Sequence and tissue distribution of the human integrin α8 subunit: a β1-associated α subunit expressed in smooth muscle cells

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SUMMARY

Integrins are a major family of cell adhesion molecules involved in cell-cell and cell-extracellular matrix interactions. Each integrin is a heterodimeric glycoprotein composed of an α and a β subunit. We now report the cDNA sequence and distribution of a new human integrin α subunit. This sequence is 78% identical to the previously reported chicken α8 integrin sequence. Thus, we have designated this subunit as human α8. By northern blot analysis, an α8 probe detects two mRNA species of approximately 6.5 and 4.0 kb in neuroglioma H4 cells. An anti-α8 polyclonal antibody precipitates a protein complex containing the β1 subunit associated with the putative α8 subunit, which has an apparent molecular mass of 180 kDa (non-reduced) or 155 kDa and 25 kDa (reduced). Immunohistochemistry with anti-α8 polyclonal antibody in adult rat tissues shows prominent staining in vascular and visceral smooth muscle. In addition, the antibody strongly stained kidney mesangial cells and a cell type in the alveolar wall of the lungs, most likely corresponding to alveolar myofibroblasts. These results suggest that in adult mammalian tissues, α8 is predominantly expressed in smooth muscle and smooth muscle-like contractile cells.

Key words: integrin, smooth muscle, cell adhesion molecule

INTRODUCTION

Integrins are a class of cell surface glycoproteins that mediate cell-cell and cell-extracellular matrix interactions (Hynes, 1992). Each integrin is composed of two non-covalently associated subunits, α and β. Each α and β subunit contains a large extracellular domain, a transmembrane domain and a cytoplasmic domain, which presumably interacts with the cytoskeleton and/or cytoplasmic signaling molecules. The extracellular domain of both subunits forms the ligand binding site and requires divalent cations such as Ca2+ or Mg2+ for functional activity. Many integrins interact with ligands through a specific binding site on the ligand containing arginine-glycine-aspartate (RGD). Some integrins appear to interact with a single ligand, whereas others can bind to a variety of different substrates.

The tissue distribution of an integrin may provide insight into its function. Although some integrin heterodimers are widely expressed by a variety of cell types (e.g. α2β1, α3β1), others have a restricted distribution. For example, the α2β1 subunit is expressed only in platelets and functions as a primary mediator of platelet aggregation. The expression of most integrins is developmentally regulated; for example, the α4β1 integrin is widely distributed during development, but is restricted to leukocytes and endothelial cells in normal adult tissues (Rosen et al., 1992).

In this paper we report the identification of a novel human integrin α subunit. A cDNA fragment was amplified by reverse-transcriptase polymerase chain reaction from human lung RNA and the complete cDNA sequence was obtained from human lung, intestine and aorta cDNA libraries. This new human α subunit shares 78% amino acid identity to a previously described chicken integrin termed α8, suggesting that we have identified the human homologue of chicken α8. We show that α8 mRNA and protein are expressed in human H4 neuroglioma cells and that α8 associates with the β1 subunit in these cells. Immunohistochemical evidence suggests that in adult mammalian tissues, α8 is primarily expressed in vascular and visceral smooth muscle cells, lung alveoli and kidney glomeruli.

MATERIALS AND METHODS

Cell lines
The human cell lines H4, U251, MG63, IMR-32 and HeLa were obtained from the American Type Culture Collection (ATCC). The human pancreatic carcinoma cell line FG was provided by D. Cheresh (Scripps Clinic, La Jolla, CA). Cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and penicillin-streptomycin.

Polymerase chain reaction amplification
Total cellular RNA was isolated using the LiCl/urea method (Harlow and Lane, 1988). Single-stranded cDNA was synthesized at 44°C for 1 hour from 20 to 40 µg of total RNA using the Superscript cDNA Synthesis System (GIBCO-BRL, Gaithersburg, MD) and random DNA hexamers.
Polymerase chain reactions (PCR) contained 1x Taq buffer (Promega, Madison, WI) with 1.5 mM MgCl2, 0.1 µM each of two primers, 0.025 units/µl Taq polymerase (Promega), 1-8 µl of DNA template, and 0.1 mM each of dATP, dGTP, dCTP, and dTTP in reaction volumes of 25-200 µl. Reactions were heated to 94°C for 4 minutes in a thermocycler (Erectorp, San Diego, CA) and then subjected to 30 cycles of PCR. The degenerate oligonucleotide primers A2AR and A14F were previously described by Erle et al. (1991b). Reactions that contained degenerate oligonucleotide primers were subjected to PCR cycles consisting of 45 seconds at 94°C, 45 seconds at 48°C, and 45 seconds at 72°C. Reactions that contained αs-specific primers or λ phage-specific primers were subjected to PCR cycles consisting of 45 seconds at 94°C, 45 seconds at 57°C, and 60 seconds at 72°C. Products of each PCR reaction were analyzed by agarose gel electrophoresis.

Cloning of DNA fragments
Restriction enzyme-digested DNA fragments were isolated on low gel temperature agarose and purified by phenol/chloroform extraction and ethanol precipitation or with the Magic PCR kit (Promega). Fragments were ligated into restriction enzyme-digested, dephosphorylated pBluescript vector (Stratagene, La Jolla, CA) with T4 DNA ligase (Bethesda Research Laboratories, Gaithersburg, MD). The ligation mixture was used to transform competent Escherichia coli (JM-109, Clontech, Palo Alto, CA). Selected plasmids were purified from liquid cultures using the Pharmacia miniprep lysis kit (Pharmacia/LKB, Pleasant Hill, CA) and sequenced using Sequenase 2.0 (United States Biochemical Corporation, Cleveland, OH), [32P]dATP (Amersham, Arlington Heights, IL), T3 primer, T7 primer, and a variety of primers specific for αs.

Library screening
Partial cDNA fragments were used as templates for the random-primed synthesis of [32P]dCTP-labeled probes (Multiprime DNA Labeling system, Amersham) or digoxigenin-labeled probes (Genius System, Boehringer Mannheim, Indianapolis, IN). These probes were used to screen an oligo(dT)-primed λgt11 cDNA library from human lung tissue including trachea and bronchioles (Clontech, catalog no. HL1066b), a mixed random hexamer and oligo(dT)-primed λgt11 cDNA library from human small intestine tissue (Clontech, catalog no. HL1133b) and a mixed random hexamer and oligo(dT)-primed λgt10 cDNA library from human aorta (Clontech, catalog no. HL1136a). Hybridizations were performed at 50°C for 16 hours in hybridization buffer (40% formamide, 50 mM sodium phosphate, pH 6.5, 800 mM NaCl, 0.05% polyvinylpyrroldione, 0.05% BSA, 0.05% Ficoll, 1 mM EDTA, 0.1% SDS, and 10 ng/ml heat-denatured sonicated salmon sperm DNA). Filters were washed twice in 1x SSC containing 0.1% SDS for 5 minutes at room temperature and once in 0.5x SSC containing 0.1% SDS for 1 hour at 50°C.

Positive library clones were isolated by further rounds of screening, and the inserts were isolated either by PCR amplification using λgt11 or λgt10 specific primers or by EcoRI digestion of purified phage DNA. The inserts were then subcloned into EcoRI-digested pBluescript and sequenced.

Northern blot analysis
Northern blot analysis was performed as previously described (Erle et al., 1991a). Briefly, total cellular RNA was electrophoresed through a formaldehyde-agarose gel and transferred to a nylon membrane (Hybond-N, Amersham). Filters were incubated with [32P]dCTP-labeled αs-specific probes at 50°C for 16 hours in hybridization buffer containing 5x SSC, 40% formamide, 20 mM Tris-HCl, pH 7.5, 0.1% polyvinylpyrroldione, 0.1% bovine serum albumin, 0.1% Ficoll, 10% dextran sulfate, and 100 µg/ml heat-denatured sonicated salmon sperm DNA and washed twice in 5x SSC containing 0.1% SDS at 50°C for 30 minutes. After washing, filters were exposed to film at −80°C with an intensifying screen.

Peptide synthesis and antibody generation
Two peptides based on the carboxyl-terminal sequence of αsα were manually synthesized using solid-phase 9-fluorenylmethoxy carbonyl (Fmoc) chemistry. Peptide synthesis reagents were purchased from Bachem Bioscience, Philadelphia, PA. The synthesis was started using Fmoc-alanine-p-alkoxybenzyl alcohol resin. Fmoc-deprotection was performed using piperdine. A solution containing benzotiazolyl-1-yl-tris (dimethylamino) phosphonium hexafluorophosphate (BOP) and 1-hydroxybenzotriazole (HOBt) was used for coupling of Fmoc-amin acid pentafluorophenyl esters. Peptides were deprotected and cleaved from the resin using reagent K (King et al., 1990), containing 82.5% trifluoroacetic acid, 5% phenol, 5% water, 5% thioanisole, and 2.5% 1,2-ethanediol.

The peptides were conjugated to the carrier proteins keyhole limpet hemocyanin or ovalbumin using m-maleimidobenzoyl-N-hydroxysulfosuccinimide (Pierce Chemical Co., Rockford, IL). Rabbits were immunized subcutaneously with conjugated peptides to generate anti-αs peptide polyclonal antiserum (CalTag Laboratories, Healdsburg, CA). Rabbits were injected on days 1, 28, 35, 42, 49, 56 and 77 and 84, 91, 98, 105. Primary injections used 150 µg of conjugated peptides with Freund’s complete adjuvant; subsequent injections used 100 µg of conjugated peptides with Freund’s incomplete adjuvant. In the experiments described here, we used serum obtained on day 91 or later. Antibodies were affinity-purified from crude antiserum using the corresponding synthetic peptide coupled to lysozyme-Affigel as previously described (Palmer et al., 1993).

Mouse monoclonal antibody P5D2 (Dittel et al., 1993) directed against the β1 integrin subunit was a gift from Elizabeth Wayner (University of Minnesota, Minneapolis, MN). Polyclonal antibody to PECAM-1 was a gift from Steve Albelda (University of Pennsylvania, Philadelphia, PA) (Albelda et al., 1990). Monoclonal antibody R6G9 directed against the β9 integrin subunit was generated in our laboratory (Weinacker et al., 1994).

Immunoprecipitations
Cells were surface labeled with 125I as previously described (Ruegg et al., 1992) and lysed in immunoprecipitation buffer (100 mM Tris-HCl, pH 7.5, 0.1% SDS, 1% Triton-X-100, 0.1% NP-40, 150 mM NaCl, 1 mM CaCl2, 1 mM MgCl2) containing the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (Gibco-BRL). Cell lysates were precleared with Protein A-Sepharose (Pharmacia-LKB Biotechnology, Piscataway, NJ) and incubated with antibodies for 1-2 hours at 4°C. Immune complexes were captured on Protein A-Sepharose and washed 5 times with immunoprecipitation buffer. Samples dissolved in Laemml sample buffer were analyzed by SDS-PAGE on 7.5% acrylamide gels and exposed to film at ~80°C with intensifying screens.

For immunodepletion experiments, the antigen of interest was removed from the lysates by 4 cycles of immunoprecipitation using a large excess of monoclonal antibody cross-linked to Protein A-Sepharose. Antibodies were cross-linked to Protein A-Sepharose as follows (Harlow and Lane, 1988): Protein A-Sepharose and an aliquot of antiserum were incubated together for 1 hour at room temperature and then washed twice with 0.2 M sodium borate, pH 9.0. Dimethylpimelimidate (6 mg/ml, Sigma, St Louis, MO) in 0.2 M sodium borate was added to Protein A-Sepharose/antisera slurry and rotated for 1 hour at room temperature. A 6 ml sample of 0.2 M ethanolamine was added and rotated an additional 10 minutes. Protein A-Sepharose cross-linked to antiserum was washed twice in PBS and resuspended in PBS with 0.02% sodium azide.

Immunoblotting
Tissue sections were minced and placed in 1 mM EDTA/150 mM NaCl/50 mM Tris-HCl for 10 minutes and centrifuged for 5 minutes at 900 rpm. The tissues were then solubilized with 200 mM octyl-β-D-glucopyranoside/100 mM Tris-HCl/1 mM PMSF for 1 hour at 4°C.
and centrifuged twice at 2000 rpm for 10 minutes each. The protein concentrations were determined by the BCA Protein Assay (Pierce). Protein samples were analyzed under reducing and non-reducing conditions. Samples (30 µg/well) were separated by SDS-PAGE on 7.5% acrylamide gel, and electrophoretically transferred to a nylon membrane (Immobilon-P, Millipore). Filters were blocked with 1% dry milk/1% BSA/0.02% Tween-20/PBS for 2 hours, probed with the anti-α8 antibody for 1 hour, rinsed in PBS, and then probed with an alkaline phosphatase conjugated goat anti-rabbit secondary antibody at a dilution of 1:3000 for 30 minutes. After rinsing in PBS, color was developed using the Protoblot alkaline phosphatase detection system (Promega). After the desired intensity was achieved, the filter was rinsed with water and photographed.

**Immunohistochemistry**

Tissue was first fixed in a 1:5 dilution of Histochoice (Amresco, Solon, OH) at room temperature, or directly immersed in 10% sucrose at 4°C for 4 hours. Tissue was embedded in OCT embedding medium (Miles, Elkhart, IN) and then frozen in isopentane that was chilled in liquid nitrogen. Cryostat sections (5 µm) of tissue were air-dried for at least 30 minutes, fixed in −18°C acetone for 5 minutes, and air dried again. Endogenous peroxidase activity was blocked with Peroxoblock Solution (Zymed Laboratories, South San Francisco, CA) for 45 seconds at room temperature. After rinsing in PBS for 30 minutes to remove the OCT, sections were incubated with 0.5% casein/0.05% Thimerosal/PBS for 15 minutes at room temperature and then incubated overnight at 4°C or 2 hours at room temperature in affinity-purified anti-α8 antibody (3 µg/ml in 0.5% casein/0.05% Thimerosal/PBS). To verify specific staining, affinity-purified anti-α8 antibody was also incubated with 0.1 mg/ml α8 peptide for 30 minutes at 4°C prior to incubation with tissue sections. After rinsing in PBS, sections were incubated in peroxidase-conjugated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA) at a dilution of 1:250 for 1 hour at room temperature in 0.5% casein/0.05% Thimerosal/PBS. Tissue sections were then incubated with di-amino benzidine (DAB Plus Kit, Zymed Laboratories) for 10 minutes at room temperature. Counterstaining was performed with hematoxylin. Sections were subsequently dehydrated in graded ethanol solutions, transferred into xylene and then mounted with Permount (Fisher Scientific, Pittsburgh, PA).

**RESULTS**

**Cloning and sequencing of human α8**

A novel 223-nucleotide partial human integrin α subunit sequence was amplified from cDNA obtained from human lung tissue using the previously described degenerate primers A14F and A2AR (see Materials and Methods). A probe generated from this partial sequence was used to screen a cDNA library from human lung. One clone (HuLu) was isolated and sequenced (Fig.1). A probe generated from this clone was used to screen aorta and intestine cDNA libraries. Seven independent clones were isolated and analyzed to obtain a series of overlapping clones encoding the full-length cDNA sequence (Fig. 1). The cDNA sequence contains a 3075 nucleotide open reading frame that is predicted to encode a 1025 amino acid mature protein (excluding the signal peptide). The predicted extracellular domain, transmembrane domain and cytoplasmic domain are 971, 25 and 29 amino acids long, respectively. One clone, HA33A contains an in-frame deletion of nucleotides 688-732. α8 has 15 asparagine residues that are potential N-glycosylation sites (NX/T/S). Like other integrin α subunits, α8 contains three repeat domains with potential metal binding activity (N/DXN/DXN/DXXXD). The primary amino acid sequence also contains a predicted site (RKR) for post-translational cleavage, located in the carboxy-terminal domain (double broken underline in Fig. 2). As with other α subunits, the short carboxy-terminal fragment is predicted to be disulfide-linked to the large amino-terminal fragment.

**Relationship of α8 to other integrins**

The composite amino acid sequence obtained from the clones shown in Fig. 1 is 78% identical to chicken α8, suggesting that this sequence encodes the human homologue of chicken α8 (Bossy et al., 1991). This degree of similarity is consistent with the similarities observed between other chicken and human integrin subunits: 76% for α6 (de Curtis et al., 1991; Tamura et al., 1990), 83% for αv (Bossy and Reichardt, 1990; Suzuki et al., 1985), and 85% for β1 (Argraves et al., 1987; Tamkun et al., 1986). The human integrins most closely related to α8 are αv (42%), α5 (43%) and αβ6 (36%), while all other α subunits are much less similar to α8 (16-26%).

**Detection of α8 mRNA by northern blotting**

We used northern blot analysis to determine the distribution of α8 in a variety of cell lines (Fig. 3). The α8 probe detects two mRNA species of approximately 6.5 and 4.0 kb in culted neuroglioma cells (H4 cells). No message was detected in U251 glioblastoma cells, FG pancreatic carcinoma cells, IMR-32 neuroblastoma cells, MG63 osteosarcoma cells, or HeLa cervical carcinoma cells (Fig. 3 and data not shown). The smaller band may represent partially degraded α8 mRNA or a second α8 mRNA species derived by alternative splicing or use of an alternative termination/polyadenylation signal.
Fig. 2. Human integrin α8 cDNA and deduced amino acid sequence. A 1025 amino acid mature protein is predicted from cDNA sequence. The putative transmembrane domain is underlined. Nucleotides 688-732 (broken underline) are not present in clone HA33A. Predicted cleavage site (RKR) is indicated by a double broken underline. 3' Untranslated sequence is presented in lower case letters. Potential N-glycosylation sites are indicated by an asterisk. The sequence data are available from GenBank under accession number L36351.

Detection of the α8 protein

Two peptides (underlined and overlined, respectively, in Fig. 4) based on the deduced sequence of the cytoplasmic domain of human α8 were synthesized and used to generate anti-α8 polyclonal antibodies. The cytoplasmic domain sequence of α8 is distinct from all other known α subunits cytoplasmic domains, including the most closely related ones, α6 and α5.

Since the H4 neuroglioma cell line was the only human cell line tested expressing detectable levels of α8 mRNA (Fig. 3), we used H4 cell lysates for immunoprecipitations with anti-α8 antibody generated against the 28-mer peptide. Surface-labeled H4 lysate was immunoprecipitated with anti-α8 antibody or with a monoclonal antibody (PSD2) against the integrin β1 subunit. Immunoprecipitation experiments using antisera generated against the 15-mer peptide yielded similar results to experiments using the antisera generated against the 28-mer peptide (results not shown). The immunoprecipitates were analyzed by SDS-PAGE under reducing and non-reducing conditions (Fig. 5). The anti-α8 antibody precipitates a protein complex consisting of 2 bands: the putative α8 subunit, which has an apparent molecular mass of 180 kDa (non-reduced) (lane 2) or 155 kDa (reduced) (lane 4), and the presumptive associated β1 subunit partner (120 kDa, non-reduced), which co-migrates with the major band pre - 

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amounts of RNA. Bromide staining was used to verify that all lanes contained similar amounts of RNA. Positions of ribosomal markers are shown to the left. Ethidium bromide staining was used to verify that all lanes contained similar amounts of RNA.

Fig. 3. Detection of α8 mRNA by northern blot analysis. Total RNA from cultured U251 glioblastoma cells, MG63 osteosarcoma cells, FG pancreatic carcinoma cells, IMR-32 neuroblastoma cells, and H4 neuroglioma cells were probed for α8 message. Two mRNA species approximately 6.5 and 4.0 kb were detected in H4 cells only.

Fig. 4. Alignment of cytoplasmic domains of human α8 with the chicken α8 and the most closely related integrin α subunits, α6 and α5. The under- and over-lined residues of α8 were used to synthesize 2 peptides for α8 antibody generation.

Immunodepleted with anti-β6 antibody (lane 3). These results indicate that β1 is the predominant β subunit partner for α8 in H4 cells.

To further confirm the specificity of the anti-peptide antibody for α8, we performed immunoblotting with the anti-α8 antibody (Fig. 7). Tissue extracts from rat aorta and lung were analyzed, since these organs showed high levels of reactivity with the anti-α8 antibody, by immunohistochemistry (see below). When samples were analyzed under non-reducing conditions, the α8 antibody detected a protein band of approximately 180 kDa in tissue extracts from aorta (lane 1) and lung (lane 2). Under reducing conditions, the 180 kDa band is no longer detected and a smaller band of approximately 30 kDa is detected (lanes 3, 4). These results are consistent with the predicted cleavage site of α8. No protein was detected from FG carcinoma cells (data not shown), as predicted from northern analysis experiments.

Tissue distribution of α8 in mammalian tissues

Immunohistochemistry with affinity-purified anti-α8 polyclonal antibody in adult rat (Fig. 8) and human tissues (data not shown) showed prominent staining in vascular smooth muscle, the kidney glomerulus, and the smooth muscle of the gastrointestinal, urogenital and respiratory tracts. Antibody staining was specific, because it could be completely abolished by pre-incubation of the antibody with the synthetic peptide that had been used to raise the antibody. A control peptide (based on the sequence of the integrin α6 cytoplasmic domain), used at the same concentration, had no effect on α8 staining.

In the kidney, glomeruli stained strongly in a pattern consistent with mesangial cells (Fig. 8B). Blood vessel walls are also positive, while kidney tubules and connective tissue cells are negative. Fig. 8C shows a high power view of stained glomeruli, demonstrating the typical distribution pattern of mesangial cells and staining of juxtaglomerular mesangial cells. These cell types
are contractile smooth-muscle-like cells. Vascular smooth muscle of arteries and veins showed prominent staining (Fig. 8E). The endothelial cells of vessels did not stain (Fig. 8F). In the rat lung, alveoli stained for \( \alpha_8 \) in a discontinuous pattern (Fig. 8H), most consistent with alveolar myofibroblasts, contractile interstitial cells (Kapanci et al., 1974). Alveolar staining with an antibody to PECAM-1, a cell adhesion molecule found on endothelium (Albelda et al., 1990), displayed a different staining pattern than \( \alpha_8 \), suggesting that \( \alpha_8 \)-positive cells in the lung are not capillary endothelium (data not shown). Airway smooth muscle stained weakly for \( \alpha_8 \). Of note, skeletal muscle (Fig. 8E) and cardiac muscle did not stain. In the gastrointestinal tract, smooth muscle staining was seen in the muscularis mucosae and muscularis externa layer, and in smooth muscle fibers extending into the villus core (Fig. 8I). The skin showed staining only in the arrecti pili, a smooth muscle cell type associated with the hair follicle (data not shown). Uterine smooth muscle stained weakly (data not shown). Staining of selected human tissues (including kidney, uterus and blood vessels) confirmed the distribution pattern observed in rat tissues (data not shown). Thus, \( \alpha_8 \) appears to have a widespread distribution in vascular and visceral smooth muscle cells (Table 1).

**DISCUSSION**

This paper reports the complete amino acid sequence of a novel human integrin \( \alpha \) subunit that forms a heterodimer with \( \beta_1 \) in human neuroglioma cells (H4) and is prominently expressed in vascular and visceral smooth muscle cells, kidney glomeruli and lung alveoli.

Several lines of evidence suggest that this \( \alpha \) subunit is the human homologue of a previously sequenced chicken integrin subunit, termed \( \alpha_8 \). The degree of sequence conservation (78%) is similar to what has been reported for the chicken homologues of other human integrins (\( \alpha_4 \), \( \alpha_6 \), \( \beta_1 \)). Antibodies to the cytoplasmic domain predicted by the human sequence immunoprecipitate a complex containing \( \beta_1 \) associated with a 180 kDa \( \alpha \) subunit. Chicken \( \alpha_8 \) also associates with \( \beta_1 \) and has a molecular mass of 160 kDa. The difference in observed molecular mass may be due to species-specific glycosylation differences. Both the human and chicken \( \alpha_8 \) subunits appear to associate exclusively with \( \beta_1 \) in the cell lines examined. In addition, both the human and chicken \( \alpha_8 \) subunits belong to the subfamily of \( \alpha \) subunits that are cleaved (see below).

Sequence homology among \( \alpha \) subunits allows one to classify various \( \alpha \) subunits into subfamilies according to sequence homology, presence or absence of I domains, presence or absence of cleavage sites, and ability to bind to arginine-glycine-aspartate (RGD). The human \( \alpha_8 \) sequence is most closely related to \( \alpha_4 \) and \( \alpha_5 \) (42 and 43% identity, respectively). \( \alpha_8 \), like \( \alpha_4 \) and \( \alpha_5 \), fits into the subclass of \( \alpha \) subunits that lack the I domain and are cleaved.

**Fig. 8.** Immunohistochemical staining of adult rat tissues with anti-\( \alpha_8 \) antibodies. Frozen sections of kidney (A,B,C), chest wall (D,E,F), lung (G, H) and small intestine (I) were stained with hematoxylin and affinity-purified anti-\( \alpha_8 \) antibodies (B,C,E,F,H,I) or hematoxylin and anti-\( \alpha_8 \) antibodies preincubated with \( \alpha_8 \) synthetic peptide (A,D,G). The anti-\( \alpha_8 \) antibodies specifically stained glomeruli (GM), alveoli, and smooth muscle (arrowhead) of vessels (V) and small intestine. Kidney tubules (T), skeletal muscle (SK), peripheral nerve (PN) and endothelium (E) were negative.

### Table 1 Immunohistochemical distribution of \( \alpha_8 \) in adult rat tissue.

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<tr>
<th>Tissue*</th>
<th>( \alpha_8 ) Expression</th>
<th>Distribution</th>
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<tr>
<td>Trachea</td>
<td>+</td>
<td>Smooth muscle</td>
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<tr>
<td>Lung</td>
<td>+</td>
<td>Alveoli; vessel and airway smooth muscle</td>
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<tr>
<td>Heart</td>
<td>–</td>
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<tr>
<td>Aorta, blood vessels</td>
<td>+</td>
<td>Smooth muscle</td>
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<td>Kidney</td>
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<td>Glomeruli (mesangial cells)</td>
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<td>Liver</td>
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</tr>
<tr>
<td>Pancreas</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Thymus</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Gastrointestinal tract</td>
<td>+</td>
<td>Muscularis mucosa</td>
</tr>
<tr>
<td>Testes</td>
<td>+</td>
<td>Myoid cells</td>
</tr>
<tr>
<td>Uterus</td>
<td>+</td>
<td>Smooth muscle (weak)</td>
</tr>
<tr>
<td>Skin</td>
<td>+</td>
<td>Arrector pili muscle</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Peripheral nerves</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

*In all tissues, blood vessel walls are positive.

The distribution of \( \alpha_8 \) in mammalian species is unique among integrins with prominent staining in vascular and visceral smooth muscle, kidney glomeruli and alveoli. In developing chicken embryos, \( \alpha_8 \) was expressed in major axon tracts in the nervous system (Bossy and Reichardt, 1990). Although we did not perform detailed staining of the nervous system, we confirmed \( \alpha_8 \) staining in neuronal extensions in the hippocampus (data not shown). Similar to our findings, chicken \( \alpha_8 \) expression was demonstrated in smooth muscle in the urogenital and gastrointestinal tract (Bossy and Reichardt, 1990). However, there are several important differences. In contrast to the chick embryo, we find the most intense staining for \( \alpha_8 \) in vascular smooth muscle and kidney mesangial cells. We also noted selective staining of the muscularis mucosae smooth muscle layers in the intestine, with the remainder of the smooth muscle layers negative. We did not detect \( \alpha_8 \) expression in the inner epithelial layers of the gastrointestinal tract and the epidermis of the skin, even though chick embryos reportedly express \( \alpha_8 \) in these tissues. In the skin of the adult rat, only the smooth muscle cells associated with the hair follicle (arrector pili) stain for \( \alpha_8 \). The differences may be due to species-specific distribution or to developmental regulation of \( \alpha_8 \) expression, which is known to occur with several other integrins (e.g. \( \alpha_4\beta_1 \), \( \alpha_5 \beta_1 \), \( \alpha_5 \beta_3 \)).

In the mammalian adult, the cell types in which \( \alpha_8 \) is located all have contractile potential. For example, the smooth muscle cells of vessel walls stain intensely for \( \alpha_8 \). These vascular smooth muscle cells maintain a contractile phenotype in the normal, uninjured vessel. The extracellular matrix proteins that surround normal vascular smooth muscle cells, and thus may be potential ligands for \( \alpha_8 \), include fibronectin, laminin, vitronectin and collagen types I and IV (de Reeder et al., 1989; Heickendorff, 1988; Ross and Klebanoff, 1971).

In another example, the mesangial cells of the kidney glomeruli stain intensely for \( \alpha_8 \). The mesangial cells share many properties with vascular smooth muscle cells. These include the ability to contract in response to a variety of stimuli, and the presence of smooth muscle myosin and actin in culture (Kashgarian and Sterzel, 1992; Schlondorff, 1987). One postulated function of mesangial cells is to regulate blood flow in the capillary bed of the glomeruli by contracting the vessels. The response of mesangial cells to injury is also analogous to the
response of vascular smooth muscle cells. In both instances, there is proliferation of contractile cells and accumulation of extracellular matrix proteins (Diamond, 1991). The extracellular matrix proteins that surround normal mesangium include fibronectin, laminin, collagen types IV and V and entactin. In view of the distribution of α6, it is possible that one of the components common to both types of matrices serves as a ligand for α6.

In the alveolar walls of the rat lung, the cells staining with anti-α6 antibody are likely to be alveolar myofibroblasts, contractile cells located in the alveolar interstitium. Alveolar myofibroblasts are situated along the capillaries and adjacent to the alveolar epithelium and basement membrane. Ultrastructurally, they contain microfilament bundles but, in contrast to alveolar pericytes, do not normally contain α-smooth muscle actin (Kapanci et al., 1992). One postulated function of alveolar myofibroblasts is regulation of capillary tone.

The staining for α6 in intestine is prominent in the muscularis mucosae layer of the intestine, a thin layer of smooth muscle cells situated between the mucosa and submucosa. The muscularis mucosae also expresses the integrins α1β1, α2β1 and α6β1 (Choy et al., 1990). Proposed functions of the muscularis mucosae include regulation of the luminal fluid microcirculation through contraction of the muscle cells and structural support of intestinal villi (Sleisenger and Fordtran, 1993).

In summary, we have characterized the human integrin α6 subunit by determining the cDNA sequence, identifying the mRNA and protein in cultured cells and determining its tissue distribution. By immunohistochemistry in adult tissue, α6 shows prominent staining in vascular and visceral smooth muscle cells, mesangial cells and alveolar myofibroblasts. The cell types expressing α6 can potentially function as contractile cells. Thus, α6 may be involved in the interactions of these cells with their environment to regulate contractile function.

We thank Elizabeth Wayner (University of Minnesota, Minneapolis, MN) for providing the monoclonal antibody P5D2; Steve Albelda (University of Pennsylvania, Philadelphia, PA) for providing the antibody PECAM-1; David Chere sh (Scripps Clinic, La Jolla, CA) for the FG cell line; Elise Palmer and David Erle for helpful discussions during the course of this work; Ronald Ferrando for his assistance with establishing our immunohistochemistry protocol; and Julianne Richter for technical assistance with peptide synthesis. This work was supported in part by National Institutes of Health grants CA53259, HL191551 (to R.P.), HL/A133259, HL/47412 (to D.S.). Dr Schnapp was supported by Clinical Investigator Development Award K08HL02874 and Institutional National Research Service Award HL07185 from the National Institutes of Health.

REFERENCES


(Received 25 May 1994 - Accepted 27 September 1994)