

Cloning of Mouse Integrin α_V cDNA and Role of the α_V -related Matrix Receptors in Metanephric Development

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Abstract. Metanephrogenesis has been a long-standing model to study cell–matrix interactions. A number of adhesion molecules, including matrix receptors (i.e., integrins), are believed to be involved in such interactions. The integrins contain α and β subunits and are present in various tissues in different heterodimeric forms. In this study, one of the members of the integrin superfamily, α_V , was characterized, and its relevance in murine nephrogenesis was investigated. Mouse embryonic renal cDNA libraries were prepared and screened for α_V , and multiple clones were isolated and sequenced. The deduced amino acid sequence of the α_V cDNA clones and hydropathic analysis revealed that it has a typical signal sequence and extracellular, transmembrane, and cytoplasmic domains, with multiple Ca^{2+} binding sites. No A(U)_nA mRNA instability motifs were present. Conformational analysis revealed no rigid long-range-ordered structure in murine α_V . The α_V was expressed in the embryonic kidney at day 13 of

the gestation, with a transcript size of ~ 7 kb. Its expression increased progressively during the later gestational stages and in the neonatal period. It was distributed in the epithelial elements of developing nephrons and was absent in the uninduced mesenchyme. In mature metanephroi, the expression was relatively high in the glomeruli and blood vessels, as compared to the tubules. Various heterodimeric associations of α_V , i.e., with β_1 , β_3 , β_5 , and β_6 , were observed in metanephric tissues. Inclusion of α_V -antisense-oligodeoxynucleotide or -antibody in metanephric culture induced dysmorphogenesis of the kidney with reduced population of the nephrons, disorganization of the ureteric bud branches, and reduction of mRNA and protein expressions of α_V . The expressions of integrin β_3 , β_5 , and β_6 were unaltered. These findings suggest that the integrin α_V is developmentally regulated, has a distinct spatio-temporal expression, and is relevant in the mammalian organogenesis.

DURING development, the interactions between cell adhesion molecules and extracellular matrix (ECM)¹ glycoproteins are believed to be essential for the morphogenesis of various organs (Hay, 1991; Humphries et al., 1991; Reichardt and Tomaselli, 1991; Ruoslahti, 1991; Toole, 1991; Hynes, 1992; Juliano and Haskill, 1993; Kramer et al., 1993; Gladson and Cheresch, 1994; Hemler et al., 1994; Quaranta et al., 1994). The maturation of the mammalian metanephros constitutes many of the steps which are prototypic of the events relevant to the development of other organ systems (Eklblom, 1993; Clapp and Abrahamson, 1994). The renal organogenesis ensues after an initial reciprocal induction between the metanephric

mesenchyme and the ureteric bud, which leads to the conversion of loose mesenchyme to a well-differentiated epithelium and dichotomization of the ureteric bud branches, with the loss of mesenchymal proteins (type I and III collagen and fibronectin) and the appearance of basement membrane proteins (type IV collagen, laminin, and proteoglycans) (Eklblom, 1993; Clapp and Abrahamson, 1994). Conceivably, during nephrogenesis, the spatio-temporal expression of various ECM glycoproteins parallels that of their receptors, i.e., integrins. Thus, it is likely that the integrins, mediating cell–matrix interactions, influence various developmental steps and are relevant in renal organogenesis.

Integrins are heterodimeric transmembrane glycoproteins, consisting of varying combinations of noncovalently bound α and β chains. To date, 16 α and 8 β chains have been described, and most of the α subunits (α_1 – α_9 , α_M , α_L , α_X , α_V , α_{IIb} , and α_E) and 8 β subunits (β_{1-8}) have been cloned and sequenced (Venstrom and Reichardt, 1995).

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1. *Abbreviations used in this paper:* ECM, extracellular matrix; ODN, oligodeoxynucleotide; RT, reverse transcriptase.

Initially, integrins were classified into three subfamilies depending upon the association of β_1 (very late antigen family), β_2 (leucam family), and β_3 (cytoadhesin family) subunits with α_{1-9} and $\alpha_{L,M,X,V,IIb}$. With the discovery and cloning of additional β subunits (β_4 – β_8), it was found that some of the α chains, e.g., α_4 , α_6 , and α_V , can also dimerize with various β subunits (Cheresh et al., 1989; Holzmann and Weissman, 1989; Horgervorst et al., 1990; McLean et al., 1990; Ramaswamy and Hemler, 1990; Sheppard et al., 1990; Suzuki and Naitoh, 1990; Suzuki et al., 1990; Erle et al., 1991; Moyle et al., 1991). The α subunit largely determines the substrate specificity with ECM glycoproteins (Yamada, 1991), while the intracytoplasmic tail of the β chain is predominantly responsible for its interaction with the cell cytoskeleton via binding to talin, vinculin, and α -actinin (Burrige et al., 1988). In addition, certain β subunits contain tyrosine phosphorylation sites in the intracytoplasmic domains, suggesting their potential role in signal transductional events (Kornberg et al., 1991). Considering the extracellular interactions of α chains with ECM proteins and those of the β chains, containing potential phosphorylation sites, with the intracellular cytoskeletal elements, the heterodimeric association of both subunits would allow the integrins to act as bidirectional signaling molecules, which are widely distributed in various tissues mediating diverse biological functions including cell polarity, migration, differentiation, and angiogenesis—the processes essential for mammalian organogenesis (Hemler et al., 1994).

In earlier immunohistochemical studies, the localization of β_1 and β_3 integrins was investigated in developing and adult human kidneys (Kerjaschki et al., 1989; Cosio et al., 1990; Korhonen et al., 1990a). Among the β_1 integrins, $\alpha_1\beta_1$ and $\alpha_4\beta_1$ were shown to be expressed in the uninucleated mesenchyme; while $\alpha_3\beta_1$ and $\alpha_6\beta_1$ had respective distributions in the podocytic and tubular plasmalemmal domains in apposition with the basement membrane. With respect to β_3 integrins, $\alpha_V\beta_3$, a vitronectin receptor, was also noted to have renal expression (Korhonen et al., 1990b, 1992). In addition to β_3 , α_V chain associates with multiple β integrins, i.e., β_1 , β_5 , β_6 , and β_8 (Bodary and McLean, 1990; McLean et al., 1990; Moyle et al., 1990; Ramaswamy and Hemler, 1990; Sheppard et al., 1990), and can exhibit diverse ligand specificities. Moreover, the integrin $\alpha_V\beta_3$ alone can bind to a number of different ligands, such as vitronectin, fibrinogen, von Willebrand factor, thrombospondin, fibronectin, osteopontin, bone sialoprotein, thrombin, laminin, and collagen type I and IV (Cheresh, 1987; Gladson and Cheresh, 1994); on the other hand, $\alpha_V\beta_1$ recognizes fibronectin or vitronectin (Bodary and McLean, 1990; Vogel et al., 1990), and $\alpha_V\beta_5$ and $\alpha_V\beta_6$ exhibit specific interactions with vitronectin and fibronectin, respectively (Cheresh et al., 1989; Sheppard et al., 1990; Smith et al., 1990; Busk et al., 1992). The multiple heterodimeric associations of α_V and various β subunits would endow this subfamily of integrins with different ligand specificities and consequential regulation of diverse biological functions, including angiogenesis (Brooks et al., 1994), retinal neurite outgrowth (Neugebauer et al., 1991), bone resorption (Davies et al., 1989), intracellular protein phosphorylation (Vuori and Ruoslahti, 1994; Rabinowich et al., 1995), phagocytosis (Blystone et al., 1994), immune re-

sponse (Biesecker, 1990; Savill et al., 1990), and metastasis of undifferentiated neoplastic cells (Felding-Haberman et al., 1992; Gehlsen et al., 1992; Seftor et al., 1992). Although a vast amount of data is available in the literature relating to the role of integrins in various biological processes, the spatio-temporal expression and developmental regulation of α_V -associated integrins have not been fully explored in tissues where epithelial:mesenchymal interactions, characteristic of embryonic development, are prevalent. This investigation relates to the cDNA cloning of murine integrin α_V , study of α_V -related integrins, i.e., $\alpha_V\beta_1$, $\alpha_V\beta_3$, $\alpha_V\beta_5$, and $\alpha_V\beta_6$, and elucidation of its role in embryonic development by using a mouse metanephric organ culture system.

Materials and Methods

Animals

ICR mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) were used. Paired male–female mating was carried out, and the appearance of vaginal plug was designated as day 0 of fetal gestation. The fetuses were removed at day 13, 17, and 19 (newborn) of gestation, and the embryonal kidneys were aseptically harvested. In addition, kidneys from 1- and 3-wk-old mice were procured.

Antibodies

Various polyclonal antibodies, directed against the cytoplasmic tails of the various integrin α and β subunits, were kindly provided by Dr. Erkki Ruoslahti, Dr. Martin E. Hemler (Dana-Farber Cancer Institute, Harvard Medical School, Cambridge, MA), Dr. Vito Quaranta (The Scripps Research Institute, CA), and Dr. Louis Reichardt. The antibodies were produced in rabbits by immunizing synthetic peptides, and their biological characteristics have been detailed previously and are briefly referred to here. Antisera to the COOH terminus of α_V (KRVRPPQEEQEREQLQPHENGEGNSET), β_3 (KFEERARAKWDTANNPLYKEATSTFTNITYRGT) (Freed et al., 1989), β_1 (EFAKFEKEKMNAKWDGTGENPIYKSAVTTVVNPKYEGK) (Argraves et al., 1987; Giancotti et al., 1990), and β_5 (KKPISTHTVDFTFNKSYNGTVD) (Vogel et al., 1990) were generated in the laboratory of Dr. Erkki Ruoslahti. Antisera to β_6 cytoplasmic domain (YKHREKQKVDLSTDC) was a generous gift from Dr. Quaranta (Lehmann et al., 1993). Antisera to chicken integrin α_V cytoplasmic domain (CKRVRPPQEEQEREQLQPHENGEGTSEA) was produced in the laboratory of Dr. Reichardt (Bossy et al., 1990). Antisera to β_5 cytoplasmic domain (CTHTVDFTFNKFNKSYNGTVD) was a generous gift from Dr. Hemler (Ramaswamy and Hemler, 1990). In addition, mAb (69-6-5) was generated by fusion of myeloma Y3 and rat spleen cells in the laboratory of Dr. Jacques Marvaldi (Lehmann et al., 1994). This antibody recognizes and inhibits the binding of $\alpha_V\beta_3$, $\alpha_V\beta_5$, and $\alpha_V\beta_6$ integrins with various cell lines. Rat anti-mouse integrin α_M (NR12C and 5C6) mAbs were purchased from Chemicon Intl., Inc. (Temecula, CA).

Construction of Embryonic Mouse Kidney cDNA Library

Total RNAs from embryonic (days 13 and 17) and newborn (day 19) kidneys were isolated by the guanidinium isothiocyanate–CsCl centrifugation method (Chirgwin et al., 1979). Poly(A)⁺ RNAs were isolated by oligo(dT)–cellulose column chromatography. First strand synthesis was carried out by using Moloney murine leukemia virus reverse transcriptase (RT) (RNase H⁻) and oligo(dT)₂₅d(G/C/A) as a primer (Clontech, Palo Alto, CA). The second strand synthesis was performed by using RNase H–DNA polymerase reaction (Okayama et al., 1982). After this, the EcoRI–NotI–Sall–phosphorylated adaptors were ligated to the double-stranded cDNAs. The cDNAs were size-fractionated, and fragments >500 bp were selected by column chromatography (CHROMA SPIN™-1000; Clontech). The selected cDNAs were ligated into λ ZAP II vector and packaged using Gigapack II gold packaging extract (Stratagene, La Jolla, CA). The packaged recombinant phage DNAs were incubated with *Escherichia coli* XL1-Blue MRF' cells for plating and amplification of the cDNA libraries.

Screening of the Libraries and Isolation of Mouse Integrin α_V cDNA Clones

About 0.5×10^6 recombinants from each of the three libraries, containing renal cDNAs from various stages of the gestation, were plated and screened with human α_V cDNA clone (gift from Drs. Pamela B. Conley and David R. Phillips, Cor Therapeutics Inc., San Francisco, CA). For screening, [α - 32 P]dCTP-labeled PstI-digested restriction fragment (bp 2354–3193) of human α_V cDNA clone (Fitzgerald et al., 1987) was used. Nitrocellulose filter lifts (Schleicher & Schuell, Inc., Keene, NH) of recombinant phage plaques were made. Prehybridization and hybridization of nitrocellulose filters were carried out in 25% formamide, $5 \times$ SSC, $5 \times$ Denhardt's solution, 50 mM sodium phosphate, pH 6.8, and 50 μ g/ml denatured salmon sperm DNA at 42°C. The filters were washed four times with $2 \times$ SSC and 0.1% SDS, followed by another four washes with $0.1 \times$ SSC and 0.1% SDS at 42°C. Several cDNA clones were isolated and purified by dilutional secondary and tertiary screenings. Twelve purified cDNA clones strongly hybridized with human α_V cDNA clone. Their identities were confirmed by restriction enzyme analyses and processed further for subcloning and nucleotide sequence analyses.

Nucleotide Sequence Analyses of cDNA Clones

The phage cDNAs of various clones were amplified and purified. After EcoRI digestion, four fragments of clone AV#3 and AV#11 (see Fig. 1) were subcloned into pBluescript phagemid KS(+) using XL1-Blue MRF' cells (Stratagene). For generation of single-stranded cDNAs, the transfected cells were propagated in the presence of VCSM13 helper phage. After ascertaining the sense and antisense orientation of various subclones, the nucleotide sequencing was performed by dideoxynucleotide chain-termination method (Sanger et al., 1977) using a DNA sequencing kit (Sequenase version 2.0; United States Biochemical Corp., Cleveland, OH). Hydrophobic (Kyte and Doolittle, 1982), nucleotide sequence homology (Lipman and Pearson, 1985), and protein structural (Chou and Fasman, 1978) analyses were performed using WISCONSIN PACKAGE 8.0.1 (Madison, WI).

Northern Blot Analysis

Total RNAs from embryonic kidneys at various stages of gestation were extracted. For a yield of 1 mg of total RNA, $\sim 1,000$, ~ 200 , and ~ 50 embryonic kidneys were used at day 13, 17, and 19 (newborn), respectively. In addition, total RNAs were extracted from kidneys of 1- and 3-wk-old mice. 5 μ g of oligo(dT)-selected poly(A)⁺ RNAs, from embryonic, neonatal, and postnatal kidneys, were glyoxalated and subjected to 1% agarose gel electrophoresis in 10 mM sodium phosphate buffer, pH 7.0. Northern blots were prepared by transferring the RNAs to nylon membranes (Pall Biosupport Corp., Glen Cove, NY), and hybridized with [α - 32 P]dCTP-labeled EcoRI fragment of mouse integrin α_V (clone AV#3, bp 1460–3086) and β -actin (GenBank accession No. M62174; American Type Culture Collection, Rockville, MD). The filters were washed at high stringency conditions with $0.1 \times$ SSC and 0.1% SDS at 60°C, and autoradiograms prepared.

Expression Studies by In Situ Hybridization

Embryonic (day 13) and newborn kidneys were fixed in 4% paraformaldehyde in PBS, pH 7.0, for 12 h at 4°C. Tissues were dehydrated in graded series of ethanol and embedded in paraffin. 3- μ m-thick sections were prepared and mounted on HCl-treated and poly-L-lysine-coated slides. The sections were deparaffinized, hydrated, deproteinized by proteinase K, treated with triethanolamine-acetic anhydride, and rehydrated. The sections were prehybridized in the presence of formamide, dextran sulfate, and Denhardt's solution at 50°C for 3 h, and hybridized with integrin α_V riboprobes at 50°C overnight. The RNA probes were prepared by using riboprobe in vitro transcription system (Promega Biotec, Madison, WI). Briefly, EcoRI fragment of clone AV#3 (1460–3086) was ligated into pBluescript KS(+) and linearized by restriction enzyme digestion. Radiolabeled antisense and sense riboprobes were synthesized by incorporating α - 35 S-dUTP (Amersham Corp., Arlington Heights, IL), using T7 and T3 RNA polymerase. The newly synthesized riboprobes were subjected to limited alkaline hydrolysis to obtain polynucleotide fragments with the size range of 100–150 bp, as ascertained by sequencing gel electrophoresis. After hybridization, the slides were successively washed with $2 \times$, $1 \times$, and $0.5 \times$ SSC in the presence of 1 mM DTT. The sections were then de-

hydrated in a graded series of ethanol, air dried, coated with emulsion (ILFORD K5; Polysciences Inc., Warrington, PA), and developed after 1–2 wk of exposure.

Immunofluorescence Studies

Embryonic (days 13 and 17) and newborn mouse kidneys were snap frozen in isopentane chilled liquid nitrogen and embedded in the OCT compound (Miles Laboratories, Inc., Elkhart, IN). 4- μ m-thick cryostat sections were prepared and air dried. The sections were washed with 0.01 M PBS, pH 7.4, incubated with antiintegrin α_V , β_1 , β_3 , β_5 , and β_6 rabbit polyclonal antibodies, with 1:100 dilution, for 30 min in a humidified chamber at 37°C, washed with PBS, and overlaid with fluoresceinated goat anti-rabbit IgG antibody for 30 min. Then they were rewashed with PBS, covered with a drop of buffered glycerol, coverslip mounted, and examined with an immunofluorescent microscope equipped with epiillumination.

Immunoprecipitation Studies

Approximately 100 embryonic kidneys were harvested from fetuses at day 13 of the gestation and maintained in an organ culture system, as detailed previously (LeLongt et al., 1988; Liu et al., 1991; Wada et al., 1993). Briefly, the dissected metanephric explants were placed on the top of an 0.8- μ m filter and floated in a serum-free medium, consisting of equal volumes of DME and Ham's nutrient mixture F12 (Sigma Chemical Co., St. Louis, MO), transferrin (50 μ g/ml), and penicillin and streptomycin (100 μ g/ml). The explants were maintained in the organ culture for 4 d, and labeled with [35 S]methionine (0.25 mCi/ml) for 12 h before the termination of the culture. The metanephroi were washed with a culture medium and lysed in an extraction buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM benzamide-HCl, 10 mM ϵ -amino-*n*-caproic acid, 2 mM PMSF, and 1% Triton X-100) by vigorously shaking at 4°C for 2 h. The extracts were centrifuged at 10,000 g for 30 min at 4°C, and the supernatants saved. Immunoprecipitations were performed by adding 0.5 ml of the supernatants ($\sim 5 \times 10^6$ dpm) to 10 μ l of each of the antiintegrin α_V , β_1 , β_3 , β_5 , and β_6 -specific rabbit antisera. The mixtures were gently swirled at 4°C in an orbital shaker. The antibody-integrin complexes were further incubated for 1 h at 4°C after the addition of 80 μ l of protein-A Sepharose 4B (Pharmacia LKB Biotechnology, Piscataway, NJ). The complexes were then centrifuged for 1 min at 10,000 g, and the pellets were washed three to five times with the extraction buffer. The immunoprecipitated complexes were dissolved in a sample buffer (4% SDS, 150 mM Tris-HCl, pH 6.8, 20% glycerol, and 0.1% bromophenol blue) and subjected to 12.5% (reducing) and 5% (nonreducing) SDS-PAGE. The gels were fixed in 10% acetic acid and 10% methanol, treated with 1 M salicylic acid, vacuum dried, and autoradiograms prepared.

Antisense Experiments

Nonsense as well as antisense phosphorothioated oligodeoxynucleotides (ODN) were synthesized by automated solid-phase synthesizer (Biotech Facility, Northwestern University, Chicago, IL), and purified by HPLC. Antisense oligonucleotide sequence (43 mer) was selected from 5' end of open reading frame of the cloned mouse integrin α_V (see Fig. 2, *dotted underline*). Its specificity for the target nucleotide sequences was established by S1 nuclease protection assays as described previously (Wada et al., 1993; Liu et al., 1994; see *Results*). Two nonsense 31-mer phosphorothioated ODNs were also synthesized, and their sequences were as follows: 5'-TAATGATAGTAATGATAGTAATGATAGTAAT-3' and 5'-GATCGATCGATCGATCGATCGATCGAT-3'. The ODNs (antisense and nonsense) did not reveal any significant homologies with other mammalian nucleotide sequences available in the GenBank database.

The embryonic kidneys were harvested at day 13 of gestation and maintained in the organ culture system for 4 d. The ODNs were added to the culture medium at a concentration range of 0.1–1.0 μ M. Up to a concentration of 1.0 μ M, most of the ODNs retain the translational blockade specificity with no discernible cytotoxic effects (Cazenave et al., 1989; Wada et al., 1993; Liu et al., 1994). The metanephric explants (30 kidneys per variable, i.e., control, nonsense, and antisense) were processed for light microscopy, and the morphological alterations were assessed. The sections from the midplane of the embryonic kidneys with a maximum number of ureteric bud iterations, including both the poles and the hilus, were evaluated for morphometric analyses, as described previously (LeLongt et al., 1988; Liu et al., 1994).

An additional 360 metanephric explants were used for immunofluores-

cence, immunoprecipitation, and competitive RT-PCR studies to ascertain the mRNA and protein expressions of integrin α_V after the treatment with the ODNs for 2 d. For immunoprecipitation, 30 metanephric explants per variable, individually treated with nonsense and antisense ODNs and untreated (control), were labeled with [35 S]methionine (0.25 mCi/ml) for 12 h before the termination of the culture. Equal amounts of incorporated radioactivities ($\sim 5 \times 10^6$ dpm) were used for immunoprecipitation with anti- α_V antibody in both the control and experimental groups. Then the entire immunoprecipitated glycoproteins were loaded onto the gels for SDS-PAGE analyses as described above. Similarly, tissue immunofluorescence was carried out on 30 explants per variable, using anti- α_V polyclonal antibodies. In addition, 90 antisense- or nonsense-treated explants were individually stained with anti- β_3 , β_5 , and β_6 .

For RT-PCR, total RNAs were isolated from 30 explants per variable, by acid guanidinium isothiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). Extracted RNAs were precipitated in the presence of RNase-free glycogen (20 μ g/ml) and pelleted by centrifugation. The pellets were resuspended in 100 μ l of DNase buffer (0.1 M sodium acetate, 5 mM MgSO₄, pH 5.0) and digested with 100 U of RNase-free DNase (United States Biochemical Corp.) in the presence of RNasin (1 U/ μ l; Promega). After another phenol-chloroform extraction, the RNAs were reprecipitated with ethanol. About 20 μ g of total RNAs, from each variable, were subjected to the first-strand cDNA synthesis using Maloney murine leukemia virus RT and oligo(dT) as a primer. The cDNAs of both groups (antisense and nonsense) were suspended in 20 μ l of deionized autoclaved water and kept at -70°C until further use.

For the competitive PCR (Gilliland et al., 1990), the following primers were synthesized: integrin α_V (5' primer: α_V -sense [α_V -SE], 5'-CAAGCTCACTCCCATCAC-3', 3' primer: α_V -antisense [α_V -AS], 5'-GGGTGTC-TTGATTCTCAAAGGG-3') (see Fig. 2), β -actin (5' primer: β -sense [β -SE], 5'-GACGACCATGGAGAAGACTCTGG-3', 3' primer: β -antisense [β -AS], 5'-GAGATGCGGCAGTGCAGAT-3') (Tokunaga et al., 1986). Expected size of the PCR products (wild type DNAs) was 859 bp for integrin α_V and 461 bp for β -actin. Competitive template for integrin α_V was prepared by site-directed mutagenesis using PCR (Ho et al., 1989). Single base pair mutation was introduced (GAACTC to GAATTC, bp 2382; see Fig. 2) using mismatch primers (sense, 5'-GAAGGCTGAATTC-CACTGC-3' [MM-SE], antisense, 5'-GCAGTTGAATTCAGCCTTC-3' [MM-AS]), containing internal EcoRI sites. The mouse integrin α_V clone AV#3 was used as template DNA at a concentration of 10 ng/ μ l and amplified by using two primer sets (α_V -SE and MM-AS, MM-SE and α_V -AS) in separate reaction mixtures. The two amplified 431- and 448-bp PCR products were electroeluted, mixed together, and reamplified by using primer set α_V -SE and α_V -AS. The resulting PCR product (859 bp), with an internal EcoRI site, was subcloned into TA cloning vector (PCRTMII; Invitrogen, San Diego, CA) and used as an "integrin α_V competitive template DNA." A competitive template for β -actin was synthesized at the Northwestern University Biotech facility and ligated into the EcoRI-digested and -dephosphorylated pBluescript using EcoRI and GC clamp linkers. This " β -actin competitive template DNA" yields a smaller PCR product with the size of 224 bp (c.f., 461 bp for wild type DNA) when β -actin primer set β -SE and β -AS is used. The nucleotide sequence of the β -actin competitive plasmid DNA is available in GenBank under the accession No. U17140.

After confirming the nucleotide sequence of various PCR products, competitive PCR analyses on cDNA from antisense and nonsense ODN-treated kidneys were performed. A fixed amount of cDNAs (1 μ l) were coamplified with serial logarithmic dilutions of competitive template DNA (500 ng/ μ l) of either integrin α_V or β -actin. The reaction mixture included 5 μ l of $10 \times$ PCR buffer (100 mM Tris-HCl, pH 8.3, 15 mM MgCl₂, 500 mM KCl, 0.01% gelatin), 250 μ M of each dNTPs, 1 μ M of 5' and 3' primers, and 1 U Taq polymerase (Perkin-Elmer Corp., Norwalk, CT) in a total volume of 50 μ l. The amplification reaction was carried out in a DNA thermal cycler (Perkin-Elmer Corp.) as follows: denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. A total of 30 thermal cycles were used. The PCR products of wild-type and mutant integrin α_V DNA were digested with EcoRI, subjected to 2% agarose gel electrophoresis, and photographed using an instant positive/

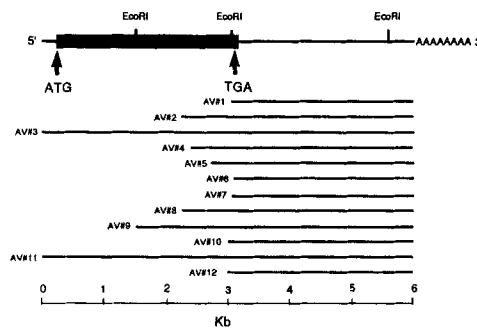


Figure 1. Schematic drawing of mouse integrin α_V renal cDNAs (AV#1–AV#12). Open reading frame flanked by 5'- and 3'-untranslated regions (closed black box), and initiation and stop codons (arrows). Three EcoRI restriction sites are also indicated. Clones AV#3 and AV#11 contain full-length open reading frame of mouse integrin α_V . AAAAAAAAAA 3', the poly(A) tail. Consensus sequence of polyadenylation site (AATAAA) starts from 20 bp upstream of the poly(A) tail.

negative film (model 665; Polaroid Corp., Cambridge, MA). The negatives were analyzed by a scanning densitometer (Hofer Scientific Instruments, San Francisco, CA), and the relative area underneath the tracings was computed. The PCR products of wild-type and mutant β -actin DNAs were similarly analyzed by agarose gel electrophoresis without prior restriction enzyme digestion. The ratios between the densitometric readings of wild-type and mutant PCR-DNA products were plotted using a logarithmic scale on the ordinate (y-axis) against the logarithmic dilutions of the competitive template DNA on the abscissa (x-axis).

Integrin α_V Blocking Antibody Experiments

The role of integrin α_V in renal development was also investigated by using the rat monoclonal (69-6-5) blocking antibody (Lehmann et al., 1994). The antibody was included in the metanephric culture medium at concentration range of 10^{-6} – 10^{-8} M. At these concentrations, optimal inhibition of integrin α_V -mediated cell adhesion to fibronectin and vitronectin has been reported (Lehmann et al., 1994). The cultures, initiated with the metanephric explants harvested from 13-d-old fetuses, were maintained for 4 d, with daily changes of the medium containing the antibody. The controls included addition of normal rat IgG and rat anti-mouse integrin α_M monoclonal blocking antibodies (NR12C and 5C6; Chemicon Intl., Inc.) in the culture media. About 30 explants per variable were processed for light microscopic examination and morphometric analyses as indicated above.

Results

Characterization of Mouse Integrin α_V Renal cDNA Clones

12 clones, isolated from embryonic cDNA libraries, had common restriction sites, suggesting that they contain identical cDNAs. Two clones, AV#3 and AV#11 (Fig. 1), had initiation and termination codons, while the remaining clones were partial-length integrin α_V cDNAs. The clones AV#3 and AV#11 revealed the translated region of 3,132 nucleotides (1,044 amino acids), which shared 92.8% and 80.6% homology to human (Suzuki et al., 1987; Fitzgerald

Figure 2. Nucleotide and deduced amino acid sequence of full-length mouse α_V renal cDNA. Dotted underlines (. . .), sequence of antisense ODN. Double underlines (= =), calcium binding domains. Underlines (—), sequence of various PCR primers. Bold underline (———), transmembrane hydrophobic domain. Arrows (\uparrow), putative cleavage sites. Asterisks (*), potential N-linked glycosylation sites. Cysteine residues are encircled. These sequence data are available from EMBL/GenBank/DBJ accession No. U14135.

cgcgagccagcagacagccggagtcgggctcgaccacgctcctcctgggtgccgc 60
ccggtcagcttcgcccggccggccgcccggccttgaccgcaagcgacagcaca 120
gctcggcagctcggggcgccagggcgccggccgcccagcagccgggacagggga 180

ctgaagctcttcggctctgccccgcccggcgccggcgatggctgctccccgggcct 240
M A A P G R L 7
gctgctacgcctcgccccggcgccctcctgctgctgctgcccgggtcctcctgctgccct 300
L L R P R P G G L L L L L P G L L L P L 27
cgccgagccttcaacctggacgtcaaaagtcccggcagatgcccgggtcccgagggaag 360
A D A F N L D V E S P A E Y A G P E G S 47
ttacttcggattcggcgtgacttcttcgagccacgctcctccaggatgtttctcct 420
Y F G F A V D F F E P S T S S R M F L L 67
ggtaggagccccaaagcagcagccagcctgggattgtagaaggagggaagttct 480
V G A P K A N T T Q P G I V E G G Q V L 87
caaatggaatgctcatccagccgggtgcccagccattgagttgattcaacaggcaa 540
K C E C S S S R R C Q P I E F D S T G N 107
tcgagattatgccaaagatgacccactggagtttaagtcaccacagtggtttggagcctc 600
R D Y A K D D P L E F K S H Q W F G A S 127
tgtgaggtcaagcagataaaatcttggcctgctcctattgtaccactggaggactga 660
V R S K Q D K I L A C A P L Y H W R T E 147
gatgaagcaggagagagaccagttggaacctgcttcttcaggacggcacaagaccgt 720
M K Q E R E P V G T C F L Q D G T K T V 167
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E Y A P C R S K N I D A D G Q G F C Q G 187
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G F S I D F T K A D R V L L G G P G S F 207
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Y W Q G Q L I S D Q V A E I I S K Y D P 227
aaatgctacagcacaataataaaccacttagcaccggactgcacaagcaatctt 960
N V Y S I K Y N N Q L A T R T A Q A I F 247
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D D S Y L G Y S V A V G D E F N G D G I E 267
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D F V S G V P R A A R T L G M V Y I Y D 287
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G K N M S S L H N F T G E Q M A A Y F G 307
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F S V A A T D I N G D D Y A D V F I G A 327
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L D K L K Q K G A I R R A L F L H N R 547
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S P V H S K T M T V F R G G Q M Q C E E 567
actggtcgcctatctcgggatgaatctgaatttagagacaagctcactccatccat 1980
L V A Y L R D E S E F R D K L T P I T I 587

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F M E Y R L D Q R T A A D A T G L Q P I 607
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Q V V C D L G N P M K A G T Q L L A G L 727
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L T L R E G D V H T L G C G I A K C L Q 907
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V F V M Y R M G F F K R V R P P Q E E Q 1027
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cacttagaacaggtacattacactacagcttgcattcataagcactga 3830

et al., 1987) and chicken integrin α_V (Bossy and Reichardt, 1990), respectively (Fig. 2; Genbank accession No. U14135). A polyadenylation site (AATAAA), 20 bp upstream of the poly(A) tail, with no A(U)_nA mRNA instability motifs, was present.

The hydrophobic analyses (Kyte and Doolittle, 1982) of the polypeptide revealed two hydrophobic domains, corresponding to the signal peptide and transmembrane segment, and they stretched between 1 and 30 and 984 and 1,012 amino acid residues (Fig. 3). The signal sequence

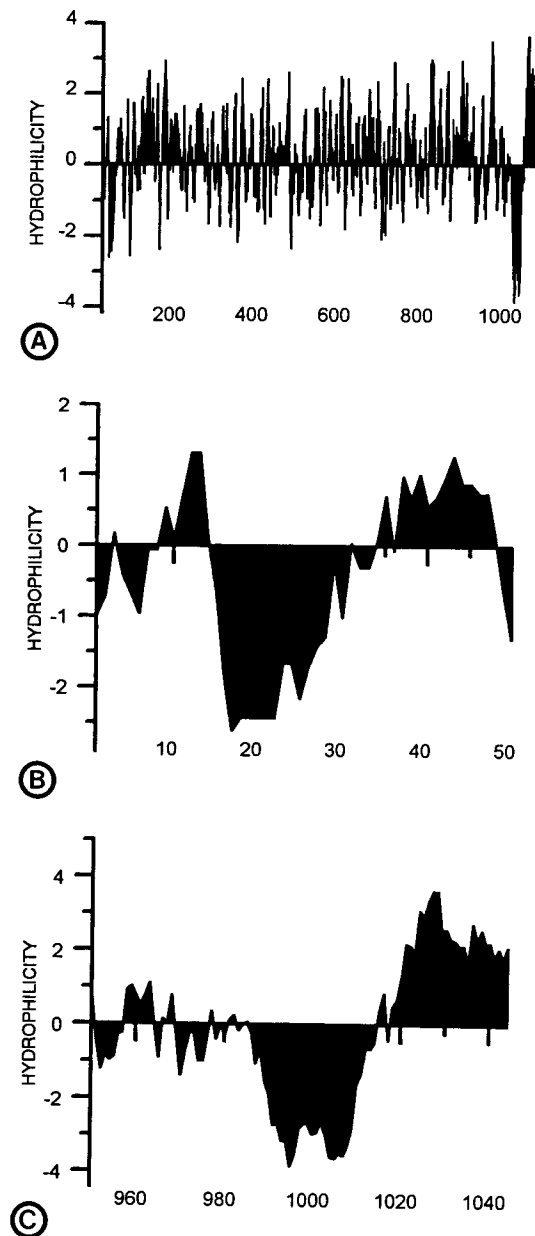


Figure 3. Kyte and Doolittle hydrophobicity/hydrophilicity plot analyses. (A) Hydrophobicity analysis of full-length mouse integrin α_V . (B and C) Analyses of putative signal peptide (1–30 amino acid residues) and transmembrane domain (984–1012 amino acid residues) of mouse integrin α_V , respectively. The hydrophobicity of the transmembrane segment approaches almost 4 (C).

had a low homology (60%) to that of human integrin α_V . Nevertheless, the amino acids located at positions -1 and -3 from the carboxy terminus of the signal peptide had characteristics to follow the “ $(-3, -1)$ rule,” and thus satisfied the requirements essential for its cleavage from the mature protein (Heijne et al., 1986).

The extracellular domain had multiple potential N-linked glycosylation sites (NXT/NXS), Ca^{2+} binding domains (DX[D/N]XDGXXD), cysteine residues, and a proteolytic cleavage site located between amino acid residue 886 and 887 (KR-G) (see Fig. 2). Although KR-G does not match the consensus cleavage site sequence, (K/R)R-(E/D) (Takada et al., 1989), the SDS-PAGE data support its location similar to that found in human and chicken integrin α_V (see below).

The transmembrane domain of mouse integrin α_V consisted of 29 amino acid residues and had hydrophobicity approaching ~ 4 . The intracellular domain was relatively short; it was highly conserved and exhibited 100% and 93.4% homologies with human and chicken integrin α_V , respectively. Like other integrin α subunits, the intracellular domain of the mouse integrin α_V included a consensus sequence of GFF(R/K)R (Hemler et al., 1994). Its two-dimensional plot analysis (Chou and Fasman, 1978) predicted no well-defined long-ordered structure of β -sheets or α -helices (Fig. 4). However, a few β -turns, α -helices, and internal coils were present.

Developmental mRNA Expression of Integrin α_V

Northern blot analyses of embryonic and neonatal kidney poly(A)⁺ RNAs revealed a major integrin α_V transcript of ~ 7 kb size (Fig. 5). The expression was detectable at day 13 of gestation, and it increased progressively during the later stages of gestation extending into the neonatal and postnatal periods. The expression of β -actin did not appear to change significantly during the embryonic and postnatal periods (Fig. 5). The in situ hybridization studies indicated that the integrin α_V mRNA expression was mainly concentrated on the developing glomerular and tubular elements at day 13 of gestation (Fig. 6 A). The expression on the ureteric bud branches was relatively less, and negligible in the metanephric mesenchyme. The expression on the tubular and glomerular elements became progressively abundant during the later stages of gestation. In the newborn kidneys, a high mRNA expression was observed on the glomeruli, while the message was relatively less abundant in tubules (Fig. 6 B), suggesting its relationship to the developing glomerular capillaries. The serial sections of metanephric tissues, hybridized with sense riboprobe, revealed background radioactivity only (Fig. 6, C and D). The in situ expression of integrin α_V and related β subunits was further investigated by immunohistochemical techniques during metanephric development.

Immunofluorescence Studies

At day 13, integrin α_V immunoreactivity was observed in the ureteric bud branches and in the induced metanephric mesenchymal components, i.e., developing glomeruli and tubules (Fig. 7, A and D). The reactivity was relatively intense in the S-shaped (Fig. 7 D) and precapillary stage glomeruli (Fig. 7 A). Unexpectedly, the intense reactivity

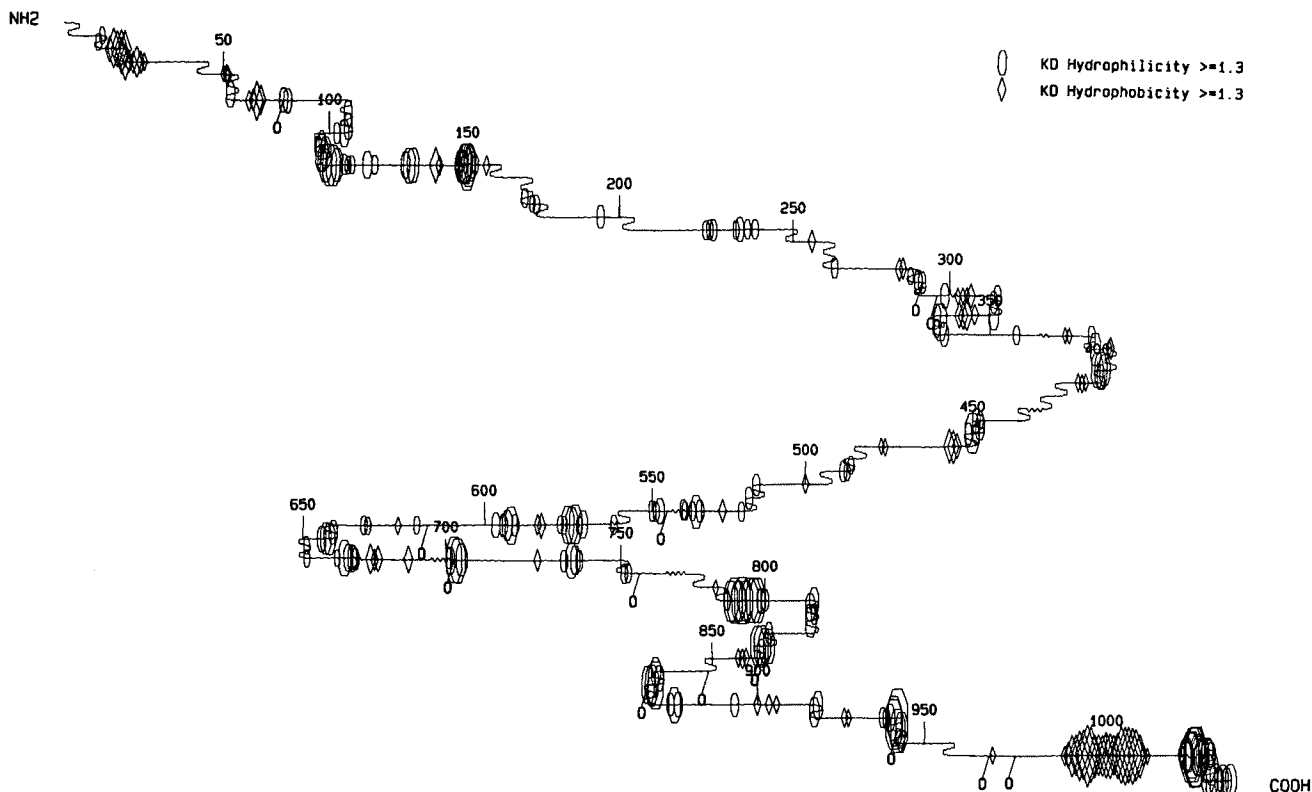


Figure 4. Predicted conformation of integrin α_v using Chou-Fasman predictive scheme from University of Wisconsin Genetics Computer Group software package. The integrin α_v does not display any long-ordered structures. However, a few α -helices (*sine waves*), β -sheets (*sharp saw-tooth waves*), coils (*dull saw-tooth waves*), and β -turn regions are present. \diamond and \circ , hydrophobicity and hydrophilicity values >1.3 , respectively. \circ , potential N-glycosylation sites.

was also observed in the cleft of the S-shaped body (Fig. 7 D)—the site where the ECM subsequently appear. No immunofluorescence staining was observed in the metanephric mesenchyme. Integrins β_3 , β_5 , and β_6 , which het-

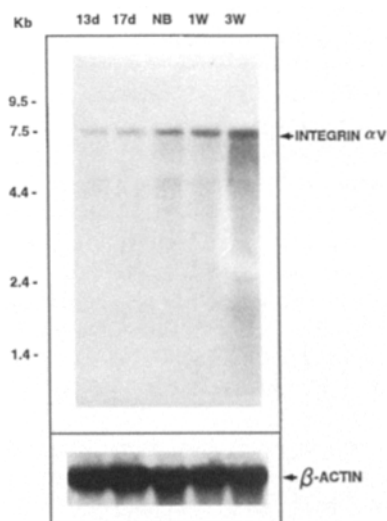


Figure 5. Northern blot analyses of mouse integrin α_v mRNA expressed in various developmental stages of the mouse kidney. Poly (A)⁺ RNAs were denatured with glyoxal, subjected to 1% agarose gel electrophoresis, capillary transferred to nylon membranes, and hybridized with [α -³²P]dCTP radiolabeled integrin α_v

erodimerize with integrin α_v , had identical codistribution with integrin α_v in the developing glomeruli and tubules. At day 17, the expression of integrin α_v increased in the cells of maturing glomeruli and tubules (Fig. 7 B). The immunoreactivity was also seen in the medullary collecting tubules. In the newborn kidneys, integrin α_v , β_3 , β_5 , and β_6 were codistributed in all the cell types of glomerulus, including parietal epithelial cells, podocytes, and mesangial and endothelial cells (Fig. 7, C and E). The integrin α_v was also expressed in the proximal and collecting tubules. An intense immunoreactivity was noted in the endothelial and smooth muscle cells of the arterial blood vessels. No interstitial staining was observed. Since integrin β_1 heterodimerizes with multiple integrin α subunits, including integrin α_v (Bodary and McLean, 1990), the distribution of integrin β_1 was also investigated during metanephric development. At days 13 and 17, integrin β_1 was seen distributed throughout the kidney tissue, i.e., undifferentiated mesenchymal cells, ureteric bud branches, and developing glomerular and tubular cells (Fig. 7 F). In the neonatal kidneys, all of the cell types of the glomerulus and the extraglomerular blood vessels exhibited intense immunoflu-

orescence. Northern blot analysis of mouse integrin α_v and β -actin cDNA inserts. A major transcript of ~ 7 kb for mouse integrin α_v is observed, and its expression increases progressively during the gestational and postnatal periods in developing kidneys. The expression of β -actin remains constant. Lanes 13d and 17d, renal poly (A)⁺ RNA from 13-d- and 17-d-old fetuses. Lanes NB, 1W, and 3W, newborn, 1-wk-, and 3-wk-old mice renal mRNAs.

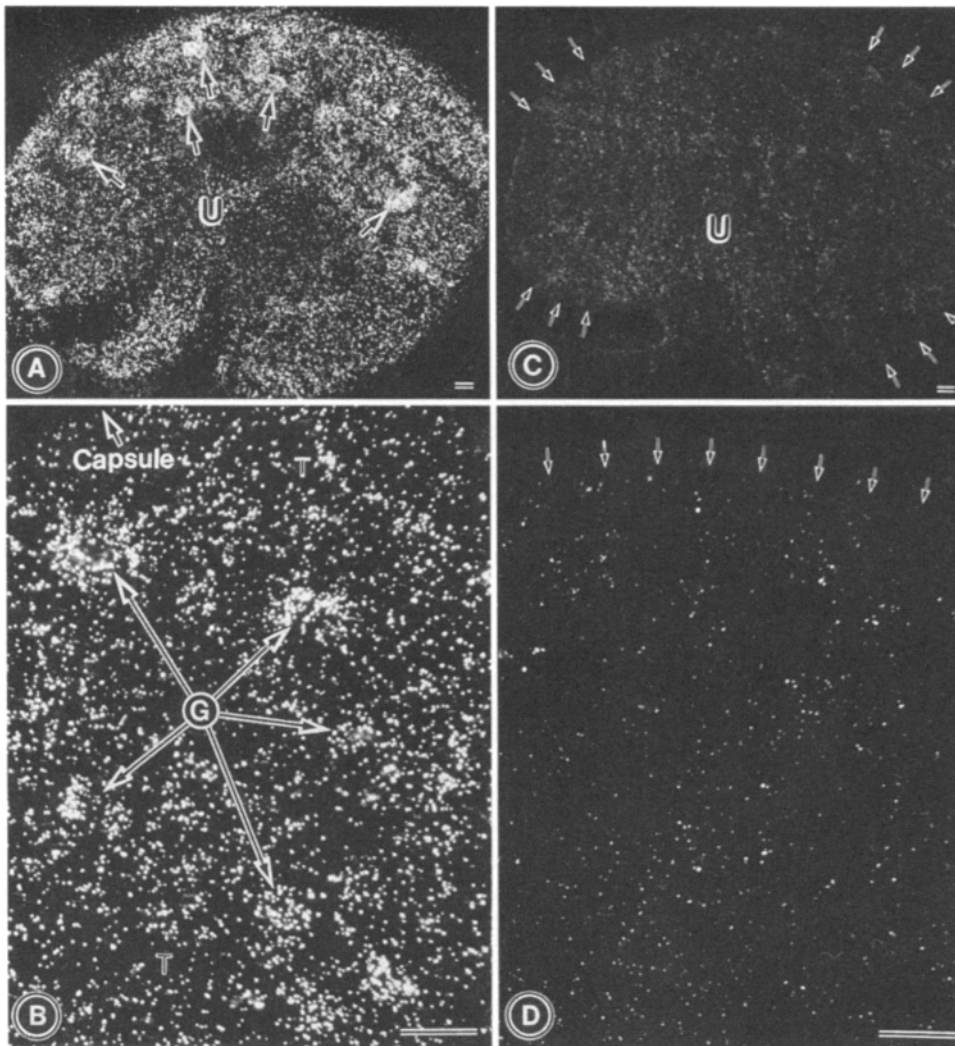


Figure 6. Dark-field light microscopic autoradiograms of in situ hybridization for integrin α_V . The tissue sections (A, day 13 mouse kidney; B, newborn mouse kidney) were hybridized with α - ^{35}S -dUTP-labeled antisense riboprobe, synthesized from mouse integrin α_V clone. In A, at day 13, the integrin α_V is expressed in the developing glomeruli (arrows) and ureteric bud branches (U). In B, the expression in the newborn kidney is concentrated mainly in the glomeruli (G), and to a lesser extent in the tubules (T). The serial tissue sections hybridized with α - ^{35}S -dUTP-labeled sense riboprobe revealed only the background radioactivity. A and C, $\times 25$; B and D, $\times 100$. Bar, 100 μm .

orescence, while a weak immunoreactivity was observed in all of the cell types of tubules (Fig. 7 G).

Association of Mouse Integrin α_V with Multiple β Subunits in the Embryonic Kidneys

To confirm the heterodimeric association of α_V with other β subunits in the embryonic kidney tissues, immunoprecipitation experiments were performed. Two (chicken and human) polyclonal antiintegrin α_V antibodies, directed against the synthetic peptide, were used. The sequence of the synthetic peptide is derived from the deduced sequence of the carboxy terminus of integrin α_V . The sequence of this carboxy terminus is highly conserved, and mismatch of only two amino acids is observed between human and chicken integrin α_V sequences. The mouse and human integrin α_V have identical sequences for the terminal 32 amino acids at the carboxy terminus. In any event, both of the antiintegrin α_V antibodies (human and chicken) yielded identical results.

Membrane glycoproteins were extracted from [^{35}S]methionine-labeled mouse metanephric kidney explants, and immunoprecipitation studies were performed. Under reducing conditions, three major bands (~ 125 kD, ~ 100 kD, ~ 25 kD) were observed in 12.5% SDS-PAGE of the

membrane extracts immunoprecipitated with integrin α_V antisera (Fig. 8 A). The 25-kD band was barely detectable by SDS-PAGE in the samples containing protein A-Sepharose-antibody-integrin complexes, which were extensively washed with the extraction buffer (Fig. 8 B). Under nonreducing conditions, three bands (~ 150 kD, ~ 110 kD, ~ 90 kD) were observed by 5% SDS-PAGE of the membrane preparations immunoprecipitated with integrin α_V antisera (Fig. 8 C). This suggested that 150-kD band of integrin α_V subunit, observed under nonreducing conditions, dissociated into two discrete bands (~ 125 kD and ~ 25 kD) upon reduction with β -mercaptoethanol, suggesting the presence of disulfide bridges between the polypeptide chains, similar to those reported in human and chicken integrin α_V (Fitzerald et al., 1987; Suzuki et al., 1987; Bossy and Reichardt, 1990).

After the characterization of embryonic renal integrin α_V , immunoprecipitation experiments were performed with β_1 , β_3 , β_5 , and β_6 antisera. Under reducing conditions (Fig. 8 B), integrin α_V subunit (~ 125 kD) coprecipitated with integrin β_3 , β_5 , and β_6 subunits (~ 100 kD). Under nonreducing conditions (Fig. 8 C), the middle ~ 110 -kD band in the antiintegrin α_V immunoprecipitated membrane extract corresponded to integrin β_1 subunit, while the lower ~ 90 -kD band corresponded to integrin β_3 , β_5 ,

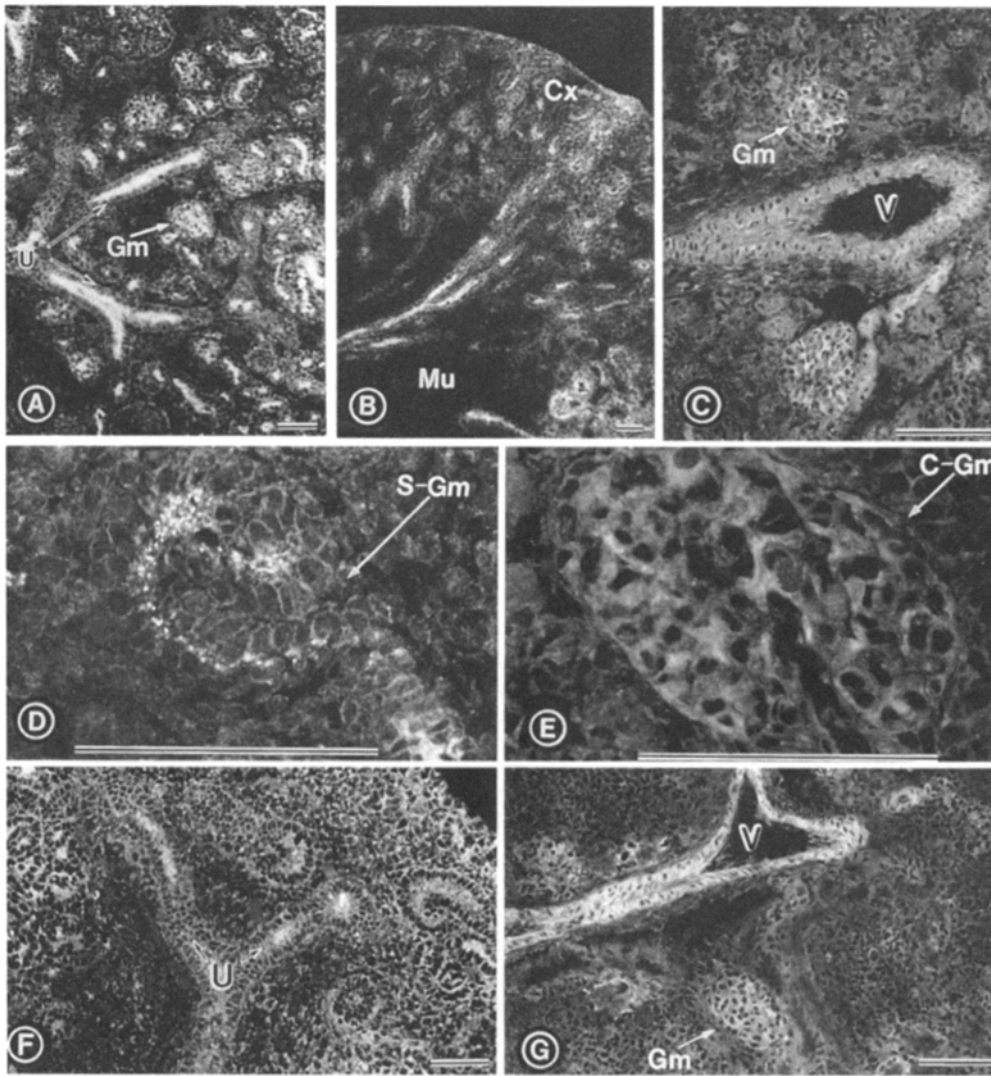


Figure 7. Immunofluorescence renal photomicrographs of integrin α_V (A–E) and integrin β_1 (F and G), from 13-d- (A, D, and F), 17-d- (B) and 19-d-old (newborn) (C, E, and G) fetuses. At day 13, the immunoreactivity of integrin α_V is seen in the developing glomeruli (Gm) and ureteric bud branches (U), but not in the metanephric mesenchyme (A). The reactivity of integrin α_V with the plasmalemma of glomerular epithelial cells of S-shaped stage glomeruli (S-Gm) and of the cleft is observed (D). At day 17 (B), integrin α_V expression is seen in the developing glomeruli and tubules of the cortex (Cx) and in the collecting ducts of the medulla (Mu), and the intensity of immunofluorescence has been relatively increased. In the newborn (C), the immunoreactivity is observed on glomeruli (Gm), proximal tubules, and maturing blood vessels (V). The integrin α_V is also localized in the plasmalemma of the various cell types of the capillary stage glomeruli (C-Gm), including glomerular epithelial and endothelial cells (E). The staining of integrin β_1 in the embryonic kidney at day 13 reveals immunoreactivity with all the components of

the metanephros, including ureteric bud branches (U), developing glomeruli, tubules, and mesenchymal cells (F). In the newborn, the expression of integrin β_1 is localized in the mature glomeruli (Gm), tubules, and vessels (V) (G). A, $\times 50$; B, $\times 35$; C, $\times 125$; D and E, $\times 500$; F, $\times 75$; G, $\times 100$. Bar, 100 μm .

and β_6 subunits. Integrin β_1 band (~ 130 kD) could not be separately visualized from integrin α_V subunit (~ 125 kD) under reducing conditions. These data suggest that integrin α_V associates with multiple integrin β subunits with the formation of various heterodimers, i.e., integrin $\alpha_V\beta_1$, $\alpha_V\beta_3$, $\alpha_V\beta_5$, and $\alpha_V\beta_6$, in the mouse embryonic kidney tissues. In addition, the integrin β_1 seems to associate with multiple α subunits, since two major bands of the size of ~ 200 and ~ 150 kD were observed (Fig. 8 C).

Role of Integrin α_V in Metanephric Renal Development (Antisense Experiments)

To assess the role of integrin α_V , antisense experiments were performed. The specificity of ODN was established by S_1 nuclease assays. A single band of radioactivity, corresponding to the size of the ODN, was observed at 40°C and 45°C hybridization temperatures (Fig. 9, lanes 1 and 3). No band was seen in the control (nonsense ODN) samples (Fig. 9, lanes 2 and 4). The morphological changes in-

duced by the antisense ODN exposure to metanephric kidneys are shown in Fig. 10. An overall moderate reduction in the size of the metanephric explants exposed to antisense ODN was compared to the control (Fig. 10, A and C; Table I) and those treated with nonsense ODN (Fig. 10, B and C; Table I). A generalized suppression in the iterations of the ureteric bud branches was observed. Also, the ureteric bud branches were disorganized, and their tips were blunted. As a result, the population of glomerular and tubular elements was decreased (Table I), and metanephric mesenchyme appeared to be loosely organized and expanded. No discernible cytotoxic effects, in the form of necrosis, were apparent (Fig. 10, B and C). Minimal reduction in the size of the metanephric explants and population of developing nephrons was observed with the exposure of nonsense ODNs (Fig. 10, A and B).

To ascertain the transcriptional and translational integrin α_V -specific blocking activities of phosphorothioated antisense ODN, competitive PCR, immunoprecipitation, and immunofluorescence studies were performed.

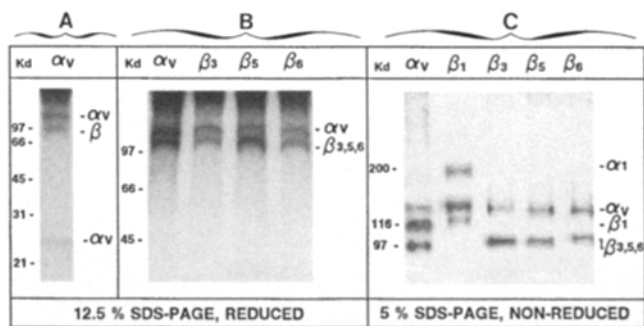


Figure 8. SDS-PAGE autoradiograms of [³⁵S]methionine-labeled glycoproteins, immunoprecipitated with specific polyclonal antibodies, directed against integrin α_V , β_1 , β_3 , β_5 , and β_6 . The kidney explants, harvested at day 13, were maintained in an organ culture and labeled with [³⁵S]methionine. Membrane proteins were extracted, and the immunoprecipitated glycoproteins were subjected to SDS-PAGE under reducing and nonreducing conditions. Under reducing conditions, three major bands (~ 125 kD, ~ 100 kD, ~ 25 kD) are observed in 12.5% SDS-PAGE of the membrane fraction immunoprecipitated with integrin α_V antiserum (A). The integrin α_V (~ 125 kD) coprecipitates with ~ 100 kD band of β_3 , β_5 , and β_6 subunits (B). Integrin β_1 (~ 130 kD) could not be separated from integrin α_V (~ 125 kD) under reducing conditions. Under nonreducing conditions, three bands (~ 150 kD, ~ 110 kD, ~ 90 kD) are observed in 5% SDS-PAGE of the membrane fraction immunoprecipitated with integrin α_V antiserum (C). The ~ 150 -kD band corresponds to integrin α_V subunit, ~ 110 -kD band to integrin β_1 , and ~ 90 -kD band to integrin β_3 , β_5 , and β_6 . The ~ 90 -kD band can be seen in membrane preparations immunoprecipitated with anti- β_3 , - β_5 , and - β_6 antibodies (C). The ~ 200 -kD glycoprotein, immunoprecipitated with integrin β_1 antibody, probably corresponds to integrin α_1 (Korhonen et al., 1990a).

The method of competitive RT-PCR was chosen to circumvent the difficulties related to the minute amount of mRNA available for Northern blot analysis from the embryonic kidneys harvested at day 13 of gestation. In the nonsense ODN-treated group (control), a linearity in the ratios of wild to mutant integrin α_V -DNA could be maintained when plotted against the 10^{-4} to $10^{-6.25}$ serial logarithmic dilutions of the competitive template DNA (Fig. 11 A). Within this range of dilution, the bands of wild-type and mutant DNA were discernible for densitometric analyses to obtain a ratio. In the samples with lower (10^{-3}) and higher (10^{-7}) dilutions, the respective bands of wild-type and mutant DNA could not be visualized, and thus no densitometric readings or ratios could be obtained. In the antisense ODN-treated group, the linearity was observed in the range of 10^{-6} – $10^{-7.5}$ dilution of the competitive template DNA (Fig. 11 A). However, for the β -actin, no significant differences in the linearity relationship to the range of logarithmic dilutions of the competitive DNA between the two groups (control and antisense) were observed (Fig. 11 B). These data suggest that the steady-state mRNA levels of integrin α_V were reduced with the exposure to antisense ODN, while that of the β -actin were unaffected.

The antisense and nonsense ODN-treated explants were radiolabeled with [³⁵S]methionine, and the extracts immunoprecipitated with antiintegrin α_V . Under reducing conditions, the SDS-PAGE autoradiographic analyses of im-

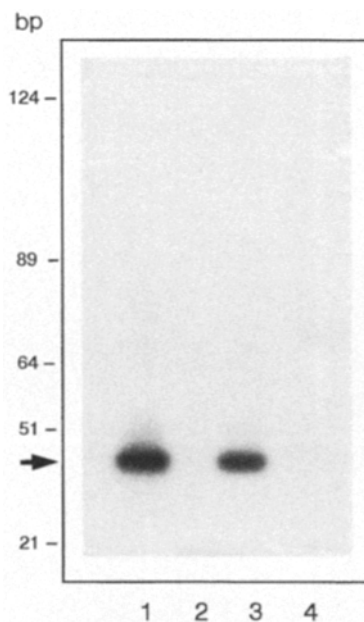


Figure 9. Autoradiograms of S_1 nuclease digests of antisense ODN:RNA hybrids. Lanes 1 and 3, $10 \mu\text{g}$ of metanephric mRNA hybridized with antisense ODN at 40°C and 45°C , respectively, followed by S_1 nuclease digestion. A single band of radioactivity, corresponding to the size of ODN (43 mer), is observed in both lanes. No band is seen in the mRNA samples incubated with nonsense ODN, indicating the susceptibility of unprotected radiolabeled ODN to S_1 nuclease digestion.

munoprecipitated proteins revealed a marked reduction in the intensity of the upper ~ 125 -kD band, corresponding to the integrin α_V in the antisense-treated explants. Minimal reduction in the intensity of 125-kD band was noted in the nonsense-treated group. In addition, the intensity of the coprecipitated β -subunit band of ~ 100 kD was decreased, to a similar degree than that of integrin α_V subunit, in the antisense-treated metanephric kidneys (Fig. 12 A). The immunofluorescence studies showed a marked reduction of the reactivity of antiintegrin α_V antibody in explants treated with the antisense ODN (Fig. 12 C), compared to those exposed to nonsense ODN (Fig. 12 B). However, no decrease in the anti- β_3 , - β_5 , and - β_6 immunoreactivities was observed in antisense ODN-treated metanephric kidneys (Fig. 12, D–F).

Integrin α_V Blocking Antibody Experiments

Inclusion of rat mAb (69-6-5) in the metanephric culture medium induced mild to moderate morphological alterations in the explants harvested at day 13 of the fetal gestation as compared to the normal rat IgG control (Fig. 13, A and B). The overall size reduction of the explants was minimal (Table I). However, a moderate reduction in the population of the developing nephrons with expansion of the mesenchyme was observed (Table I). Also, a mild swelling of the ureteric bud branches with deformities in their iterations was seen. The metanephroi exposed to rat anti-mouse integrin α_M monoclonal blocking antibodies (NR12C and 5C6) did not induce any significant alterations (Fig. 13, C and D; Table I). No discernible cytotox-

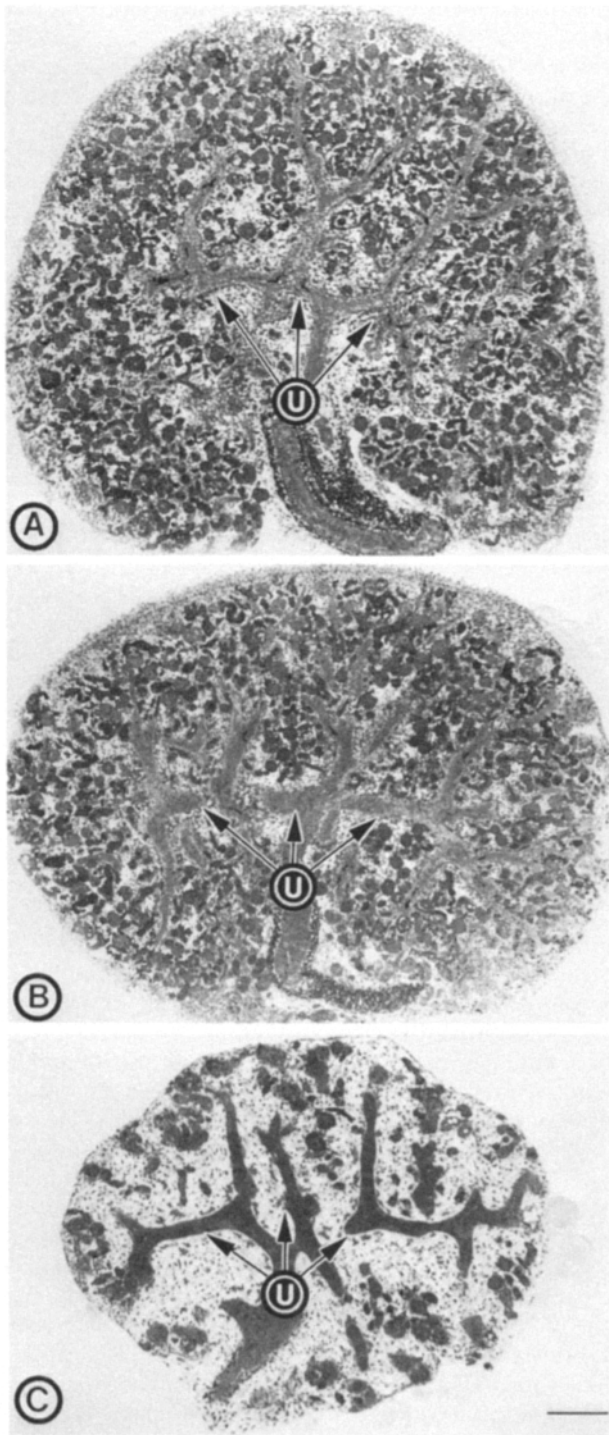


Figure 10. Light photomicrographs of the control (A), nonsense (B), and integrin α_v antisense ODN-treated (C) metanephric explants, harvested at day 13 of the fetal gestation. The antisense ODN-treated kidney shows reduction in size, dysmorphogenesis of the ureteric bud branches (U), and decreased population of glomerular and tubular elements in the loose metanephric mesenchyme. Minimal reduction in the explant size is noted with the treatment of nonsense ODN. $\times 35$. Bar, 200 μm .

Table 1. Effects of Integrin α_v , Antisense ODN, Antiintegrin α_v , and α_M Blocking Monoclonal Antibodies on the Metanephric Development

	Number of glomeruli*	Relative area
Control	28.72 \pm 2.23	98.20 \pm 9.34
Nonsense ODN	26.53 \pm 1.62	88.37 \pm 8.65
Antisense ODN	10.26 \pm 0.95 [‡]	62.42 \pm 6.39 [‡]
Rat IgG	27.32 \pm 2.45	90.84 \pm 8.35
Rat antiintegrin α_v mAb (69-6-5)	19.34 \pm 1.34 [‡]	85.06 \pm 7.23
Rat antiintegrin α_v mAb (NR12C)	28.43 \pm 2.40	89.20 \pm 8.62
Rat antiintegrin α_v mAb (5C6)	28.02 \pm 2.53	92.34 \pm 9.01

Values are expressed as mean \pm SD; $n = 30$.

Metanephric explants were harvested at day 13 of the gestation and maintained for 4 d in the culture.

*Number of glomeruli represents the nephron population in S-shaped body and pre-capillary stages.

[‡]Significantly different from the control ($P < 0.001$).

icity of the antibodies in the metanephric explants was noted.

Discussion

In this investigation, the metanephric culture, a model system of mammalian organogenesis in which epithelial:mesenchymal or ECM:matrix receptor (integrins) interactions, characteristic of certain embryonic developmental processes, is presented. Also, cloning, nucleotide sequencing, and structural analyses of the mouse integrin α_v are described, and its role in embryonic renal development is elucidated.

The subfamily of α_v -associated integrins, like β_1 integrin subfamily, is distributed in embryonic and adult tissues in heterodimeric forms, where α subunit pairs with multiple β subunits (Gladson and Cheresh, 1994). Along these lines, it is interesting to note that the sequence analyses of various clones, isolated from embryonic cDNA libraries, revealed no base-pair substitutions, suggesting that the α_v is expressed early in fetal life with no alternatively spliced variants in different stages of gestation, thus implying that various α_v heterodimeric pairs, distributed in the embryonic kidney, have identical integrin α_v subunits. Moreover, given the absence of rigid long-range-ordered structure in the integrin α_v molecule, there may be minimal constraints in the pairing of various heterodimers, and as a result, a large number of integrins, constituting α_v subfamily, would be expressed in the embryonic tissues. Conceivably, this heterodimerization would also diversify integrin α_v interactions, stabilized by the Ca^{2+} binding domains, with multiple ligands (Gladson and Cheresh, 1994) distributed in ECM of the embryonic kidney. Such a diversity of ligand interactions, related to the extracellular domain of the integrin α_v , would be highly amenable to sustain various morphogenetic steps in which different sets of necessary molecules interact with one another at a given stage of an organ development.

The conserved sequences of the cytoplasmic domain imply that the integrin α_v may have some functional significance across interspecies lines and in various cellular processes, e.g., signal transduction, cell migration, and cell differentiation—the processes prevalent during embryogenesis and morphogenesis of different organ systems. Given the structure of integrin α_v , the signal transduc-

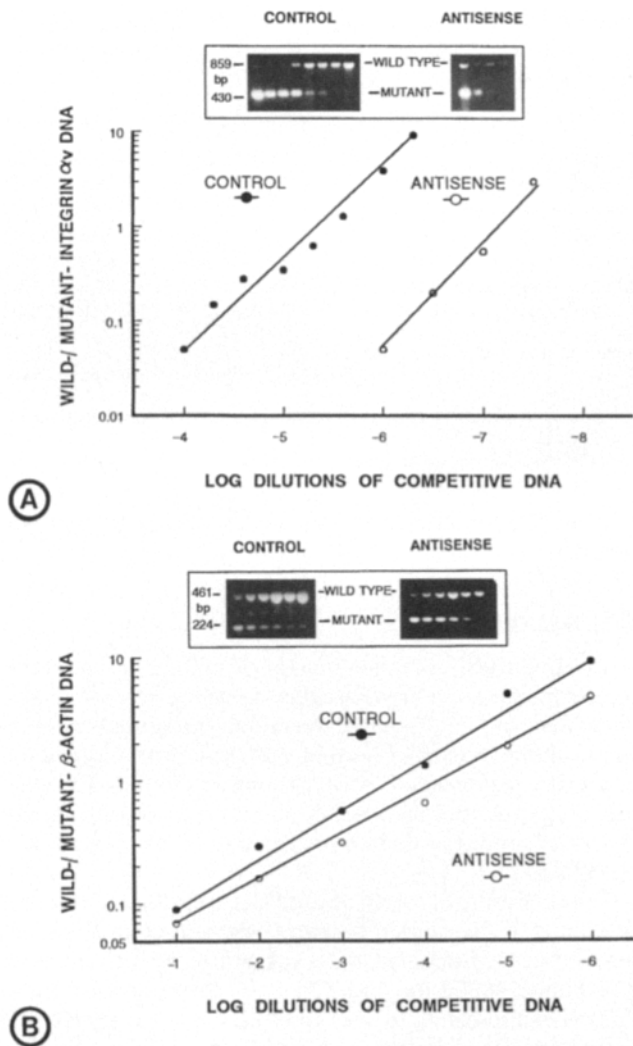


Figure 11. Competitive RT-PCR of integrin α_V (A) and β -actin (B) cDNAs, prepared from nonsense and integrin α_V antisense ODN-treated kidney explants. Serial logarithmic dilutions of mutated competitive DNA template of integrin α_V and β -actin (see *Materials and Methods*) were coamplified with fixed amount (1 μ l) of first-strand cDNA prepared from kidney explants, and ratios between the densitometric readings of the wild-type and mutant PCR products were plotted on logarithmic scales on the ordinate (y-axis) against the logarithmic dilutions of competitive DNA on the abscissa (x-axis). A reduction in the amplification of wild-type integrin α_V DNA is observed in antisense-treated kidney explants compared with the control (A). The amplification product of wild-type integrin α_V DNA is barely detectable even at the highest dilution ($10^{-7.5}$) of mutant competitive integrin α_V DNA. No differences in the amplification of β -actin between two groups are observed (B).

tional processes would certainly be facilitated by the presence of dibasic KR residues in the highly conserved GFFKR motifs involved in anchoring of α_V with the cytoplasmic glycoproteins (Hemler et al., 1994). Lastly, the β turn in the cytoplasmic tail (PPQEE), as predicted from Chou-Fasman analysis, may further aid in the ligand interaction with the intact $\alpha_V\beta_3$ heterodimer—the interaction essential for various transductional processes operative

during mammalian organogenesis (Filardo and Cheresh, 1994).

The mRNA expression of integrin α_V could be detected in the metanephroi as early as day 13 (Figs. 5 and 6), and it increased progressively during later stages of gestation extending into the postnatal period. The progressive rise in the integrin α_V expression may partly be related to the absence of A(U)_nA mRNA instability motifs, and it would also suggest that integrin α_V is developmentally regulated and, conceivably, plays a role in the organotypic differentiation processes, e.g., metanephrogenesis. In line with such a contention, the regulation of α_V has also been reported during late stages of avian retinal development, where $\alpha_V\beta_{100K}$ -dependent neuronal attachment and neurite outgrowth was observed (Neugebauer et al., 1991). As to the differentiation, an increased expression of α_V has been observed in K562 leukemia cell lines in response to tetradecanoylphorbol acetate exposure (Stockbauer et al., 1985). Similarly, induction of $\alpha_V\beta_1$ and $\alpha_V\beta_3$ heterodimeric expressions in murine embryonal carcinoma cell lines has been noted with the treatment of retinoic acid (Dedhar et al., 1991). The contention that the integrins are relevant to various differentiation processes is further supported by their spatio-temporal distribution during different stages of the embryonic development. The best evidence for the position-specific developmental expression of integrins has been provided by the *in vivo* studies in amphibian vertebrate embryos (DeSimone, 1994). These studies yield clues as to the role of integrins in specific tissue interactions during the early morphogenetic events of gastrulation and neurulation. In the developing kidneys, the α_1 , α_2 , α_3 , α_4 , α_5 , α_6 , β_1 , and β_3 display overlapping expressions (Korhonen et al., 1990a, 1992; Rahilly and Fleming, 1992). But with maturity of the metanephroi, their distribution gets confined to distinct segments of the nephrons, suggesting a potential role in nephrogenesis. Before vascularization, the integrin α_V displays a restricted distribution in developing metanephroi, including in the cells of nonvascular origin, i.e., tubular and glomerular epithelia. The expression in the epithelial components increased with progression of the gestation and vasculogenesis of the kidney. In mature kidneys, the expression was concentrated in the glomerular and extraglomerular vasculature, with codistribution of β_3 , β_5 , and β_6 . In contrast, β_1 integrin was diffusely distributed in the immature metanephroi, including in the uninduced and induced mesenchymal elements. These *in vivo* expression studies imply that α_V is developmentally regulated in a spatio-temporal manner and may be relevant to the vascularization of the kidney—the processes akin to $\alpha_V\beta_3$ -dependent angiogenesis of chick chorioallantoic membrane and human wound granulation tissue (Brooks et al., 1994).

The $\alpha_V\beta_3$ has been regarded as a “promiscuous” receptor recognizing more than one ligand, e.g., fibronectin, vitronectin, types I and IV collagens, and laminin (Gladson and Cheresh, 1994). The fact that the heterodimer of α_V can bind to various ligands and maintain its sustained expression immediately after the induction and extending into the neonatal life supports the notion that integrin α_V may be relevant to nephrogenesis throughout the metanephric development. Accordingly, the $\alpha_V\beta_3$ could interact with interstitial ECM glycoproteins, e.g., fibronectin

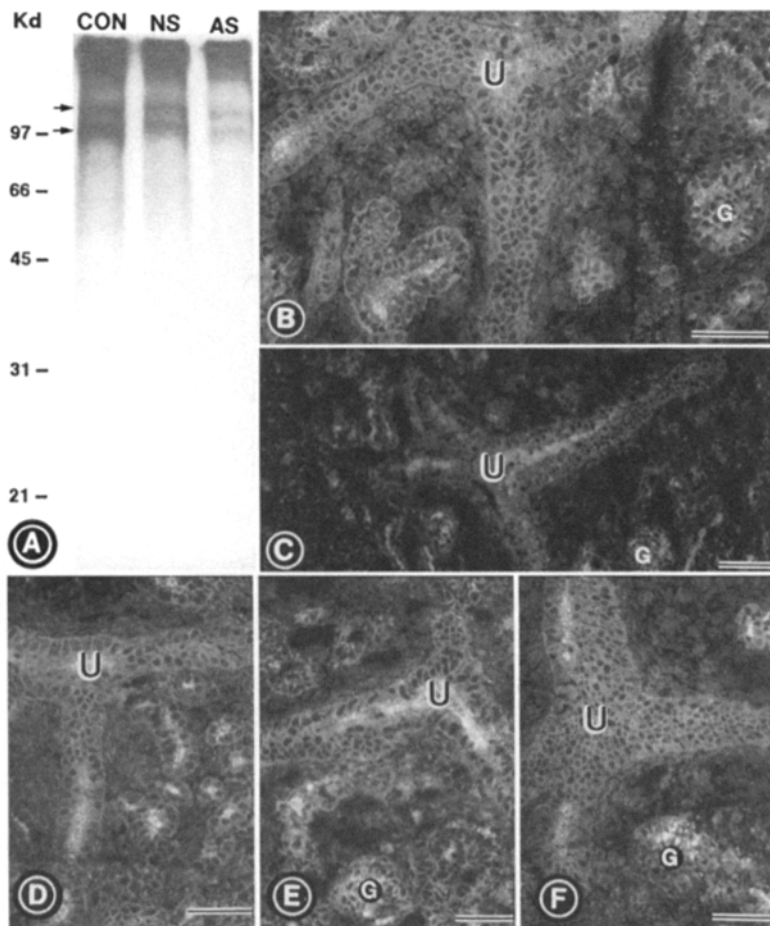


Figure 12. Immunoprecipitation (A) and immunofluorescence (B–F) studies of the control, nonsense, and integrin α_v antisense ODN-treated kidney explants. (A) 12.5% SDS-PAGE autoradiogram (reducing conditions) of integrin α_v related receptors immunoprecipitated with polyclonal anti- α_v antibodies. For immunoprecipitation, equal amounts of radioactivities ($\sim 5 \times 10^6$ dpm) were used in both the control and experimental groups. Band density of both integrin α_v and coprecipitated β subunits are decreased in antisense ODN-treated group (AS) compared with control (CON) and nonsense ODN-treated group (NS). A relative loss of integrin α_v immunostaining is observed in antisense ODN-treated (C) kidney explants compared to the control (B). The immunoreactivities of integrin β_3 (D), β_5 (E), and β_6 (F) were unaffected in the explants treated with antisense ODN. B, $\times 100$; C–F, $\times 75$. Bar, 100 μm .

and type I collagen up to the inductive stage, and thereafter in the postinductive period, it would bind to basement membrane ECM glycoproteins, e.g., laminin and type IV collagen, thus carrying out its signal transductional functions during the entire span of nephrogenesis with subsequent vascularization of the metanephroi. Such sustained expression of various integrin heterodimers throughout the fetal life has been postulated to play a role in amphibian embryogenesis as well (DeSimone, 1994). In addition to β_3 , the α_v may exhibit its functional activities by pairing with other β subunits distributed in various embryonic tissues or cell lines. Certainly, the current data suggest that, at least in the metanephric tissues, the α_v exists in heterodimeric association with β_1 , β_3 , β_5 , and β_6 . Here, one could speculate that the relevant heterodimers of α_v may interact with various ligands, i.e., ECM glycoproteins, expressed in a spatio-temporal manner in metanephric tissues during different stages of the development. In general, the coexpression of a given ECM glycoprotein and the corresponding integrin in spatio-temporal manner has been regarded as being ideally suited to maintain various morphogenetic processes (Reichardt and Tomaselli, 1991). The coexpression and colocalization of fibronectin and $\alpha_4\beta_1$ during the preinductive phase and E8 fragment of laminin and $\alpha_6\beta_1$ in the postinductive period are the good examples of such a biological precept applicable to the morphogenetic steps involved in various stages of metanephric development (Sorokin et al., 1990; Korhonen et

al., 1992). The fact that inclusion of anti- α_6 or antilaminin antibodies in the metanephric culture, during the time when both of the corresponding antigens are coexpressed, inhibits the epithelial polarization of the developing kidney tubules affirms the interactive role of ECM glycoproteins: integrins in nephrogenesis (Klein et al., 1988; Sorokin et al., 1990). However, treatment of metanephroi in the preinductive period with antifibronectin antibodies had no effect on the nephrogenesis, and the reason for this remains unexplained. Here, it is worth mentioning that the fibronectin-binding integrin heterodimers, $\alpha_3\beta_1$, $\alpha_4\beta_1$, and $\alpha_5\beta_1$, often do not colocalize with their putative ligand in the embryonic or adult kidney tissues (Korhonen et al., 1992). Nevertheless, the localization of α_v in the glomerular and extraglomerular vasculature and previously reported expression of vitronectin with similar spatio-temporal distribution (Hayman et al., 1983) would be certainly suggestive of the role of $\alpha_v\beta_3$ in metanephrogenesis. Intriguingly enough, although the presence of vitronectin, a serum-spreading factor, has been well documented, the evidence for its mRNA expression in the developing or mature nephrons is lacking (Seiffert et al., 1991). However, the present studies do establish the role of α_v in metanephric development, which may be accomplished by interacting with vitronectin or other putative ligands.

The role of integrins in various biological processes has been elucidated either by truncation/mutation of the sequences of the receptor or by interference in its functional

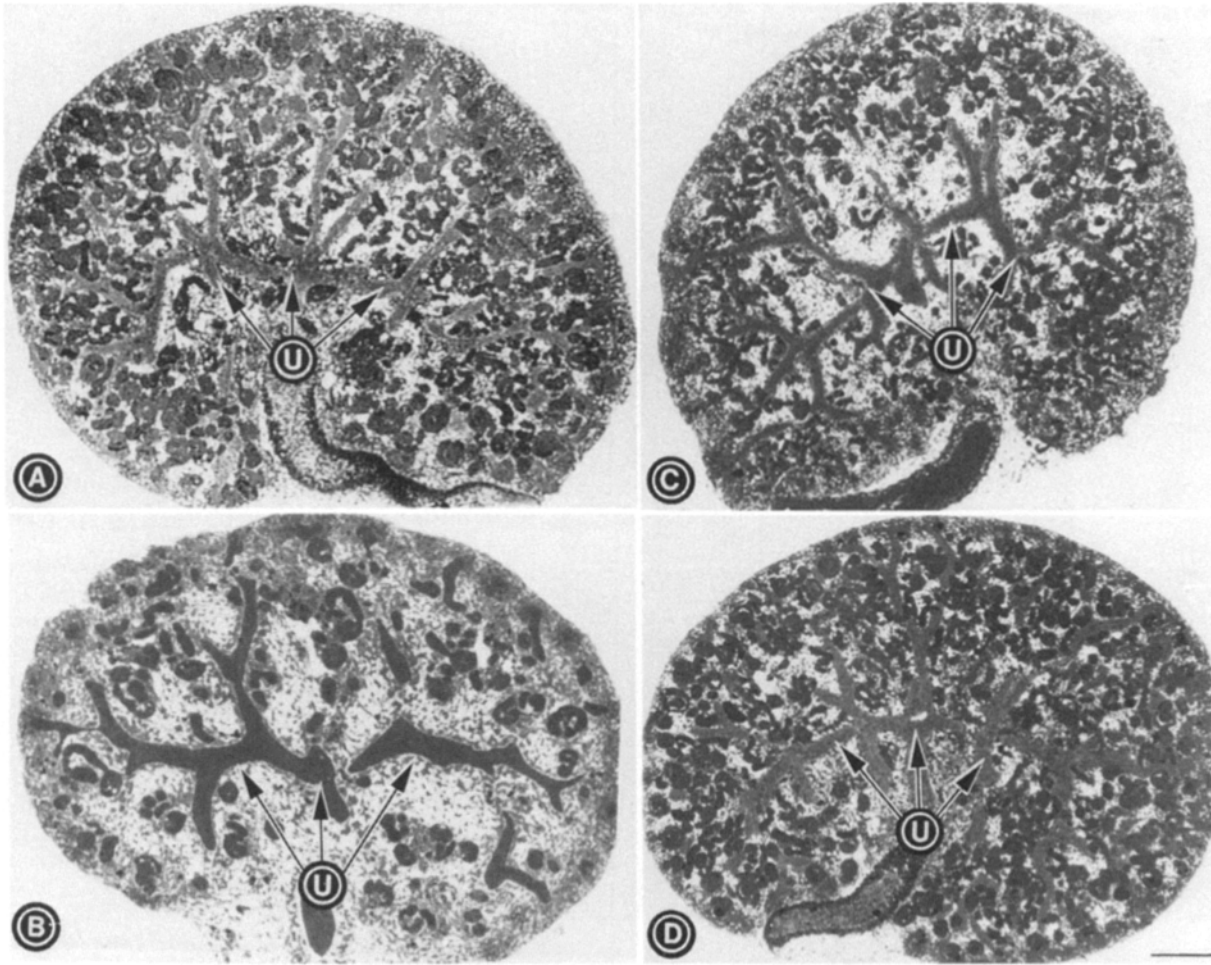


Figure 13. Light photomicrographs of the metanephric explants treated with normal rat IgG (A), rat antiintegrin α_v (69-6-5) (B), and -integrin α_M (NR12C and 5C6) (C and D) blocking monoclonal antibodies. The blocking antibodies were individually included in the culture media at a concentration range of 10^{-6} – 10^{-8} M. The overall size reduction is minimal with the treatment of the antiintegrin α_v antibody, but a moderate reduction in the population of the developing nephrons and mild dysmorphogenesis of the ureteric bud branches are observed. No alterations are observed in the metanephroi treated with normal rat IgG or antiintegrin α_M antibodies. $\times 35$. Bar, 200 μ m.

epitopes with blocking peptides and antibodies or by translational blockade with antisense ODNs. Mutation in the PS3 antigen (a common β -like subunit) in *Drosophila* weakens the muscle attachment sites (MacKrell et al., 1988). The β_1 -related RGD-blocking peptides have been shown to perturb mesencephalic neural crest migration (Boucaut et al., 1984). The neurite outgrowth and neural crest migration can also be perturbed by anti- β_1 antibodies or by corresponding antisense ODNs (Hall et al., 1987; Bronner-Fraser, 1994; Galileo et al., 1992). Besides β integrins, the functional activities of α subunits have been elucidated by such methods as well. Truncation of α_v , presumably, leads to the alteration in its vitronectin- and fibrinogen-related adhesive properties (Filardo and Cheresh, 1994), and anti- α_v antibodies block the tumor- or cytokine-induced angiogenesis (Brooks et al., 1994), oligodendrocyte differentiation (Milner and French-Constant, 1994), and neural crest cell adhesion and migration (Delannet et al., 1994). Using some of these techniques, the role α_v plays in the metanephrogenesis was established in this investigation. The inclusion of α_v -related antisense

ODN caused marked inhibition of nephrogenesis and dysmorphogenesis of metanephroi, reduced gene expression, and translational blockade of α_v . Since no cytotoxicity was observed, and antisense ODN was used at a concentration below 1 μ M, this would indicate that the effects are indeed related to inhibition in the de novo synthesis of integrin α_v . Moreover, the absence of morphological and biochemical alterations in tissues exposed to nonsense ODN would support the assumption that the antisense ODN-induced dysmorphogenesis of the metanephroi is indeed specific. Furthermore, the data of S_1 nuclease protection assay certainly confirm the specificity of the antisense ODN towards the target mRNA in the embryonic tissues. Although the antisense ODN caused remarkable alterations in the developing metanephroi, the integrin α_v antibody-induced changes were rather less impressive. It is quite possible that unlike antisense ODN, the antibody, being a high molecular weight glycoprotein, is not uniformly accessible to the renal subcortical region. However, the effects were certainly discernible, as reflected by the reduced population of nephrons, and were also specific since

irrelevant antiintegrin antibodies, like anti- α_M , failed to induce any morphological alterations. In any instance, the results of the antisense ODN and antibody studies do complement one another, and either probe seems to be capable of specifically perturbing the integrin α_V biological activities, conceivably related to the epithelial:mesenchymal or ECM:integrin interactions, that are necessary to sustain morphogenesis during mammalian development.

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