (*B*) an aliquot of CSAT antigen before gel filtration (*C*) were run on 7% nonreducing SDS PAGE and visualized by autoradiography.

from a region of fibronectin that does contain the cell binding sequence and a hexapeptide (gly-arg-gly-glu-ser-pro) containing a single substitution in the cell binding region (27), had no effect on the formation of either the laminin or fibronectin complexes (Figs. 5 and 7). Pre-equilibrating the column with 400 μg/ml BSA did not alter the antigen elution profile. Finally, the presence of 400 μg/ml fibronectin or laminin did not alter the elution of catalase, a Stokes radius standard.

The unexpected observation that the fibronectin cell-binding tetrapeptide inhibits the interaction of the purified CSAT antigen with both laminin and fibronectin prompted a preliminary examination of its significance by assaying for the effect of this peptide on cell adhesion to wells coated with these two substrata. Fig. 8 shows that the tetrapeptide but not the hexapeptide (or nonapeptide) inhibited adhesion of tendon fibroblasts to fibronectin. We also observed similar effects of these peptides on the adhesion of the cardiac fibroblasts on fibronectin. These specificities confirm the previous observations by others (26, 27). The effect of the tetrapeptide and hexapeptide on adhesion of tendon fibroblasts to laminin is also shown in Fig. 8. A partial inhibition was observed but appeared significant when compared with the hexapeptide (or nonapeptide) control, which did not show comparable effects even at 3–5-fold higher concentrations. The cell binding tetrapeptide showed only a very small effect on the adhesion of the cardiac fibroblasts to laminin. A more thorough examination of specificities will be required before these observations are fully understood.

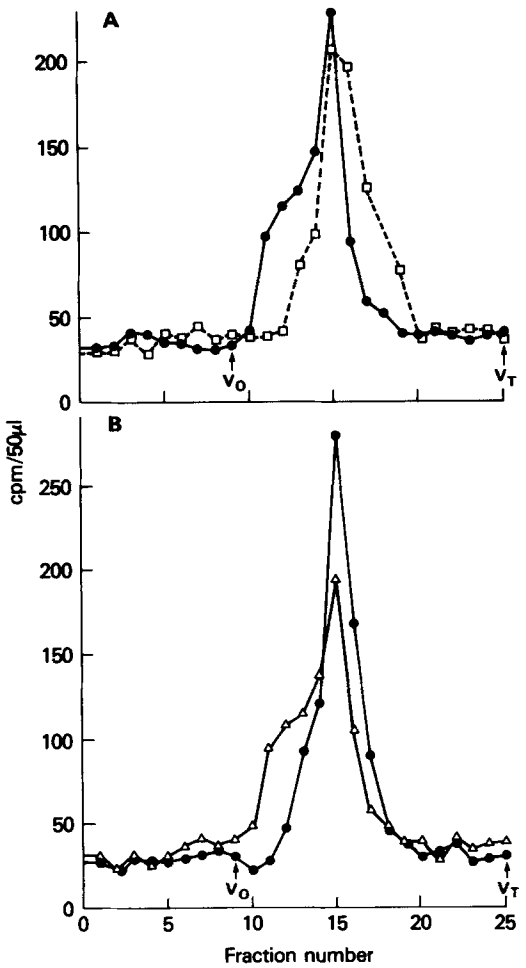

FIGURE 7 Equilibrium gel filtration elution profile of CSAT antigen from a column pre-equilibrated with fibronectin (*A*) or fibronectin and cell binding tetrapeptide from fibronectin (*B*). Gel filtration was performed as described in the legend to Fig. 5. (*A*) (□) CSAT antigen alone; (●) CSAT antigen passed through column pre-equilibrated with 200 μg/ml fibronectin. (*B*) (●) CSAT antigen passed through column pre-equilibrated with 200 μg/ml fibronectin and 1 mg/ml cell binding tetrapeptide; (Δ) CSAT antigen passed through column pre-equilibrated with 200 μg/ml fibronectin and 1 mg/ml fibronectin 9-mer.

The Oligomeric Nature of the CSAT Antigen Complex

The presence of all three glycoproteins comprising the CSAT antigen in the CSAT antigen–laminin and CSAT antigen–fibronectin complexes raises the possibility that the antigen itself is an oligomeric complex. We have explored this by studying the behavior of the antigen during gel filtration and velocity sedimentation in sucrose density gradients. As reported above, the antigen elutes as a single peak on Ultrogel AcA22, a molecular sieve. Subfractions from the leading and trailing edges and the center of the peak were analyzed by SDS PAGE. The gel profiles from the subfractions were indistinguishable and revealed the same three bands in the proportions seen in the unfractionated antigen. The CSAT antigen was also studied by sedimentation velocity in 5–20% linear sucrose gradients. The antigen resolved into a major peak and a small minor peak. Both were analyzed on SDS PAGE (Fig. 9). The antigen present in subfractions of the leading and trailing edges and center of the major peak were indistinguishable from each other and revealed the three bands characteristic of the original antigen. The minor peak contained only a single component that migrated like band 3 on SDS PAGE. Purified band 3 also migrated in this position (Buck, C., and A. Horwitz, unpublished observation). Although the origin and significance of this satellite peak is unclear, its presence demonstrates that the gradient can resolve and separate monomers from oligomers. These data together with our previous observations that the three bands comprising the CSAT antigen tend to co-purify using several different kinds of purification procedures suggests that the antigen resides as an oligomer (7).

TABLE II. *Binding Equilibria of CSAT Antigen with Different Ligands*

Ligand	K_d	K_i
	M	
CSAT antibody	6×10^{-9}	—
Laminin	2×10^{-6}	—
Fibronectin	10^{-6}	—
Tetrapeptide (inhibition of laminin binding)	—	2×10^{-4}
Tetrapeptide (inhibition of fibronectin binding)	—	10^{-3}–10^{-4}
Nonapeptide (inhibition of laminin binding)	—	$>5 \times 10^{-1}$
Nonapeptide (inhibition of fibronectin binding)	—	$>5 \times 10^{-1}$

The binding equilibria for the interaction of the purified CSAT antigen in TNC with the CSAT monoclonal antibody, laminin, and fibronectin were estimated using the equilibrium gel filtration method described in the text. The inhibition constants were measured from concentrations required to inhibit interaction of the purified antigen with fibronectin or laminin.

The gradient and gel filtration data were used to estimate the sedimentation coefficient, Stokes radius, and molecular weight of the oligomeric complex (20–22): $S_{w,20} = 8.6$; a = 6.0 nm; and mol wt = 235 kD (detergent + antigen) and 212 kD (antigen alone), respectively.

DISCUSSION

In previous studies we have shown that the CSAT antigen participates in the adhesion of several different kinds of cells to extracellular matrices and that not all cell types respond similarly to the CSAT monoclonal antibody. We have extended these observations by addressing three related issues: the extracellular molecules with which adhesions involving the CSAT antigen might interact; the role played by the CSAT antigen in the adhesion process; and the origin of the different responses to the CSAT monoclonal antibody displayed by different cell types.

The effect of the CSAT monoclonal antibody on fibroblasts and myoblasts plated on laminin demonstrates that the antigen participates in their adhesion to this extracellular molecule. Short term cultures of skeletal myoblasts and cardiac and tendon fibroblasts plated on laminin-coated dishes round and detach from the substratum when the CSAT monoclonal antibody is added. This effect of the CSAT monoclonal antibody is seen with well spread cells as well as with cells in earlier stages of adhesion. The observation that the residual adhesive strength of cells treated with the CSAT monoclonal antibody does not withstand a force of 1 *g* (10^{-7} dynes) is particularly important. It demonstrates that the antigen plays a very dominant role in the adhesion of these cells to laminin. Other adhesive interactions, if they were to exist, must be very weak or involve only a few molecules. Recent observa-

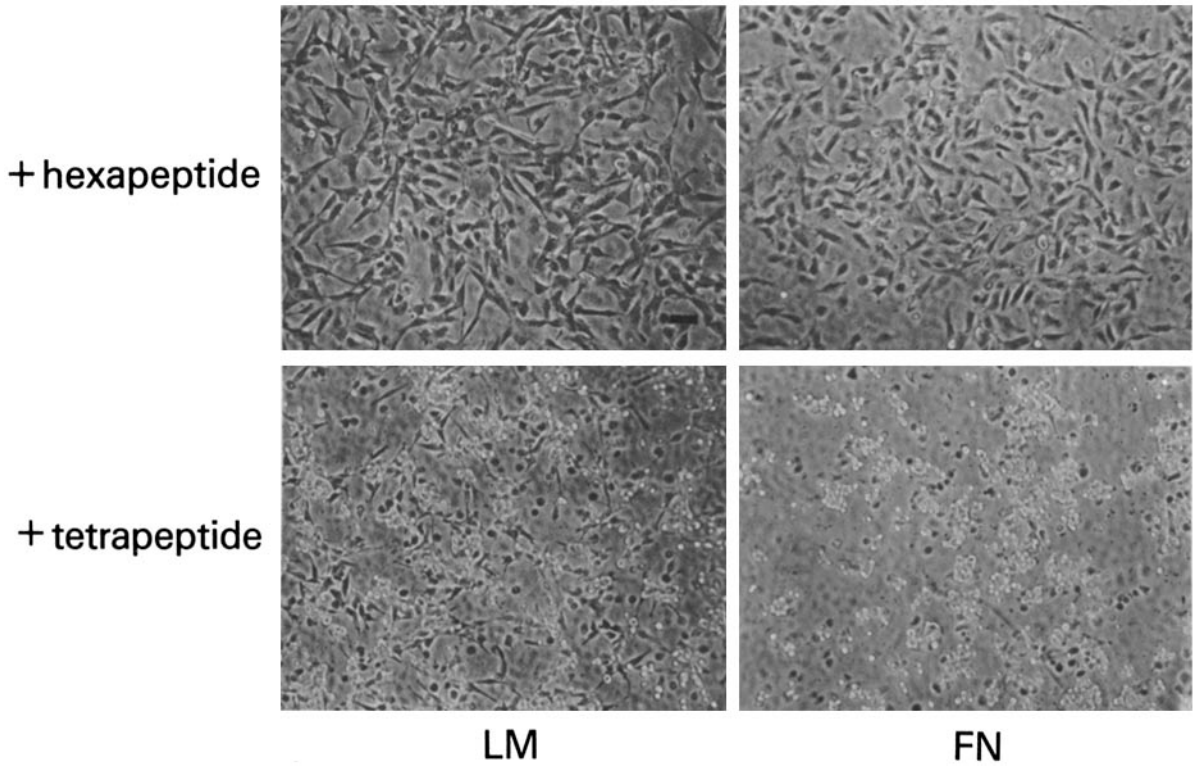

FIGURE 8 Effect of fibronectin peptides on adhesion of tendon fibroblasts. Tendon fibroblasts were plated in serum-free DME onto laminin- or fibronectin-coated wells (200 mm^2) in 10-μl spots at a density of 4×10^5 cells/ml. The peptides were added at the time of plating at a concentration of 1 mg/ml (tetrapeptide) and 1.6 mg/ml (hexapeptide). The cultures were flooded after 2 h of incubation and then photographed. Bar, 40 μm.

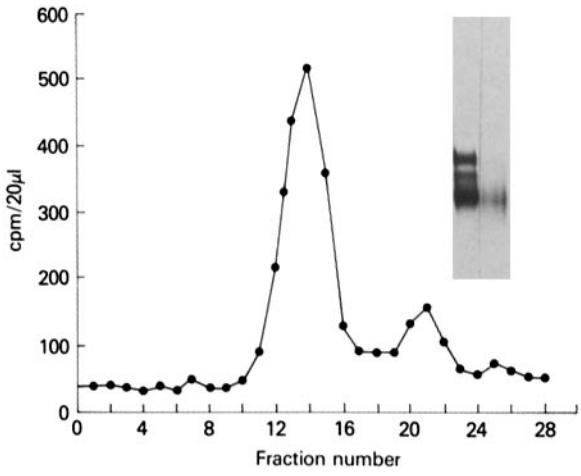

FIGURE 9 SDS PAGE analysis of CSAT antigen in fractions from sucrose density gradients. ^{35}S-labeled antigen in TNC was layered onto a 5–20% sucrose density gradient and sedimented for 8–9 h at 45,000 rpm at 4°C in a SW60 rotor. 150-μl fractions were collected. The first fraction corresponds to the bottom of the tube. The material in the main peak (fractions 11–16) and minor peak (fractions 19–32) were pooled separately and analyzed on SDS PAGE run under nonreducing condition. The left and right profile in the insert correspond to the main and minor peaks, respectively.

tions on the localization of the antigen are consistent with its role in adhesion to laminin. The antigen co-localizes with laminin in regions of acetylcholine receptor hot spots on cultured myotubes (Bozyczko, D., and A. Horwitz, unpublished observations). These are thought to be sites of interaction between the basal lamina, the cell surface, and the cytoskeleton (28, 29).

The observations reported here also implicate the CSAT antigen in adhesion to fibronectin. The adhesion of the tendon fibroblasts and skeletal myoblasts is inhibited substantially by the presence of the CSAT monoclonal antibody. Although the adhesion of the cardiac fibroblasts is not inhibited significantly by the CSAT monoclonal antibody, prior treatment with trypsin in the presence of calcium renders their adhesion sensitive to the antibody. The total inhibitions of adhesion by the CSAT monoclonal antibody seen for cells on laminin was not observed for cells on fibronectin. This observation along with the minimal effects of the CSAT monoclonal antibody on cardiac fibroblasts point to differences between adhesion to fibronectin and laminin. Morphological studies also implicate the CSAT antigen in adhesion to fibronectin. On fibroblasts, the CSAT antigen co-localizes with fibronectin along portions of stress fibers, at the cell periphery, and in regions surrounding the focal contact as visualized by vinculin staining (5, 8). Other investigators, using the JG-22 monoclonal antibody that is directed against the same antigen, have also localized the antigen to fibronectin-rich adhesion sites on fibroblasts (11, 11a).

The question arises concerning the role that the CSAT antigen plays in adhesion to these matrix molecules (30). Two obvious hypotheses are that (*a*) it functions as a structural molecule to organize the adhesion complex or (*b*) it functions directly in adhesion as a receptor for extracellular matrix ligands. We have explored the latter hypothesis—that it functions as their cell surface receptor. Our observation that the purified antigen interacts with laminin and fibronectin provides direct evidence supporting this hypothesis. It is strength-

ened further by the inhibitory effect of the CSAT monoclonal antibody and the cell-binding peptides from fibronectin on formation of the antigen–laminin and antigen–fibronectin complexes, respectively. The K_d for the interaction of the antibody with the purified antigen and the antibody concentrations required for inhibition of the antigen–laminin complex are identical with those reported previously for the dissociation constant for the antibody binding to antigen on cell surfaces and for inhibition of cell adhesion by the antibody (5). Yamada and co-workers (31, 32) have investigated the binding of fibronectin and its fragments to baby hamster kidney fibroblasts. They report that the receptor is resistant to trypsin in the presence of calcium. They also report 5×10^5 fibronectin binding sites per cell with a K_d of 8×10^{-7} M and a K_i of 10^{-3}–10^{-4} M for tetrapeptide inhibition of adhesion to fibroblasts. Despite the differences in species, these data are very similar to those that we have estimated for the CSAT antigen. We find 5×10^5 CSAT monoclonal antibody binding sites per fibroblast and a K_d of $\sim 10^{-6}$ M for the antigen–fibronectin interaction. We also find the CSAT-mediated adhesion resists treatment with calcium and trypsin.

The observation that the CSAT antigen participates in adhesion to both laminin and fibronectin was unexpected but of particular potential importance. The antigen also may interact with other extracellular ligands and thus may serve as a multifunctional extracellular matrix receptor. The inhibition of antigen interaction with both laminin and fibronectin by the cell binding tetrapeptide from fibronectin suggests that these extracellular molecules may have a common sequence and all bind to a common receptor site. However adhesions to laminin and fibronectin appear to display different susceptibilities to the CSAT antibody, trypsin, and the fibronectin cell-binding tetrapeptide. Therefore, it would seem that adhesion to these matrix molecules is more complex than the simple interaction of a single receptor binding site with its ligands (33). Hypotheses for the function of the CSAT antigen and its interactions with ligands and inhibiting molecules must consider the nature of the antigen itself. It is an oligomeric complex composed of three distinct glycoproteins. Where the laminin, fibronectin, and other adhesion-promoting and inhibiting molecules interact with this complex is a problem that remains to be addressed.

Several papers have implicated proteins migrating in the molecular weight range of 140,000 D in adhesion to extracellular substrates (34–36). Molecular complexes with average molecular weight of 140,000 D that can block the effect of adhesion-perturbing antibodies have been isolated from mouse and hamster fibroblasts as well as from epithelial cells. More recently, Pytela et al. (36) have identified fibronectin-binding proteins from human fibroblasts. These proteins, like the CSAT antigen, migrate in the 140-kD range on SDS PAGE and resolve into distinct bands when run on nonreduced gels. These proteins further resemble the CSAT antigen in that their interaction with fibronectin is competed specifically by the tetrapeptide fragment from the cell-binding site of fibronectin.

Three laboratories have described a 67,000-D molecule as a laminin receptor on mammalian muscle, human breast carcinoma, and mouse melanoma cells (37–40). Since the CSAT antigen is a complex of three polypeptide chains that migrate on reduced SDS PAGE in the molecular weight range of 140,000, it would appear that these putative receptors are different. This is also suggested by the higher affinity of the mammalian receptor for laminin (41). Lesot et al. (38), however, report two bands in the molecular range of 150,000 that are recognized by laminin on immunoblots. Recent studies on neurite extension using antibodies against different laminin domains suggest that there may be more than one kind of molecule that interacts with laminin (42). In this regard we have found that the CSAT antigen is present on nerve and have demonstrated its role in neuron adhesion and axonal extension (43).

The final issue that we have addressed is why different cell types respond characteristically to the CSAT monoclonal antibody. We have proposed elsewhere that the different responses reflect a progressive display of additional adhesive molecules and their organizational complexes on increasingly adhesive cell types (6). The data reported here extend these earlier observations. The inhibition of cardiac fibroblast adhesion to laminin and to fibronectin demonstrates that the CSAT antigen is not only present but functional on these cells. It follows then that they adhere to fibronectin by a more complex mechanism than that used on the tendon fibroblasts or skeletal myoblasts. An obvious hypothesis is that there is more than one kind of mechanism involved in adhesion to fibronectin. Recently evidence has been provided for multiple fibronectin adhesion mechanisms on neurons (43).

In summary three lines of evidence argue strongly for the CSAT antigen functioning as a receptor for extracellular molecules. (*a*) Monoclonal antibodies directed against the antigen inhibit adhesion to both fibronectin and laminin; (*b*) the antigen localizes in putative regions of cell–matrix adhesion; and (*c*) the purified CSAT antigen binds to both fibronectin and laminin to form a physiologically meaningful association. Although only fibronectin and laminin were studied in detail, it is likely that CSAT antigen functions more generally as a cell surface receptor for extracellular molecules.

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