Distribution of the Cell Substratum Attachment (CSAT) Antigen on Myogenic and Fibroblastic Cells in Culture

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ABSTRACT Previous studies (Neff et al., 1982. *J. Cell. Biol.* 95:654–666; Decker et al., 1984. *J. Cell. Biol.* 99:1388–1404) have described a monoclonal antibody (CSAT Mab) directed against a complex of three integral membrane glycoproteins of 120,000–160,000 mol wt (CSAT antigen [ag]) involved in the cell matrix adhesion of myoblasts and fibroblasts. In localization studies on fibroblasts presented here, CSAT ag has a discrete, well-organized distribution pattern. It co-aligns with portions of stress fibers and is enriched at the periphery of, but not directly beneath vinculin-rich focal contacts. In this last location, it co-distributes with fibronectin, consistent with the suggestion that the CSAT ag participates in the mechanism by which fibroblasts attach to fibronectin. In prefusion myoblasts, which are rapidly detached by CSAT Mab, CSAT ag is distributed diffusely as are vinculin, laminin, and fibronectin. After fusion, myotubes become more difficult to detach with CSAT Mab. The CSAT ag and vinculin are organized in a much more discrete pattern on the myotube surface, becoming enriched at microfilament bundle termini and in lateral lamellae which appear to attach myotubes to the substratum. These results suggest that the organization of CSAT ag-adhesive complexes on the surface of myogenic cells can affect the stability of their adhesive contacts. We conclude from the sum of the studies presented that, in both myogenic and fibroblastic cells, the CSAT ag is localized in sites expected of a surface membrane mediator of cell adhesion to extracellular matrix. The results from studies that use fibroblasts in particular suggest the involvement of CSAT ag in the adhesion of these cells to fibronectin.

CSAT monoclonal antibody (CSAT Mab),[1] raised against chick embryo myoblast surface membrane vesicles, rapidly rounds and detaches 48-h cultured chick myoblasts from their extracellular matrices (ECM). With additional time in culture, developing myotubes become increasingly resistant to detachment. CSAT Mab binds equally well to the skeletal fibroblasts present in the myoblast cultures, but does not detach them (24). The CSAT antigen (ag) is clearly relevant to myoblast adhesion in vivo since a population of cells is released when 11-d chick embryo breast muscle explants are treated with CSAT Mab. When subsequently cultured in the absence of CSAT Mab, these cells attach and undergo fusion (24). Studies on the effects of CSAT Mab on cultured cells isolated from different embryonic tissues have shown that CSAT Mab can perturb the adhesion of a number of other cell types that include cardiac myocytes and some kinds of embryonic fibroblasts; however, the extent to which the adhesion is perturbed depends on the particular cell type studied and the culture conditions used (14).

The ag to which CSAT Mab binds has been identified by immunoaffinity chromatography using immobilized CSAT Mab (24) and has been partially characterized (21). The ag consists of a complex of three distinct integral membrane glycoproteins with molecular weights after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS PAGE) under nonreducing conditions of 160,000 (band 1), 135,000 (band 2), and 110,000 (band 3). These glycoproteins cannot be separated from one another by selective extraction of the cell surface nor can they be individually eluted from the

[1] *Abbreviations used in this paper*: ag, antigen; anti-GP-3, polyclonal antiserum raised in rabbits against the third glycoprotein band of immunoaffinity-purified CSAT antigen complex; CSAT, cell substratum attachment; ECM, extracellular matrix; FITC, fluorescein isothiocyanate; Fn, fibronectin; GP-3, third glycoprotein band; Lm, laminin; Mab, monoclonal antibody; Vn, vinculin.

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CSAT Mab column (21). They follow one another throughout a multistep purification procedure and travel together in a centrifugal field (3, 21). These data suggest that the CSAT ag exists as a complex. All cell types examined contain all three bands in approximately the same ratio. Therefore, the differential effects of the CSAT Mab on their adhesion cannot be readily explained by a gradient of antigen or a reduction in one or more of the bands (14). The particular function in cell matrix adhesion of the CSAT ag complex as a whole, or of its individual components, is as yet unknown. However, based on the effect of CSAT Mab on the cell-matrix adhesion and morphology of myoblasts and fibroblasts, and its lack of effect on other physiologic processes such as myoblast fusion (19, 24), it seems likely that the ag participates directly in cell-matrix adhesion, perhaps as a transmembrane link between the cytoskeleton and the ECM. To perform such a role, the CSAT ag should be localized in the vicinity of cell-substratum contact sites.

The cell-matrix attachment sites of myogenic cells in culture have not been well studied to date. However, the difference in the susceptibility of myoblasts and fibroblasts to detachment by CSAT Mab may reflect significant differences in both the organization and the molecular composition of adhesive sites in the two cell types.

Fibroblasts in culture have been shown to interact with the substratum at several kinds of sites: focal contacts, and close contacts where cells approach the substratum to within 10–20 and 30–50 nm, respectively (18, 20), and ECM contacts where cells closely approach large fibrils of ECM (10). In immunoelectron microscope studies of well-spread normal fibroblasts, the cytoskeletal protein vinculin (Vn) has been shown to be highly enriched at focal contact sites and a subset of ECM sites, whereas fibronectin (Fn) is enriched at the external surface of the cell at the periphery of focal contacts and at a different subset of ECM sites from that of Vn (10). The cytoskeletal proteins a-actinin (31) and, more recently, talin (5), are also enriched at cell-matrix adhesion sites. Since none of these molecules is integrated into the plasma membrane, it has been assumed for some time that transmembrane components would mediate adhesion at cell-matrix contact sites and promote the organization of relatively stable transmembrane adhesive complexes (10, 12). The CSAT ag complex is a candidate to fulfill such a role, and therefore its distribution with respect to adhesion-related matrix and cytoskeletal molecules in both myoblasts and fibroblasts is a major question addressed by the experiments reported here.

With these questions in mind, we have localized the CSAT ag in both myogenic and fibroblastic cells with respect to cytoskeletal markers for Vn and F-actin, and with respect to the extracellular adhesive glycoproteins Fn and laminin (Lm).

MATERIALS AND METHODS

Cells: Myoblasts were obtained from 10-d chick embryos and plated on collagen as described by Neff et al. (24). Prefusion myoblasts (24–48 h), small myotubes (48–96 h), and 6-d myotubes were used for the studies reported below. Tendon fibroblasts were obtained from 15–18-d and cardiac fibroblasts from 14-d embryos as described by Decker et al. (14). These were used between the third and tenth passage.

Antibodies: CSAT Mab was produced as described previously (24). A polyclonal antiserum was raised in a rabbit against the third glycoprotein band of the immunoaffinity-purified CSAT ag complex excised from SDS polyacrylamide gels (anti-GP-3) run according to Laemmli (23) under nonreducing conditions (21). Its specificity was evaluated by the Western immunoblot procedure (4) using an NP40 extract of eviscerated, decapitated 10-d chick embryos and protein A-purified anti-GP-3 IgG at 2 μg/ml. Mouse Mabs against chick Fn and chick Lm were gifts of Dr. D. Fambrough (Carnegie Institute of Washington: see reference 11). Guinea pig anti-chicken gizzard Vn was provided by Dr. Irwin Singer (Merck Sharpe & Dohme Research Laboratories, Rahway, NJ). Fluorescein isothiocyanate (FITC) phalloidin was the gift of Dr. Guido Tarone, University of Torino. Secondary antibodies: rhodamine- and fluorescein-conjugated goat anti-mouse, fluorescein goat anti-guinea pig, and fluorescein or rhodamine goat anti-rabbit were purchased from Cooper Biomedical, Inc., Malvern, PA). All were absorbed on fibroblasts fixed and permeabilized with methanol at −20°C as described by Rorschneider (28) to reduce nonspecific staining.

Except as noted specifically in the text, fibroblasts were plated on glass coverslips in medium containing 2% fetal calf serum and used 8–16 h later. This procedure produced a population of fibroblasts with a very flat, well-spread morphology. Myoblasts were plated on collagen-coated coverslips and used 24–48 h, 48–96 h, or 6 d later. The overall protocol for immunofluorescence was as described in Neff et al. (24). Cells were stained live, after fixation with methanol 3–5 min at −20°C, or after fixation with 3% paraformaldehyde for 20 min and permeabilization with acetone at −20°C. Protein A-purified CSAT Mab was used at 25 μg/ml, whereas protein A-purified IgG from rabbit anti-GP-3 serum was used at 2 μg/ml, which was the same concentration as that used for immunoblotting. A nonrelevant monoclonal antibody or preimmune rabbit IgG at corresponding concentrations was used as controls. Primary and secondary antibodies were diluted in phosphate-buffered saline with 2 mg/ml normal goat globulin and incubated with cells for 1 h. When two antigens were localized in the same cells (e.g., CSAT and Vn), the secondary antibodies (rhodamine ~ goat anti-mouse and fluorescein ~ goat anti-guinea pig) were cross-absorbed on heterologous immunoglobulin affinity columns to remove cross-reacting antibodies.

Cells were observed in a Zeiss phase-epifluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with filters appropriate for double immunofluorescence, and a × 63 planapochromat oil immersion lens numerical aperture 1.4. Cells were photographed using Tri-X-film which was push-developed with Accufine (Eastman Kodak Co., Rochester, NY).

Video Image Processing: Negatives of photomicrographs were projected onto a screen and the image was digitized by using a Panasonic (CD10) television camera equipped with a 12.5–75-mm f/1.8 lens and interfaced, via a digitizing card, to an Apple IIe microcomputer with 128k extended memory (Apple Computer, Inc., Cupertino, CA). The digitizing card (Microworks DS-65, Delmar, CA) had a resolution of 256 × 256 × 6 bits. Assembler level programs were developed for processing the images and manipulating the digital data. A gray scale output code was developed for visualizing the digital images; it had a resolution of 28 gray levels.

For comparisons of large regions of two related micrographs, the backgrounds were first subtracted from each digitized image and the intensely staining regions were assigned a single, arbitrary gray value code. The gray value for each of the two pictures was different, thus producing a code for each picture. Regions less intense than the critical intensity were assigned to zero. The digitized images from pairs of related micrographs were then lined up by superimposing the edges of the frame and sprocket tracks. The superimposed, encoded images were then added together. The resulting output had three different gray levels: one corresponding to intensely staining regions of each of the two different cell images and one corresponding to their regions of superimposition.

For quantitative comparisons of small regions, the digitized images, at a full resolution of 64 gray levels with backgrounds subtracted, were lined up as described above. The pixel values along a row of pixels that included the region of interest were plotted as a function of distance. The distance per pixel was calibrated with digitized photographs of a micrometer ruling.

RESULTS

CSAT ag was localized on cardiac and tendon fibroblasts and on myoblasts and well-developed myotubes. Live cells, cells fixed and permeabilized with methanol at −20°C, and cells fixed with paraformaldehyde and permeabilized with acetone were used. CSAT Mab was used to stain live and methanol-permeabilized cells. However, the CSAT determinant does not survive paraformaldehyde fixation. To study such cells, a polyclonal antiserum that was raised in rabbits against the third glycoprotein band (GP-3) excised from SDS gels of immunoaffinity-purified CSAT ag complex run under nonreducing conditions (Fig. 1*A*) was used. When an unfractionated NP40 extract of chick fibroblasts is analyzed by SDS

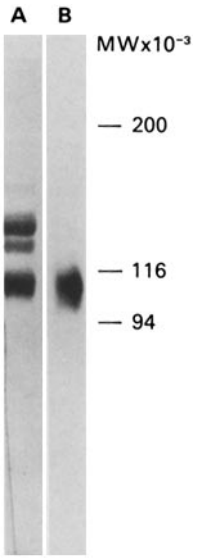
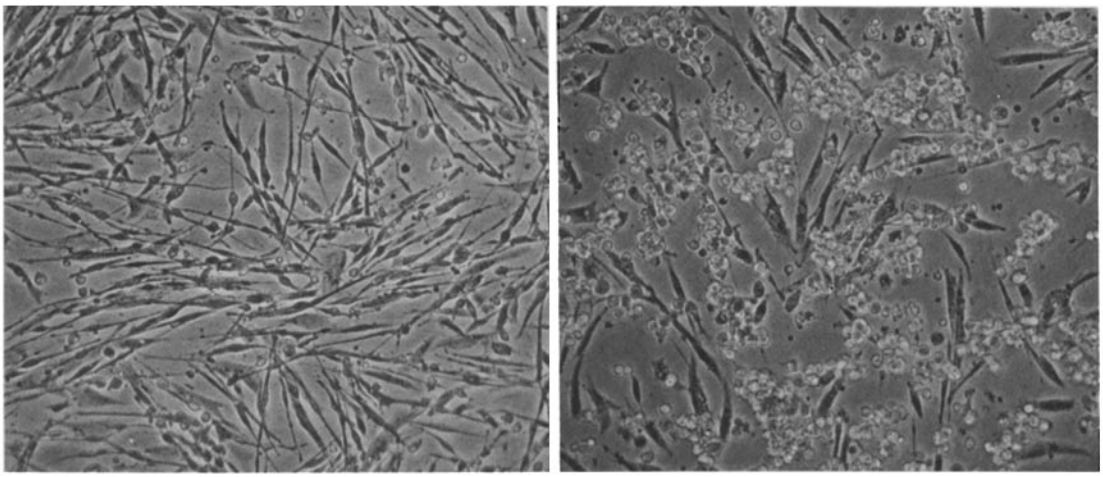


FIGURE 1 (*A*) SDS PAGE of immunoaffinity-purified CSAT ag transferred to nitrocellulose and stained with amido schwartz. (*B*) Immunoblot of an NP40 extract of chick tendon fibroblasts analyzed by SDS PAGE, transferred to nitrocellulose, exposed to rabbit anti-GP-3 IgG at 2 μg/ml, and detected with ^{125}I-protein A. (*C*) Control and (*D*) anti-GP-3-treated 48-h myoblast cultures. Culture was treated with anti-GP-3 IgG at 2 μg/ml for 1 h. Most of the cells have detached. These will go on to fuse, forming multinucleate myoballs in the presence of anti-GP-3 (not shown; see also reference 24). The remaining attached cells retain a fibroblastic morphology and do not fuse later. × 100.

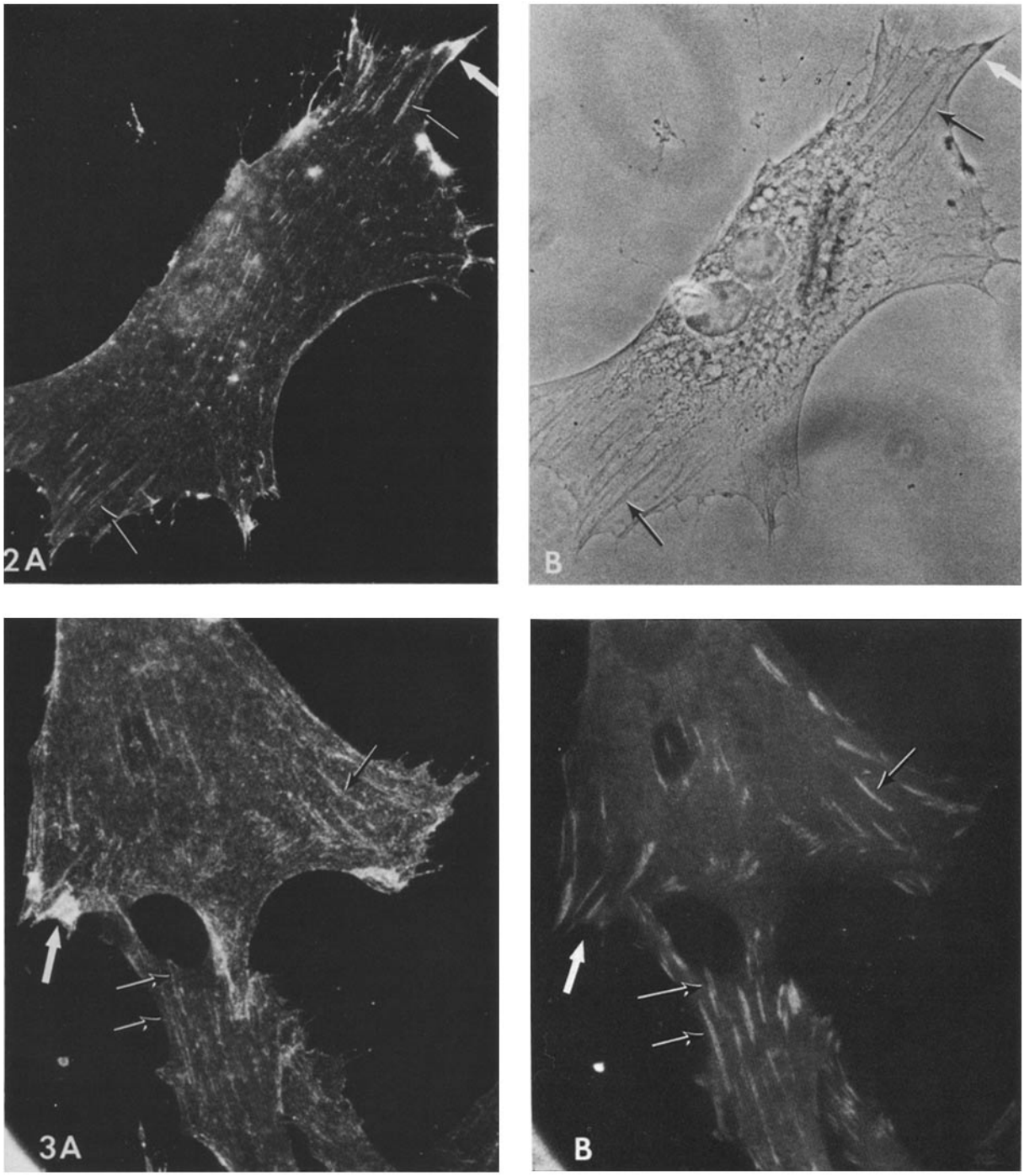


PAGE and the immunoblot procedure, anti-GP-3 binds only material in the region of GP-3 (Fig. 1 *B*). This antiserum detaches chick myoblasts at high dilution (1 μg/ml IgG) but does not round and detach the skeletal fibroblasts present in the culture (Fig. 1, *C* and *D*). Anti-GP-3 perturbs the morphology of some but not all kinds of fibroblasts under some culture conditions in a manner resembling CSAT Mab. The staining patterns obtained using CSAT Mab and anti-GP-3 under the fixation conditions used were indistinguishable.

CSAT ag on Fibrogenic Cells

Fig. 2, *A* and *B* illustrates the staining pattern of CSAT Mab on a well-spread live tendon fibroblast (see also Table I). The CSAT ag co-aligns with portions of stress fibers visible by phase-contrast microscopy. Tips of cell processes in the vicinity of stress fiber termini are also prominently stained. The staining pattern on live cells was found when cells were incubated with CSAT Mab at either room temperature or 4°C in the presence of azide indicating that the pattern obtained was not caused by antibody-induced ag redistribution. The staining pattern observed with CSAT Mab on live cells was the same as that observed on fixed permeabilized cells incubated with either CSAT Mab or anti-GP-3. This pattern was typical of ~75% of the cells in cultures grown under these conditions.

The relationship of CSAT ag staining to cell-substratum contact sites was examined more closely by employing double-label fluorescence techniques to localize, in the same cell, CSAT ag and cytoskeletal components known to be enriched at cell-substratum contacts. FITC phalloidin was used to stain F-actin. This reagent visualizes particularly well the stress fibers of well-spread cells. Guinea pig anti-Vn was used as a marker for focal contact sites (6, 15). When well-spread tendon or cardiac fibroblasts were stained with CSAT Mab or anti-GP-3 in conjunction with anti-Vn, both staining patterns were enriched near the termini of stress fibers visible by phase-contrast microscopy. However, the patterns were often not superimposable. Instead, CSAT ag was localized primarily at the periphery of the discrete patches of Vn staining that mark focal contacts (Fig. 3, *A* and *B*). This relationship is particularly clear in substratum-attached material left behind after mechanical detachment of cells (Fig. 4). The CSAT ag staining pattern outlines a series of needle eyes, whereas Vn staining appears to fill in the needle eyes. Fig. 5 illustrates the relationship of CSAT ag staining and that of F-actin as identified by FITC phalloidin. CSAT ag is aligned with portions of F-actin bundles and appears to surround their termini. This pattern was observed using all three cell preparation methods described and was characteristic of both CSAT Mab and anti-GP-3.



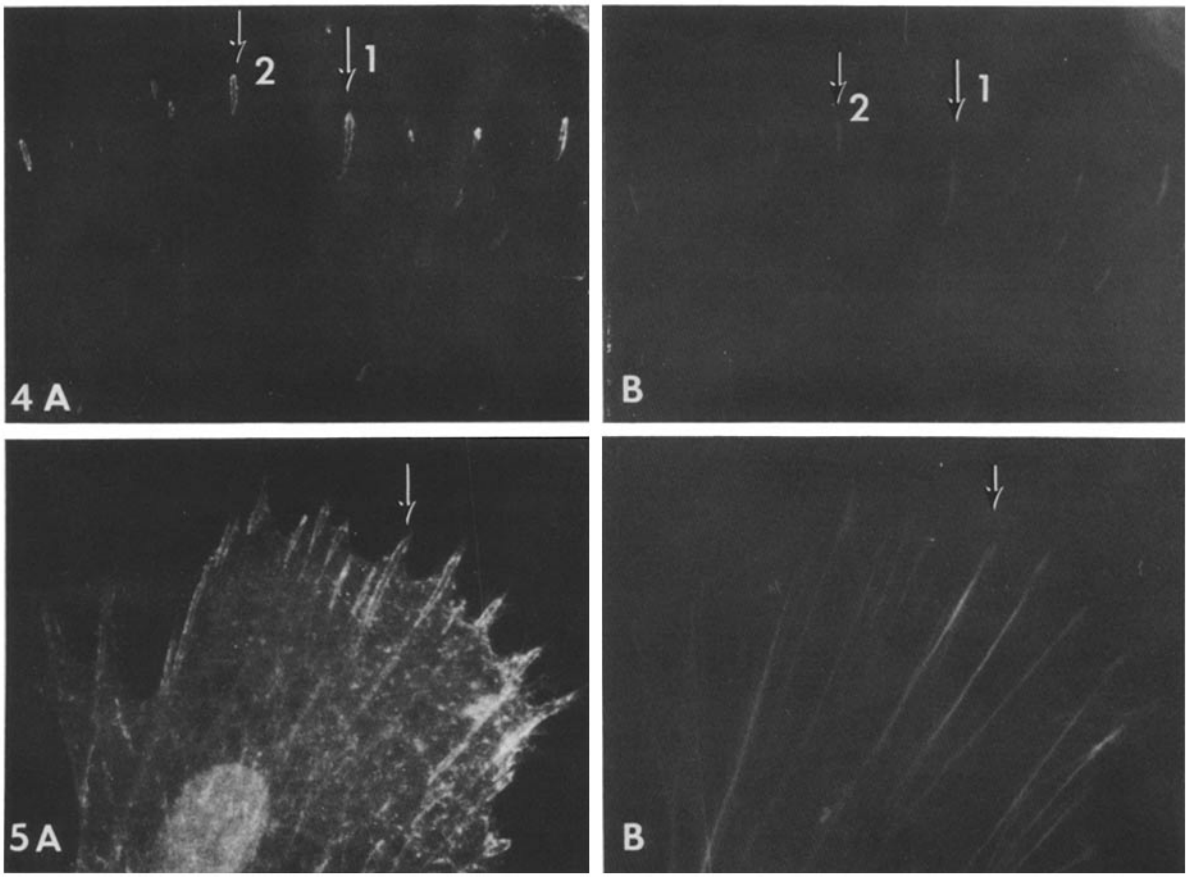
FIGURES 2 and 3 Fig. 2: (*A*) Tendon fibroblast incubated live with 25 μg/ml CSAT Mab, fixed with paraformaldehyde, permeabilized with acetone, and stained with rhodamine-conjugated goat anti-mouse (R-GaM). (*B*) Phase-contrast micrograph of same field. CSAT Mab stains portions of phase-dense stress fibers and is enriched at their termini. Controls using a nonrelated Mab and using CSAT Mab preabsorbed with immunoaffinity-purified CSAT ag complex show only very dim, diffuse fluorescence (not shown). × 800. Fig. 3: chick cardiac fibroblast permeabilized with methanol at −20°C and incubated with (*A*) CSAT Mab and (*B*) rabbit anti-Vn. Cell was stained with R-GaM and fluorescein-conjugated goat anti-guinea pig. Although CSAT Mab consistently stains in the vicinity of Vn-rich streaks, it is frequently restricted to the periphery of these regions (thin arrows). CSAT Mab also stains ruffled membranes (white arrow). × 1,600.

The relationship of CSAT ag and microfilament bundle termini was confirmed by digital processing of video images. Two separate samples were analyzed: a cell double-labeled with CSAT Mab and FITC phalloidin (Fig. 5, *A* and *B*), and substrate-attached material labeled with CSAT Mab and anti-Vn (Fig. 4, *A* and *B*). The respective negatives of each micrograph pair were digitized and the large regions including areas of intense staining encoded. The two images were superimposed and output such that the fluorescence from each cell type was uniquely coded as were regions of overlap (see Materials and Methods). At low resolution, microfilament bundle termini as marked by Vn or FITC phalloidin staining always coincided with regions of CSAT staining (not shown). However, the converse was not true: CSAT staining was

TABLE I

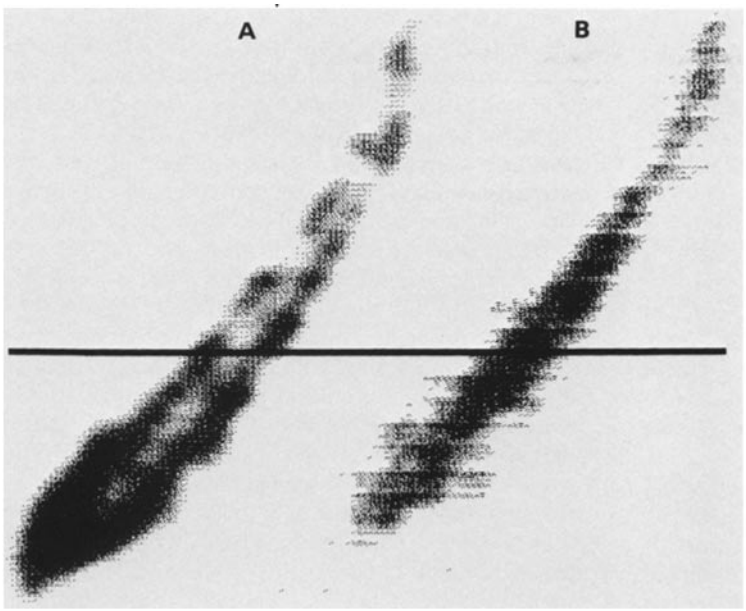
Distribution of Adhesion-related ag on Myogenic and Fibroblastic Cells in Culture

	CSAT	Vn	Fn	Lm
Fibroblasts	Enriched at stress fiber termini; frequently confined to periphery Aligns with portions of stress fibers Membrane ruffles	Stress fiber termini Portions of stress fibers	Enriched at stress fiber termini; frequently confined to periphery Aligns with portions of stress fibers Extra cellular matrix fibrils	Not detected
Myoblasts	Surface diffusely stained	Diffuse cytoplasmic staining	Surface diffusely stained	Surface diffusely stained
Myotubes				
72 h	Enriched at lateral lamellae Aligns with portions of F-actin bundles and their termini	Enriched at lateral lamellae Aligns with portions of F-actin bundles and their termini	Little or no staining	Surface diffusely stained
6 d	Enriched at lateral lamellae Discontinuous surface streaks Myotube termini	Enriched at lateral lamellae Discontinuous subsurface streaks	Little or no staining	Diffuse staining with additional patchy staining



FIGURES 4 and 5 Fig. 4: material left behind after mechanical disruption of cells. (*A*) CSAT Mab stain is displayed as a series of needle's eyes (arrows). (*B*) Anti-Vn, same field. The needle's eyes appear to be filled with anti-Vn staining. (The sets of arrows labeled 1 and 2 represent corresponding needle eye structures in the two prints). × 1,600. Fig. 5: co-localization of CSAT ag and FITC phalloidin on tendon fibroblasts. (*A* and *B*) Live cell stained with CSAT Mab, fixed with paraformaldehyde, permeabilized with 0.1% NP40 in phosphate-buffered saline and stained with FITC phalloidin. CSAT ag staining (*A*) appears to surround the termini of bundles of F-actin visualized by FITC phalloidin (*B*, arrow). × 1,600.

present in some areas where phalloidin or anti-Vn staining were not detected. A higher resolution view of the region of co-localization of Vn and CSAT ag indicated by arrow set 1 in Fig. 4, *A* and *B* was particularly revealing (Fig. 6, *A* and *B*). The Vn staining was surrounded by CSAT ag staining. This is more clearly seen in the output of a linear section through a region of Vn and CSAT staining (Fig. 6*C*). In this figure, the relative intensity of staining is plotted against a distance axis. The Vn lies within the region delineated by the CSAT ag.



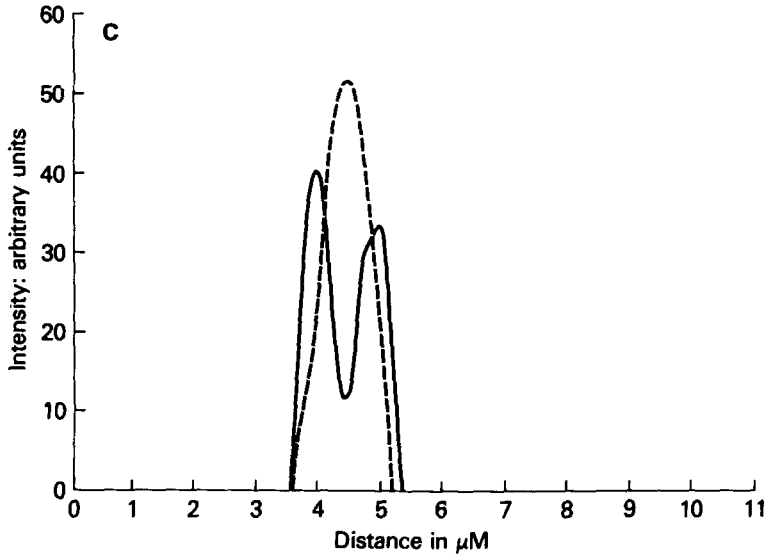


FIGURE 6 High-resolution output of digitized images of (*A*) CSAT ag and (*B*) Vn fluorescence in regions indicated with set 1 arrows in Fig. 4, *A* and *B*. (*C*) Output of a linear section through region of the CSAT ag and Vn staining is indicated by line through *A* and *B*. Intensity along a distance axis of CSAT ag (——) and Vn (------) is plotted.

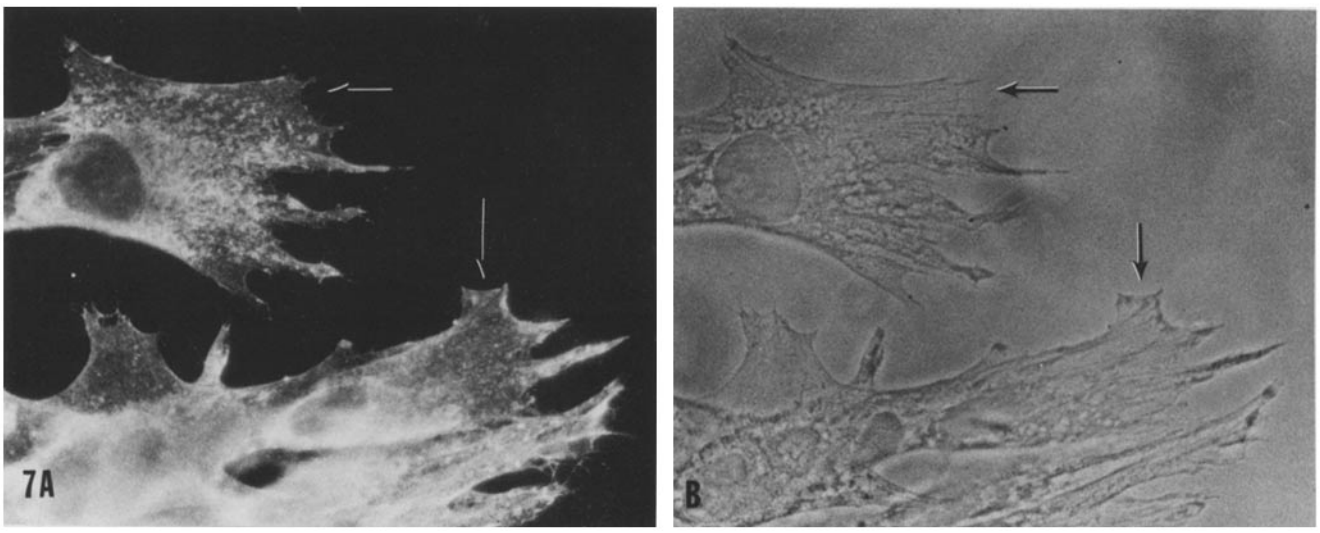


FIGURE 7 Control for accessibility of antibodies to all regions of the cell surface. (*A*) Tendon fibroblast fixed with paraformaldehyde, permeabilized with acetone, and incubated with rabbit anti-concanavalin A IgG. Concanavalin A staining was visualized with rhodamine ~ goat anti-rabbit. Staining has a uniform, diffuse distribution with no accentuation or depletion along stress fibers visible in the accompanying phase image (*B*) or at their termini (thin arrows). × 1,800.

These results indicate that CSAT ag exists in the vicinity of microfilament bundle termini and, in particular, at the periphery of focal contacts as monitored by Vn staining. However, the possibility exists that poor accessibility of antibodies to the CSAT ag might be responsible for the lack of staining directly underneath all of the focal contact sites stained by anti-Vn. To control for this, concanavalin A and rhodamine-conjugated anti-concanavalin A were used to label the cell surface nonspecifically. Cells stained with these reagents showed a uniform fluorescence with no detectable pattern of staining in the vicinity of stress fibers visible by phase-contrast microscopy (Fig. 7, *A* and *B*). Thus the enrichment of CSAT ag at the periphery of Vn-rich contact sites, and its frequent absence directly beneath these sites, cannot be explained in a simple way by the lack of accessibility of reagents to these regions of the cell surface.

When the distribution of CSAT ag was compared with that of Fn, both were co-extensive in the vicinity of sites of cell matrix contacts: that is, whenever CSAT ag revealed a needle eye pattern at the termini of stress fibers, Fn was superimposed on this pattern as well (Fig. 8, *A* and *B*). The CSAT and Fn distributions were not coincidental in other locations, however. Fn was not found on the edges of the leading lamella and CSAT was not coextensive with the network of Fn-rich ECM fibrils. Anti-Lm did not stain tendon or cardiac fibroblast cultures above background level (not shown), suggesting that these cells produce negligible levels of this adhesion glycoprotein. All studies described above were performed using cells plated on glass coverslips for 8–16 h in 2% serum-containing medium. Under these conditions, neither tendon nor cardiac fibroblasts were detachable by CSAT Mab.

CSAT Antigen on Myogenic Cells

CSAT Mab and anti-GP-3 IgG were used to stain myogenic cells at various times during their development in culture (see also Table I). CSAT ag is diffusely distributed on prefusion myogenic cells. It appears as fine punctate staining over the surfaces of these cells (Fig. 9, *A–C*). In contrast to fibroblasts in the same culture, CSAT ag distribution on myoblasts appears much less discrete (Fig. 9*A*). The Vn staining pattern on myoblasts is diffuse throughout the cytoplasm without a strong concentration beneath the cell surface (Fig. 9*D*). The very different staining patterns of Vn and CSAT ag on myoblasts and fibroblasts in the same culture suggest that cell-matrix contact sites are organized differently in the two cell types. This extends as well to the ECM adhesion glycoproteins. Myoblasts stain diffusely but strongly with anti-Lm (Fig. 9*E*) and, to a lesser extent, anti-Fn (not shown). Anti-Lm staining is not detectable on the fibroblasts (Fig. 9, *E* and *F*).

In a 72-h myogenic culture, most myoblasts have fused and the culture consists primarily of myotubes of various sizes as well as cells with a fibroblastic morphology. Fig. 10, *C* and *D* shows the distribution of both CSAT ag and F-actin in two small myotubes. The pattern of staining of CSAT ag in these myotubes appears more discrete than that found in myoblasts, becoming enriched at the termini of lateral lamellae which attach the myotube to the matrix and co-aligning with portions of F-actin bundles. Vn in these cells also becomes enriched at lateral lamellae and along portions of F-actin bundles (Fig. 10, *A* and *B*). Fn staining is difficult to detect on myotubes, although the fibroblasts stain intensely (Fig. 10, *E* and F). After 6 d in culture, large myotubes with prominent sarcomeric cross-striations are present (Fig. 11). The CSAT ag staining pattern is now highly organized. It is prominent at the termini of myotubes, along the lateral lamellae, and in discontinuous streaks at the cell surface (Fig. 11, *A* and *E*). The staining is particularly striking at the ventral surface of the myotube, where it appears as a myriad of small dots and patches (Fig. 11, *A* and *B*). Fn staining appears restricted to the ECM material between the large myotubes (Fig. 11, *F* and *G*). Lm has a patchy distribution at the cell surface of myotubes, but is not always coextensive with CSAT Mab staining at lateral lamellae or along the surface streaks stained by CSAT (Fig. 11, *E* and *F*). It is also absent from CSAT rich-fibroblasts and from the extracellular matrix fibres of the kind stained by Fn (see Fig. 11*C*).

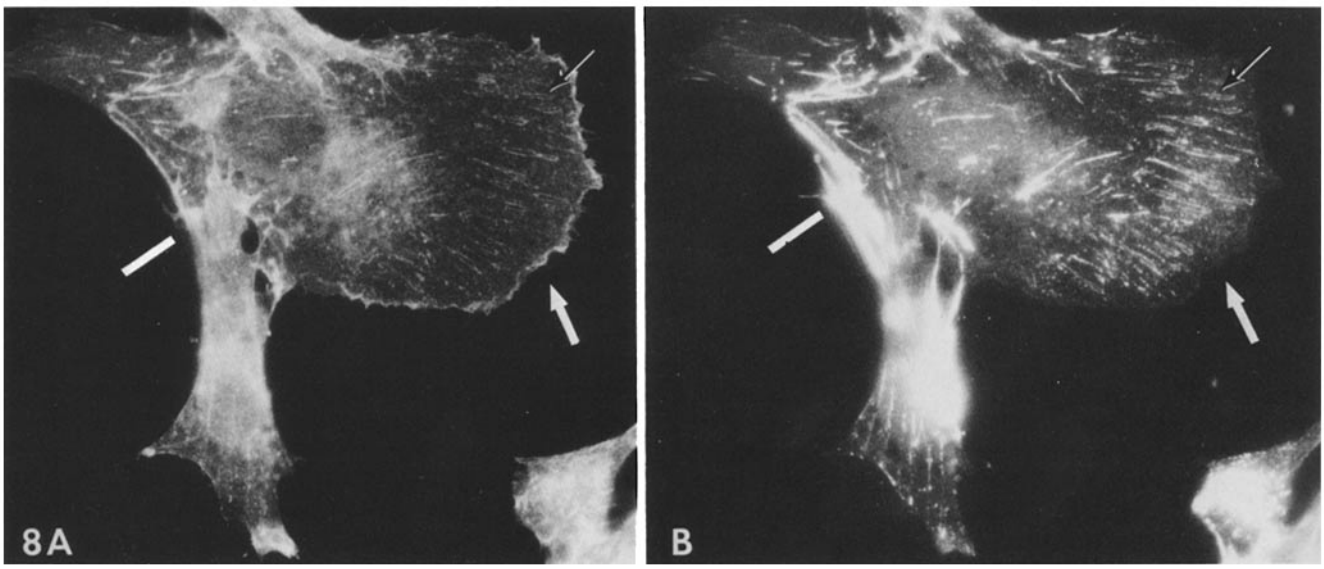


FIGURE 8 Cardiac fibroblast fixed and permeabilized as described for Fig. 7 and stained with (*A*) anti-GP-3 and (*B*) anti-Fn Mab. Fn co-localizes with CSAT ag in the needle eye pattern (thin arrows; see Figs. 2–5) but is absent from the edges of leading lamella (white arrows). On the other hand, CSAT ag staining is not co-extensive with Fn-rich fibers as they extend from the cell surface (white bar). × 800.

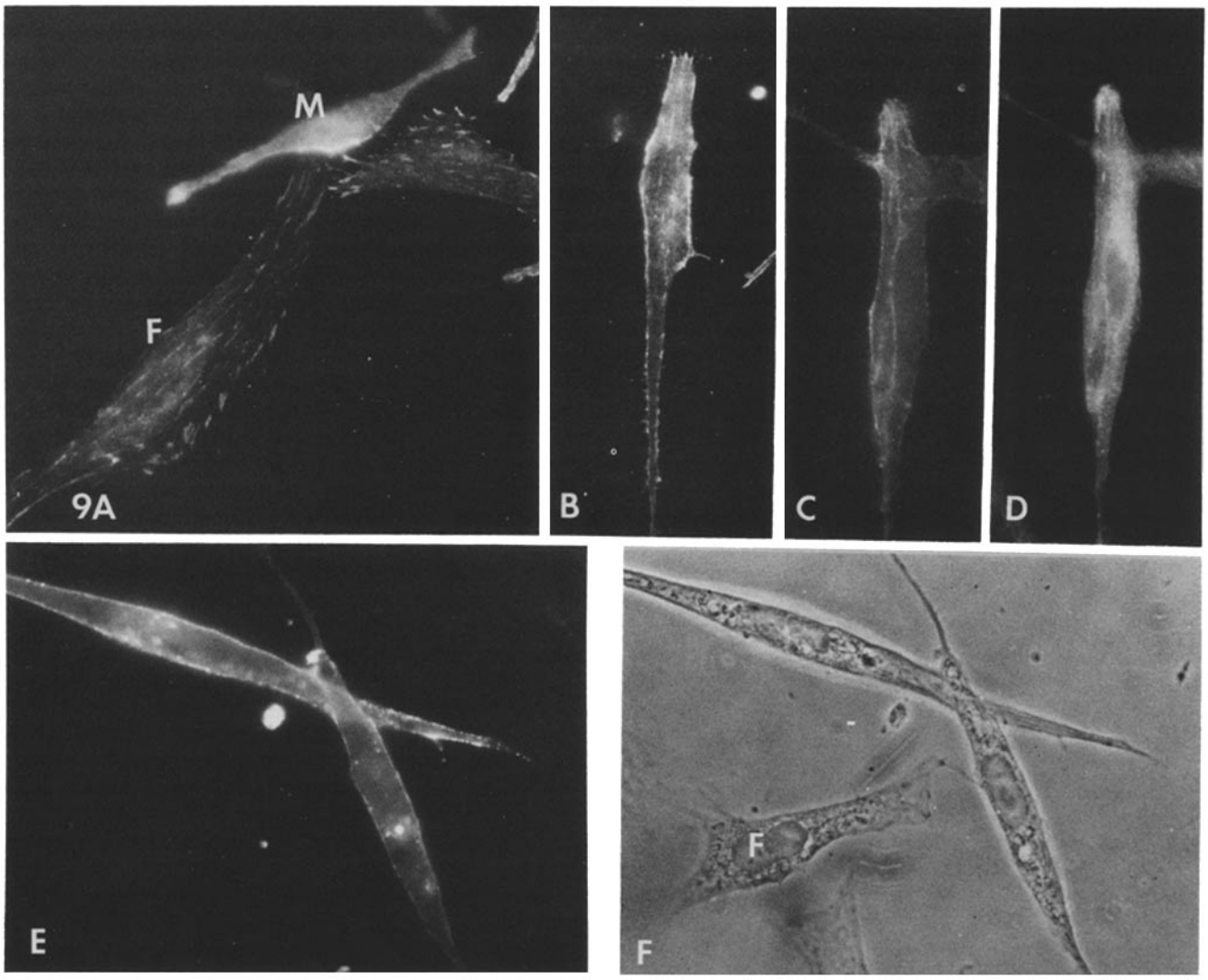


FIGURE 9 24–48-h myoblast cultures. (*A* and *B*) Cells permeabilized with methanol at −20°C and stained with CSAT Mab. CSAT ag distribution is diffuse on the surface of the myoblast. Fibroblasts in the culture stain in a highly organized pattern. (C and *D*) Colocalization of CSAT ag and Vn in myoblasts prepared as in *A*. Myoblasts have aligned preparatory to fusion. CSAT ag (*B*) has a uniform surface membrane distribution. Vn (*D*) distribution is diffuse and largely cytoplasmic. (*E* and *F*) Distribution of Lm on myoblasts. (*E*) LM stains the surface of myoblasts but not the fibroblast visible in the accompanying phase-contrast micrograph (*F*). × 800.

DISCUSSION

The present localization studies were undertaken to determine how CSAT ag was distributed on the cell surface of myoblasts and fibroblasts with respect to cytoskeletal and ECM components considered to be involved in cell-matrix adhesion and to determine whether the susceptibility of a particular cell type to detachment by CSAT Mab could be related to the distribution pattern of CSAT ag on that cell. The relationship of Vn and Fn to one another, and to morphologically distinguishable cell-matrix contact sites in fibroblasts, has been particularly well studied by many laboratories. These studies have shown that Vn is an excellent marker for the termini of microfilament bundles and co-distributes with focal contact sites (6, 10, 15). The relationship of Fn to Vn-rich sites has been somewhat more controversial. Fn has been shown to co-localize with Vn-rich sites in some situations (29, 30), but in other cases, it has been shown to be enriched adjacent to the Vn-rich subset of contact sites (1, 9, 10). In particular, the studies of Chen and Singer (10), using frozen thin section immunoelectron microscopy, which minimizes problems of antibody accessibility, have indicated that Fn resides in a different subset of contact sites than Vn under their culture conditions.

In our studies of fibroblasts, CSAT ag was consistently found to be enriched at stress fiber termini in the vicinity of Vn-rich sites (Figs. 2–5). When co-localized with Vn or Fn, CSAT ag, along with Fn, was found to be enriched at the periphery but generally not directly beneath these Vn-rich sites (Figs. 3–5, 8). This is in contrast to the attachment inhibiting Mab, FC-1 (25), whose antigen does co-localize with Vn in focal contacts. Control experiments using concanavalin A suggest that the absence of CSAT ag is not due to inaccessibility of reagents beneath focal contact areas. Taken together, our studies show quite clearly that in fibroblasts CSAT ag is localized in positions consistent with its playing an important role in mediating cell-matrix adhesion. Its striking relationship to fibronectin in these cells (Fig. 8) suggests that it participates directly in the particular class of adhesion sites involved with cell attachment to Fn. This is further

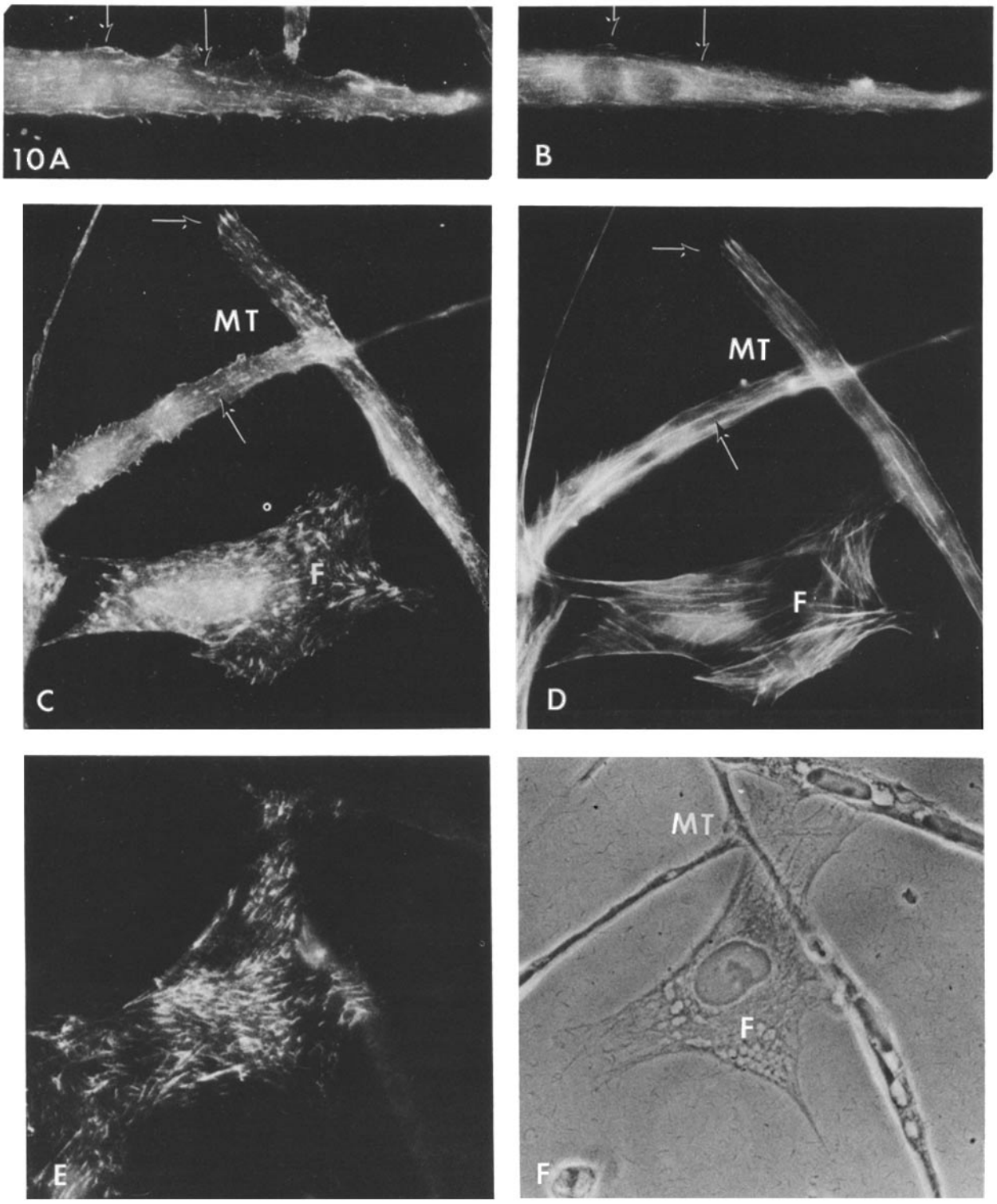


FIGURE 10 48–72-h myotube cultures. (*A* and *B*) Co-localization of CSAT Mab and anti-Vn on methanol permeabilized 48–72 h myotube. CSAT ag and Vn codistribute along surface streaks which probably represent bundles of F-actin (see C and *D*). There is considerable diffuse cytoplasmic anti-Vn staining as well. (*C* and *D*) Co-localization of anti-GP-3 and FITC phalloidin on two 72-h myotubes and a fibroblast, prepared as described for Figs. 7 and 8. CSAT ag staining has become much more discrete on myotubes, resembling more closely the staining pattern of fibroblasts (compare Fig. 9). CSAT ag stains portions of F-actin bundles (arrows) and their termini and is enriched in lateral projections which may anchor myotube to the substratum. (*E* and *F*) Localization of Fn Mab on 48–72-h myotubes and fibroblast. Fn stains the fibroblast strongly, extending out beyond the boundaries of the phase image of the cell. Fn staining is difficult to detect on myotubes. × 800.

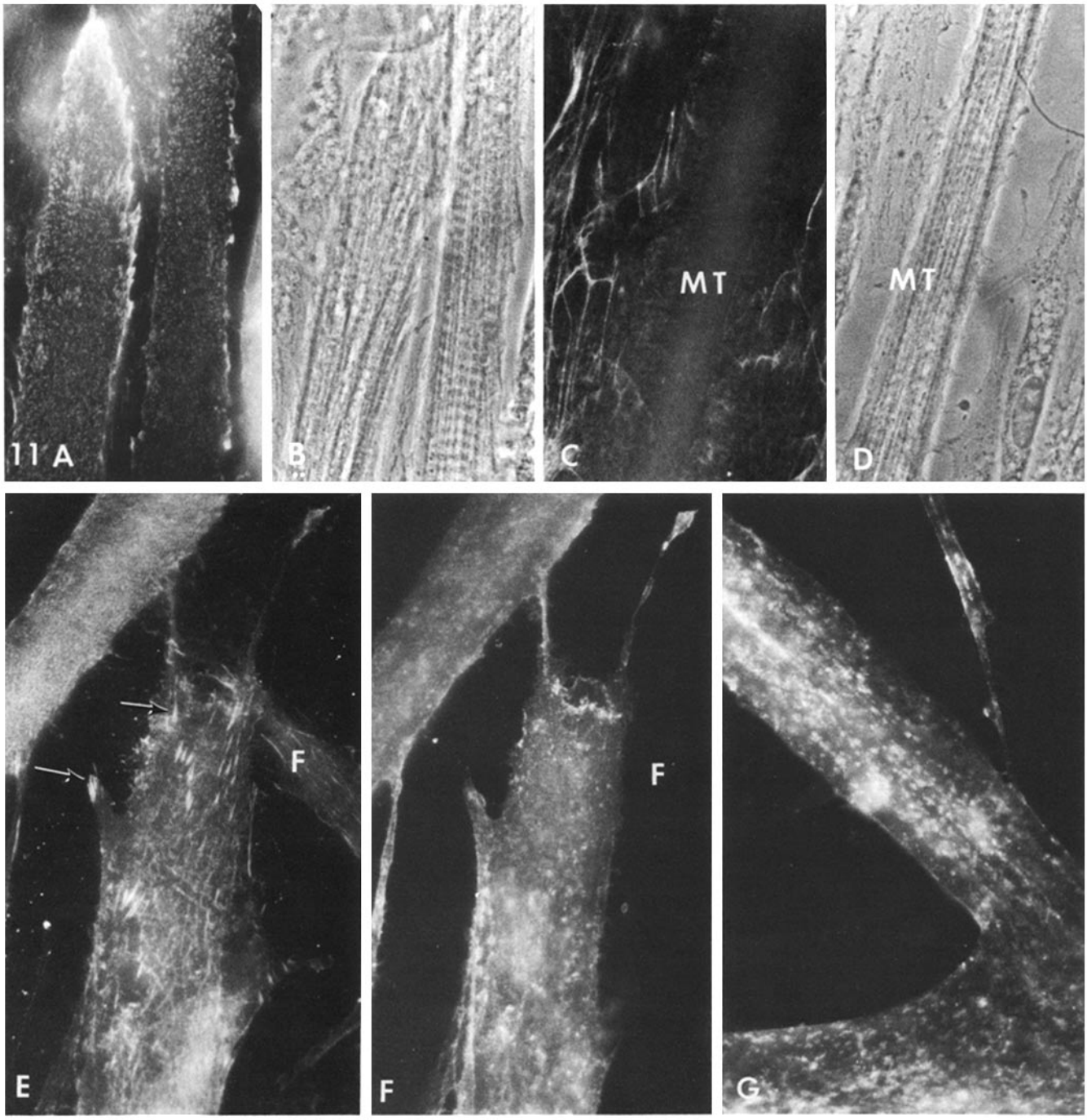


FIGURE 11 6-d myotubes fixed with paraformaldehyde and permeabilized with acetone before staining with (*A*) anti-GP-3; (*B*) phase-contrast micrograph of same field; (*C*) Fn Mab; (*D*) phase-contrast micrograph of same field; (*E*) anti-GP-3; (*F*) Lm Mab of same field; (*G*) Lm Mab. × 800.

supported by the observation that CSAT Mab inhibits the attachment of tendon fibroblasts to Fn (14).

Studies using developing myoblasts reveal several interesting differences between these cells and the various fibroblasts examined. In contrast to fibroblasts, prefusion myoblasts, which are the most susceptible of all the cell types tested to detachment by CSAT Mab, have a diffuse distribution of both CSAT and Vn. In addition, myoblasts, unlike fibroblasts, stain prominently with Lm. At 72 h, Fn is not readily detectable on myotubes, and the distribution patterns of both CSAT and Vn are more highly organized. This increased organization of CSAT ag correlates with a significant change in the kinetics of the response of myotubes to CSAT Mab. By 6 d, CSAT ag has an elaborate organizational pattern with particular enrichment in lateral lamellae, myotubes termini, and in streaks along the surface of the myotube. Vn accompanies CSAT in the lateral lamellae and surface streaks. Thus, in muscle cells throughout their development, as well as in fibroblasts, CSAT ag and Vn have considerable similarity in their distribution patterns.

Lm and CSAT ag are both abundant on the myotube surface and there is considerable overlap in their staining

patterns. However, distribution is not coextensive. In particular, Lm is not enriched along lateral lamellae. Fn is difficult to detect at all on the developing myotubes. In 6-d myotube cultures, Fn appears confined to fibroblast-rich regions between myotubes. The paucity of Fn on the surface of myotubes, and of Lm on fibroblasts, suggests differential cell surface expression of these molecules by the different cell types. This, however, does not bear on the ability of either cell type to interact with these ligands in ECM. Both cell types are able to attach and spread on either Fn or Lm in culture suggesting that receptors for both are present.

The studies reported here have encompassed cells with three distinctive responses to CSAT Mab. Fibroblasts from three different embryonic tissues, plated in serum either on uncoated glass coverslips or on gelatin-coated coverslips, will not round and detach when exposed to CSAT ag. In contrast, 48–72-h myoblasts plated in the presence of serum are readily detached by CSAT Mab. Larger myotubes require a much longer time to respond to CSAT Mab but will eventually detach. The increased resistance to detachment by CSAT Mab of myoblasts as they develop correlates with the development of a much more discrete localization pattern of CSAT ag, suggesting that in myogenic cells, changes in the organizational pattern of cell surface adhesion molecules can affect the stability of adhesive contacts. This is probably not true for all cell types, however. Recent experiments (14; Horwitz, A. F., R. Greggs, and C. Decker, manuscript in preparation) have shown that fibroblasts isolated from various embryonic tissue sources and plated on different defined substrata in the absence of serum vary in their responses to CSAT Mab. Preliminary localization studies (Damsky and Horwitz, unpublished experiments) have shown that in some cases cells susceptible to CSAT Mab display a highly organized distribution of the CSAT ag in association with well-defined adhesive structures. Thus, a major change in the organizational pattern of CSAT ag is not necessarily required for all cell types to exhibit an increased sensitivity to morphologic perturbation by CSAT Mab. This suggests that an increased organization of CSAT ag complexes on the cell surface may make some contribution to adhesive stability, but is not likely by itself to account for all the differential effects of CSAT Mab. These observations add further support to the proposal of Decker et al. (14) that these differential effects are due to a progressive display of different kinds of adhesion-related molecules and their organizational complexes on increasingly adhesive cells. According to this proposal, the CSAT ag, which is present on all cells examined, makes some contribution to the adhesion strategy of each cell type. However, the extent to which cells can employ additional adhesion mechanisms will determine the susceptibility of that cell type to CSAT Mab. The existence of additional mechanisms will depend both on the properties of the specific cell type and the molecules that are available in the environment at any particular time. Thus, the CSAT ag participates in the dominant adhesion mechanism in myoblasts under all culture conditions. In fibroblasts plated in serum-containing medium, its contribution is overriden by the additional mechanisms that these cells are able to express. This differential display and organization of adhesive molecules by different cell types should be of particular significance during development, permitting a graded or selective response by different cell types present, to migratory or other environmental stimuli to which all are exposed.

The CSAT ag is a complex of three integral membrane glycoproteins migrating in the 140,000-mol-wt region of SDS-polyacrylamide gels. The ability of antibodies specific for this complex to perturb cellular adhesion plus its co-localization in fibroblasts with fibronectin in known adhesive structures suggest that it plays a direct role in the adhesion process. A second possible, but less direct role for CSAT ag is that it stabilizes some kinds of ligand-receptor interactions once they have occurred and contributes to the organization of functional, transmembrane adhesive complexes. Thus, it might be able to serve a subset of the total repertoire of possible ligand-receptor interactions that a particular cell type is able to form. This role would still require the antigen to be enriched at sites of cell-matrix interaction. Experimental approaches taken thus far have not been able to distinguish convincingly between these two possibilities.

Other laboratories have identified similar 140,000-mol-wt region glycoproteins as being involved in cell-matrix adhesion. In fact, the antigen recognized by the monoclonal antibody JG22 (17) is identical to CSAT-antigen in its biochemical properties (7, 8) and its cellular distribution (8) as well as its immunologic properties (19). Similar 140,000-mol-wt glycoproteins have been purified from mammalian cells utilizing their ability to inhibit adhesion disrupting antisera (13, 22). Using selective proteolysis in the presence and absence of reducing agents, Giancotti et al. (16) have implicated a 135,000-mol-wt glycoprotein in the adhesion of murine sarcoma cells to a fibronectin-coated matrix. When subjected to SDS PAGE, this band behaves in a manner similar to band 3 of the CSAT ag complex in that its mobility on the gel decreases upon exposure to reducing agents. Corresponding proteolysis studies on chick fibroblasts also suggest that the CSAT band 3 glycoprotein is required for adhesion to fibronectin (Damsky, Gruber, and Buck, unpublished observations).

Pytela et al. (27) have reported that 140,000-mol-wt glycoprotein(s) can bind to immobilized Fn and are eluted with the cell binding tetrapeptide of Fn (26). Finally, Brown and Juliano (2) have reported a monoclonal antibody, which prevents adhesion of Chinese hamster ovary cells to fibronectin but not to other ligands and whose antigen migrates in the 140,000-mol-wt region of reduced SDS polyacrylamide gels. Thus, although direct comparisons of all these molecules are not available, several independent laboratories have now identified 140,000-mol-wt CSAT-like cell surface antigens. By virtue of their localization, their interaction with adhesion perturbing antibodies and their binding to extracellular matrix molecules, these glycoproteins become highly likely candidates for a class of cell surface molecules directly involved in the adhesion of cells to the extracellular matrix, and in this case, to fibronectin.

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