Integrin (The CSAT Antigen): Functionality Requires Oligomeric Integrity

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Abstract. Integrin, the cell–substrate attachment (CSAT) antigen, is a complex of integral membrane glycoproteins whose apparent function is to mediate cell–substratum adhesion by serving as a transmembrane link between the extracellular matrix and elements of the cytoskeleton. Previous attempts to separate the members of this complex under nondenaturing conditions have been successful. We have now produced a monoclonal antibody "G" that is specific for the lower molecular mass cysteine-rich band 3 of the complex. Using an antibody affinity column containing this monoclonal antibody, it is possible to dissociate integrin into two fractions, one containing band 3, the other containing bands 1 plus 2. Neither fraction will by itself bind fibronectin, laminin, or talin. However, when the fractions are combined, the reconstituted integrin elutes from a gel filtration column in the same position as the native complex, and binding activity to these molecules returns. Further, it is shown by gel filtration that the recognition site for the adhesion-disrupting monoclonal antibodies CSAT and JG22 is on band 3, supporting the contention that integrin is an oligomer. The data presented here is consistent with integrin being either a mixture of heterodimers, each with a common subunit and reacting with a particular extracellular matrix molecule, or a single heterotrimer capable of binding to several different extracellular matrix molecules.

THE adhesion of cells to an extracellular matrix is the end result of a complex series of interactions involving specific elements of the extracellular matrix, receptors on the cell surface, and constituents of the cytoskeleton. A group of cell surface glycoproteins originally designated the cell–substrate attachment (CSAT)[1] complex, but more recently named integrin (26), has been implicated as the transmembrane linker mediating these molecular interactions during cell–substrate adhesion (13). Integrin was isolated using a monoclonal antibody that inhibits cellular adhesion to either fibronectin or laminin (14, 18). Integrin as isolated from chick cells consists of at least three glycoproteins with an average molecular mass of 140 kD (15). It interacts directly with fibronectin and laminin. This interaction can be inhibited with either a monoclonal antibody against integrin or the cell-binding tetrapeptide from fibronectin (14). Integrin also binds to talin, a molecule thought to be important in linking elements of the cytoskeleton with the cell surface. This talin–integrin interaction involves a site on integrin different from that which binds fibronectin and laminin (13). A complex with identical properties has been independently isolated from chick cells using the monoclonal antibody JG22 (1, 9, 11).

The glycoproteins of which integrin is composed differ from one another in size, peptide composition, and serological reactivity (15, 11). The gene for band 3 of integrin has been cloned and sequenced (26). The data show it to consist of 803 amino acids arranged in such a way as to have a large external NH2-terminal domain with cysteine-rich regions, a single transmembrane segment, and a short COOH-terminal cytoplasmic domain. This component and band 2 of integrin are both phosphorylated after viral transformation (12). It has not been possible to separate integrin glycoproteins except under the denaturing conditions of nonreduced SDS PAGE. This has led to the suggestion that these glycoproteins reside and function as an integral membrane complex (3). To test this hypothesis, we have produced a monoclonal antibody that allows the fractionation of integrin. Using this monoclonal antibody, we have been able to show that integrin exists as an oligomer and to evaluate the requirement of its integrity for both antibody and ligand binding. The results of these experiments show that the oligomeric nature of integrin is required for binding to fibronectin, laminin, and talin, and that the epitope recognized by the adhesion-disrupting monoclonal antibody CSAT is on the cysteine-rich band 3 glycoprotein of the complex.

Materials and Methods

Chick Fibroblast Cultures

Fibroblasts were prepared and cultured as previously described (6). Cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum.

Purification of Integrin

[35S]methionine labeling of cell cultures was carried out in methionine-free Eagle's minimum essential medium as previously described (13). The inte-

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1. *Abbreviation used in this paper:* CSAT, cell substrate attachment.

grin was purified from nonionic detergent extracts of [³⁵S]methionine-labeled cells or decapitated and eviscerated 10-d-old chick embryos as previously described (15). Briefly, embryos were extracted in 10 mM Tris-acetate buffer, pH 8.0, containing 0.5 mM Ca^{++}, 2 mM phenylmethylsulfonyl fluoride, and 0.5% NP-40 (TNC). After centrifugation at 50,000 g for 30 min, the supernate was precipitated with acetic acid (16), neutralized, and applied to an antibody affinity column containing 10 mg of CSAT monoclonal antibody per millileter of packed volume (18). The antigen was eluted with 50 mM diethylamine, pH 11.5, immediately neutralized, dialyzed extensively against TNC, and stored at −70°C until used.

Monoclonal Antibody Production

Monoclonal antibodies were produced as described previously (18). Intraperitoneal injections of 50 µg/ml of purified integrin mixed with equal volumes of incomplete Freund's adjuvant were administered to BALB/c mice three times at 10-d intervals. 5 d after the final injection, spleens were harvested and cells fused with plasmacytoma cell line SP 2/0-Ag 14. Supernates from the resulting hybridomas were screened for antibody production by an ELISA assay performed in microtiter wells coated with purified integrin. Hybridomas selected in this manner were cloned by limiting dilution, the clones rescreened by the ELISA assay, and positive clones recloned. After the final ELISA assay, positive clones were assayed for their ability to immunoblot purified integrin, and selected hybridomas were characterized further as described in Results. The following monoclonal antibodies were used in the experiments presented here: CSAT, characterized as described previously (18); G, specific for band 3 of integrin; and B, specific for band 1 of integrin. B and G monoclonal antibodies are of the class IgG₁.

Immunoprecipitation

200 µl of [³⁵S]methionine-labeled integrin in TNC buffer containing 0.15 M NaCl (TNC-NaCl) and 0.2% BSA were preabsorbed first with 25 µl packed volume of protein A Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) prepared according to manufacturer's instructions and then with 25 µl packed volume of protein A Sepharose saturated with affinity-purified rabbit anti–mouse heavy and light chain IgG (Cappel Laboratories, Div. Cooper Biomedical, Inc., Malvern, PA). The preabsorbed antigen was then mixed with 20 µg purified monoclonal antibody and allowed to stand for 60 min at 4°C. 50 µg of rabbit anti–mouse IgG was then added and the mixture incubated another 60 min at 4°C, after which time it was added to a 50-µl packed pellet of protein A Sepharose and mixed constantly at 4°C for another 60 min. The immune complex absorbed to the protein A Sepharose was centrifuged and washed five times in TNC-NaCl, finally resuspended in sample buffer, and analyzed by SDS PAGE under nonreducing conditions (14).

Antibody Affinity Chromatography

Purified immunoglobulin was bound to cyanogen bromide–activated Sepharose (Pharmacia Fine Chemicals) at a concentration of 10 mg/ml packed volume of hydrated cyanogen bromide Sepharose, according to the manufacturer's instructions. Ethanolamine was used to block all activated sites not saturated with immunoglobulin. Water-jacketed columns were packed with 1-3 ml bed volume of solid-phase material. Material was applied to each column in TNC-NaCl at a flow rate of 0.3-0.5 ml/min. The column was washed with at least 50 column volumes of TNC-NaCl, and eluted with 50 mM diethylamine, pH 11.5, containing 0.5% NP-40 and 0.15 M NaCl. Samples were immediately brought to pH 7.5 with 0.1 M HCl and dialyzed against TNC-NaCl.

Gel Electrophoresis

Samples were analyzed by SDS PAGE using 7% acrylamide gels by the method of Laemmli (17) without the addition of reducing agents. Materials used for immunoblots were transferred to nitrocellulose paper as previously described (15). Alternatively, proteins were detected by silver staining (19).

Immunoblotting

Nitrocellulose paper containing the samples was cut into strips and incubated with 4% BSA in PBS for 60 min and then exposed to the appropriate monoclonal antibody for 60 min at room temperature. The strips were then extensively washed with PBS containing BSA and then exposed to affinity-purified rabbit anti–mouse heavy and light chain IgG. After another 60 min, the samples were washed once again and exposed to ¹²⁵I-protein A (ICN

Radiochemicals, Irvine, CA). The strips were subsequently washed and autoradiographed using an enhancing screen and Kodak X-Omat AR film.

Equilibrium Gel Filtration Assay for Macromolecular Associations

This assay was carried out as previously described (14) using a 0.2 × 30-cm column of Ultrogel AcA22 (LKB Instruments, Inc., Gaithersburg, MD). The void volume of this column was 0.54 ml and the included volume was 1.5 ml. The flow characteristics and calibration of this column have been previously described (14, 18). When interactions between integrin or any of its derivatives with other macromolecules were assayed, the column was saturated with one column volume of the desired macromolecule at the appropriate concentration in TNC-NaCl before addition of the test mixture. 60-µl fractions were collected and radioactivity counted in a scintillation counter (Intertechnique, Plaisir, France). The reproducibility of this column has been previously documented (13, 14).
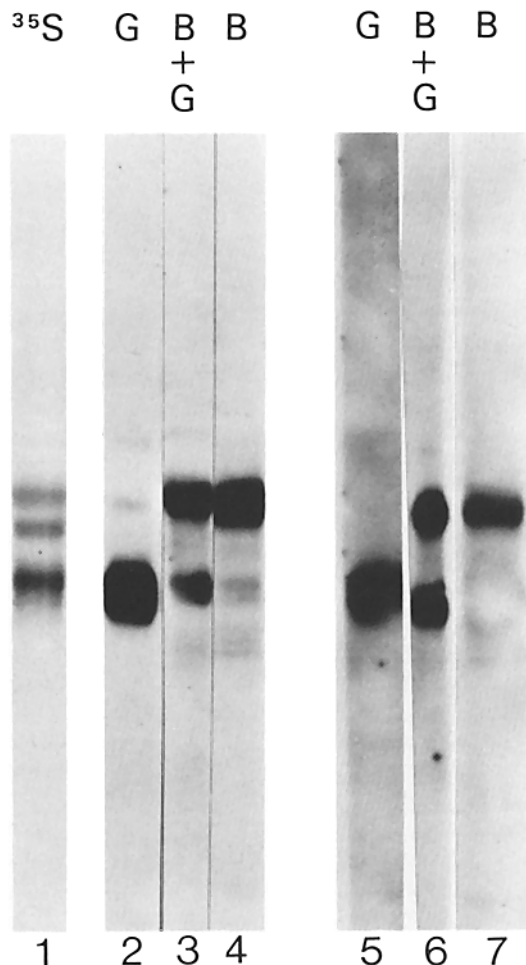

Figure 1. Specificity of monoclonal antibodies for individual integrin glycoproteins. Purified integrin, first four lanes, or an NP-40 extract of chick embryos, last three lanes, were subjected to SDS PAGE under nonreducing conditions. Material was transferred to nitrocellulose paper, and strips were cut and exposed to the appropriate monoclonal antibody. Monoclonal antibody binding was detected using rabbit anti–mouse IgG and ¹²⁵I-protein A as described in Materials and Methods. [³⁵S]Methionine-labeled integrin (lane 1); immunoblot of integrin using G monoclonal antibody (lane 2); mixture of G and B monoclonal antibody (lane 3); and B monoclonal antibody (lane 4); immunoblot of chick embryo NP-40 extract using G monoclonal antibody (lane 5); mixture of B and G monoclonal antibody (lane 6); and B monoclonal antibody (lane 7).

Results

Characterization of Monoclonal Antibody Specificity

Hybridomas were selected as described in Materials and Methods after injection of mice with purified, nondenatured integrin. After screening for integrin specificity, ascites fluid was prepared with selected hybridomas. Antibodies isolated from the ascites fluid were used in Western blots to test for reactivity with individual glycoproteins. The results of such a screen using two monoclonal antibodies designated B and G are shown in Fig. 1. Either purified integrin (first four lanes) or an NP-40 extract of chick cells (last three lanes) was subjected to SDS PAGE under nonreducing conditions and transferred to nitrocellulose. Individual lanes were then reacted with the appropriate monoclonal antibody. ^{35}S-la-
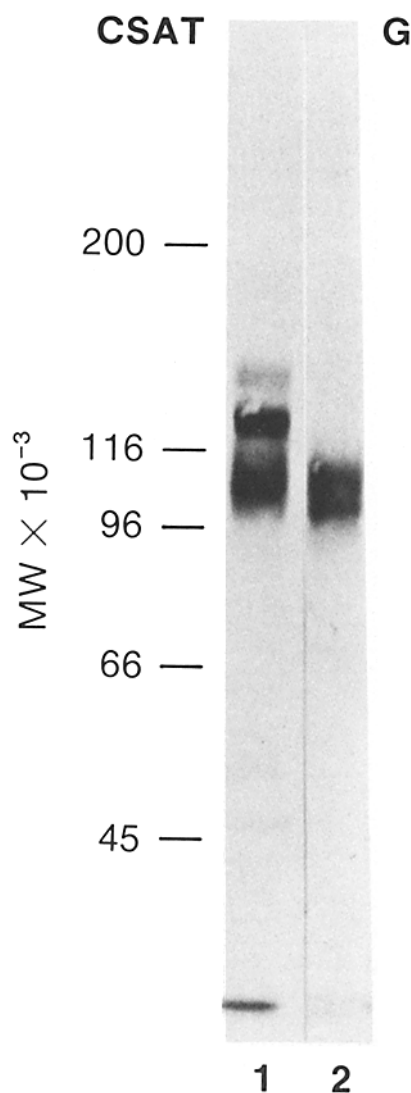

Figure 2. Selective immunoprecipitation of [35S]methionine-labeled integrin with G monoclonal antibody. Radioactive integrin was mixed with CSAT monoclonal antibody (lane *1*) or G monoclonal antibody (lane 2). Rabbit anti–mouse heavy and light chain was added to each mixture, and the complex absorbed to protein A Sepharose. Absorbed complex was eluted with sample buffer and separated on nonreduced SDS PAGE.

beled antigen was electrophoresed in an adjacent lane for use as a position marker. It is clear that the G monoclonal antibody reacts predominantly with the lower molecular mass member of the complex designated band 3. There is some trace reactivity in the vicinity of the highest molecular mass band 1 region of the blot and also in a still higher molecular mass region. These probably represent aggregates, as they do not appear in blots of whole NP-40 extracts of cells (last three lanes). A second monoclonal antibody designated B reacts primarily with the higher molecular weight band of integrin. However, again, there is trace reactivity with lower molecular mass material. This is insignificant when compared with that of band 1 and may well represent proteolytic fragments. Immunoblots of an unfractionated NP-40 extract from chicken embryos (last three lanes) further demonstrate the specificity of these two monoclonal antibodies. Here, each antibody reacts only with material migrating in the appropriate molecular mass region of the gel. Thus, G monoclonal reacts only with material in the band 3 region of the gel and B monoclonal only with material from the band 1 region of the gel. This was further confirmed when both B and G antibodies were included in the blotting procedure.

To examine the specificity of the G monoclonal antibody under nondenaturing conditions, NP-40 extracts from chick fibroblasts labeled with [35S]methionine were immunoprecipitated with both CSAT monoclonal antibody and G monoclonal antibody. The results are shown in Fig. 2. Lane *1* contains material immunoprecipitated with the CSAT monoclonal antibody. Lane 2 contains material immunoprecipitated with the G monoclonal antibody. It immunoprecipitates only material migrating in the region of band 3. Identical results were obtained if the G monoclonal antibody was used to immunoprecipitate purified integrin.

Fractionation of Integrin Using a Monoclonal Antibody

The immunoprecipitation results indicated that the G monoclonal antibody might be used to separate integrin into two component parts, one enriched in band 3 glycoprotein and the other containing a mixture of bands 1 and 2. To do this, an immunoaffinity column was constructed using the G monoclonal antibody. The results of passing purified integrin over such a column are shown in Fig. 3. Silver staining of the CSAT complex before passage over the G affinity column is shown in lane *1*. Lane 2 shows a silver stain of the material eluted from the G affinity column. It is clearly enriched in band 3 glycoprotein. Lane *3* shows the material that did not bind to the affinity column. It contains primarily band 1 material with traces of band 2 material. Because of the dilution of material during the process, band 2 is not clearly visible on the gel. However, when ^{35}S-labeled integrin is fractionated in an identical manner, both band 1 and 2 material are readily visible (see Fig. 4). Variations in the relative amount of material in each band are due to differences in specific activity and sensitivity to staining procedures. Material used in these studies has been submitted to silver staining and autoradiography after nonreduced SDS PAGE to monitor the possible contamination by other proteins and the amount of proteolytic degradation of integrin that may occur during isolation. The latter may be responsible for the occasional impression of more than one component in the band 3 region
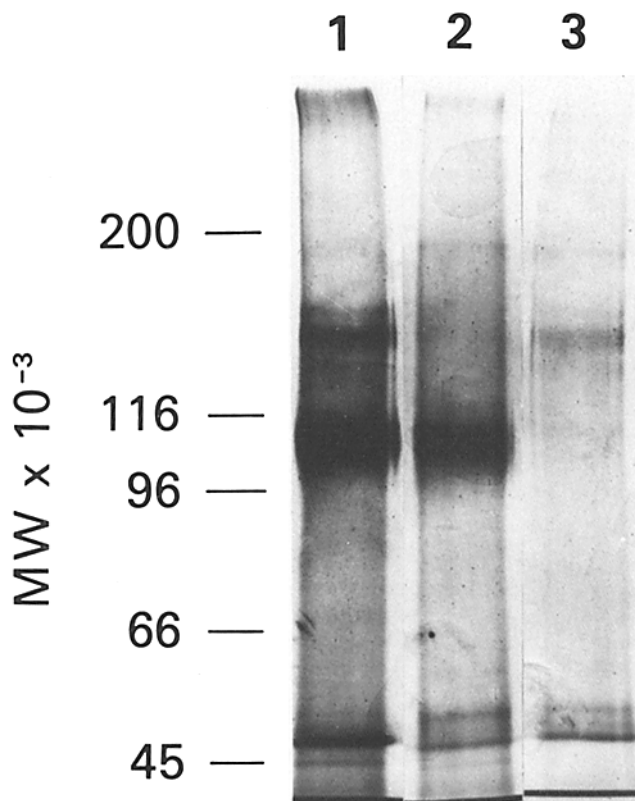
Figure 3. Fractionation of integrin on a G monoclonal antibody affinity column. Integrin purified from chick embryos was passed over a 1-ml column containing G monoclonal antibody conjugated to cyanogen bromide Sepharose at a concentration of 10 mg/ml packed volume of Sepharose. Absorbed material was eluted with 50 mM diethylamine, pH 11.5, and immediately neutralized as detailed in Materials and Methods. Silver-stained nonreduced SDS PAGE of integrin applied to column (lane *1*), material eluted from column (lane *2*), and material not bound to column (lane *3*).

of a gel. The material seen near the bottom of each lane is a contaminant in the tracking dye. These results demonstrate that the G monoclonal antibody can be used to produce two fractions, one enriched in band 3 glycoprotein, and the other containing primarily band 1 and 2 glycoproteins.

The elution profiles of [³⁵S]methionine-labeled band 1 plus 2 and band 3 fractions from an AcA22 gel filtration column are shown in Fig. 4. The reproducibility of this column has been previously documented (13, 14). The elution profile for band 1 plus 2 is rather broad, with a peak just behind that of integrin (V_Ag). This more than likely reflects the heterogeneity of the sample in that it contains primarily band 1 and 2 glycoproteins, with a small amount of contaminating band 3. This is shown in the insert, the first lane of which shows an SDS PAGE separation of pooled fractions 14–20. The band 3 glycoprotein elutes from this same column in a position behind that of the band 1 plus 2 mixture, producing a sharper elution profile. SDS PAGE separation of material eluting in the band 3 region shows this to contain primarily band 3 glycoprotein material (second lane in insert).

The Binding Site for CSAT and JG22 Monoclonal Antibodies Is on Band 3

The CSAT monoclonal antibody and an independently iso-

lated monoclonal antibody, JG22, have been used to identify and isolate integrin (9, 18). The antigenic site recognized by these antibodies is sensitive to denaturation; therefore, it has not been possible to determine which band of the integrin complex contains the antibody-binding epitope, or if it consists of a configuration requiring all three members of the complex. To test these possibilities, CSAT monoclonal antibody was mixed with [³⁵S]methionine-labeled band 1 plus 2, or band 3 (see Fig. 4) before addition to the AcA22 gel filtration column. The results are shown in Fig. 5. In Fig. 5 *A*, a slight shift, though within experimental error, of the ³⁵S-labeled band 1 plus 2 elution profile is seen in the presence of CSAT monoclonal antibody. This is probably due to the small amount of contaminating band 3 in the preparation (see insert, Fig. 4) as Fig. 5 *B* shows a major change in the elution profile of the band 3 material in the presence of CSAT monoclonal antibody. The same results were obtained with the JG22 monoclonal antibody. Denaturing the band 3 glycoprotein by boiling or by reduction with dithiothreitol before the addition of CSAT or JG22 monoclonal antibodies abolished all recognition of the glycoprotein by either antibody. It appears that band 3 contains the major epitope recognized by the CSAT or the JG22 monoclonal antibodies. This epitope would appear to be sufficiently far removed from the sites involved in the association of band 3 with bands 1 and 2 in the complex, so as not to effect the integrity of the complex in a manner similar to that of the G monoclonal antibody.
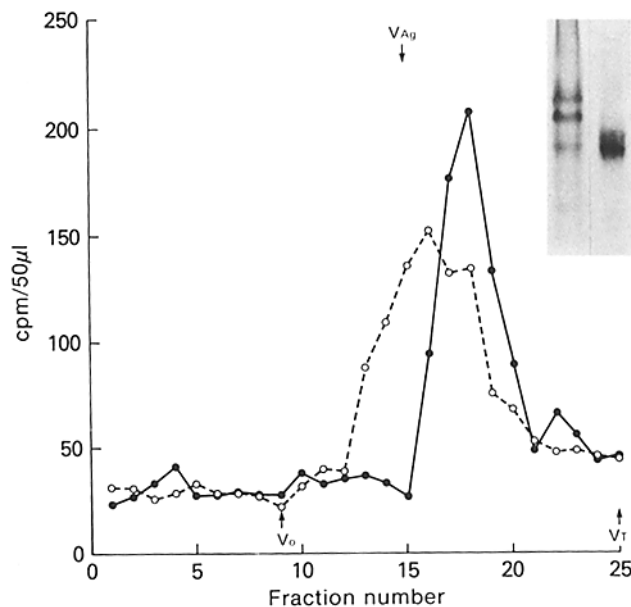

Figure 4. AcA22 gel filtration pattern of band 1 plus 2, and band 3 material from a G monoclonal antibody affinity column. [³⁵S]methionine-labeled integrin was fractionated on a G monoclonal antibody affinity column to yield fractions containing predominantly band 1 plus 2, or band 3. The samples were applied to a 0.2 × 30-cm AcA22 column and 60-μl fractions collected. Band 1 plus 2, (*open circles, broken line*); band 3, (*solid circles, solid line*); V_Ag, peak elution position for intact integrin. *Insert,* nonreduced SDS PAGE of material from bands 1 plus 2, fractions 14–20 (first lane), and band 3, fractions 16–20 (second lane).

Requirement of Integrin Oligomeric Integrity for Binding to Extracellular Matrix and Cytoskeletal Constituents

To determine whether integrin must remain intact for it to bind to elements of the extracellular matrix, band 1 plus 2, band 3, or reconstituted integrin were mixed with laminin and applied to a gel filtration column pre-equilibrated with laminin at 400 µg/ml. With either band 1 plus 2 or band 3, there was no change in the elution profile (Fig. 6, A and B). However, when the complex was reconstituted by mixing band 1 plus 2 and band 3 in the presence of the ligand just before addition to the gel filtration column, a peak of radio-activity appeared in the void volume (Fig. 6 C), demonstrating that reconstituted integrin binds laminin. The formation of this complex was inhibited by both the CSAT monoclonal antibody and by the RGDS cell-binding tetrapeptide from fibronectin, as shown previously (14).

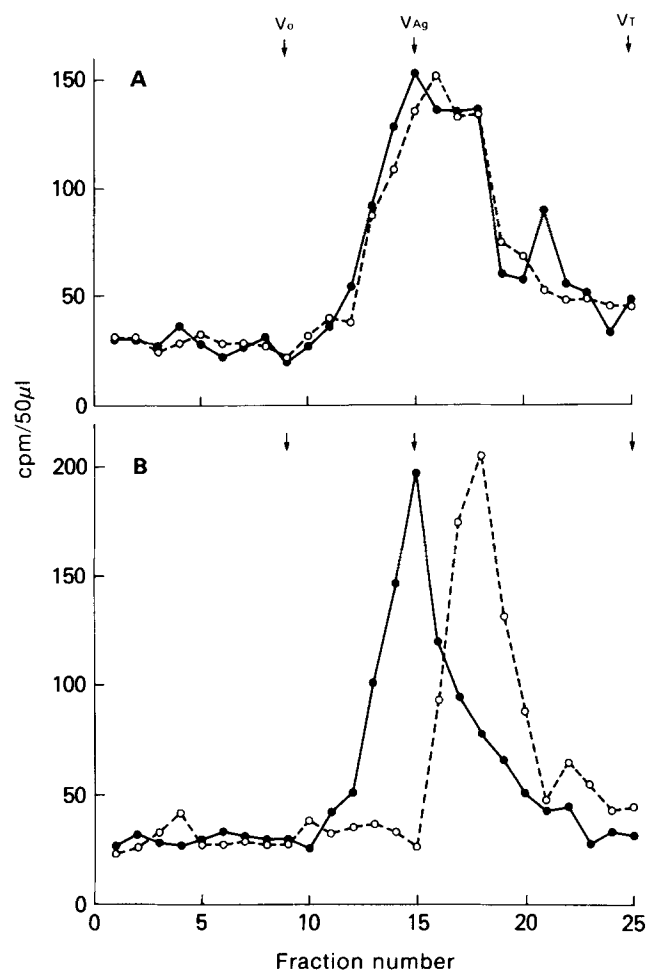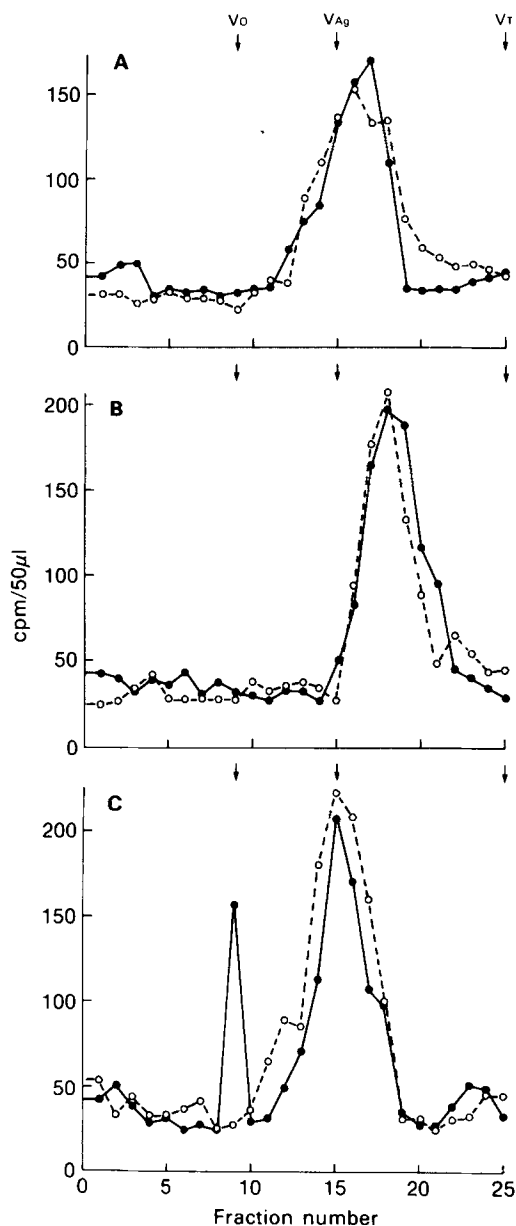The elution profile of reconstituted integrin differed from

Figure 6. Laminin binding to reconstituted integrin. [35S]methio-nine-labeled band 1 plus 2, band 3, or reconstituted integrin were mixed with 400 µg/ml laminin and applied to a 0.2 × 30-cm AcA22 column pre-equilibrated with the same concentration of laminin; 60-µl fractions were collected. (A) Bands 1 plus 2 alone (open circles, broken line); bands 1 plus 2 plus laminin (solid circles, solid line); (B) band 3 alone (open circles, broken line); band 3 plus laminin (solid circles, solid line); (C) reconstituted integrin plus laminin (solid circles, solid line); Vo, void volume, VAg, elution position of integrin, VT, total inclusion volume of the column.

Figure 5. The binding of CSAT monoclonal antibody to [35S]me-thionine-labeled band 3 glycoprotein. Band 1 plus 2, or band 3 glycoprotein preparations shown in Fig. 4 were mixed with 20 µg/ml CSAT monoclonal antibody and applied to a 0.2 × 30-cm AcA22 column; 60-µl fractions were collected. (A) Bands 1 plus 2 alone (open circles, broken line), and bands 1 plus 2 plus CSAT monoclonal antibody (solid circles, solid line); (B) band 3 alone (open circles, broken line), band 3 plus CSAT monoclonal antibody (solid circles, solid line). Vo, void volume; VAg, elution position of integrin; VT, total inclusion volume of the column.

that of either band 3 or the mixture of bands 1 plus 2 (cf. Fig. 6, A–C). The reconstituted complex reproducibly eluted as a relatively sharp peak with a higher effective Stokes radius in precisely the same position as native integrin. SDS PAGE of the leading and trailing fractions showed all three bands present as in the original integrin preparation. Parallel experiments were performed with sucrose gradient centrifugation with analogous results (not shown). These results, together with previously reported hydrodynamic (12, 14) and
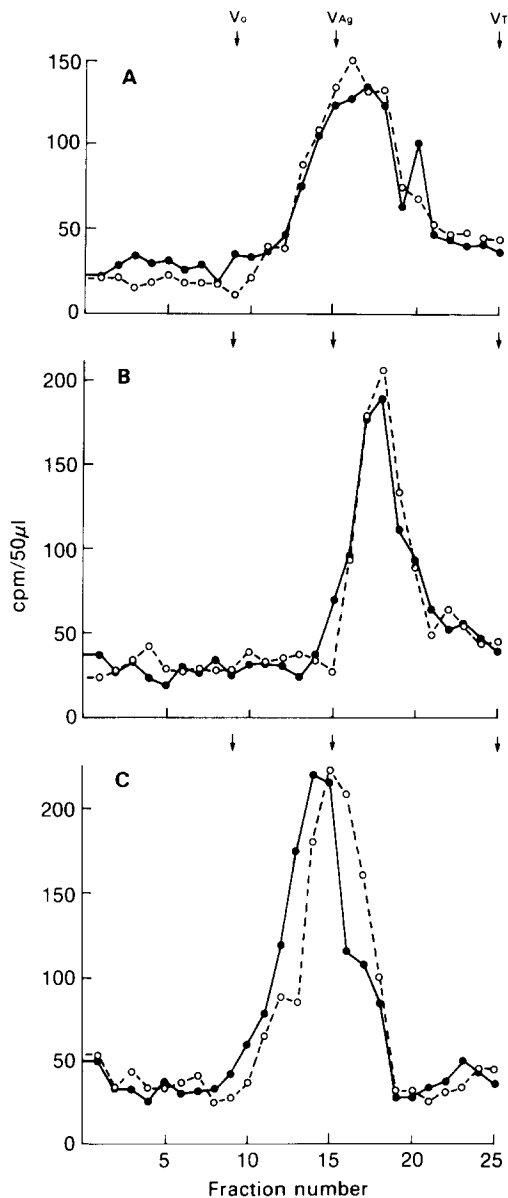
Figure 7. Fibronectin binding to reconstituted integrin. [^{35}S]methi-onine-labeled band 1 plus 2, band 3, or reconstituted integrin were mixed with 400 μg/ml fibronectin and applied to a 0.2 × 30-cm AcA22 column pre-equilibrated with the same concentration of fibronectin; 60-μl fractions were collected. (*A*) Bands 1 plus 2 alone (*open circles, broken line*), bands 1 plus 2 plus fibronectin (*solid circles, solid line*); (*B*) band 3 alone (*open circles, broken line*), band 3 plus fibronectin (*solid circles, solid line*); (*C*) reconstituted integrin alone (*open circles, broken line*), reconstituted integrin plus fibronectin (*solid circles, solid line*), Vo, void volume; VAg, elution position of integrin; VT, total inclusion volume of column.

biochemical data (15), show unequivocally that these glyco-proteins exist as an oligomeric complex.

The ability of the fractionated integrin to bind fibronectin was similarly tested. When band 1 plus 2 or band 3 alone was mixed with 400 μg/ml of fibronectin, there was no change in the elution profile (Fig. 7). However, the profile of the reconstituted complex mixed with fibronectin was shifted toward the void volume of the column in a manner similar to that previously reported (14). This change in elu-

tion profile was inhibited by the RGDS cell-binding tetra-peptide from fibronectin (14).

Integrin also binds talin (13), a 215-kD protein thought to play a role in the attachment of cytoskeletal elements to the cytoplasmic surface of the plasma membrane (4, 5). Again, when either band 1 plus 2 or band 3 alone was mixed with talin before running on a gel filtration column equilibrated with talin, no alteration in the elution profile of the ^{35}S-labeled glycoprotein was detected (Fig. 8, *A* and *B*). The ability to bind talin was restored upon reconstituting the
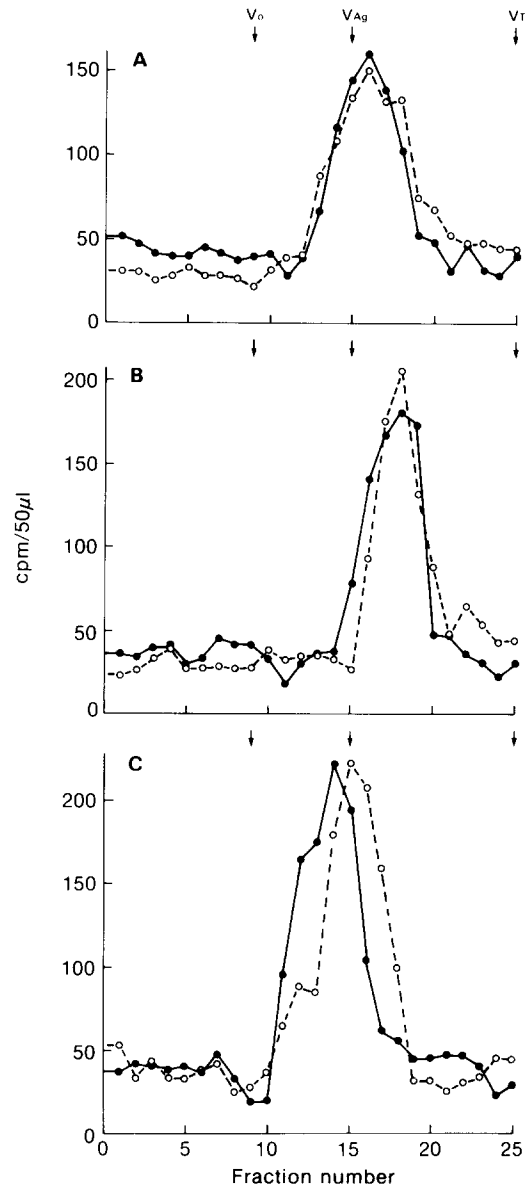

Figure 8. Talin binding to reconstituted integrin. [^{35}S]methionine-labeled band 1 plus 2, band 3, or reconstituted integrin were mixed with 400 μg/ml talin and applied to a 0.2 × 30-cm AcA22 column pre-equilibrated with the same concentration of talin; 60-μl fractions were collected. (*A*) Bands 1 plus 2 alone (*open circles, broken line*), bands 1 plus 2 plus talin (*solid circles, solid line*); (*B*), band 3 alone (*open circles, broken line*), band 3 plus talin (*solid circles, solid line*); (*C*) reconstituted integrin alone (*open circles, broken line*), reconstituted integrin plus talin (*solid circles, solid line*). Vo, void volume; VAg, elution position of integrin; VT, total inclusion volume of the column.

complex (Fig. 8 C). This shift in the elution profile is similar to that noted when the reconstituted complex was mixed with fibronectin. The binding site for talin is, however, distinct from that of fibronectin in that talin-binding is insensitive to the presence of the fibronectin cell-binding tetrapeptide (13).

Discussion

Integrin consists of at least three glycoproteins that probably serve as a transmembrane link between the extracellular matrix and cytoskeletal-associated proteins required for cell–substratum adhesion and cell migration (1, 9, 11, 13–15, 18). The members of this complex have not previously been separated, except under denaturing conditions. Therefore, it has not been possible to determine whether the three glycoproteins of the complex co-purify because they exist as an oligomer, or if they represent three glycoproteins with a common antigenic site recognized by the adhesion-disrupting monoclonal antibodies CSAT and JG22 (9, 18). Further, if they do exist as a true oligomer, the question arises as to the functional requirement for the oligomeric configuration. We have now produced a monoclonal antibody designated G that is able to dissociate the complex under nondenaturing conditions. When the purified CSAT complex was passed over a G monoclonal antibody affinity column, band 3 glycoprotein bound to the column while bands 1 and 2 passed through. Each of these fractions eluted from an Ultrogel AcA22 column nearer the included volume than the intact complex. However, when the two fractions were combined, the reconstituted complex co-eluted with the native oligomer, suggesting that the dissociated and reconstituted complex could be used to address these questions.

The ability of band 1 plus 2 and band 3 fractions from a G antibody affinity column to bind the CSAT and JG22 monoclonal antibodies was compared by gel filtration. Only band 3 glycoprotein was recognized by these two antibodies. The fact that the CSAT and JG22 antibodies bound only to band 3 and yet could be used to co-purify band 1 and 2 glycoproteins further confirms the oligomeric nature of integrin and eliminates the possibility that the glycoproteins are co-purified because they share a common epitope recognized by these two monoclonal antibodies. The portion of band 3 recognized by the CSAT and JG22 antibodies is important to the function of the complex as a receptor for extracellular matrix molecules. Blocking this site prevents direct binding to fibronectin or laminin (1, 14), thus inhibiting cell adhesion and spreading. Polyclonal antibodies raised against purified band 3 glycoprotein also inhibit cell attachment and spreading on defined substrata (unpublished data), further substantiating that band 3 glycoprotein contains regions controlling the binding of integrin to extracellular matrix molecules. This site is distinct from that recognized by the G monoclonal antibody, which has no effect on cellular adhesion. The monoclonal antibodies, CSAT and G, should be useful in identifying peptides from the regions of band 3 that control complex integrity and receptor function.

To determine if integrin must remain an oligomer to act as a receptor for fibronectin, laminin, or talin, the dissociated and reconstituted complex was subjected to equilibrium gel filtration in the presence of each of these ligands. This method has proven extremely reproducible in our hands (13, 14). The experiments reported here were repeated at least three times using three independent integrin preparations with essentially the same results. That is, the shift in elution profile upon reconstitution of integrin and addition of the various ligands was reproducible to the fraction using the column calibrated as previously described (13, 14). When bands 1 plus 2 or band 3 glycoproteins were passed over the column in the presence of any of these ligands, no ligand receptor interaction was detected. However, when band 1 plus 2 glycoproteins were mixed with band 3 glycoprotein, the molecules were functionally and hydrodynamically reconstituted. They could now bind the various ligands, and they eluted in a position on the AcA22 column characteristic of intact integrin. The binding activity of the reconstituted complex resembled that of nondissociated integrin in that it was sensitive to the fibronectin cell-binding tetrapeptide, RGDS, and to the CSAT monoclonal antibody. These experiments support the contention that integrin must be oligomeric to function as a receptor.

While these data show that neither band 3 by itself nor band 1 plus 2 can bind any of the ligands tested, the quaternary structure required for activity is not clear. In addition to a heterotrimer of bands 1, 2, and 3, other possible combinations, such as heterodimers comprised of band 2 plus 3 or band 1 plus 3, might also produce active configurations. Relevant to this point is the fact that, while preparations of integrin always contain all three glycoproteins, there is a discrepancy between the sedimentation data and the theoretical molecular mass of a heterotrimer (3). As pointed out previously, when corrections are made for detergent binding, the calculated molecular mass of the material migrating in a sucrose gradient with a $s_{20, w}$ of 8.6 is between that of a heterodimer and a heterotrimer. While the molecular mass estimates from such data are subject to error due to incomplete knowledge of the partial specific volume of the complex, especially in the presence of detergent, and the inaccuracy of the estimation of the apparent molecular mass of the various integrin glycoproteins by SDS PAGE, it is quite possible that the minimum functional unit is a heterodimer containing band 3 plus one of the others.

There are both similarities and differences between integrin and extracellular matrix–binding receptors from mammalian cells. The integrin molecules from mammalian cells that bind fibronectin consist of two glycoproteins, not three (2, 7, 8, 20–23, 25). Further, in mammalian cells, different receptors appear to be involved in fibronectin, laminin, vitronectin, and collagen adhesion. These receptors are distinguished genetically (10), serologically (2), and biochemically (2, 7, 8, 20–23, 25). In contrast, integrin from avian cells may serve as a universal receptor for fibronectin, laminin (14), collagen, and vitronectin (Horwitz, A. F., and C. A. Buck, unpublished observations). However, the binding of all integrin-like molecules regardless of their source to their respective ligands is similar in that it is blocked by the RGDS cell-binding tetrapeptide. Also, the lower molecular mass constituent of each of these complexes is a cysteine-rich glycoprotein with a great deal of internal disulfide bonding, suggesting, perhaps, a common subunit in each of these receptors. If this were the case in the avian system, integrin could be a mixture of receptors each having the band 3 subunit in common. The individual receptors in the mixture isolated with the CSAT or JG22 monoclonal antibodies would then resemble the heterodimeric complexes from

mammalian cells. Supporting this idea are recent data suggesting that band 3 of both integrin and the collagen receptor from rat cells contains a common serologically cross-reactive epitope (C. Altmann, T. Borg, A. F. Horwitz, and C. A. Buck, unpublished observations). If these data can be extended to surface membrane receptors for other extracellular matrix molecules, it may be that there is indeed a family of integrin-like molecules all sharing a common band 3–like subunit, and, as proposed by Ruoslahti and Pierschbacher (24), all sensitive to the arg-gly-asp cell recognition sequence from fibronectin. A similar suggestion has been made for position-specific antigens expressed during Drosophila development (27). Immunoprecipitation studies suggest that these antigens represent a family of developmentally regulated molecules whose tissue specificity is achieved by combining different molecules with a common subunit (28). In this case, the common subunit is also a cysteine-rich protein whose apparent molecular mass increases upon SDS PAGE performed under reducing conditions.

If, on the other hand, integrin functions as a heterotrimer capable of binding more than one extracellular matrix molecule, it would resemble the gpIIb/IIIa complex from platelets. This complex binds several ligands, including fibronectin, fibrinogen, and von Willebrand factor, by an arg-gly-asp–mediated mechanism. Whatever the precise nature of the quaternary structure of integrin, its oligomeric integrity is required for it to function as a receptor for extracellular matrix and cytoskeletal-associated molecules.

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