Down-regulation of the chicken $\alpha_5\beta_1$ integrin fibronectin receptor during development

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Summary

We have characterized the diversity of the chicken $\beta_1$ integrin family and studied the expression of individual receptors during development. The diversity of the $\beta_1$ integrin family was investigated by affinity purifying the $\beta_1$ integrins from a variety of adult and embryonic tissues. These purifications reveal the relative levels of expression and also the differential expression of the $\alpha$ subunits in those tissues. Monoclonal antibodies were generated against the prominent 'band 1' of the embryonic chicken integrins and used to characterize the expression of this $\alpha$ subunit in embryonic and adult tissues. This $\alpha$ subunit is shown to be the chicken homologue of human $\alpha_5$ fibronectin receptor. The chicken $\alpha_5\beta_1$ integrin is the most prominent $\beta_1$ integrin in the embryo and is expressed on the majority of cell types through the day 17 stage. The distribution of this receptor in the embryo closely parallels the distribution of its ligand, fibronectin. In adult tissues, expression of this receptor is greatly diminished relative to the expression of other $\alpha$ subunits. The cell type distribution is highly restricted; limited primarily to the vasculature and to connective tissue regions. These studies reveal a prominent role for the $\alpha_5\beta_1$ integrin in embryonic cell types and a down-regulation of this receptor on many cell types during development.

Abbreviations: ECM, extracellular matrix; FN, fibronectin; LM, laminin; Col, collagen; mAb, monoclonal antibody.

Key words: chick $\alpha_5\beta_1$ integrin, receptor, fibronectin, laminin, collagen, monoclonal antibody.

Introduction

The $\beta_1$ integrins are a family of cell surface receptors with specificity for molecules in the extracellular matrix (ECM) (Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Buck and Horwitz, 1987). These receptors also associate with the cytoskeleton via their cytoplasmic domain and therefore function as transmembrane links between the cytoskeleton and the ECM (Horwitz et al. 1986; Otey et al. 1990; Argraves et al. 1989). The integrins are $\alpha\beta$ heterodimers formed by two non-covalently linked glycoproteins both of which have a small cytoplasmic domain, a single membrane spanning region, and a large extracellular domain (Tamkun et al. 1986; Argraves et al. 1987). Diversity in the $\beta_1$ integrin family is generated by a variety of $\alpha$ subunits, which associate with the common $\beta_1$ subunit to form unique $\alpha\beta$ heterodimers. As the $\beta$ subunit is constant in each receptor, the ligand specificity of the receptor is conferred by the variable $\alpha$ subunit. The $\beta_1$ integrin family is best characterized in humans where at least seven different receptors (i.e. different $\alpha$ subunits) have been identified. Each heterodimer appears to function as a receptor for ECM molecules. Among them are receptors for collagen types I and IV, laminin and fibronectin (for reviews see Hemler, 1990; Albeda and Buck, 1990).

Many functions have been attributed to the $\beta_1$ integrin family. By mediating the interaction of the cellular cytoskeleton with the ECM, the $\beta_1$ integrins are central to issues of cell migration, proliferation, differentiation, morphology, and the maintenance of tissue architecture and integrity. Antibodies against the $\beta_1$ subunit reveal its presence on virtually every cell type in the animal systems studied thus far (Buck and Horwitz, 1987). Anti-$\beta_1$ antibodies have been shown to perturb cell adhesion to matrix molecules (Neff et al. 1982), alter cell morphology (Greve and Gottlieb, 1982), perturb cell migration (Bronner-Fraser, 1986) and neurite outgrowth (Bozycko and Horwitz, 1986; Tomasselli et al. 1986), and inhibit cell differentiation (Menko and Boettiger, 1987; Adams and Watt, 1989). The $\beta_1$ integrins are also thought to play an important role in the morphogenetic events of embryonic development by conferring selective adhesive properties to cells.

Despite the many functions attributed to the $\beta_1$ integrin family, little is known about the details of $\beta_1$ integrin function as played out by the individual $\alpha\beta$ heterodimeric receptors. The localization, expression pattern and in vivo role of each individual receptor is only beginning to be addressed. Recently, progress in this area has accelerated as antibodies for many of the human $\alpha$ subunits have been described (Hemler, 1990).
However, a comparable library of antibodies directed against α subunits in systems appropriate for embryologic studies has not yet been developed. Consequently, our understanding of β1 integrin function during embryonic development is still relatively primitive.

We have initiated a study to characterize the diversity of the chicken β1 integrin family and study the regulation and functions of the individual αβ heterodimers during development. In this paper, we use affinity purification of the β1 integrins to reveal the diversity of α subunits expressed in several embryonic and adult tissues. We then use monoclonal antibodies generated against the chick embryo ‘band 1’ (Knudsen et al. 1985; Chen et al. 1985) to identify a chicken α subunit homologous to the human α5 subunit (fibronectin receptor) (Pytel, 1985) and to study the expression of this receptor in embryonic and adult tissues. The relative expression of the different α subunits seen in the β1 integrin purifications reveals the prominence of this chicken α subunit in tissues of the embryo, and its general down-regulation in the adult. Immunohistochemical studies reveal the widespread cell type distribution of the α5β1 integrin in the embryo, a distribution comparable to its ligand, fibronectin. These studies also reveal the restricted expression of the α5β1 integrin in adult tissues, and its down-regulation on many cell types during development.

Materials and methods

Purification of integrins

Chicken integrins were purified by the method of Knudsen et al. (1985) with minor modifications. One volume of tissue was added to four volumes of extraction buffer (10 mM Tris-HOAc pH 8.0, 0.15 M NaCl, 0.5 mM CaCl2, 0.5 mM MgCl2, 0.5% NP-40, 0.1 mM PMSF, 1.0 mM o-phenanthroline, 15 mM pepstatin, 2.1 mM leupeptin) and homogenized twice for 30 s in a Waring blender. The homogenate was centrifuged at 27,000g for 20 min at 4°C. The supernatant was brought to 20 mM pH 8.0 with 1.0 M HOAc, pooled, and dialyzed against complete adjuvant was used. Two weeks after the second immunization the sera from both mice tested positive for ‘band 1’ on immunoblots. A fusion was performed using one of these mice three days after the third immunization. The fusion was performed using an SP2/0 myeloma cell line by the methods of Kennett et al. (1982). Six hybridomas were identified as specific for protein within the chicken embryo integrin ‘band 1’. Three were used in this study; they are designated α5A2, α5B2 and α5D7. Having been generated against denatured protein, these antibodies immunoblot well, immunostain fixed tissue sections best when the sections are unprocessed, and they do not appear to precipitate the native receptor. The final supernatant was neutralized to pH 8.0 with 1.0 M Tris base and, if necessary, filtered through Whatman No. 1 filter paper (Whatman Inc., Clifton, NJ). This extract was then passed over a 3 ml CSAT mAb affinity column (Neff et al. 1982) at a flow rate of 30 ml/h at 4°C. The column was washed with 20 column volumes of extraction buffer and eluted with 50 ml diethylamine in extraction buffer, pH 11.5, with the detergent decreased to 0.05% NP-40. Fractions were neutralized to pH 8.0 with 1.0 M HCl, pooled, and dialyzed against extraction buffer. Samples were often concentrated before dialysis using Centriprep 10 concentrators (Amicon, Danvers, MA).

The 150×10^6 Mf chicken α subunit was purified by first denaturing the purified chick embryo integrin heterodimers and passing these proteins over an affinity column constructed from the anti-chick α subunit monoclonal antibody (α5A2, see below). The embryonic integrin heterodimers, stored in extraction buffer, were denatured by adding guanidine isothiocyanate (Sigma, St Louis, MO) to a final concentration of 6.0 M and heating the sample to 60°C for 15 min. The sample was dialyzed once against 200 volumes of extraction buffer before passing over the affinity column. The column was then washed and eluted by the method described above for the CSAT column.

CSAT and α5A2 affinity columns were constructed by coupling 5 mg/ml 1 pure monoclonal antibody to CNBr-activated Sepharose 4B (Pharmacia, Piscataway, NJ). The day 11 chicken embryos were prepared from decapitated and eviscerated embryos rinsed in cold PBS and stored at −70°C. The individual embryonic tissues were dissected, rinsed and stored similarly. Most adult tissues were obtained from Pel-Freeze Biologicals (Rogers, Ark) and stored at −20°C. The only exception was the adductor femoris skeletal muscle and small intestine which were dissected in our laboratory from adult K-strain Leghorn chickens.

**Protein electrophoresis**

SDS–PAGE was performed by the method of Laemmli (1970). Separating gels were 7% acrylamide and 0.12% bis-acrylamide. Samples were prepared in sample buffer (62.5 mM Tris–HCl pH 6.8, 2.0% SDS, 10% glycerol) and heated at 60°C for 15 min. All gels were run at 4°C and were visualized by silver staining using Bio-Rad Silver Stain kit (Bio-Rad, Richmond, CA).

**Electroelution**

Electroelution was performed according to the method of Hankapiller et al. (1983) using the procedure described for elution at 4°C. The electroelution apparatus was obtained from Isoce, Inc. (Lincoln, NE). The eluant removed from the sample collection chamber was lyophilized in a Speedvac evaporator (Savant Instruments, Inc., Farmingdale, NY), resuspended in 100 μl deionized water and precipitated with 4 volumes methanol/acetone (1:1) overnight at 4°C to remove salts. Protein yield was estimated by comparison on silver-stained protein gels with samples of known concentration.

**Antibody production**

Nine-week-old female Balb/c mice were immunized at 21 day intervals with 10–25 μg of protein electroeluted from the ‘Band 1’ region of the chick embryo integrins (Knudsen et al. 1985). For the first immunization the antigen was mixed (1:1) with Freund's complete adjuvant (Gibco Laboratories; Life Technologies Inc., Gränd Island, NY) to a final volume of 0.5 ml/mouse and injected into the interperitoneal cavity. Subsequent immunizations were identical except Freund's incomplete adjuvant was used. Two weeks after the second immunization the sera from both mice tested positive for ‘band 1’ on immunoblots. A fusion was performed using one of these mice three days after the third immunization. The fusion was performed using an SP2/0 myeloma cell line by the methods of Kennett et al. (1982). Six hybridomas were identified as specific for protein within the chicken embryo integrin ‘band 1’. Three were used in this study; they are designated α5A2, α5B2 and α5D7. Having been generated against denatured protein, these antibodies immunoblot well, immunostain fixed tissue sections best when the sections are first heat denatured, and immunoprecipitate the monomeric α subunit after the dimeric receptor has first been denatured, but, they do not appear to precipitate the native receptor.

**Hybridoma screening**

The hybridoma supernatants were screened using a dot blot apparatus (Bio-Dot Apparatus; Bio-Rad) and immunoblot detection. A nitrocellulose filter (Schleicher and Schuell, Inc., Keene, NH) was assembled into the apparatus and the purified day 11 embryo integrins were aliquotted into the wells at a concentration of 0.5 μg/well and drawn onto the filter by vacuum. The filter was then removed, blocked with
wash buffer (10 mM Tris-HOAc pH 8.0, 0.15 mM NaCl) containing 3% gelatin, and reassembled into the apparatus. Individual supernatants to be screened were applied to the wells at a dilution of 1:1 with wash buffer containing 0.5% gelatin, and incubated with the filters for 30 min. After rinsing the supernatants from the wells the filter was removed and developed according to the remaining immunoblot procedure described below.

**Western immunoblots**

SDS-PAGE was performed as described above. The proteins were electrophoretically transferred from the gels to nitrocellulose membranes (Schleicher and Schuell, Inc.) at 4°C overnight at 40 volts using a Bio-Rad Trans-Blot Cell (Bio-Rad). The transfer buffer consisted of 25 mM Tris, 19 mM glycine, 20% methanol. Filters were blocked in wash buffer containing 3% gelatin for 1 h. All antibodies were diluted in wash buffer containing 0.5% gelatin and incubated with the filters for 30 min. Primary antibody concentrations ranged from 10 to 25 μg ml⁻¹. The filters were washed for 30 min at 37°C with three changes of wash buffer. 0.05% Tween-20 (Bio-Rad) was included in the buffer of the second wash. The biotinylated secondary antibody, biotinylated alkaline phosphatase and avidin were obtained from the Vectastain ABC-AP Kit (Vector Laboratories, Burlingame, CA) and used at half the concentration recommended in the kit instructions. The color developing reagent was the Alkaline Phosphatase Substrate Kit (Vector).

**Immunofluorescence staining**

Sections of the day 4 chick embryo were provided by M. Bronner-Fraser (UC, Davis, CA). For other sections the tissues were fixed in liquid nitrogen, embedded in O.C.T Compound (Tissue-Tek; Miles Scientific, Naperville, IL) and sectioned on a Tissue-Tek cryostat at a thickness of 6–10 microns. Sections were placed on gelatin-coated slides and blocked with 10% goat serum (Sigma) in PBS for 1 h. Primary antibodies were diluted with 5% goat serum in PBS. For the primary antibody incubations, the slides were placed on a heating plate set at 60°C. The antibodies were added and incubated for 30 min on the heating slides which were covered to prevent excessive evaporation. This procedure was required for good staining with the anti-chicken α5 antibodies and did not appear to inhibit the staining with other antibodies. After incubation with the primary antibodies, the slides were removed from the heat and washed several times with PBS at room temperature. The FITC-conjugated anti-mouse secondary antibody (Cappel; Oregonon Teknika, West Chester, PA) was diluted with 5% goat serum in PBS to 40 μg ml⁻¹. The slides were incubated with the secondary antibody for 30 min and washed extensively with PBS before mounting. The primary antibodies were generally used at a concentration of 25 μg ml⁻¹. The best staining for chicken α5 subunit was obtained when three monoclonal antibodies were pooled (α5A2, α5D7 and α5B2) each at a concentration of 25 μg ml⁻¹ and incubated on the heated sections. The negative control for all section staining was the P3 mAb used at 75 μg ml⁻¹ to match the total IgG concentration in the α5 staining. The negative controls were photographed and printed with exposures identical to those used for the α5 staining.

**Antibodies**

W1B10 mAb was prepared as described by Hayashi et al. (1990). CSAT mAb was prepared as described by Neff et al. (1982). Rabbit anti-human α3 cytoplasmic domain serum was a gift of C. Buck (Wistar Institute, Philadelphia, PA). Monoclonal anti-FN (B3/D6) was purchased from the Developmental Studies Hybridoma Bank (Johns Hopkins Univ. School of Medicine, Baltimore, MD) and prepared as described by Gardner and Fambrough (1983).

**Results**

*Adult tissues show diversity and differential expression of the chicken β1 integrins*

The diversity of the chicken β1 integrin family was investigated by purifying the β1 integrins from embryonic and adult chicken tissues. Extracts of tissues were passed over a CSAT (anti-β1) mAb affinity column which purifies the β1 subunit and associated α subunits. These purifications provide a profile of the β1 integrins expressed in each tissue. Previous purifications of the chicken β1 integrins from day 11 chick embryos and from chick embryo fibroblasts (CEFs) showed only three major protein bands: two putative α subunit bands at 150 and 130×10^3 Mr, (bands 1 and 2, respectively) and the common β1 subunit at 95–110×10^3 Mr (band 3) (see lanes 1 and 2, Fig. 1A) (Knudsen et al. 1985; Chen et al. 1985).

The purifications of the β1 integrins from chicken tissues are shown in Fig. 1A. The common β1 subunit migrates as a broad band between 95 and 110×10^3 Mr. Differences are evident in the apparent molecular weights of the β1 subunits from different tissues: the β1 subunits of the gizzard and brain migrate faster than those of the other tissues, and some of the β1 subunit bands are broader than others. These differences among the β1 subunits appear to arise primarily by variable glycosylation as only one message is seen on northern blots and glycosidase-treated integrins migrated similarly on protein gels (data not shown).

All other bands of the β1 integrin purifications in Figure 1A are putative integrin α subunits or integrin-associated proteins by virtue of their association with the β1 subunit. The only major exception is the 200×10^3 Mr protein which purifies on control columns. An estimated 1 to 5 different α subunits, in the molecular weight range of 120 to 180×10^3 Mr, are seen in the purifications from each tissue. It is possible that some of these bands represent different glycosylated forms of a common α subunit; however, antibodies that react with chicken α subunits on immunoblots detect only single bands in each purification (see below). A greater number of α subunits may be present in these purifications than visualized in Fig. 1A. Some α subunits may co-migrate with other bands, and others may be present at levels too low to be detected. The number of distinct α subunits present in these purifications appears to exceed the seven identified in the human system thus far.

Unique sets of α subunits are expressed in each tissue in Fig. 1A. The day 4 and day 11 embryos each has the prominent α subunits at 150 and 130×10^3 Mr, (bands 1 and 2 respectively). More bands are resolved in the day

**Chicken αβ integrin expression**

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The integrin profile from the gizzard contrasts all other minor bands migrating lower than the embryo band 1. The brain and sciatic nerve also have 70×10^3 Mr band appears to be an integrin-associated protein. The subunit is virtually absent from the α integrins purified from the gizzard.

Identical samples to those shown in Fig. 1A were run on 7% SDS-PAGE gels (non-reduced), transferred to nitrocellulose and probed with an anti-band 1 mAb against an 18 amino acid polypeptide corresponding to the cytoplasmic domain of the human α$^1$ subunit (at 155×10^3 Mr), and band 1 is essentially absent. In addition, skeletal and cardiac muscle both express a 70×10^3 Mr protein that co-purifies with the β1 integrins but whose molecular weight is not characteristic of integrin α subunits. Some of this 70×10^3 Mr protein migrates with the integrin heterodimer when examined by gel filtration, suggesting that it is an integrin-associated protein (data not shown).

The most prominent α subunit in the chick embryo is homologous to the human α3 subunit

As band 1 represents the major β1 integrin α subunit(s) in the embryo and is less prominent among the β1 integrins of the adult, we initiated a study of its expression and regulation during development. We first generated several (6) mAbs specific for band 1. All of these mAbs were subsequently shown to recognize a single protein. Immunoprecipitation experiments show this one protein to account for the majority (>75%) of the protein in the embryonic band 1 (not shown). Fig. 1B shows an immunoblot of the purified β1 integrins using one of these mAbs (α5A2). These antibodies detect a single band out of the many putative α subunits present in each tissue. The immunoblot signal corresponds closely in position and in relative intensity to the silver stained band seen at 150×10^3 Mr in all of the integrin purifications of Fig. 1A.

Immunofluorescent staining of fibroblasts using the anti-150×10^3 Mr α subunit mAbs shows that this receptor concentrates in the adhesion plaques of fibroblasts adhering to a fibronectin substrate (not shown). Several integrins, including the human fibroblast receptor (α5β1), localize in the adhesion plaques of fibroblasts which are adhering to the receptors' respective ligands (Burridge, 1986; Roman et al. 1989; Singer et al. 1988). Therefore, the observed localization of the chicken α subunit suggested that it functions as the α subunit of a fibronectin receptor. To test if the chicken protein was homologous to the human fibronectin receptor (α5β1), we obtained an antiserum against an 18 amino acid polypeptide corresponding to the cytoplasmic domain of the human α3 subunit (Tomaselli et al. 1988). The anti-human α3 antiserum recognizes specifically the 150×10^3 Mr α subunit purified using our antibodies (Fig. 2). Thus, by immunologic relatedness and by localization in focal contacts of cells adhering to fibronectin, we conclude that the 150×10^3 Mr α subunit purified by our antibodies is the chicken homolog of the human α3 and will be referred to as the chicken α3 subunit.

The α3β1 integrin is widely distributed in embryonic tissues

The integrin purification results described above suggest that the chicken α3 subunit is the major α subunit expressed in the embryo. It follows, therefore, that the α3 subunit is expressed either widely on most cell types or strongly on a few cell types that are prominent in their respective tissues. We have investigated the cell type distribution of the α3β1 integrin in

11 embryo than in the day 4 embryo, but band 1 is the predominant α subunit band in both. Most of the α subunits from the adult tissues appear to represent receptors not detected in the purifications from the embryo. All of the adult tissues have prominent bands migrating at molecular weights higher than that of the embryo band 1. The brain and sciatic nerve also have minor bands migrating lower than the embryo band 1. The integrin profile from the gizzard contrasts all other

Fig. 1. β1 integrin purifications and immunoblot detection of the 'band 1' 150×10^3 Mr α subunit. (A) β1 integrin purifications from adult chicken tissues. Extracts from various tissues were applied to a CSAT (anti-β1) mAb affinity column. The purified proteins were run on 7% SDS–PAGE gels (non-reduced) and visualized by silver staining. Lane 1, whole day 4 embryo; Lane 2, eviscerated day 11 embryo; lane 3, adult adductor femoris (fast twitch) skeletal muscle; lane 4, adult breast (slow twitch) skeletal muscle; lane 5, adult heart; lane 6, adult gizzard; lane 7, adult sciatic nerve; lane 8, adult brain. The β1 subunit migrates as a broad band between 95 and 110×10^3 Mr. The putative α subunits are bracketed between 120 and 180×10^3 Mr. The 200×10^3 Mr protein purifies on control columns and is not an integrin-associated protein. The 70×10^3 Mr α subunit is expressed in the embryo. It follows, therefore, that the chicken α3β1 integrin subunit(s) is expressed either widely on most cell types or strongly on a few cell types that are prominent in their respective tissues. We have investigated the cell type distribution of the α3β1 integrin in...
the chick embryo by immunofluorescent staining of tissue cryosections with antibodies specific for the α5 subunit.

Fig. 3 shows a transverse section of a stage 24 (day 4) chick embryo stained with mAbs specific for the α5 subunit. Weak to moderate α5 staining can be seen throughout most of the embryo with the exception of the neural tissues (neural tube, dorsal root ganglia (DRG) and ventral horn) which show only background levels of fluorescence. Bright α5 staining can be seen on cells that outline the neural tube and DRG, and on the walls of the aorta. Moderate staining is seen along the lateral and dorsal side of the embryo, and extremely weak but detectable staining is seen in central portion of the embryo. Fig. 4 shows α5 staining in the limb bud from the same embryo section. Here too α5 staining is seen throughout the mesenchyme. The α5 subunit is absent only from the ectodermal cell layer of the limb bud. This distribution of the α5β1 integrin seen in the day 4 embryo is very similar to the reported distribution of its ligand, fibronectin (Kosher et al. 1982; Rogers et al. 1986).

Later stages of development (days 4–17) show a similar distribution of the α5β1 integrin. Despite extensive cell differentiation and tissue formation, the α5 subunit continues to be expressed on the majority of cell types in the embryo with the exception of neuronal and epithelial cells. Fig. 5 illustrates this widespread α5 staining pattern on a section of the small intestine from a day 16 chick embryo. This section shows α5 staining over the majority of cell types including the smooth muscle layer, but only background staining is seen on the intestinal epithelium. In sections from all muscle types (smooth, cardiac and skeletal muscle) in the day 11 embryo, the α5 subunit is detected on essentially all cells including desmin-positive muscle cells, desmin-negative muscle precursors, fibroblasts and endothelial cells (not shown). In the day 14 kidney, α5 staining is observed on all cell types with the exception of the tubules (i.e. epithelial cells) (not shown). The continued absence of the α5 subunit from neurons is illustrated in a section of the day 11 sciatic nerve (Fig. 6). In this section, the α5 staining is limited primarily to the nascent perineurium surrounding the neurons. The neurons themselves do not stain detectably for the α5 subunit, although some punctate α5 staining is seen among them. The co-presence of fibronectin in these areas (Fig. 6) suggests that this staining may correspond to regions of emerging connective tissue and capillaries. Sections of the embryonic retina (days 7–17) and ciliary ganglion (days 8 and 13) show α5 staining on capillaries and connective tissues but do not show detectable staining on the neurons (not shown).

As in the day 4 embryo, the distribution of the α5β1 integrin in the later embryo generally parallels that of its ligand, fibronectin. This is illustrated in Figs 5B and 6B where the staining for fibronectin mirrors the
Fig. 3. Fluorescent photomicrograph of a cross-section through a stage 24 (day 4) chick embryo showing the localization of the α5 subunit. The neural tube (NT), dorsal root ganglia (D) and ventral roots (arrows) do not stain for the α5 subunit. Bright staining is seen on the cells that outline the neural tube and dorsal root ganglia, and on the walls of the aorta (A). In general, α5 staining can be detected throughout the entire mesenchyme.
staining for $\alpha_5$. This co-distribution of the $\alpha_5\beta_1$ integrin with fibronectin was observed in all embryonic tissues surveyed.

The $\alpha_5\beta_1$ integrin expression is restricted to a few cell types in the adult

In contrast to the widespread distribution of the $\alpha_5$ subunit in tissues from the embryo, its distribution in the adult is limited to a few cell types. The $\alpha_5$ subunit is found mainly in regions of connective tissue and vasculature. This restricted expression is illustrated in a section of smooth muscle from the adult gizzard (Fig. 7A). In this section, the $\alpha_5$ subunit is detected only on capillaries and in some fibronectin-rich connective tissue regions. The smooth muscle cells, which comprise the major cell type in this tissue, do not stain detectably for the $\alpha_5$ subunit. This restricted expression was seen in all tissues surveyed including all muscle types, the small intestine and the kidney (not shown). The $\alpha_5$ subunit remains absent from the neurons in adult tissues as observed on sections of the adult sciatic

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**Fig. 4.** Fluorescent photomicrograph of a cross-section through the limb of a stage 24 (day 4) chick embryo showing the localization of the $\alpha_5$ subunit. The $\alpha_5$ subunit is found throughout the mesenchyme but absent from the ectoderm layer (arrows). The patches of brighter staining consist of endothelial cells.

**Fig. 5.** Fluorescent photomicrographs of cross-sections through the small intestine of a day 16 chick embryo showing the localization of the $\alpha_5$ subunit and fibronectin. Tissue sections stained with anti-$\alpha_5$ mAbs (A), an anti-fibronectin mAb (B) and a negative control mAb (C). The smooth muscle layer (SM) and epithelial layer (E) are labelled in frame (A). (bar=100 $\mu$m).
Fig. 6. Fluorescent photomicrographs of cross-sections through the sciatic nerve of an day 11 chick embryo showing the localization of the $\alpha_5$ subunit and fibronectin. Tissue sections stained with anti-$\alpha_5$ mAbs (A), an anti-fibronectin mAb (B), and a negative control mAb (C). The neurons (N) and perineurium (P) are labelled in frame A. (bar=100 $\mu$m).

Fig. 7. Fluorescent photomicrographs of sections through the gizzard of an adult chicken showing localization of the $\alpha_5$ subunit and fibronectin. Tissue sections stained with anti-$\alpha_5$ mAbs (A), an anti-fibronectin mAb (B), and a negative control mAb (C). Some capillaries are designated with arrows in frame A. (bar=50 $\mu$m).
nerve and retina (not shown). This restricted expression of the \(\alpha_5\beta_1\) integrin in adult tissues is observed in the same tissues which, in the embryo, expressed this receptor on the majority of cells. This points to a dramatic down-regulation of the \(\alpha_5\beta_1\) integrin on many cell types during the later stages of cell differentiation.

The fibronectin distribution in adult tissues differs significantly from that of the \(\alpha_5\beta_1\) integrin, in contrast to the strong co-localization seen in the embryo. Although the \(\alpha_5\beta_1\) integrin always co-localizes with fibronectin in the adult, fibronectin is also found in many areas where the \(\alpha_5\beta_1\) integrin has disappeared. Such differences are apparent in the section of the adult gizzard (Fig. 7) where the \(\alpha_5\) subunit is not detectable on the smooth muscle cells, but, bright fibronectin staining surrounds these cells.

Discussion

The \(\beta_1\) integrins mediate the interactions between cells and molecules in the extracellular matrix (ECM) (Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Buck and Horwitz, 1987). They also mediate linkages between the cell surface and the cytoskeleton (Horwitz et al., 1986). The specificity of the different integrins for ECM ligands is determined by the variable \(\alpha\) subunits that associate with the common \(\beta_1\) subunit. Although the ligand specificities for the different \(\alpha\beta\) heterodimers have not been characterized completely, the following specificities have been assigned: \(\alpha_2\beta_1\), Col, LM (Kramer and Marks, 1989; Ignatius and Reichardt, 1988); \(\alpha_3\beta_1\), Col, LM (Wayner and Carter, 1987; Staatz et al. 1989; Elices and Hemler, 1989); \(\alpha_5\beta_1\), FN, LM, (Col) (Wayner and Carter, 1987; Gehlsen et al. 1988); \(\alpha_6\beta_1\), FN (CS-1 region) (Wayner et al., 1989); \(\alpha_7\beta_1\), FN (Pytel et al. 1985); \(\alpha_8\beta_1\), LN (Sonnenberg et al. 1988); \(\alpha_9\beta_1\), FN (Vogel et al. 1990).

The expression of different \(\beta_1\) integrins confers selective adhesive properties to a cell. Therefore, the differential expression of integrin \(\alpha\beta\) heterodimers provides a potential mechanism for morphogenetic events during development. Until recently, it has been difficult to address the issue of differential integrin expression during development due to a lack of probes for integrin \(\alpha\) subunits in systems amenable to embryologic studies. We have approached the problem of \(\beta_1\) integrin expression during development in two ways. First, we obtained profiles of \(\alpha\) subunit expression by affinity purifying the sets of \(\beta_1\) integrins from various embryonic and adult chicken tissues. These profiles offer a glimpse at the diversity and differential expression of the \(\beta_1\) integrins. Second, we generated monoclonal antibodies against chicken \(\alpha\) subunits. These antibodies can be used as probes for the expression of the individual \(\beta_1\) integrins during chick development.

In the present work, we describe mAbs raised against a 150 \(\times 10^3M_\alpha\) subunit in the prominent ‘band 1’ of the chicken embryo integrins and characterize its expression in embryonic and adult tissues. We find that this 150 \(\times 10^3M_\alpha\) subunit is homologous to the human integrin \(\alpha_5\beta_1\) subunit. It is the most prominent \(\beta_1\) integrin \(\alpha\) subunit in the embryo and is found on most cell types in the day 4–17 chick embryo. In the adult, however, the level of \(\alpha_5\) expression is dramatically reduced relative to other \(\alpha\) subunits. Its distribution in the adult is restricted primarily to connective tissue and vasculature. These observations reveal a down-regulation of \(\alpha_5\) on many cell types and also imply an up-regulation of other \(\alpha\) subunits during development.

The 150 \(\times 10^3M_\alpha\) \(\alpha\) subunit purified using our antibodies was identified as the avian homologue of the human \(\alpha_5\beta_1\) fibronectin receptor by two criteria: (1) its concentration in adhesion plaques of cells adhering to fibronectin, and (2) its cross-reaction with antisera specific for the cytoplasmic domain of the human \(\alpha_5\) subunit. During the course of this work, Hofer et al. (1990) described a mAb directed against a 150 \(\times 10^3M_\alpha\) avian integrin \(\alpha\) subunit. They identified this \(\alpha\) subunit as a homolog of the human \(\alpha_5\) by its localization in adhesion plaques of fibroblasts adhering to fibronectin, and by comparing its amino terminal protein sequence with that of the human \(\alpha_5\) subunits. Hynes et al. (1989) have also identified a 150 \(\times 10^3M_\alpha\) protein in the chicken integrin band 1 as the \(\alpha\) subunit of a fibronectin receptor related to the human \(\alpha_5\) subunit. This was demonstrated by the cross-reaction of an antiserum raised against the human \(\alpha_5\) cytoplasmic domain and by the binding of this receptor to a fibronectin affinity column.

The cumulative evidence suggests that we have all identified the same \(\alpha\) subunit and that this \(\alpha\) subunit is the chicken homolog of the human \(\alpha_5\) integrin.

The dominant and widespread expression of the \(\alpha_5\) subunit in the chick embryo was a striking, unexpected observation. Restricted \(\alpha_5\) expression in adult tissues has been reported in human kidney (Korhonen et al., 1990) but widespread expression in embryonic tissues has not been observed. The presence of the \(\alpha_5\) subunit on most cells in diverse tissues in day 4–17 chick embryos points to a major role for the \(\alpha_5\) subunit during development. The loss of this receptor in adult cell types may be of equal significance. The observed extinction of the \(\alpha_5\) subunit seems to accompany the terminal differentiation of many cell types. Loss of the \(\alpha_5\beta_1\) integrin upon terminal cell differentiation has been described previously during the terminal differentiation of keratinocytes (Adams and Watt, 1990). In addition, Vuillet-Gaugler et al. (1990) and Cardarelli et al. (1988) report the loss of integrin-dependent binding to fibronectin upon terminal differentiation of erythrocytes and thymocytes, respectively.

The observed changes in the relative distribution of the \(\alpha_5\beta_1\) integrin and fibronectin during development may also be significant. Fibronectin and \(\alpha_5\beta_1\) integrin distribution are both widespread and nearly identical in the embryo, but in the adult their distributions differ significantly. Several other \(\beta_1\) integrins are reported to serve as receptors for fibronectin in addition to the \(\alpha_5\beta_1\) integrin. These include the \(\alpha_5\beta_1\), \(\alpha_6\beta_1\) and \(\alpha_9\beta_1\) integrins. As seen in the purification profiles, the \(\alpha_5\beta_1\) is
clearly the predominant $\beta_1$ integrin fibronectin receptor in the embryo; however, the relative expression of the other fibronectin receptors appears to increase at later stages of development. Intriguingly, it has become apparent that the alternatively spliced fibronectin isoforms are more prevalent in adult tissues whereas a single fibronectin isoform is most prevalent in the embryo (French-Constant and Hynes, 1988, 1989). Therefore, the observed pattern of fibronectin receptor expression may be related to the expression of alternate fibronectin isoforms. The $\alpha_4\beta_1$ is one integrin known to serve as the receptor for an alternatively spliced domain of fibronectin (Wayner et al. 1989). Other possible functions for the different fibronectin receptor $\alpha$ subunits may reside in their cytoplasmic domains, which appear to be unique (Hemler, 1990; Hynes et al. 1989) and may specify associations with different cytoskeletal components or transduce specific signals.

The question now arises as to the role(s) of the $\alpha_5\beta_1$ integrin in development. A complete answer should address its nearly ubiquitous presence across diverse embryonic cell types, its presence in adult connective tissue, its possible up-regulation during wound healing and inflammation (Clark, 1990; Holers et al. 1989), and its apparent extinction on many terminally differentiated cell types. Possible functions of the $\alpha_5\beta_1$ integrin include: matrix assembly, cytoskeletal organization, cell migration, and transduction of differentiative or proliferative signals. A role as an organizer of the fibronectin ECM (Giancotti and Ruoslahti, 1990; Akiyama et al. 1989; Roman et al. 1989) is consistent with the widespread distribution of this receptor, including its presence in the loose mesenchyme of the embryo and its persistence in connective tissue regions of the adult. The role of $\alpha_5\beta_1$ integrin in cell migration is not clear. Some migratory cells, such as fibroblasts and macrophages, do express this receptor (Holers et al. 1989); however, in some studies, the presence of the $\alpha_5\beta_1$ integrin is reported to inhibit cell migration on fibronectin (Akiyama et al. 1989; Giancotti and Ruoslahti, 1990). The $\alpha_5\beta_1$ integrin might function to mediate signals pertaining to differentiation. The differentiation of both muscle and keratinocytes are inhibited by fibronectin binding (von der Mark and Ocalan, 1989; Adams and Watt, 1989). Erythrocyte differentiation, on the other hand, requires fibronectin binding (Patel and Lodish, 1987). Werb et al. (1989) have shown induction of specific genes as the result of signal transduction through the human $\alpha_5\beta_1$ integrin. Finally, there is a possible relationship between $\alpha_5\beta_1$ integrin expression and cell proliferation. This receptor appears to be preferentially expressed on proliferating cell types (Cardarelli et al. 1988). Most recently, Giancotti and Ruoslahti (1990) have implicated the $\alpha_5\beta_1$ integrin in the control of cell proliferation and suggest that loss of this receptor contributes to anchorage-independent growth by transformed cells.

We would like to acknowledge the generous gifts of the anti-human $\alpha_5$ antiserum from Dr L. F. Reichardt, of the antihuman $\alpha_5$ antiserum from Dr C. Buck, and of the stage 24 embryo sections from Dr M. Bronner-Fraser. We would like to thank Steve Miklasz for his assistance in monoclonal antibody production, and also Rhett Miller for his technical assistance throughout the course of this work.

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(Accepted 7 May 1991)