Integrin Heterodimer and Receptor Complexity in Avian and Mammalian Cells

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Abstract. We report data showing that the integrin receptor complex in chickens contains several discrete heterodimers all sharing the β_1-integrin subunit combined separately with different α-subunits. Using antisera to synthetic peptides based on cDNA sequences of chicken and human α-integrin subunits to analyze the integrin complement of avian and mammalian cells, we show that band 2 of the chicken integrin complex contains α-subunits related to both α_3- and α_5-subunits of human integrins. $\alpha_3\beta_1$ and $\alpha_5\beta_1$ have both previously been shown in human cells to be fibronectin receptors and $\alpha_3\beta_1$ can also act as a receptor for laminin and collagen. We also provide evidence for the presence, in band 1 of the chicken integrin complex, of a third integrin α-subunit which is also α_5 related. This integrin subunit exists in a separate heterodimer complex with β_1 and binds to fibronectin-affinity columns. These results provide explanations for published data showing that the avian integrin complex contains receptor activity for a variety of extracellular matrix proteins. We conclude that the chicken integrin complex comprises a set of β_1-integrin heterodimers equivalent to the human VLA antigens and includes at least two fibronectin receptors. Finally, we show that chicken embryo fibroblasts also contain a β_3-class integrin related to the RGD receptors defined in various human cells.

The family of cell surface receptors known as integrins includes multiple receptors for extracellular matrix proteins and the interactions of integrins with these extracellular ligands play important roles in cellular adhesion (Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Buck and Horwitz, 1987). Integrins are transmembrane proteins composed of two subunit types, α and β. They fall into at least three major subclasses, each characterized by a common β-subunit and a set of α-subunits.

The ligand specificities of some $\alpha\beta$-complexes have been defined but others remain unclear. In particular, a complex of proteins was isolated several years ago from chicken cells using antibodies which block adhesion to extracellular matrix proteins (Neff et al., 1982; Greve and Gottlieb, 1982; Knudsen et al., 1985). This chicken integrin complex contains proteins which interact with fibronectin, collagen, and laminin (Horwitz et al., 1985; Akiyama et al., 1986; Buck and Horwitz, 1987), but it remains unclear whether there is a single complex with multiple affinities or whether there is a mixture of several different heterodimers each with distinct ligand specificity.

The β-subunit of the chicken integrin complex has been cloned (Tamkun et al., 1986) and is homologous in sequence with the β-subunit of an integrin receptor from human cells, isolated by its affinity for fibronectin (Pytela et al., 1985*a*; Argraves et al., 1987). Comparison of these sequences with the sequences of other integrin β-subunits (Fitzgerald et al., 1987; Kishomoto et al., 1987; Law et al., 1987; DeSimone and Hynes, 1988) has allowed classification based on three β-subunits (Hynes, 1987; Ruoslahti and Pierschbacher, 1987). We designate the β-subunit of the chicken integrin complex β_1; as mentioned, it is clearly the avian equivalent of the β-subunit of the so-called human "fibronectin receptor." Immunological evidence indicates that these two β_1-subunits are equivalent to the β-subunit of a set of human antigens known as VLA antigens which have at least six distinct α-chains (α_1-α_6) (Hemler et al., 1987, 1988; Takada et al., 1987, 1988).

These data lead to a picture in which the integrin β_1-subclass comprises at least six separate $\alpha\beta$-complexes. Some of these complexes have been assigned ligand specificities in human cells. The so-called human fibronectin receptor appears to be $\alpha_5\beta_1$ (Pytela et al., 1985*a*; Takada et al., 1987; Argraves et al., 1987; Wayner et al., 1988). However, there is at least one other fibronectin receptor in the same integrin subclass, namely $\alpha_3\beta_1$. This heterodimer mediates adhesion to fibronectin, laminin, or collagen (Wayner and Carter, 1987; Wayner et al., 1988; Takada et al., 1988). Very recently, laminin receptors from the β_1-class of integrins have been isolated (Gehlsen et al., 1988; Ignatius and Reichardt, 1988). Finally, $\alpha_2\beta_1$ appears to be a collagen receptor (Nieuwenhuis et al., 1985; Kunicki et al., 1988). Other β_1-integrins are not well defined as to their ligand specificity and it is unclear how most of the human α-subunits are related

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to the α-subunits of the avian integrin complex, which is generally defined as comprising three bands, with bands 1 and 2 being α-subunits and band 3 being the β_1-subunit.

In this paper we clarify the identity of several of the chicken α-subunits, show that they exist as separate αβ-heterodimers, and relate them to their mammalian counterparts and ligand specificities.

Materials and Methods

Cells

Chicken embryo fibroblasts (CEFs)[1] were prepared from day 11 embryos (Spafas, Inc., Norwich, CT) as described (Rein and Rubin, 1968). Cells were grown in DME plus 5% FCS (Gibco Laboratories, Grand Island, NY) and used between passages 3 and 9. MG63 human osteosarcoma cells (Billiau et al., 1977) were grown in DME plus 10% FCS.

Purification of Chicken Integrin Complex

Chicken integrin complexes were purified from chicken embryos following the procedure of Knudsen et al. (1985) with minor modifications. Chicken embryos from day 10 to 14 were decapitated, eviscerated, and their limbs removed while on ice. The tissue was weighed, and then homogenized with 10 ml per 30 embryos of PBS in a Waring blender (Waring Products; Dynamics Corp., New Hartford, CT) at 4°C twice for 15 s. Solubilization buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM $CaCl_2$, 2 mM PMSF, 0.03 U/ml aprotinin, and either 0.5% NP-40 or 50 mM β-octylglucoside [BOG; Calbiochem-Behring Corp., La Jolla, CA]) was added in the amount of 3 ml/g original wet weight of tissue. The homogenate was filtered through three layers of cheesecloth, stirred for 1 h at 4°C, then spun at 3,000 g for 10 min and the supernatant further spun at 60,000 g for 45 min. White filmy material was aspirated off the top of the supernatant and the latter was passed through Whatman Inc. (Clifton, NJ) 3M filter paper, acidified to 20 mM acetic acid (pH 5.5), and left at 4°C for 45 min. Insoluble material was removed by centrifugation at 12,000 g for 10 min and the supernatant neutralized to pH 8.0 with 1 M Tris-HCl. The resulting extract was clarified when necessary by centrifugation (60,000 g for 30 min) and/or by passage through a 0.45-μm Nalgene filter. The extract was either used immediately or stored frozen at −35°C.

CSAT monoclonal antibody affinity columns were prepared by coupling of 10 mg/ml isolated IgG from CSAT hybridomas (Neff et al., 1982) to CNBr-activated Sepharose 4B. Chicken embryo extract was passed over a 1- or 2-ml column at a flow rate of 5-10 ml/h at 4°C. The column was then washed with 50 column vol of wash buffer (10 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.5 mM $CaCl_2$, and either 0.5% NP-40 or 50 mM BOG). The column was eluted with 50 mM diethylamine buffer, pH 11.5, with 0.15 M NaCl, 0.5 mM $CaCl_2$, and either 0.5% NP-40 or 50 mM BOG. Fractions were immediately neutralized to pH 8.0 with 1 M Tris, pH 6.8, and analyzed on SDS-PAGE gels. The peak fractions were pooled and frozen at −35°C.

Peptide Synthesis

Synthetic peptide corresponding to the COOH-terminal 14 residues of chicken band 2 (α_3) was kindly provided by C. Buck (Wistar Institute, Philadelphia, PA). The *cys*-23mer corresponding to the human α_5 COOH terminus was synthesized by Peninsula Laboratories, Inc. (Belmont, CA). GRGESP, GRGDSP, RNGESQ (a peptide analogous to GRGDSP but from the next type III repeat, III-11; Schwarzbauer et al., 1983; Kornblihtt et al., 1985), and a peptide corresponding to the COOH-terminal 13 amino acids of the human α_5-sequence were synthesized using a peptide synthesizer (model 430A; Applied Biosystems, Inc., Foster City, CA) using solid-phase t-boc chemistry. Peptides were deprotected and cleaved from the solid support by treatment with anhydrous TFMSA and were partially desalted by gel filtration through Sephadex G10. Peptides used to elute affinity columns were further purified before use by reverse-phase HPLC chromatography on a Vydac C_{18} column (Rainin Instruments Co., Woburn, MA), eluted with a 0-60% acetonitrile gradient in 0.1% trifluoroacetic acid.

Production of Antipeptide Sera

The α_3-14mer peptide was coupled to keyhole limpet hemocyanin (KLH; Calbiochem-Behring Corp.) using 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC; Sigma Chemical Co., St. Louis, MO) essentially as described (Staros et al., 1986). 2 mg of KLH and 15 mg of peptide were mixed in 4 ml of PBS. After adjusting the pH to 5.5 with HCl, EDC was added to a final concentration of 0.1 M. The reaction was stirred overnight at room temperature and quenched with 0.5 M ethanolamine for 1 h. After exhaustive dialysis against 0.05 M NH_4HCO_3, pH 7.5, the mixture was lyophilized and resuspended in 1 ml of water.

α_5-*Cys*-23mer was conjugated to KLH with *m*-maleimidobenzoylsulfosuccinimide ester (Pierce Chemical Co., Rockford, IL) using a procedure described previously (Marcantonio and Hynes, 1988).

For injection, 200 μg of peptide conjugate in PBS were mixed with 1 ml of complete Freund's adjuvant (Gibco Laboratories) by sonication and injected subcutaneously at multiple sites along the dorsal midline of rabbits. At 3-4-wk intervals, booster injections of 100 μg of protein in incomplete adjuvant were given. Significant antibody titers were observed after two boosts for the α_5-23mer, and after five boosts for the α_3-14mer.

Other Antibodies

Rabbit anti-β_1 cytoplasmic domain sera were prepared as described (Marcantonio and Hynes, 1988). Rabbit anti-α_5 COOH terminus (13mer) serum was donated by W. S. Argraves and E. Ruoslahti (La Jolla Cancer Research Foundation, La Jolla, CA). Monoclonal anti-extracellular matrix receptor (ECMR) I (P1B5) (Wayner and Carter, 1987) was gift of W. G. Carter and E. Wayner (Fred Hutchinson Cancer Research Center, Seattle, WA) and CSAT antibody (Neff et al., 1982) was a gift of C. Buck (Wistar Institute) and R. Horwitz (University of Illinois, Urbana, IL). Monoclonal anti-RGD receptor (LM609; Cheresh and Spiro, 1987) was a gift of D. Cheresh (Research Institute of Scripps Clinic, La Jolla, CA).

Iodination and Immunoprecipitation

Cells were labeled with ^{125}I and lactoperoxidase (Sigma Chemical Co.) in monolayers as described (Hynes, 1973). 10^7 cells and 1.0 mCi/ml were used per experiment. Cells were washed three times with PBS with 1 mM $CaCl_2$ and 1 mM $MgCl_2$, resuspended in 1 ml of extraction buffer (50 mM Tris, pH 8.0, 0.15 mM NaCl, 0.5mM $CaCl_2$, 0.5% NP-40), incubated for 15 min on ice, and then sedimented for 10 min at 10,000 g. The supernatant was preincubated with 50 μl of protein A–Sepharose for 5 min and the beads sedimented for 2 min at 10,000 g. The resulting supernatant was used for immunoprecipitation. Typically, 5×10^6 TCA-precipitable counts were used per sample.

Integrin complex purified from day 11 chicken embryos was labeled with ^{125}I using Iodobeads (Pierce Chemical Co.) and desalted using a 10×0.5-cm G-50 column (Pharmacia Fine Chemicals, Piscataway, NJ) with extraction buffer. The peak fractions in the void volume were pooled and used directly for immunoprecipitation.

Immunoprecipitation of native and denatured labeled cell extracts or of purified integrin complex was performed as described previously (Marcantonio and Hynes, 1988).

Western Immunoblotting

Samples of chicken embryo extract or purified integrin were solubilized in electrophoresis sample buffer and run on 7% nonreducing SDS-PAGE gels with 4% stacking gels (Laemmli, 1970). Proteins were electrophoretically transferred to nitrocellulose filters (Schleicher & Schuell, Inc., Keene, NH) overnight at 500 mA (Towbin et al., 1979). Filters were India Ink stained, blocked in blocking buffer (2% hemoglobin in PBS with 0.05% Tween-20 and 0.05% NaN_3) for 1 h to overnight, then mounted in a miniblotter apparatus (Immunetics, Cambridge, MA). Primary antibodies were diluted in blocking buffer, introduced into miniblotter channels, and filters were incubated with rocking for 1-3 h. For peptide competition experiments, synthetic peptides were added at 100 μg/ml. Each filter was washed three times for 15 min in blocking buffer with detergents (blocking buffer plus 0.1% SDS and 0.5% NP-40) then twice for 10 min in blocking buffer. ^{125}I-labeled protein A (DuPont Co., Wilmington, DE) was diluted to 50 μCi/ml (2.5 μCi/50-ml filter) in blocking buffer and applied to the filter for 2 h with agitation. Filters were washed four times for 10 min in blocking buffer plus 0.1% SDS and 0.5% NP-40, and twice for 10 min in PBS, dried on 3M paper, and exposed to XAR-5 film (Eastman Kodak Co., Rochester, NY) with an enhancing screen.

1. *Abbreviations used in this paper*: BOG, β-octylglucoside; CEF, chicken embryo fibroblast; FNf, fibronectin 120-kD cell-binding domain fragment; KLH, keyhole limpet hemocyanin.

Metabolic Labeling

CEFs or MG63 cells were labeled in 35-mm dishes while subconfluent. Cells were incubated for 30 min in DME minus methionine plus 10% FCS, followed by incubation in methionine-free medium plus 10% FCS containing 500 μCi/ml of [^{35}S]methionine (Translabel; 1,200 Ci/mmol, ICN Radiochemicals, Irvine, CA). After 30 min, the radioactivity was removed and the cells incubated in DME plus 10% FCS for the chase times listed in the figure legends. The cells were then washed three times with ice-cold PBS, followed by extraction and immunoprecipitation as described above.

Gel Electrophoretic Analysis

SDS-PAGE was performed by the method of Laemmli (1970). Separation gels were 7% acrylamide with a 4% stacking gel. Samples were prepared in sample buffer (2-5% SDS, 100 mM Tris-HCl, pH 6.8, 10 mM EDTA, 10% glycerol, and bromphenol blue) and boiled for 3 min. Reduced sample buffer contained 50 mM DTT. The following were used as reduced molecular mass markers: myosin (200 kD); β-galactosidase (116 kD); phosphorylase B (97 kD); and BSA (66 kD).

Affinity Chromatography

Purified human plasma fibronectin was purchased from the New York Blood Center (New York, NY). The 120-kD fragment of fibronectin containing the cell-binding domain (FNf) was purified after digestion with chymotrypsin (Millipore Corp., Freehold, NJ) essentially as described (Pierschbacher et al., 1981). Affinity columns were prepared by coupling the desired ligand (FNf or GRGDSPC) to either Affi-gel 10 (Bio-Rad Laboratories, Richmond, CA) or CNBr-activated Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) as suggested by the manufacturers. 0.5-1.0 $\times$ 10^8 cells were surface labeled with 1-2 mCi of ^{125}I using lactoperoxidase/glucose oxidase as described. For GRGDSPC columns, the labeled cells were extracted with 1 ml of 200 mM BOG in PBS containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 3 mM PMSF as described by Pytela et al. (1985*a*,*b*, 1986). For analysis on FNf columns, labeled cells were extracted either with 200 mM BOG in PBS with $CaCl_2$ and $MgCl_2$ as above, or with 200 mM BOG in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 3 mM PMSF, 1 mM $MnCl_2$ (TNM). $MnCl_2$ was included to enhance receptor binding to FNf as described by Gailit and Ruoslahti (1988). After allowing lysis to proceed for 10 min on ice, detergent-insoluble material was removed by centrifugation at 10,000 *g* for 30 min. The resulting supernatant was loaded onto 1.0-ml columns of either immobilized GRGDSPC or FNf over the course of 1 h. Columns were washed extensively with wash buffer (50 mM BOG in PBS or TNM) until radioactivity eluting from the columns had fallen to a constant level. Columns were then eluted with 1 column vol of wash buffer supplemented with 1 mg/ml control peptide (GRGESP or RNGESQ) followed by 1 column vol of wash buffer containing 1 mg/ml GRGDSP. Both elution buffers were applied to the columns over the course of 1 h. Column fractions were analyzed either by immunoprecipitation with antibodies as described in the text or directly on 7% SDS gels.

Affinity chromatography of purified integrins was performed on NP-40-extracted material prepared as described above. Integrin-containing fractions eluted from the CSAT column were dialyzed against 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5 mM $CaCl_2$, 0.5% NP-40 as described by Buck et al. (1986). The dialyzed sample was supplemented with $MnCl_2$ to a final concentration of 1 mM and chromatographed on 1.0-ml columns of immobilized FNf as described above for labeled cell extracts. Each column fraction was iodinated after collection with 1-5 μCi of ^{125}I using Iodobeads (Pierce Chemical Co.) and analyzed by immunoprecipitation and SDS-PAGE as described in the text.

Results

cDNA Clones and Sequence of an Avian Integrin α-Subunit

In our original isolation of chicken integrin cDNA clones using a polyclonal antibody (Tamkun et al., 1986) we reported three clones, two of which were shown to encode the β_1-subunit. The third clone, I, was used to isolate related cDNA clones and several of these were sequenced. Fig. 1 shows a partial amino acid sequence deduced from the sequence of these clones. The sequence comprises the COOH-terminal 179 residues of an integrin α-subunit and corresponds with the light chain of an α-subunit, as can be seen by comparison with the sequences of several other α-subunits (Fig. 1). The sequence, while clearly homologous with α-subunits in general, is not closely related to the sequence of any specific one of the human α-subunits which have been published. In particular, the sequence is distinct from that of the human fibronectin receptor α-subunit (α_5), the only published sequence for an α-subunit of the β_1 subclass (Fig. 1). Data to be presented below lead us to conclude that the chicken α-sequence corresponds with the human α_3-subunit and, for clarity, we will refer to this subunit as α_3 (see Discussion).

α-Subunit-specific Antisera

To identify the α-subunit encoded by this cDNA, we obtained a synthetic peptide corresponding with the COOH-terminal 14 residues (Fig. 1). We also obtained a synthetic peptide corresponding with the COOH-terminal 23 residues of the human α_5-sequence (Fig. 1). Polyclonal antisera were raised against each of these peptides conjugated to KLH and tested by Western blotting and immunoprecipitation.

Fig. 2 *A* shows that antiserum to the chicken cDNA α_3-sequence recognizes predominantly band 2 of the chicken integrin complex on Western blots. Fig. 3 (lane *f*) shows immunoprecipitation by this antiserum under denaturing conditions: it precipitates a band comigrating with band 2. Under nondenaturing conditions, the antiserum precipitates material comigrating with both band 2 (α_3) and band 3 (β_1) indicating the presence of an $\alpha_3\beta_1$-heterodimer (Fig. 3, lane *b*). On Western blots this antiserum variably stains higher molecular weight bands which we suspect arise artefactually during isolation and concentration of purified integrin (Fig. 2), since they are not observed in blots of whole embryo homogenates (data not shown). The presence of this α_3-subunit in band 2 of the chicken integrin complex agrees with immunological data showing cross-reaction of anti-human α_3-antibodies with band 2 (Takada et al., 1987).

Antisera raised against human α_5-23mer peptide cross-reacted with both bands 1 and 2 of the purified chicken integrin complex on Western blots (Fig. 2 *B*) or by immunoprecipitation (Fig. 3). Two different antisera gave similar results, although one of them showed a stronger bias toward band 1 than the other in immunoprecipitation assays (Fig. 3, lanes *d* and *e*). This cross-reactivity with two different bands was somewhat surprising. Accordingly, we obtained a third antiserum raised against a shorter α_5-peptide (a generous gift of S. Argraves and E. Ruoslahti, La Jolla Cancer Research Foundation). This antiserum was raised against the COOH-terminal 13 residues of the human α_5-sequence (see Fig. 1). As shown in Fig. 2 *A* and *B*, this antiserum recognized a single α-subunit band on Western blots. This α_5-cross-reactive subunit (α_{5a}) comigrated with the lower band recognized by the two antisera against the 23 residue α_5-peptide and was slightly larger than the band recognized by the putative α_3-antiserum. In immunoprecipitation experiments, this antiserum precipitated two bands comigrating with bands 2 and 3 of the integrin complex (Fig. 3, lane *c*). This indicates the existence of a distinct $\alpha_{5a}\beta_1$-dimer recognized by this antiserum.

These results lead to several conclusions. First, at least

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CHK  α3  TSRCWRMGPPLGTDGSWGGAEPGEPPITLATGKK...............AKSEVLLSCSQGTARCIWFEC
FNR  α5  GLNCTTNHPINPKGLELDPEGSLHHQQKR............EAPSRSSASSGPQILKCPE..AECFRLRC
VNR  αv  PMNCTSDMEINPLRIKISSLQTTEKNDTVAGQGERDHLITKR.DLALLSEGDIHTLGCGV..AQCLKIVC
IIB      GLQCFPQPPVNPLKVDWGLPIPSPSPIHPAHHKRDRRQIFLPEPEQPSRLQDPVLVSCDS..APCTVVQC

         PI..PAAQHPATFRVRARVWNSTFIEEYRSFD....RVKVDGTATLFLRTHIPTINMRNHTFSVDVDSEL
         ELGPLHQQESQSLQLHFRVWAKTFLQREHQPFS..LQCEAVYKALKMPYRILPRQLPQKERQVATAVQWT
         QVGRLDRGKSAILYVKSLLWTQTFMNKQNQNHSTSLKSSASFNVIEFPYKNLPIENITDSTLVTTNVTWG
         DLQEMARGQRAMVTVLAFLWLPSLYQRPLDQFVLQSHAWFNVSSL..PYAVPPLSLPRGEAQVWTQLLRA

     α3  TEEQPPQVALWLVLVAAAAGLLLLGLIIVLLWKCGFFRRASTGAMYEAKGQKAEMRIQPSETERLTDDY
     α5  KAEGSYGVPLWIIILAILFGLLLLGLLIYILYKLGFFKRSLPYGTAMEKAQLKPPATSDA
         IQPAP..MPVWVIILAVLAGLLLLAVLVFVMYRMGFFKRVRPPQEEQEREQLQPHENGEGNSET
         LEERA..IPIWWVLVGVLGGLLLLTILVLAMWKVGFFKRNRPPLEEDDEEGE
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Figure 1. Comparison of chicken integrin α-subunit with several human α-subunits. The COOH-terminal 179 amino acid residues of a chicken integrin α-subunit cDNA clone (chicken α_3) are compared with sequences of three human α-subunits. Fibronectin receptor (*FNR*, α_5; Argraves et al., 1987; Fitzgerald et al., 1987), vitronectin receptor (*VNR*, α_v; Suzuki et al., 1987), and platelet glycoprotein IIb (*IIB*; Poncz et al., 1987; Fitzgerald et al., 1987). Several homologies are highlighted; including a set of three cysteine residues (*), the transmembrane domain (*heavy lines*), and the dibasic residues which denote the start of the cytoplasmic domains (+). The known or suggested cleavage sites of α_5, α_v, and platelet glycoprotein IIb are marked (*arrowheads*). These cleavages generate the COOH-terminal light chain of these α-subunits. The sequence of α_3 also suggests a cleavage site after a dibasic sequence. The peptides used as immunogens are underlined: α_3-14mer, α_5-23mer, and α_5-13mer.

two different β-subunits are present in the band 2 region of the gels. One (α_{5a}) is recognized by three different antisera against the human α_5-sequences and a second, slightly smaller one is recognized by antisera against the chicken α_3-peptide. Second, antisera against a longer human α_5-peptide recognize, in addition, a third α-subunit which migrates in the band 1 region (α_{5b}). Since the sera were tested in parallel on the same preparations of integrins, the reaction with band 1 of our two antisera raised against the α_5-23mer cannot be due to aggregation or anomalous migration of the smaller α_5 cross-reactive species so that it migrates in the band 1 region. Any such aggregates should also be recognized by Argraves' antiserum to the α_5-13mer which does not cross-react with band 1. This suggests, rather, that some epitope in the first 10 residues of the 23mer peptide is present in a different, larger α-subunit in the band 1 region.

To test the epitope specificity of the antisera, we performed peptide competition experiments. Fig. 2 *C*, antiserum *1*, shows that the anti-α_3-serum is blocked by the α_3-peptide but not by either of the α_5-peptides. The different anti-α_5-

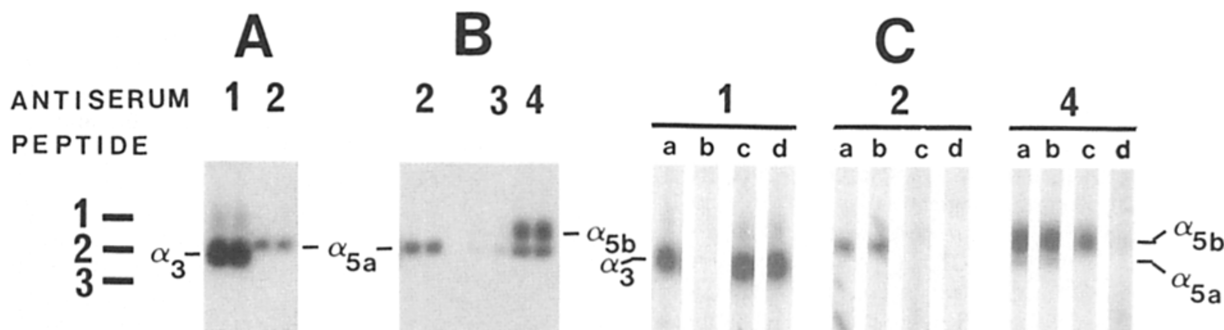

Figure 2. Western immunoblotting of chicken integrins with antipeptide sera. In *A–C*, purified chicken integrin complex was separated by electrophoresis on SDS gels, transferred to nitrocellulose, and individual lanes were stained with different antisera in a miniblot apparatus as described in Materials and Methods. (*A*) Pairs of lanes were stained with antisera to the α_3-14mer peptide (antiserum *1*) or the α_5-13mer peptide (antiserum *2*). The migration positions of bands *1*, *2*, and *3* as detected by India ink staining of the blot are marked. The two antisera stain distinct bands in the band *2* region of the gel marked α_3 and α_{5a}. (*B*) Pairs of lanes were stained with three different antisera to α_5-peptides: antiserum *2*, anti-α_5-13mer as in *A*; antisera *3* and *4*, two different antisera (#160 and #161) to α_5-23mer peptides. Note that the two antisera to the larger peptide stain two bands, one comigrating with the α_{5a}-band stained by the anti-α_5-14mer and a larger one (α_{5b}) which migrates in the band *1* region of the gel. (*C*) Three of the antipeptide sera were tested in the presence of different competitor peptides. The sera are numbered as in *A* and *B* and *a–d* denote competition by different peptides. (*a*) β_1-peptide failed to block reaction of any of the anti-α-sera; (*b*) α_3-14mer blocked reaction of antiserum *1* (anti-α_3-serum) but of none of the anti-α_5-sera (*2* and *4*); (*c*) α_5-13mer had no effect on anti-α_3-serum (*1*) but blocked reaction of the anti-α_5-sera (*2* and *4*) with the α_{5a}-band; and (*d*) α_5-23mer was also without effect on the anti-α_3-serum (*1*) but blocked reaction of the anti-α_5-sera (*2* and *4*) with both α_{5a}- and α_{5b}-bands.

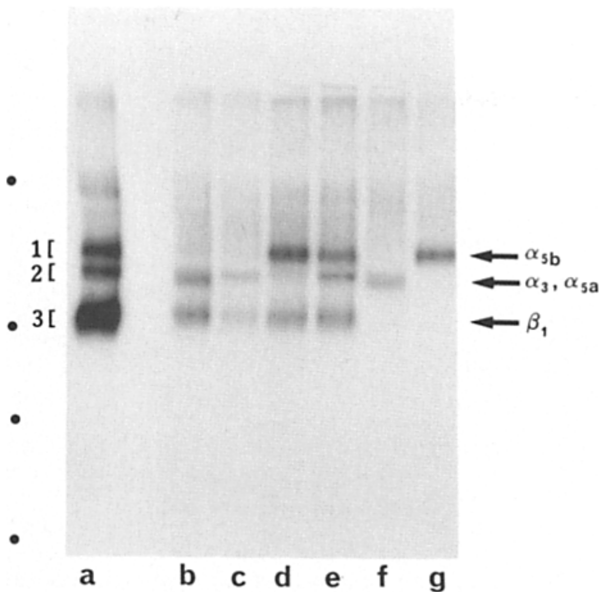

Figure 3. Immunoprecipitation of integrins purified from chicken embryo extract. Integrin complexes were purified from chicken embryo extract using a CSAT-Sepharose column. Purified integrins were ^{125}I-labeled and incubated with (lane *a*) anti-β_1-peptide serum; (lane *b*) anti-α_3-peptide serum; (lane *c*) anti-α_5-13mer peptide serum; (lane *d*) anti-α_5-23mer peptide serum #160; (lane *e*) anti-α_5-23mer peptide serum #161; (lane *f*) anti-α_3-peptide serum after SDS denaturation of integrins; or (lane *g*) anti-α_5-23mer antiserum #160 after SDS denaturation of integrins. The samples were immunoprecipitated using protein A-Sepharose and analyzed by SDS-PAGE (nonreduced) followed by autoradiography of the dried gels. Specific sets of $\alpha\beta$-heterodimers are precipitated under nondenaturing conditions with the α-antisera, while only α-subunits are recovered under denaturing conditions. Molecular mass markers indicated by dots at left are from top to bottom: myosin (200 kD), β-galactosidase (116 kD), phosphorylase B (93 kD), and BSA (68 kD).

sera are not blocked by the α_3-peptide and are differentially affected by the two α_5-peptides (Fig. 2 *C*, antisera *2* and *4*). The α_5-13mer effectively blocks Argraves' antiserum raised against this peptide and also blocks the reaction of the anti-23mer sera with the smaller α_5 cross-reactive subunit (α_{5a}) but not that with the larger one (α_{5b}). The α_5-23mer peptide blocks reaction with both α_5-related α-subunits. Similar results were obtained by immunoprecipitation of ^{125}I-labeled chicken embryo integrins (data not shown). These peptide competition data are consistent with the hypothesis that antisera to the α_5-23mer peptide recognize epitopes in the first 10 residues which are present on the larger α_{5b}-subunit in the band 1 region but not on the smaller α_{5a}-subunit in the band 2 region.

Therefore, these immunological data establish the existence of at least three distinguishable α-subunits (α_3, α_{5a}, and α_{5b}) in the chicken integrin complex. Furthermore, these α-subunits can be separately immunoprecipitated, each in complex with the common β_1-subunit (Fig. 3, lanes *b–d*), demonstrating clearly that the chicken integrin complex consists of a mixture of several different heterodimers.

Mammalian α-Subunits

To relate these results on chicken integrins to those of other groups working on mammalian cells, we also tested the antipeptide sera on extracts of surface-labeled MG63 cells (Fig. 4).

All three anti-α_5-peptide sera reacted with a broad fuzzy band and immunoprecipitated the β_1-subunit in a complex with this α_5-subunit (Fig. 4, *A*, lane *f*, and *B*). The α_5-band frequently appeared as a closely spaced doublet with all three anti-α_5-sera but we did not observe differential reactivities among the three anti-α_5-sera when tested on human cells (Fig. 4 *B*). All three were blocked by either the α_5-23mer peptide or the α_5-13mer peptide suggesting that the major epitopes recognized by these three antisera in human cells lie in the COOH-terminal 13 residues. There was no evidence for the presence in MG63 cells of larger α_5-related subunits reactive only with antisera to the large peptide.

The anti-α_3-peptide serum precipitated a sharp α-band which was also complexed with the β_1-subunit (Fig. 4 *A*, lane *b*). The major α-band in MG63 cells was not α_5-related but reacted with the anti-α_3-serum (compare Fig. 4, *A*, lane *a*, and *B*). These results are consistent with previous findings (Hemler et al., 1987; Takada et al., 1987) showing that α_3 is a major subunit on these cells while α_5 is present in relatively lower quantities. The human α_3-subunit is also recognized by a monoclonal antibody, PIB5 (Wayner and Carter, 1987), as shown in Fig. 4 *A* (lane *d*) and preclearing of the extracts with PIB5 abolished subsequent precipitation with the antiserum to the chicken α_3-peptide (Fig. 4 *A*, lane *e*) but not with the anti-α_5-peptide (Fig. 4 *A*, lane *f*). These results confirm the identity of the chicken α-subunit as α_3.

Formation of Discrete $\alpha\beta$-Complexes during Biosynthesis

Data presented in the preceding sections provide evidence that the chicken integrin complex contains several different $\alpha\beta$-heterodimers and argue strongly that the complex is not a heterotrimer or some higher oligomer containing all three of bands 1–3. To investigate this question further, we examined the formation of $\alpha\beta$-heterodimers during biosynthesis. CEFs were incubated in [^{35}S]methionine for 30 min and then chased in unlabeled methionine for varying periods before extraction and immunoprecipitation with antisera against β_1 or α_3. The results are shown in Fig. 5. After the pulse, one can detect precursor to α_3 using the anti-α_3-14mer serum (Fig. 5 *C*, lane *0*). At the same time the anti-β_1-serum precipitates precursor β_1 and what appear to be at least two different precursors to α-subunits (Fig. 5 *B*, lane *0*). After 1 h of chase, antisera to β_1 precipitate precursor β_1, a small amount of mature β_1, and mature α's of both band 1- and band 2-size classes. The anti-α_3-serum precipitates only the mature α_3-subunit (in the band 2 region) along with some precursor β_1 and some mature β_1. Rather slowly over the next few hours, the precursor β_1 matures to its final form which is precipitated by both antisera (β_1 and α_3). Comparison of Fig. 5, *B* and *C*, shows clearly that the antibody against α_3-peptide selectively precipitates an $\alpha_3\beta_1$-complex at all chase times while the β_1-antibody precipitates a mixture of several different α-subunits along with the β_1-subunit. This provides further evidence in support of independent, discrete $\alpha\beta_1$-heterodimers in chicken cells. The complex pattern of bands precipitated by antibodies reactive with β_1 (including CSAT and JG22 monoclonals; Buck et

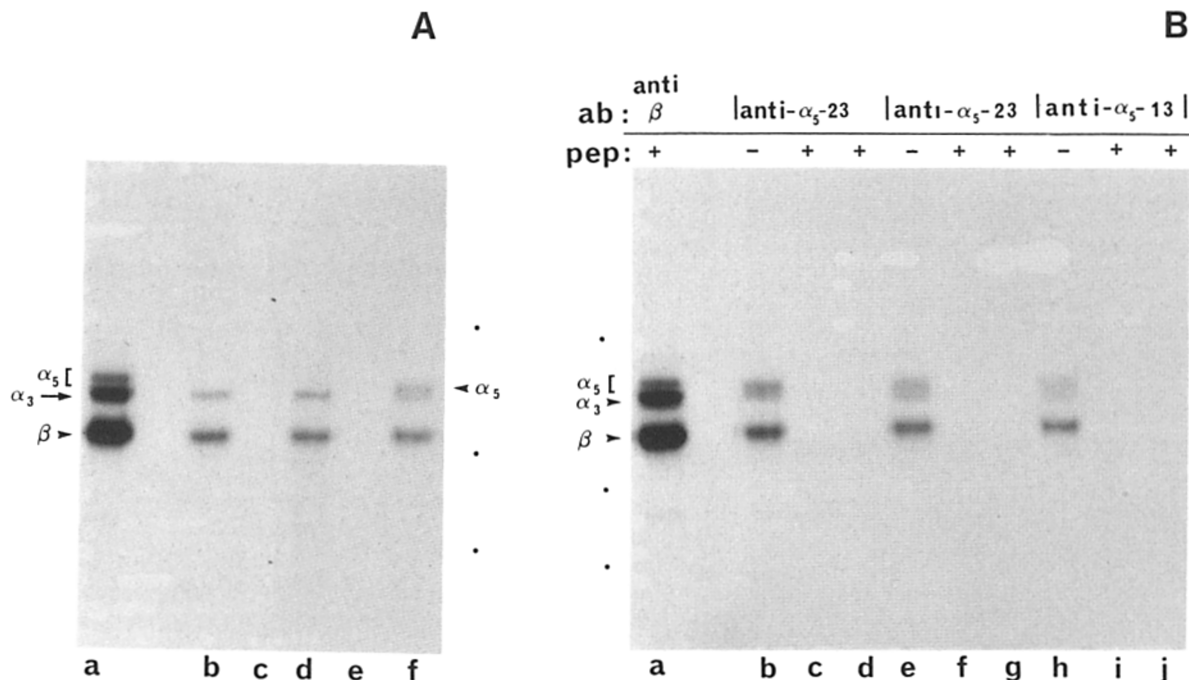

Figure 4. Immunoprecipitation of integrin complexes from MG63 cells. (*A*) Identification of $\alpha_3\beta_1$-complex. Extracts of ^{125}I surface-labeled MG63 cells were incubated with (lane *a*) anti-β_1-peptide serum with 10 μg of α_3-peptide added; (lane *b*) anti-α_3-peptide serum; (lane *c*) anti-α_3-peptide serum with 10 μg of α_3-peptide; (lane *d*) anti-ECMR I antibody, PIB5, plus rabbit anti-mouse IgG–Sepharose; (lane *e*) anti-α_3-peptide serum after two cycles of preclearing with anti-ECMR I antibody plus rabbit anti-mouse IgG–Sepharose; and (lane *f*) anti-α_5-23mer peptide serum after preclearing with anti-ECMR I antibody PIB5. The samples (*a–c*, *e*, and *f*) were immunoprecipitated using protein A–Sepharose and analyzed by SDS-PAGE (nonreduced) followed by autoradiography. Molecular mass markers shown by dots at the right as in Fig. 3. (*B*) Immunoprecipitation of $\alpha_5\beta_1$-complexes. Extracts of ^{125}I surface-labeled MG63 cells were incubated with (lane *a*) anti-β_1-peptide serum with 10 μg each (+) of both α_5-13mer and α_5-23mer peptides added; (lane *b*) anti-α_5-23mer peptide serum #160; (lanes *c* and *d*) anti-α_5-23mer peptide serum #160 with 10 μg (+) of either α_5-13mer peptide (lane *c*) or α_5-23mer peptide (lane *d*); (lane *e*) anti-α_5-23mer peptide serum #161; (lanes *f* and *g*) anti-α_5-23mer peptide #161 serum with 10 μg (+) of either α_5-13mer peptide (lane *f*) or α_5-23mer peptide (lane *g*); (lane *h*) anti-α_5-13mer peptide serum; (lanes *i* and *j*) anti-α-13mer peptide serum with 10 μg(+) of either α_5-13mer peptide (lane *i*) or α_5-23mer peptide (lane *j*). The samples were immunoprecipitated using protein A-Sepharose and analyzed by SDS-PAGE (nonreduced) followed by autoradiography. Molecular mass markers are shown by dots at left. The three α_5-antisera all react with $\alpha_5\beta_1$-complexes and this precipitation is blocked by either α_5-13mer or α_5-23mer peptides.

al., 1986) is due to precipitation of a variety of different $\alpha\beta$-complexes.

The data in Fig. 5 also indicate that $\alpha\beta$-complexes form in the cells before complete processing of the subunits. Thus, antibodies against β_1 precipitate precursor α-subunits at early times (Fig. 5 *B*, lane *0*) and, conversely, antibody against α_3 precipitates precursor β_1 (Fig. 5 *C*, lane *1*). This is more clearly seen in a similar experiment (Fig. 6) performed on MG63 cells. These cells have a simpler pattern of α-subunits among which α_3 predominates (Fig. 4). At 0, 1, and 2 h of chase the precursor form of α_3 is evident. The precursor form of β_1 is coprecipitated by antibodies to α_3 (Fig. 5 *C*, lanes *1* and *2*) although it is not labeled stoichiometrically with the α_3-subunit. This is because mammalian cells contain a larger, intracellular pool of presynthesized β_1-subunit which can also combine with α-subunits but does not appear as a labeled species; it is seen by immunoblotting with anti-β_1-sera (data not shown). The presence of this large pool of immature β_1-subunits is reflected in the very slow processing during the chase of the precursor to β_1 (Fig. 6 *B*). Labeled precursor β_1 is still in excess over mature β_1 at 12 h of chase and still present after 24 h (Fig. 6). In contrast, the avian β_1-subunit is largely in the mature form by 6 h of chase and completely matured by 12 h (Fig. 5). However, apart from these differences in kinetics, the process of $\alpha\beta$-assembly appears similar in avian and mammalian cells (see also Discussion).

Ligand Specificity of Chicken Integrins

Previous attempts to demonstrate direct interaction of purified chicken integrins with affinity columns of candidate ligands have been complicated by the low affinity of the interactions (Akiyama et al , 1985). To demonstrate these low affinity interactions, Horwitz and colleagues (Horwitz et al., 1985; Buck et al., 1986) have used equilibrium-gel filtration chromatography. We have attempted to fractionate purified, iodinated, chicken integrin complex by affinity chromatography and have similarly observed low levels of interaction. However, we have been able to demonstrate interaction of chicken integrins with affinity columns using either total extracts of surface-labeled CEFs, or purified integrins bound directly without prior iodination.

Fig. 7 *A* shows fractionation of an extract of surface-labeled CEFs on a column of GRGDSPC–Sepharose. Bound integrins were eluted by GRGDSP but not by GRGESP, as shown for other cell types (Pytela et al., 1985*b*, 1986;

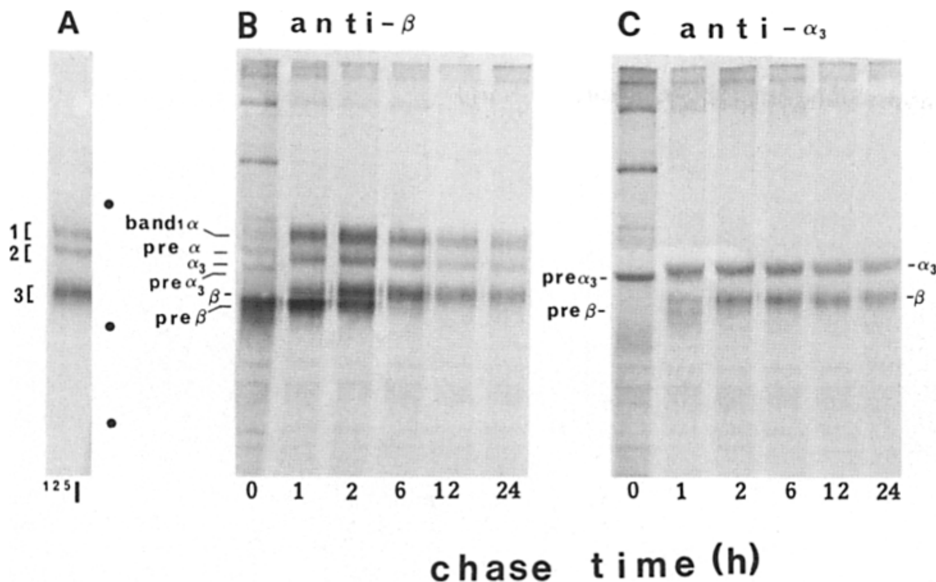

Figure 5. Biosynthesis and assembly of integrin complexes in CEF. CEFs were labeled with [^{35}S]methionine for 30 min and then chased for various times (*h*) with growth medium as indicated at the bottom of the figure. Labeled cells were then washed, extracted, and immunoprecipitated using anti-β_1-peptide serum (*B*) or anti-α_3-peptide serum (*C*) followed by SDS-PAGE (nonreduced) and fluorography. Molecular mass markers (as in Fig. 3) are indicated by dots on the left and integrin bands *1*, *2*, and *3* are marked on the ^{125}I surface-labeling profile (*A*).

Cheresh, 1987; Cheresh and Spiro, 1987; Lawler et al., 1988). The bound material was analyzed by immunoprecipitation with various antibodies (Fig. 7 *B*). The major avian integrin complex selected by the GRGDSP column appears distinct from the chicken integrin complex defined by the CSAT monoclonal antibody (data not shown) or by antisera against the cytoplasmic domains of β_1 or α_3 (Fig. 7 *B*). The avian RGD-binding complex is, however, precipitated by a monoclonal antibody (LM609) raised against a β_3-class integrin complex isolated from human melanoma cells (Cheresh and Spiro, 1987) as shown in Fig. 7 *B*.

Therefore, the GRGDSP column selects a β_3-integrin from extracts of chicken cells. This integrin is presumably the avian homologue of human receptors which bind to GRGDSP columns (Pytela et al., 1985*b*, 1986; Cheresh, 1987; Cheresh and Spiro, 1987; Lawler et al., 1988). Avian β_1-integrins are poorly bound by this column, although we frequently detected a trace amount of what appears to be β_1 in the GRGDSP eluates of this column (data not shown).

We turned next to affinity chromatography on columns coupled with the FNf (FNf–Sepharose). In early experiments using buffers containing $MgCl_2$ and $CaCl_2$, we observed only a small amount of binding of chicken integrins as reported by others (Akiyama et al., 1985). However, substitution of $MnCl_2$ as suggested by Gailit and Ruoslahti (1988) greatly enhanced the binding. Fig. 8, *A* and *B*, shows fractionation of BOG extracts of iodinated CEFs on columns of FNf–Sepharose. Much more material bound and was eluted specifically by GRGDSP when the buffers contained $MnCl_2$. The bound species migrated in the positions of bands 1 and 3. We repeated the experiment with integrins purified from chicken embryo extracts. While the starting sample contained all three of bands, 1, 2, and 3, and subunits reacting with each of the antisera to α_3 and α_5 (Fig. 9 *A*),

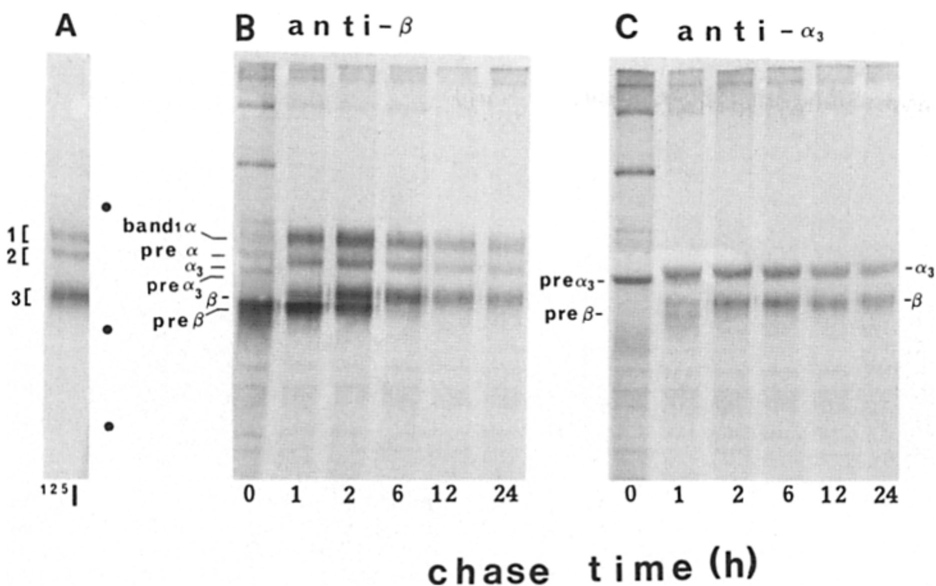

Figure 6. Biosynthesis and assembly of integrin complexes in human MG63 cells. MG63 cells were labeled with [^{35}S]methionine for 30 min and chased for various times (*h*) with growth medium as indicated at the bottom of the figure. Labeled cells were washed and extracts were immunoprecipitated with either anti-β_1-peptide serum (*B*) or anti-α_3-peptide serum (*C*) followed by SDS-PAGE (nonreduced) and fluorography. (*A*) The mature integrin, ^{125}I-surface-labeled profile is shown.

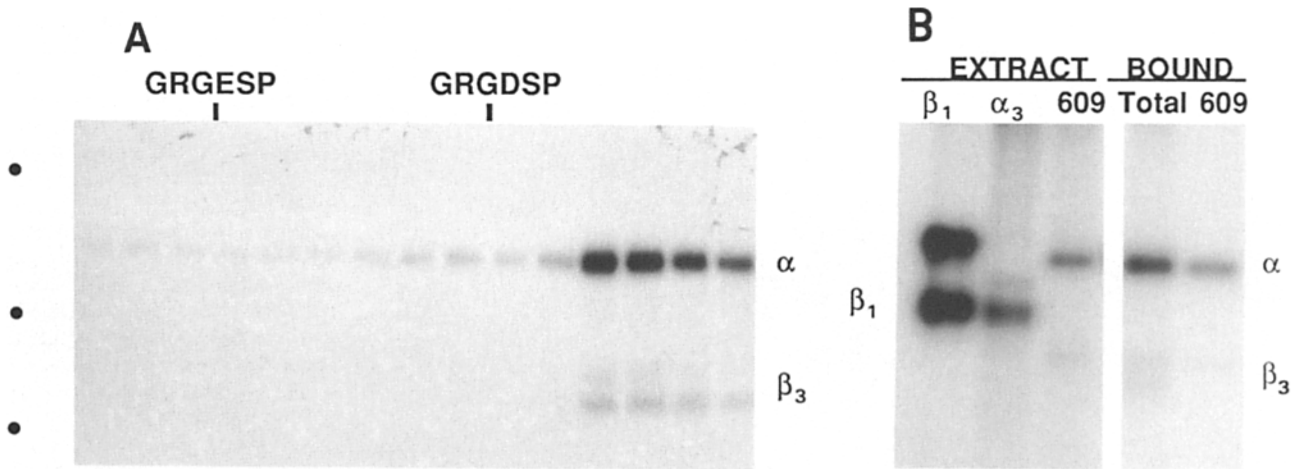

Figure 7. Affinity chromatography on GRGDSPC–Sepharose. (*A*) CEFS were iodinated and extracted with BOG buffer. The extracts were passed through a column of GRGDSPC–Sepharose; the column was then washed and eluted sequentially with GRGESP and GRGDSP. The latter peptide selectively eluted bands of approximate molecular masses of 130, 80, and 75 D. Molecular mass markers are indicated by dots from top to bottom: myosin (200 kD), phosphorylase B (97 kD), and BSA (68 kD). (*B*) The total extract or the GRGDSP-eluted material was immunoprecipitated with the antibodies indicated. The bound material was precipitated by LM609, a monoclonal antibody against β_3-integrins. The lowest band was variable in different experiments and probably represents a degradation product.

the specifically bound and eluted material contained predominantly bands 1 and 3 (Fig. 9 *B*), which could be immunoprecipitated by antibodies to β_1 or to human α_5-23mer (Fig. 9 *C*). No material reactive with antisera to α_3 or to α_5-13mer was detected in the bound fractions (Fig. 9 *C*), although cross-reactive species were detected in the starting sample (Fig. 9 *A*) and in the unbound material (data not shown). When a similar experiment was performed with MG63 human cells, $\alpha_5\beta_1$, which was reactive with all three of the anti–α_5-peptide sera, bound to the column, while $\alpha_3\beta_1$ did not (Fig. 10).

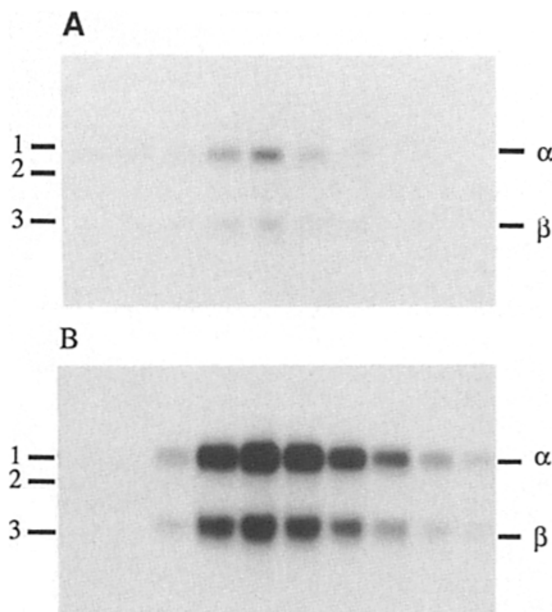

Figure 8. Fibronectin affinity chromatography of CEF extracts. Cells were iodinated, extracted with BOG in the presence of $MgCl_2$ and $CaCl_2$ (*A*) or $MnCl_2$ (*B*). Extracts were passed over columns of FNf–Sepharose. The columns were washed well and eluted with GRGDSP. Two bands were bound and eluted under both salt conditions, but much more material was recovered using buffers containing $MnCl_2$. Migration positions of bands *1*, *2*, and *3* are marked.

Thus, both human and chicken $\alpha_3\beta_1$-integrins fail to bind to FNf–Sepharose at physiological salt concentrations. α_5-related integrins from both humans and chickens do bind to FNf–Sepharose but we have been unable to detect binding of the smaller α_5 cross-reactive species (α_{5a}) from chicken integrins. Whether this reflects the low levels present or another technical problem, or whether this integrin does not interact with FNf is currently unclear (see Discussion). However, it is clear that the larger α_5-related β_1-integrin from chickens ($\alpha_{5b}\beta_1$) does bind to FNf–Sepharose and is, therefore, a fibronectin receptor by this criterion.

Discussion

We have presented data that establish the following points. (*a*) The chicken integrin complex originally defined by adhesion-blocking antibodies contains multiple different α-subunits each of which occurs as a heterodimer with the common β_1-subunit. (*b*) The α-band originally described as band 2 contains at least two different α-species: a minor α_5-reactive subunit; and another, slightly smaller, α-subunit which reacts with antibodies against a peptide defined by a chicken cDNA clone. We designate these two "band 2 α-subunits" α_{5a} and α_3 (see below). (*c*) Band 1 contains another α-subunit (α_{5b}) which is cross-reactive with some but not all antisera raised against human α_5-sequences. This α-subunit complexed with β_1 binds to columns of fibronectin cell-binding fragments. The α_{5b}-species appears to be distinct from α_{5a}. (*d*) CEFs also bear yet another integrin, from the β_3-class, which binds to GRGDSP columns and is immunologically cross-reactive with human β_3-integrin receptors isolated in similar fashion. (*e*) In both avian and mammalian cells, discrete $\alpha\beta$-complexes form before complete processing of the carbohydrates of the subunits, that is, in a *pre*-Golgi compartment.

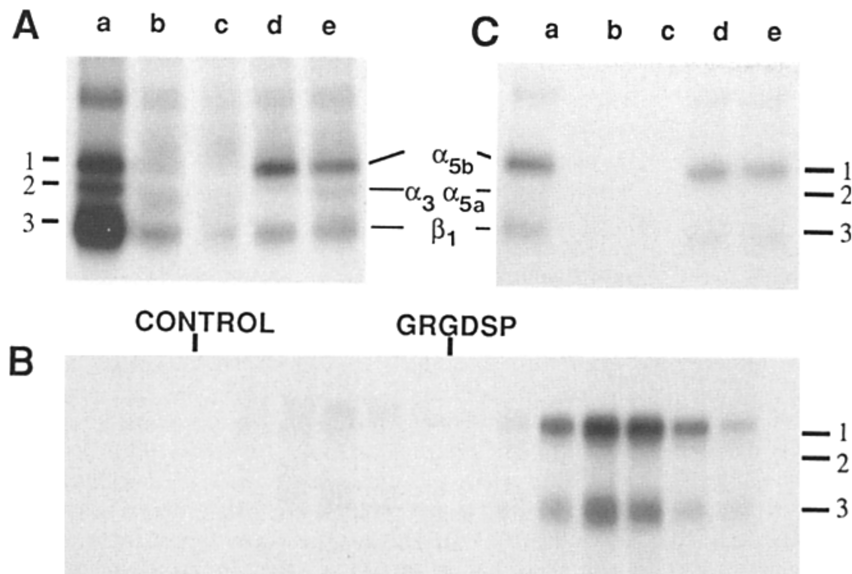

Figure 9. Fibronectin affinity chromatography of purified chicken embryo integrins. Purified integrins from chicken embryo extracts were chromatographed on a column of FNf-Sepharose. After washing, the column was eluted with RNGESQ followed by GRGDSP. Starting material and eluted fractions were iodinated with Iodobeads and some samples were immunoprecipitated. (*A*). Starting material immunoprecipitated with the following antisera: lane *a*, anti-β_1; lane *b*, anti-α_3; lane *c*, anti-α_5-13mer; lane *d*, anti-α_5-23mer #160; and lane *e*, anti-α_5-23mer #161. Migration positions of bands *1-3* and specific α- and β-integrin subunits are marked. (*B*) Elution profile. The total samples are shown without immunoprecipitation. Migration positions of bands *1-3* are marked. (*C*) Immunoprecipitation of peak eluted fractions (see *B*) with the same antisera as in *A*. The eluted bands comprise an $\alpha_{5b}\beta_1$-heterodimer. α_3 and α_{5a} are not detectable.

Integrin Heterodimer Complexity

One message derived from our data is that the integrins on avian cells are very similar to those on mammalian cells. Both β_1- and β_3-class integrins are present and the previously defined chicken integrin complex is the avian equivalent of the human VLA antigens. Both the VLA antigens and the chicken integrin complex have a common β_1-subunit; the β_1-subunits of the two species are 85% identical in sequence (Tamkun et al., 1986; Argraves et al., 1987; DeSimone and Hynes, 1988). The β_1-subclass of integrins in both species is complex; at least six α-subunits have been defined on human cells and we present direct evidence here for three different α-subunits in chickens each of which forms a separate heterodimer with the β_1-subunit (Fig. 3).

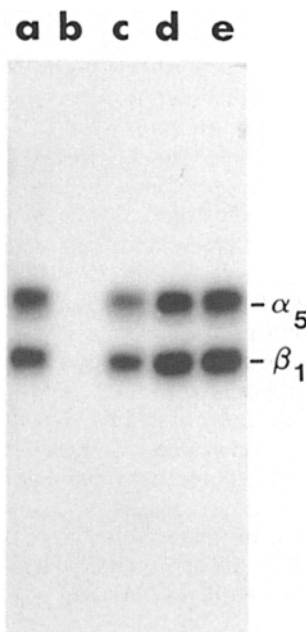

Figure 10. Fibronectin-binding integrins from MG63 cells. Material isolated from extracts of iodinated MG63 cells by affinity chromatography on a column of FNf was immunoprecipitated with the following antisera: lane *a*, anti-β_1; lane *b*, anti-α_3; lane *c*, anti-α_5-13mer; lane *d*, anti-α_5-23mer #160; and lane *e*, anti-α_5-23mer #161. Note the presence of an $\alpha_5\beta_1$-heterodimer reactive with all antisera against α_5-peptides and the absence of $\alpha_3\beta_1$.

How do the avian α-subunits relate to the better defined human VLA α-subunits? The chicken α-subunit cDNA clone (Fig. 1) defines an α-subunit which we have referred to as α_3. The evidence is as follows. (*a*) Antibodies to a peptide defined by this sequence react with avian integrin band 2 which is known also to cross-react with antibodies to human VLA-α_3 (Takada et al., 1987). (*b*) Antibodies to the chicken peptide cross-react with the major α-subunit on MG63 cells (Fig. 4 *A*); α_3 is the major VLA α-subunit on these cells (Hemler et al., 1987; Takada et al., 1987). (*c*) A monoclonal antibody, P1B5, which reacts with an extracellular matrix receptor (ECMR I) with affinity for fibronectin, laminin, and collagen (Wayner and Carter, 1987) has been shown to react with VLA-α_3 (Takada et al., 1988) and preclears material reactive with our anti-chicken α_3-peptide serum (Fig. 4 *A*). (*d*) Comparison of the chicken sequence with a partial sequence of a human cDNA clone isolated with antibodies to VLA-α_3 (Hemler, M. E., and Y. Takada, personal communication) shows strong homology; 24 out of 30 identical residues in the COOH-terminal cytoplasmic domain sequence. These two clones also cross-react on Southern blots (Stepp, M. A., Y. Takada, M. E. Hemler, and R. O. Hynes, unpublished data) whereas α_3 and α_5 cDNA clones do not cross-react with each other. These data provide a reasonably strong argument that this is indeed the chicken α_3-subunit, which is one constituent of band 2.

A slightly larger α-subunit (α_{5a}) is recognized by three antisera raised against two different peptides from human α_5 (Figs. 1-4). A complication arises from the fact that two of the antisera raised against human α_5-peptides recognize, in addition, an even larger α-subunit in chickens (Figs. 2 and 3). This subunit (α_{5b}) migrates in the band 1 region and is not recognized by Argraves' antiserum raised against a shorter α_5-peptide. Chicken α_{5a} shares epitopes with human α_5 which are contained in the last 12 residues of the cytoplasmic domain (EKAQLKPPATSDA; Fig. 1). In contrast, chicken α_{5b} shares epitopes with human α_5 which are contained in the first 10 residues of the cytoplasmic domain (KRSLPYGTAM; Fig. 1) but does not share epitopes con-

tained in the sequence EKAQLKPPATSDA (Fig. 2 *C*). The simplest interpretation of these results is that α_{5a} and α_{5b} in chickens represent two α-subunits with different amino acid sequences, each of which happens to be related to the sequence of human α_5.

Alternative explanations are that some form of posttranslational modification or experimental artefact (aggregation, degradation, or conformational change) distinguishes the two forms, α_{5a} and α_{5b}. Since all the antisera are raised against cytoplasmic domain peptides, glycosylation seems an unlikely possibility. Since antibodies against the shorter peptide, which is the COOH terminus, recognize the smaller subunit (α_{5a}) but not the larger one (α_{5b}), degradation is also an unlikely explanation. As discussed in the Results section, the three different sera react differentially with α_{5a} and α_{5b} even when all are tested in parallel (on the same integrin preparations on the same gel) under both native and denatured conditions (Figs. 2 and 3). Therefore, it is difficult to see how aggregation or conformational change could account for the results. Nonetheless, it is impossible to eliminate completely the possibility that α_{5a} and α_{5b} share the same primary sequence but differ in some other way, although this seems unlikely.

The biosynthetic data (Figs. 5 and 6) indicate that the different $\alpha\beta_1$-heterodimers appear to assemble independently from a pool of precursors of β_1 and the various α-subunits. The presence of $\alpha\beta_1$-complexes containing the precursor forms of both subunits suggests that the complexes form before completion of the carbohydrate side chains; that is, in an early Golgi or *pre*-Golgi compartment. Similar results have been described for a β_3-integrin in melanoma cells (Cheresh and Spiro, 1987). There appears to be a large steady-state pool of β_1 precursor, particularly in mammalian cells. This pool of β_1 precursor can combine with the various α precursors. When the rate of biosynthesis of α-subunits is elevated by transforming growth factor-β, the rate of processing of β_1 precursor is accelerated (Ignotz and Massague, 1986; Heino et al., 1988).

Ligand Specificity

Turning to the question of ligand specificity, we conclude that the chicken β_1-integrin complex contains at least two fibronectin receptors and possibly more.

The argument is as follows. $\alpha_3\beta_1$ in humans has been shown to be a receptor for fibronectin, laminin, and collagens (Wayner and Carter, 1987; Wayner et al., 1988; Takada et al., 1988). In humans, $\alpha_3\beta_1$ does not bind to fibronectin affinity columns at physiological salt concentrations (Fig. 10), although it will bind in low salt (Wayner and Carter, 1987). We also observe that chicken $\alpha_3\beta_1$ does not bind to fibronectin affinity columns (Fig. 9). Comparison of the antibody blocking and affinity chromatography data suggests that human $\alpha_3\beta_1$ is a low affinity receptor for fibronectin and also a receptor for other ligands. We presume that the same is true in chickens, although formal proof requires cross-reactive α_3-specific blocking antibodies which are not yet available.

In humans, a different fibronectin receptor was defined originally by affinity chromatography and liposome binding assays (Pytela et al., 1985*a*, 1986). cDNA clones isolated using antibodies to this fibronectin receptor (Argraves et al., 1987) predict an NH_2-terminal sequence which is the same as that published for human VLA-5 (Hemler et al., 1987). It has, therefore, been widely assumed that α_5 is the α-subunit of the original fibronectin receptor.

Which of the two α_5-related chicken subunits is the true homologue of human α_5? The answer depends somewhat on the definition of α_5. We started from published sequences (Argraves et al., 1987; Fitzgerald et al., 1987) which were obtained from cDNA clones isolated from placental or endothelial cell cDNA libraries using either polyclonal antibodies or degenerate oligonucleotides. It has been assumed that this sequence corresponds with the α-subunit of the integrin heterodimer which binds to columns of FNf. Indeed, all three antipeptide antibodies used in this paper do react with the α-subunit of human integrin isolated from MG63 cells on such columns (Fig. 10). If one defines the human fibronectin-binding α-subunit as α_5, it appears most similar in size to chicken α_{5a} (compare Figs. 3 and 4). However, we have been unable to demonstrate binding of chicken α_{5a} to affinity columns of FNf (Figs. 8 and 9; and unpublished data). This may be because of its rarity on the surfaces of CEFs and in preparations of integrins from chicken embryos, or it could be a technical problem. As noted above, $\alpha_3\beta_1$ does not bind to such columns under similar conditions even though it is a fibronectin receptor by the criterion of antibody inhibition of cell adhesion to fibronectin (Wayner and Carter, 1987; Wayner et al., 1988). In contrast, $\alpha_{5b}\beta_1$ from either fibroblasts or embryos binds well and specifically to fibronectin-affinity columns (Figs. 8 and 9). By this criterion, α_{5b} appears functionally analogous with human α_5, defined by its affinity for fibronectin.

Johansson et al. (1987*a*,*b*) have described three different fibronectin receptors isolated from different rat cell types on affinity columns of fibronectin cell-binding fragments. They appear to be integrins and to share a common β-subunit but have different α-subunits. The α-subunit from rat endothelial cells is significantly larger than the ones from hepatocytes or fibroblasts. It is conceivable that these various α-subunits could include the rodent homologues of α_{5a} and α_{5b} in chickens. We frequently observe a doublet of two α_5-related bands also on human cells (e.g., Fig. 4).

For the present, we think it wisest to designate the two chicken α-subunits α_{5a} and α_{5b}, to denote their cross-reactivity with antibodies to human α_5. It is currently unclear which is the true structural homologue of the sequenced human α-subunit and, we feel, it is also uncertain whether the published human sequence can be unambiguously related to a single fibronectin receptor.

Further research is obviously necessary to elucidate the nature of the various fibronectin receptors. In the context of the present work, the main point which we wish to make is that the chicken integrin β_1-complex contains avian homologues of two known human integrins, $\alpha_5\beta_1$ and $\alpha_3\beta_1$. In addition, like the mammalian integrins, the avian integrins are discrete heterodimers. The original complex of proteins defined by the monoclonal antibodies, CSAT and JG22, comprises a mixture of several such heterodimers. Included in the mixture are at least two fibronectin receptors and possibly more.

Direct binding experiments with purified chicken integrins (Horwitz et al., 1985; Buck et al., 1986) and antibody blocking results with the monoclonal antibodies CSAT and JG22 (Decker et al., 1984; Tomaselli et al., 1988), both of which

react with the β_1-subunit (Buck et al., 1986), demonstrate that the β_1-integrins in chickens also include receptors for laminin and collagen. These could include polyspecific receptors such as $\alpha_3\beta_1$ (Wayner and Carter, 1987) or specific receptors for collagen (Wayner and Carter, 1987) or laminin (Gehlsen et al., 1988; Ignatius and Reichardt, 1988). It is not clear from our results whether chicken $\alpha_{5b}\beta_1$ is a fibronectin-specific receptor like human $\alpha_5\beta_1$ or a polyspecific receptor like $\alpha_3\beta_1$. However, it is clear that the β_1-class of integrins in both humans and chickens is complex both structurally and functionally.

CEFs express, in addition, a β_3-class integrin (Fig. 7) which is functionally and immunologically related to RGD receptors isolated from a variety of human cell types (Pytela et al., 1985*b*, 1986; Cheresh, 1987; Cheresh and Spiro, 1987; Lawler et al., 1988). It is currently unclear whether all these human receptors are identical or merely similar. The β_3-integrin isolated from MG63 cells on RGD columns appears specific for vitronectin (Pytela et al., 1985*b*, 1986), whereas those isolated in similar fashion from melanoma, endothelial, or smooth muscle cells have a much wider ligand specificity, including fibrinogen, von Willebrand factor, and thrombospondin, in addition to vitronectin (Cheresh, 1987; Cheresh and Spiro, 1987; Lawler et al., 1988). These β_3-integrins are not constituents of the original chicken integrin complex isolated by monoclonal antibodies, but they clearly contribute to the complexity of the integrin receptor complement on chicken cells.

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