Sequence and Tissue Distribution of the Integrin α9 Subunit, a Novel Partner of β1 That Is Widely Distributed in Epithelia and Muscle

Elise L. Palmer, Curzio Ruegg, Ronald Ferrando, Robert Pytela,* and Dean Sheppard

The Lung Biology Center, the Cardiovascular Research Institute, the Center for Occupational and Environmental Health, and the Departments of Medicine and * Pharmacology, University of California, San Francisco, California 94143

Abstract. The integrin family of adhesion receptors consists of several heterodimeric glycoproteins, each composed of one α and one β subunit. A novel integrin α subunit partial cDNA isolated from TGF-β stimulated guinea pig airway epithelial cells has previously been reported (Erle, D. J., D. Sheppard, J. Bruess, C. Ruegg, and R. Pytela. 1991. Am. J. Respir. Cell Mol. Biol. 5:170-177). We have now determined cDNA and amino acid sequence for the human homolog of this subunit, named α9, from a human lung cDNA library, a human small intestine cDNA library, and cDNA from the cell lines U937, HL-60 and Tera-2. This sequence is predicted to encode a 1006-amino acid mature protein that shares 39% identity with the previously identified integrin subunit α4. By Northern blot analysis, α9 mRNA was detected in the human carcinoma cell lines Tera-2 and Caco-2. Anti-peptide antibodies against the predicted COOH-terminal sequence of α9 immunoprecipitated a heterodimer (140 kD/115 kD nonreduced; 150 kD/130 kD reduced) from Tera-2 lysates. Immunodepletion of β1-containing integrins with Tera-2 lysates removed α9 immunoreactivity, suggesting that β1 is the principal β subunit partner for α9 in these cells. α9 was detected by immunohistochemistry in airway epithelium, in the basal layer of squamous epithelium, and in smooth muscle, skeletal muscle, and hepatocytes.

The integrins are a large family of cell surface glycoproteins that mediate cell-cell and cell-matrix adhesion (21). All known members of this family are heterodimers consisting of an α and a β subunit that bind non-covalently to each other. Published reports suggest the existence of 8 β subunits (β1-β8) (2, 12, 14, 20, 26, 30, 32, 34, 37, 40, 41) and 16 α subunits (2-5, 9, 10, 13, 16, 23, 25, 28, 33, 38, 39, 42-47). 13 of these have been completely sequenced (α1-α8, αδ, αM, αL, αX, αIIb). The existence of αIL and αIRI has been established on the protein level. We have previously reported identification of partial integrin α subunit cDNA sequences using degenerate oligonucleotide primer pairs and the PCR (13). Primers were designed based on two highly conserved regions, separated by 72-92-amino acid residues, located within the fifth and sixth repeated domains of the previously reported integrin α subunits. PCR amplification of cDNA using these primers resulted in the identification of several integrin α subunit partial cDNAs. From guinea pig airway epithelial cells six different sequences were identified. Five of these were 88 to 92% identical to the sequences of α1-α3, α5 and αV from other species and were presumed to encode the guinea pig homologs of those subunits. One of the amplified sequences was only 24-60% identical to previously reported α subunits. This novel cDNA was predicted to encode a 71-amino acid fragment of an integrin α subunit, provisionally designated αA. We now report the predicted coding sequence of the human homolog of this novel subunit. We have identified mRNA encoding this subunit and the corresponding protein in two cell lines, including the human teratoma cell line Tera-2, and demonstrate that this α subunit associates principally with the integrin β1 subunit in these cells. Based on the convention of sequentially numbering newly identified partners of β1, we have called this α subunit α9. We also demonstrate wide distribution of α9 in epithelia, smooth muscle, skeletal muscle, and hepatocytes.

Materials and Methods

Cell Lines, RNA Purification, and cDNA Synthesis

ATCC human cell lines were maintained in DME (HeLa, Tera-2, Caco-2,) or RPMI 1640 media (U937, HL-60, Raji, HuT 78, MOLT-4) containing 10% FBS. Media were obtained from BioWhittaker, Inc. (Walkersville, MD).

mRNA was isolated from resected human lung tissue using the Fast Track mRNA isolation kit (Invitrogen, San Diego, CA). Total cellular RNA was isolated from resected human lung tissue using the Fast Track mRNA isolation kit (Invitrogen, San Diego, CA).
was isolated using the LiCl/Urea method (12). Single-stranded cDNA was synthesized at 4°C for 1 h from 1 to 5 μg of mRNA or 20-40 μg of total RNA using the Superscript cDNA Synthesis System (GIBCO-BRL, Gaithersburg, MD) and random DNA hexamers. In some cases cDNA was synthesized as above using a previously described sequence tagged random DNA hexamer primer (CGAAGGGGATGGCTACGGAAAAGCCGAC-NNNNNNT) (15).

**Amplification and Purification of Lambda Library DNA**

Aliquots of λgt1 libraries containing 1-5 million plaque forming units were mixed with 500 μl of LE392 bacteria (OD = 0.5) in SM buffer (100 mM NaCl, 8 mM MgSO₄, 50 mM Tris-HCl, pH 7.0, 0.01% gelatin), incubated 15 min at 37°C, and shaken at 250 rpm overnight at 37°C in Luria broth containing 10 mM MgCl₂ and 0.2% maltose. The cultures were incubated at 37°C and 250 rpm for 30 min with 5% chloroform. Aliquots of the aqueous layer were subjected to three cycles of min in dry ice/ethanol followed by 3 min at 90°C, phenol/chloroform extraction, and precipitation with ethanol.

**PCR Amplification**

PCR reactions were performed in 25-200 μl reaction volumes and contained 1 X Taq buffer (Promega Corp., Madison, WI) with 1.5 mM MgCl₂, 0.1 μM each of two primers, 0.025 U/μl Taq Polymerase (Promega Corp.), 1-8 μl of DNA template, and 0.1 mM each of dATP, dGTP, dCTP, and dTTP. Reactions were heated to 94°C for 4 min in a thermocycler (Eppendorf, Inc., San Diego, CA) and then subjected to 30 cycles of PCR followed by 10 min at 72°C. Reactions that contained degenerate oligonucleotide primers were subjected to PCR cycles consisting of 45 s at 94°C, 45 s at 48 or 53°C, and 45 s at 72°C. Reactions that contained a specific primer pairs designed to amplify DNA fragments less than 700 nucleotides were subjected to PCR cycles consisting of 45 s at 94°C, 45 s at 57°C, and 60 s at 72°C. Reactions that contained an a-specific primer, a λgt1-specific primer (Aglt1F: CACTACTCTACGGTCGACGGAAGCGACC or Aglt1R: CCGTACCGAACGCCGCGAGCGAC), and purified restriction-digested total library DNA were subjected to PCR cycles consisting of 45 s at 94°C, 45 s at 57°C, and 60 s at 72°C. All other PCR reactions were subjected to cycles consisting of 45 s at 94°C, 45 s at 57°C, and 120 s at 72°C. Products of each PCR reaction were analyzed by agarose gel electrophoresis using standard or low gel temperature agarose.

PCR reactions designed to amplify 3' sequence from sequence tagged cDNA, described above, contained a-specific forward primers and primers complementary to the sequence tagged random hexamer (GTGTCGACGGAACGCACGC) and were performed as above.

**Cloning of DNA Fragments**

Restriction-digested DNA fragments were isolated on low gel temperature agarose and purified by phenol/chloroform extraction and ethanol precipitation. Fragments were ligated into restriction-digested, dephosphorylated pBlueScript vector (Strategene, La Jolla, CA) with T4 DNA ligase (GIBCO-BRL). 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 mini prep lysis kit (Pharmacia Fine Chemicals/LKB, Pleasant Hill, CA) and sequenced using Sequenase 2.0 (Amersham Corp., Arlington Heights, IL), 3′- or 5′-end, random primed Xgt1 I cDNA library from human lung tissue including trachea and bronchi (catalog No. HL1333; Clontech) and a mixed random and oligo-dT primed λgt1 I cDNA library from human small intestine tissue (catalog No. HL1133b; Clontech). Hybridizations were performed at 50°C for 16 h in hybridization buffer containing 5 × SSC, 40% formamide, 20 mM Tris, pH 7.5, 0.1% polyvinylpyrrolidone, 0.1% BSA, 0.1% ficoll, 10% dextran sulfate, and 100 μg/ml heat-denatured, sonicated salmon sperm DNA, and washed in 5 × SSC containing 0.1% SDS at 50°C for 30 min. After washing, filters were exposed to film at -80°C with an intensifying screen.

**Antibodies and Immunoprecipitations**

Polyclonal antiserum was generated against the α9 peptide CRMKEDNDSW-DWHVQKO. Peptide synthesis, conjugation to KLH, and injection of rabbits performed by Immunodynamics, Inc. (La Jolla, CA). α9 antibodies were affinity purified from crude antiserum on a peptide-lysozyme-agarose column as follows. Affigel-10 slurry (4 ml bed volume) (Bio Rad Labs., Richmond, CA) was washed three times with cold 10 mM sodium acetate, pH 4.5; rinsed once with 0.1 M potassium phosphate, pH 7.5; mixed with 100 ng lysozyme (Sigma Immunochemicals, St. Louis, MO) in 8 ml 0.1 M potassium phosphate, pH 7.5, for 2 h at room temperature; washed twice with 0.1 M potassium phosphate, pH 7.5; mixed with 8 ml 0.2 M ethanolamine, pH 8.0, for 2 h at room temperature; and washed three times with PBS. Lysozyme-agarose (600-μl bed volume) was washed with 0.05 M sodium phosphate, pH 8.0; mixed with 2.5 μg sulfo-maleimidobenzoyl-N-hydroxy-succinimide ester (Pierce Chemicals, Rockford, IL), 1 ml 0.05 M sodium phosphate, pH 8.0, for 30 min at room temperature; washed with 0.05 M sodium phosphate; pH 8.0; mixed with 3 μg α9 peptide in 500 μl 0.05 M sodium phosphate, pH 8.0, for 3 h at room temperature; and washed with (a) 0.05 M sodium phosphate, pH 7.0, (b) 200 mM glycine, pH 1.5, (c) 0.05 M sodium phosphate, pH 7.0, (d) 8 M urea, and finally (e) 0.05 M sodium phosphate, pH 7.0. 5-10 μl aliquots of antiserum were mixed with the peptide-lysozyme-agarose overnight at 4°C. The slurry was transferred to a column and washed with 0.05 M sodium phosphate, pH 7.0, until the OD 280 of the wash buffer was less than 0.01. Antibodies were eluted in 1.5 ml fractions with 200 mM glycine, pH 1.5, and collected in tubes containing 250 μl 1 M sodium phosphate, pH 8.0. Pooled fractions were dialyzed overnight at 4°C against PBS containing 0.02% azide.

mAb PS52 (11) directed against the β1 integrin subunit was a gift from Richard Wagner (University of Minnesota, Minneapolis, MN). mAb R699 directed against the β6 integrin subunit was generated in our laboratory. For some experiments antibodies were cross linked to protein A-Sepharose with dimethylpimelimidate (17).

Cells were surface labeled with [3H]I, lysed in immunoprecipitation buffer (100 mM Tris-HCl, pH 7.5, 0.1% SDS, 1% Triton X-100, 0.1% NP-40, and 300 mM NaCl), and immunoprecipitated by standard techniques. Samples were analyzed by SDS-PAGE on 7.5% acrylamide gels and exposed to film at -80°C with intensifying screens.

**Tissue Staining**

Frozen sections (5 μm) of tissue were fixed in either 2% paraformaldehyde (mouse tissue) at room temperature or in -18°C acetone (human tissue) for 5 min and subsequently rinsed in PBS. Sections were blocked for endogenous peroxidase activity with Peroxoblock Solution (Zymed Laboratories, Inc., South San Francisco, CA) for 45 s at room temperature. After rinsing, sections were preblocked with 0.5% casein/0.05% thimerosal/PBS for 15 min at room temperature and then incubated overnight at 4°C in primary antibody (1:200 in 0.5% casein/0.05% thimerosal/PBS) that either was or was not preincubated with 0.1 mg/ml α9 peptide for 30 min at 4°C. After rinsing in PBS, sections were incubated in either biotinylated donkey anti-rabbit secondary antibody (Amersham Corp.) at a dilution of 1:200 or peroxidase-conjugated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA) at a dilution of 1:250 for 1 h at room temperature in 0.5% casein/0.05% thimerosal/PBS. Sections incubated with biotinylated secondary antibodies were then rinsed and incubated in ABC avidin/peroxi-

dase reagent (Vector Laboratories) for 1 h at room temperature. Chromagen
was developed using the DAB Plus Kit from Zymed Laboratories. Reactions were monitored until suitable color development was achieved. The signal was enhanced with 0.5% nickel chloride, and the sections were rinsed in distilled water. Sections were subsequently air dried and then mounted with Permount (Fisher Scientific, Pittsburgh, PA) onto clean slides.

**Protein Sequencing**

Human uterine tissue was homogenized in immunoprecipitation buffer and cleared by centrifugation at 200 g. The supernatant was incubated with affinity-purified α9 anti-cytoskeletal peptide antiseraum cross-linked to protein-A-Sepharose CL4B (Pharmacia Fine Chemicals, Piscataway, NJ) overnight at 4°C. The beads were then washed extensively with immunoprecipitation buffer and heated to 95°C for 5 min in 2% SDS, 300 mM 2-mercaptoethanol, 80 mM Tris, pH 6.8. The supernatant was concentrated on a Centricon-10 Concentrator (Amicon, Beverly, MA), subjected to 7.5% SDS-PAGE, transferred to PVDF membrane (Bio Rad Labs. Hercules, CA) for 3 h at 50 V in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid, pH 11/10% methanol, and quantified by staining with 0.1% Coomassie blue R-250 in 40% methanol/1% acetic acid. A prominent band at 150 kD was microsequenced by the USCF Biomedical Resource Center (San Francisco, CA).

**Results**

**Cloning and Sequencing of Human α9**

A novel 223-nucleotide partial guinea pig integrin α subunit cDNA (αA) was previously reported by Erle et al. (13). To obtain the corresponding human sequence of the novel subunit, we used the previously described degenerate integrin α subunit forward primer (A14F: CGGAATTCGGIG-obtain the corresponding human sequence of the novel cDNA (t~A) was previously reported by Erie et al. (13). To

A novel 223-nucleotide partial guinea pig integrin α sub unit, we used the previously described degenerate inte-rogen reverse primer based on a human α sequence (AN2R: CAAGTCGACAA(AG)TGIGC(AG)TT(GC)TA-IGGC(GT)/(GT)/(AT), and cDNA made from human lung tis

A band of the predicted size (204 nucleotides) was ob-

ained (data not shown), subcloned into pBluescript, and sequenced. The resulting nucleotide sequence was 88% identical to the sequence of guinea pig αA and 39–54% identical to other known human integrin α subunits. For reasons discussed later, we have named this novel human integrin α subunit α9.

The 204-nucleotide α9 cDNA fragment was used to screen a human lung cDNA library. One hybridizing clone, L1 (Fig. 1), was identified, purified, subcloned and sequenced. This 1,678-bp clone contained a 1,123-nucleotide open reading frame that included the probe sequence.

We were able to amplify α9 from cDNA from leukocyte cell lines U937 (clones U1–U3) and HL-60 (HI) by PCR using α9-specific primers. To isolate additional sequence from these cell lines, we used a previously described 3′-sequence extension technique (15) which employs PCR. One clone from HL-60 cDNA, H2, and one clone from U937 cDNA, U4, obtained by this method contained 117 nucleotides of α9 sequence.

PCR was performed on amplified purified human lung cDNA library DNA using α9-specific forward oligonucleotide primers and λgt11-specific primers (λgt11F and λgt11R). Individual bands were isolated, reamplified, subcloned, and sequenced. One clone, L2a, consisted of 300 nucleotides of α9 sequence. Clone L2a was used as a probe for screening the human lung library and one hybridizing clone, L2b, was isolated, purified, subcloned, and sequenced. Both clone L2a and clone L2b ended at nucleotide 1817, suggesting that clones L2a and L2b represented the same library clone.

We screened a human duodenal cDNA library with probes from clones L1 and L2. 12 independent hybridizing clones were identified, isolated, subcloned, and analyzed. Six of these were determined to contain additional α9 cDNA and were sequenced (D1–D6). D2 and D6 contained a predicted transmembrane domain, cytoplasmic domain, and translation stop codon. D6 also had an additional 1.1 kb, 3′ of the stop codon, which did not extend to the poly(A) tail.

To complete the α9 sequence, α9 protein was purified from human uterus using anti-α9 antiseraum, described later, and microsequenced. The amino terminal sequence was unequivocally determined to be YNLDPQ (Q/E). A series of degenerate forward oligonucleotide PCR primers were designed based upon these possible amino-terminal sequences. PCR amplifications were performed using each of the degenerate forward primers paired with α9-specific reverse oligonucleotide primers and Tera-2 cDNA (data not shown). Reactions using a degenerate forward primer based upon the sequence YNLDPQ and two different α9-specific reverse primers resulted in intense bands which were cloned and sequenced (T1–T3).

We have sequenced 3,139 nucleotides (Fig. 2) of α9 cDNA that contains a 3,000-nucleotide open reading frame that includes a termination signal but lacks an initiation codon. Based on this cDNA sequence and the amino terminal se-

quence we obtained, we predict the mature α9 protein to be 1,006 amino acids in length with a 947-amino acid extracellular domain, a 26-amino acid transmembrane domain, and
Figure 2. Human integrin α9 cDNA sequence and deduced amino acid sequence. The amino acid sequence is shown in one-letter code below the first nucleotide of each codon. The putative transmembrane domain is shown by a double underline. Asparagine residues (N) that are potential sites for N-glycosylation are indicated by dotted underline. Conserved putative metal binding domain are indicated by a single underline. Nucleotide 36 was determined to be A from some clones and G from others (indicated by a G in parentheses above the nucleotide location). Nucleotide 2974 was C in some clones and A in others. None of these nucleotide variations changed the deduced amino acid sequence. This sequence data is available from EMBL/GenBank/DDBJ under accession number L24158.

a 33-amino acid cytoplasmic domain. α9 has 12 asparagine residues that are potential N-glycosylation sites (NXT/S).

The Relationship of α9 to Other Integrin α Subunits

Comparison of α9 to the general structure of integrin α subunits is indicated in Fig. 3A. The consensus structure includes: a large NH₂-terminal extracellular domain containing seven conserved repeats (four partial and three complete putative metal binding domains) and in some cases the I domain, an insertion of approximately 200 amino acids, a single transmembrane-spanning domain, and a short COOH-terminal cytoplasmic domain. Some subunits are cleaved into two disulfide-linked fragments near the transmembrane domain. The second subfamily consists of the seven conserved repeats found in all known integrin α subunits. α9 does not contain an I domain nor is it cleaved. The cytoplasmic domain of α9, as with all α subunits, contains the highly conserved sequence GFF(R/K)R.

Sequence, structural, and functional relationships between the integrin α subunits are shown in Fig. 3B. The integrin α subunit family has three distinct subfamilies. The first subfamily consists of the seven α subunits that undergo cleavage near the transmembrane domain. The second subfamily includes the five α subunits that contain an I domain. The third subfamily consists of α4, which contains neither the I domain nor undergoes cleavage yielding disulfide-linked fragments. The deduced partial amino acid sequence of α9 is 39% identical to the integrin α4 subunit sequence and 18–22% identical to the other known human integrin α subunit sequences. Based on its sequence and structural similarity to α4, α9 is clearly a member of the third integrin α subunit subfamily.

Fig. 4 shows the alignment of the predicted α9 amino acid sequence with α4. All 23 cysteine residues found in α9 align with those found in α4 (α4 contains one additional cysteine).
This probe hybridized with an ~7.0-kb mRNA band (size markers not shown) that was present in two of the cell lines tested, i.e., Tera-2, and Caco-2 cells (lanes 1 and 2). The RNA was isolated from a variety of cell lines and analyzed by Northern blot analysis. Total RNA (30-40 µg/lane) isolated from human cell lines Caco-2 (lane 1), Tera-2 (lane 2), HeLa (lane 3), Raji (lane 4), HuT 78 (lane 5), MOLT-4 (lane 6), and HL-60 (lane 7) was probed for α9 mRNA using 2.1 kb of rIP-labelled α9 32P-labeled α9 cDNA. The positions of the 28- and 18-S ribosomal bands are indicated on the left.

Figure 4. Alignment of α9 with its closest integrin α subunit relative, α4. The deduced amino acid sequences of human α9 and guinea pig αA (reference 3) and the previously reported sequence of the mature form of α4 (reference 46) are shown aligned using the one-letter amino acid code. Cysteine residues conserved between α9 and α4 are indicated by an asterisk (*). The conserved metal binding repeats common to all integrin α subunits are underlined. The predicted transmembrane domains are indicated by double underline. The cleavage site in α4 (++), between Arg558 and Ser559, is not present in α9.

including the 19 cysteines generally found in integrin α subunits. α4 contains a cleavage site following Lys557-Arg558 which is absent in α9.

α9 Is Expressed by Teratoma and Colon Carcinoma Cells and Forms a Heterodimer with β1

To demonstrate expression of α9 mRNA and to identify cell lines that express α9, Northern blots were performed. Total RNA was isolated from a variety of cell lines and analyzed by Northern blotting with a 2.1-kb α9 cDNA probe (Fig. 5). This probe hybridized with an ~7.0-kb mRNA band (size markers not shown) that was present in two of the cell lines tested, i.e., Tera-2, and Caco-2 cells (lanes 1 and 2). The smaller band may represent partially degraded α9 mRNA. Alternatively, a second α9 mRNA species derived by alternative splicing or an alternative polyadenylation signal may be present in these cells. Tera-2 cells are derived from an embryonal carcinoma, and Caco-2 cells from a colon carcinoma.

Figure 5. Identification of α9 mRNA by Northern blot analysis. Total RNA (30-40 µg/lane) isolated from human cell lines Caco-2 (lane 1), Tera-2 (lane 2), HeLa (lane 3), Raji (lane 4), HuT 78 (lane 5), MOLT-4 (lane 6), and HL-60 (lane 7) was probed for α9 mRNA using 2.1 kb of 32P-labelled α9 cDNA. The positions of the 28- and 18-S ribosomal bands are indicated on the left.

Figure 6. Alignment of integrin α subunit cytoplasmic domains. The COOH-terminal cytoplasmic sequences of the known human integrin α subunits are shown (references 2, 5, 9, 10, 28, 33, 39, 42-45). The amino acid sequence of the peptide used for production of α9-specific rabbit antiserum is underlined and labeled α9 Peptide.

Figure 7. Immunoprecipitation of α9- and β1-containing integrins from lysates of Tera-2 cells. Aliquots of 125I-surface-labeled lysates from Tera-2 cells were immunoprecipitated with a monoclonal anti-β1 antibody (lanes 1 and 4), affinity purified polyclonal antiserum raised against a portion of the cytoplasmic domain of α9 (lanes 2 and 5), and preimmune serum (lanes 3 and 6). The proteins were analyzed by SDS-PAGE under nonreducing conditions (Fig. 7). Immunoprecipitation with anti-β1 yielded major proteins.
Figure 8. Immunodepletion of β1 containing integrins. Aliquots of 125I-surface-labeled lysates from Tera-2 cells were subjected to four rounds of immunodepletion with an anti-β1 monoclonal antibody or an anti-β6 mAb cross-linked to protein A-Sepharose. The resulting supernatants were immunoprecipitated with affinity purified α9 polyclonal antisera. The immunoprecipitated material (anti-β1 first round immunoprecipitate, lane 1; anti-β1 followed by α9 antiseraum, lane 2; anti-β6 followed by α9 antiseraum, lane 3) was analyzed by SDS-PAGE under nonreducing conditions followed by autoradiography. The positions of molecular size markers (in kD) are shown to the right.

nonreduced bands at 140 and 115 kD and a minor band at 180 kD (lane 1). These bands changed upon reduction to 190, 150, and 130 kD (lane 4). α9 antiseraum immunoprecipitated a heterodimer consisting of 140 and 115 kD subunits nonreduced (lane 2) and 150 and 130 kD subunits reduced (lane 5). The upper band migrated with the same apparent molecular mass as several other previously reported integrin α subunits (19). The lower band migrated with the same apparent molecular mass as β1 (19). The band present in α9 immunoprecipitations at 110 kD is also present in immunoprecipitations using preimmune serum (lanes 3 and 6), and hence is nonspecific.

To determine if β1 was a β subunit partner for α9, Tera-2 lysates were subjected to four rounds of immunodepletion with either anti-β1 or anti-β6 antibody followed by immunoprecipitation with affinity purified α9 antiseraum. The results (Fig. 8) show that α9 could not be immunoprecipitated from lysate depleted of β1 (lane 2). Immunodepletion with the anti-β6 antibody (lane 3) did not interfere with subsequent precipitation of α9, demonstrating that nonspecific loss of α9 does not occur during multiple rounds of immunodepletion. These results suggest that β1 is the predominant β subunit partner for α9 in Tera-2 cells. This justifies our terminology, following the convention of sequentially numbering the α subunit partners of β1.

α9 is Widely Distributed in Normal Tissues

Because α9 was initially isolated from airway epithelial cell cDNA, we attempted to identify α9 in human airway tissue by immunohistochemistry. Fig. 9 A, shows a human airway biopsy section, including epithelium and submucosa, stained with hematoxylin. α9 antiseraum intensely stained the airway epithelium (B). When the antiseraum was incubated with α9 peptide prior to tissue staining, staining of the epithelium was substantially blocked (C).

Because we were able to detect α9 immunoreactivity in airway tissue, we examined the expression of α9 in other tissues. Sections of mouse tissue were stained with hematoxylin (Fig. 9, D, G, and J) α9 antiseraum (E, H, and K), or α9 antiseraum preblocked with α9 peptide (F, I, and L). In mouse esophagus (D–F), the basal layer of the epithelium (arrow) was stained by α9 antiseraum. Skeletal muscle (M) present in this section also showed α9 immunoreactivity. In mouse small intestine (G–I) both the longitudinal and circumferential smooth muscle layers (SM) reacted with the α9 antiseraum. Hepatocytes in the liver (J, K, and L) demonstrate cell surface localization of α9. A variety of other mouse tissues were analyzed for the presence of α9. Table I summarizes these results. The tracheal epithelium; basal epithelium of the larynx, pharynx, esophagus, skin, and cornea; smooth muscle of trachea, veins, duodenum, colon, stomach, and esophagus; skeletal muscle; hepatocytes; and splenic giant cells all demonstrated α9 immunoreactivity. In all of these cases, staining was blocked by incubation of the α9 antiseraum with α9 peptide prior to tissue staining.

Discussion

This report presents three novel findings. First, we present the complete amino acid sequence, deduced from cDNA and amino terminal sequencing, of a new member of the human integrin α subunit family, α9. Second, we demonstrate that α9 forms an integrin heterodimer with the known β subunit, β1. Third, we show that α9 is expressed in a variety of cell types in vivo including airway epithelial cells, the basal layers of squamous epithelium, smooth muscle, skeletal muscle, and hepatocytes.

Comparison of the deduced amino acid sequence of α9 with the previously published sequences of human integrin α subunits clearly demonstrates that this protein is a member of the integrin α subunit family. α9 has high sequence homology with other human integrin α subunits and has predicted structural features common to the integrin α subunits including (a) a large extracellular domain containing four partial and three complete metal binding domains, 19 consensus cysteine residues, and several potential N-glycosylation sites, (b) a single transmembrane spanning domain, and (c) a short cytoplasmic domain containing the sequence GFF(K/R)R. Although α9 is the only human integrin α subunit to have the alternative sequence GFFRR, chicken α3 contains GFFRR (22). Both variations of this sequence are also found in the DNA binding domain of the members of the steroid hormone receptor superfamily (29). The functional significance of this sequence is not known, although it has been reported that a GFFKR-containing peptide can interact with the Ro(SS-A) antigen (calreticulin) (35).

There are reports of four other integrin α subunits for which the human sequence has not been published. We do not believe that any of these four are identical to the subunit we describe based on the following evidence. The integrin subunits α7 and α8 have been cloned from other species, and

Figure 9. Immunohistochemical localization of α9. Frozen sections of human airway biopsies (A–C) and mouse esophagus (D–F), duodenum (G–I), and liver (J–L) were stained with hematoxylin (A, D, G, J), with α9 antiseraum (B, E, H, K), or with α9 antiseraum preincubated with α9 peptide (C, F, I, L). The α9 antiseraum specifically stained airway epithelium, esophageal skeletal muscle (M) and basal cells of the squamous epithelium (arrow), duodenal smooth muscle (SM), and hepatocytes.
the resulting sequences have less than 21% amino acid identity to α9 (4, 38). Partial cDNA sequences of the human homologs of α7 and α8 are clearly distinct from the α9 sequence (our unpublished observations). Both of the subunits of a novel integrin expressed on activated leukocytes, termed the leukocyte response integrin, have different electrophoretic mobilities than does α9 (16). The previously identified HML-1 antigen is an integrin composed of a novel α subunit, αIEL, paired with β1 (7, 25, 47). The HML-1 antigen has been detected in intraepithelial lymphocytes and a subset of lymphocytes located in the submucosal layer of the intestine (8), whereas α9 was not detected in either the intestinal epithelial layer or submucosa.

We screened several cell lines by Northern blotting for expression of α9 and identified two cell lines, Tera-2 and Caco-2, that expressed significant levels of α9 mRNA. Immunoprecipitation of lysates from these cell lines with α9 anti–peptide antiserum precipitated a heterodimer consisting of a larger subunit of similar size to many other α subunits and a smaller subunit that comigrated with β1 (Caco-2 data not shown). Immunodepletion of β1 from Tera-2 lysates removed all detectable α9 immunoreactivity, suggesting that β1 is the principal β subunit partner for α9 in these cells. This increases the number of α subunits known to associate with β1 to 10. Three of these α subunits (αv, α4, and α6) can also form heterodimers with other β subunits. We cannot exclude the possibility that α9 is expressed in low abundance with another β subunit in Tera-2 cells or that α9 has other β subunit partners in other cell lines or in vivo.

The closest known relative of the α9 subunit is the α4 subunit. α9 and α4 share 39% amino acid identity and are both equally divergent from the other known α subunits. Consistent with their similarity, α9, like α4, is not composed of two disulfide-linked fragments and does not contain an I domain. Integrin α subunits generally demonstrate low correlation of sequence similarity with tissue distribution. Therefore, even though α9 and α4 are closely related, we would not necessarily expect them to have similar expression patterns. α4 was first identified on leukocytes (19) and has more recently been shown to be expressed on endothelial cells (31) and in developing, but not adult, skeletal muscle (36). In contrast, α9 was detected in adult skeletal and smooth muscle and in a subset of epithelial cells and is not generally expressed in lymphocytes or in endothelium. Although α9 was detected by PCR in two leukocyte cell lines, it could not be detected by Northern blotting in these cells. The overwhelming majority of lymphocytes present in sections of mouse spleen did not stain with the α9 antibody. However, rare, splenic giant cells did demonstrate α9 immunoreactivity, suggesting that α9 may be expressed in at least some leukocytes in vivo.

Our results show widespread expression of α9 in vivo. In airway epithelium and the basal layer of squamous epithelium, α9 appears to be distributed at cell–cell borders as well as at cellular contacts with basement membrane. In smooth muscle, skeletal muscle, and hepatocytes, α9 appears to be uniformly distributed over the cell surface. In these tissues, α9 is expressed diffusely at sites of homotypic cell to cell contact in cells that are not actively involved in spreading, migration, or any other obvious dynamic interaction with the extracellular matrix. These results suggest that α9 may be involved in homotypic cell–cell interactions. Although integrins are not generally thought to be involved in homotypic cell–cell adhesion in non-leukocyte cells, localization of integrins to cell–cell contacts has been shown for α2β1 in bronchial epithelial cells (1), cultured keratinocytes (6), and endothelial cells (27); for α3β1 in bronchial epithelial cells (1), cultured keratinocytes (6), and a variety of transformed cell lines (24); and for α5β1 in endothelial cells (27). Furthermore, contacts between cultured keratinocytes have been shown to be disrupted by antibodies directed against α3β1 and β1 (6). Mediation of cellular cohesion by integrins could result from direct binding of integrins to counter-receptors present on adjacent cells or by neighboring cells jointly binding to extracellular matrix proteins deposited in the intercellular space.

The results of this study demonstrate the existence of a previously unrecognized member of the integrin family that is widely expressed in vivo in differentiated cells that are not actively involved in migration, proliferation, or heterotypic interactions with other cells. These data suggest that this integrin may function in some aspect of normal tissue cohesion or homeostasis. More definitive functional characterization will require identification of the ligand or ligands for this receptor and the development of reagents that specifically interfere with its function.

We thank Elizabeth Wayner (University of Minnesota, Minneapolis, MN) for providing the mAb PS/D2, Ann Weinacker and Aileen Chen for developing the mAb R6G9, David Erie and Lynn Schnapp for helpful discussions during the course of this work, and Angela Wang for her assistance with establishing our immunohistochemistry protocol.

This work was supported by grants HL/AI33259, HL47412, CA53259, and HL191551 from the National Institutes of Health, and grant RT338 from the University of California Tobacco-Related Disease Research Program. C. Rüegg was supported by a fellowship from the American Heart Association, California Affiliate.

Received for publication 21 June 1993 and in revised form 27 August 1993.

References

1. Albelda, S. M. 1991. Endothelial and epithelial cell adhesion molecules. Am. J. Respir. Cell Mol. Biol. 4:195–203.
2. Angervais, W. S., S. Suzuki, H. Araki, K. Thompson, M. D. Pierschbacher, and E. Ruoslahti. 1987. Amino acid sequence of the human fibronectin receptor. J. Cell Biol. 105:1183–1190.
3. Arnaout, M. A., O. E. Remold, M. W. Pierce, P. Harris, and D. G. Tenen.
26. Kimishima, T. K., K. O'Connor, A. Lee, T. M. Roberts, and T. A. Springer. 1986. Cloning of the beta subunit of the leukocyte adhesion proteins: homology to an extracellular matrix receptor defines a novel supergene family. Cell. 48:681–690.

27. Lampugnani, M. G., M. Renziti, E. Dejana, and P. C. Marchisio. 1991. The role of integrins in the maintenance of endothelial monolayer integrity. J. Cell Biol. 112:479–490.

28. Larson, R. S., A. L. Corbi, L. Berman, and T. Springer. 1989. Primary structure of the leukocyte function-associated molecule-1 alpha subunit: an integrin with an embedded domain defining a protein superfamily. J. Cell Biol. 108:703–712.

29. Laudet, V., C. Hantti, J. Coll, P. Catzeflis, and D. Stelhien. 1992. Evolution of the nuclear receptor gene superfamily. EMBO (Eur. Mol. Biol. Organ.) J. 11:1003–1013.

30. Law, S. K., J. Gagnon, J. E. Hildreth, C. E. Well, C. E. Willis, and J. S. Bennett. 1992. Structure of the platelet membrane glycoprotein IIb. Homology to the alpha subunits of the vitronectin and fibronectin membrane receptors. J. Biol. Chem. 262:8474–8482.

31. Law, S. K., J. Gagnon, J. E. Hildreth, C. E. Well, C. E. Willis, and J. S. Bennett. 1992. Structure of the platelet membrane glycoprotein IIb. Homology to the alpha subunits of the vitronectin and fibronectin membrane receptors. J. Biol. Chem. 262:8474–8482.

32. Moyle, M., A. M. Napier, and J. W. McClean. 1991. Cloning and expression of a divergent integrin subunit beta 8. J. Biol. Chem. 266:19650–19658.

33. Poonc, R. R., E. Riesman, R. Heidenreich, S. M. Silver, G. Viuione, S. Sorens, E. Schwartz, and J. S. Bennett. Structure of the platelet membrane glycoprotein IIb. Homology to the alpha subunits of the vitronectin and fibronectin membrane receptors. J. Biol. Chem. 262:8474–8482.

34. Rameswaran, H., and M. E. Hemler. 1990. Cloning and primary structure and properties of a novel human integrin beta subunit. EMBO (Eur. Mol. Biol. Organ.) J. 9:1561–1568.

35. Rojiani, M. V., B. Finlay, V. Gray, and S. Dedhar. 1991. In vitro interactions of a polypeptide hormone, RoSS-A antigen (calciferulin) with a highly conserved amino acid sequence in the cytoplasmic domain of integrin alpha subunits. Biochemistry. 30:9859–9866.

36. Rosen, G. D., J. R. Sones, R. LaChance, J. M. Cunningham, J. Roman, and D. C. Dean. 1992. Roles for the integrin VLA-4 and its counter receptor VCAM-1 in myogenesis. Cell. 69:1107–1119.

37. Sheppard, D., C. Rozzo, L. Starr, V. Quaranta, D. J. Erle, and R. Rytel. 1990. Complete amino acid sequence of a novel integrin beta subunit (beta 6) identified in epithelial cells using the polymerase chain reaction. J. Biol. Chem. 265:11502–11507.

38. Song, W. K., W. Wang, R. F. Foster, D. A. Bielsler, and S. J. Kaufman. 1992. H36-alpha 7 is a novel integrin alpha chain that is developmentally regulated during skeletal myogenesis. J. Cell Biol. 117:643–657.

39. Suzuki, S., W. S. Argraves, H. Arai, L. R. Languino, M. D. Pierschbacher, and E. Rossolashvily. 1987. Amino acid sequence of the vitronectin receptor alpha subunit and comparative expression of adhesion receptor mRNAs. J. Biol. Chem. 262:14480–14485.

40. Suzuki, S., J. Z. Huang, and H. Tanhara. 1990. Cloning of an integrin beta subunit exhibiting high homology with integrin beta 3 subunit. Proc. Natl. Acad. Sci. USA. 87:5534–5538.

41. Suzuki, S., and Y. Naitoh. 1992. Amino acid sequence of a novel integrin alpha 4 subunit and primary expression of the mRNA in epithelial cells. EMBO (Eur. Mol. Biol. Organ.) J. 9:757–763.

42. Takada, Y., J. M. Elies, C. Crouse, and M. E. Hemler. 1989. The primary structure of the alpha 7 subunit of VLA-4: homology to other integrins and a possible cell-cell adhesion function. EMBO (Eur. Mol. Biol. Organ.) J. 8:1361–1368.

43. Takada, Y., and M. E. Hemler. 1989. The primary structure of the VLA-2/collagen receptor alpha 2 subunit (platelet GPIIa): homology to other integrins and the presence of a possible collagen-binding domain. J. Cell Biol. 109:397–407.

44. Takada, Y., E. Murphy, P. Piil, C. Chen, M. H. Ginsberg, and M. E. Hemler. 1990. Molecular cloning and expression of the CD24 antigen: homology to alpha 3 subunit of human alpha 3 beta 1 (VLA-3), an integrin receptor for fibronectin, laminin, and collagen. J. Cell Biol. 115:257–266.

45. Tamura, R. N., C. Rozzo, L. Starr, J. Chambers, L. F. Reichardt, H. M. Cooper, and V. Quaranta. 1990. Epithelial integrin alpha 6 beta 4 is a complete primary structure of alpha 6 and variant forms of beta 4. J. Cell Biol. 111:1593–1604.

46. Tsuji, T., F. Yamamoto, Y. Minra, K. Takio, K. Titani, S. Pawar, T. Oohara, and S. Hakomori. 1990. Characterization through DNA cloning of galactoprotein b3 (Gap b3), a cell surface membrane glycoprotein showing enhanced expression on oncogenic transformation. Identification of Gap b3 as a member of the integrin superfamilly. J. Biol. Chem. 265:7016–7021.

47. Yuan, Q., W. M. Jiang, D. Hollander, E. Leung, J. D. Watson, and G. W. Krissansen. 1991. Identity between the novel integrin beta 7 subunit and an antigen found highly expressed on intraepithelial lymphocytes in the small intestine. Biochem. Biophys. Res. Commun. 176:1443–1449.