

Identification of Osteopontin as a Novel Ligand for the Integrin $\alpha 8 \beta 1$ and Potential Roles for This Integrin–Ligand Interaction in Kidney Morphogenesis

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Epithelio–mesenchymal interactions during kidney organogenesis are disrupted in integrin $\alpha 8 \beta 1$ -deficient mice. However, the known ligands for integrin $\alpha 8 \beta 1$ —fibronectin, vitronectin, and tenascin-C—are not appropriately localized to mediate all $\alpha 8 \beta 1$ functions in the kidney. Using a method of general utility for determining the distribution of unknown integrin ligands in situ and biochemical characterization of these ligands, we identified osteopontin (OPN) as a ligand for $\alpha 8 \beta 1$. We have coexpressed the extracellular domains of the mouse $\alpha 8$ and $\beta 1$ integrin subunits as a soluble heterodimer with one subunit fused to alkaline phosphatase (AP) and have used the $\alpha 8 \beta 1$ -AP chimera as a histochemical reagent on sections of mouse embryos. Ligand localization with $\alpha 8 \beta 1$ -AP in developing bone and kidney was observed to be overlapping with the distribution of OPN. In “far Western” blots of mouse embryonic protein extracts, bands were detected with sizes corresponding to fibronectin, vitronectin, and unknown proteins, one of which was identical to the size of OPN. In a solid-phase binding assay we demonstrated that purified OPN binds specifically to $\alpha 8 \beta 1$ -AP. Cell adhesion assays using K562 cells expressing $\alpha 8 \beta 1$ were used to confirm this result. Together with a recent report that anti-OPN antibodies disrupt kidney morphogenesis, our results suggest that interactions between OPN and integrin $\alpha 8 \beta 1$ may help regulate kidney development and other morphogenetic processes.

INTRODUCTION

Integrins are a family of transmembrane glycoproteins that serve as receptors for a wide variety of ligands, including extracellular matrix (ECM)¹ constituents, Ig and cadherin class cell adhesion molecules, and cell surface-associated and soluble members of the disintegrin family (Sonnenberg, 1993; Wolfsberg *et al.*,

1995). Integrins are noncovalently linked heterodimers with α and β subunits, which play an important role in development, wound healing, and immune responses (Hynes, 1992). We have previously shown that the integrin $\alpha 8$ subunit is expressed as a heterodimer with the integrin $\beta 1$ subunit (Bossy *et al.*, 1991). Affinity chromatography with detergent-solubilized integrins and cell adhesion assays have been used to identify fibronectin (FN), vitronectin (VN), and tenascin-C (TN-C) as ligands for the integrin $\alpha 8 \beta 1$ (Müller *et al.*, 1995; Schnapp *et al.*, 1995; Varnum-Finney *et al.*, 1995).

Recently, we have shown that the integrin $\alpha 8 \beta 1$ mediates essential steps in metanephric kidney development, both ingrowth of the ureteric bud and formation of epithelial tubular structures from metanephric mesenchyme (Müller *et al.*, 1997). Although this integrin is strongly expressed in metanephric mesen-

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¹ Abbreviations used: AP, alkaline phosphatase; E, embryonic day; ECM, extracellular matrix; FN, fibronectin; FN120, 120-kDa fragment of FN; GST, glutathione S-transferase; mAb, monoclonal antibody; OPN, osteopontin; PMSF, phenylmethylsulfonyl fluoride; TBS, Tris-buffered saline; TN-C, tenascin-C; VN, vitronectin.

chyme, none of the known $\alpha 8\beta 1$ ligands appears to have an appropriate expression pattern to account alone for the requirement for this integrin during kidney morphogenesis. FN is expressed in the metanephric mesenchyme before invasion by the ureteric bud and is down-regulated upon induction (Ekblom, 1981; Aufderheide *et al.*, 1987). VN is not expressed in the embryonic kidney (Seiffert *et al.*, 1995), and TN-C is expressed too late (Aufderheide *et al.*, 1987) to account for the phenotype in $\alpha 8\beta 1$ -deficient animals. Consistent with these observations, mice with targeted mutations in the VN and TN-C genes develop without kidney abnormalities (Saga *et al.*, 1992; Zheng *et al.*, 1995). Mice lacking FN die too early to analyze kidney development, but antibodies to FN do not perturb kidney development in organ cultures (Klein *et al.*, 1988; Sariola *et al.*, 1988; George *et al.*, 1993). Antibodies to TN-C also have failed to perturb kidney development (Talts *et al.*, 1997).

Motivated in part by these observations, we developed a strategy to identify and characterize ligands that are coexpressed *in vivo* with their cognate integrin receptor(s) and that may mediate essential developmental functions. Previous strategies for detection of integrin ligands based on *in vitro* assays are limited to use of purified proteins as candidate ligands. In addition, some receptor–ligand interactions may not occur *in vivo*, because the ligand and receptor are not codistributed. To search for $\alpha 8\beta 1$ ligand(s) *in vivo*, we chose a combination of biochemical and histochemical approaches using a recombinant soluble integrin heterodimer expressed as a fusion protein with alkaline phosphatase (AP). Although AP chimeras have been used to identify the ligands for receptor tyrosine kinases (Flanagan and Leder, 1990; Cheng and Flanagan, 1994), they have not been previously used to identify ligands of heterodimeric receptor molecules such as integrins. Using the $\alpha 8\beta 1$ -AP chimera as a histochemical reagent, we observed codistribution of $\alpha 8\beta 1$ ligand(s) and osteopontin (OPN) in areas of bone formation and within the developing kidney. Using the $\alpha 8\beta 1$ -AP chimera as a probe in far Western blots of tissue extracts and in solid-phase binding assays with purified proteins, we were able to confirm that OPN is an additional $\alpha 8\beta 1$ ligand. Based on the recent finding that anti-OPN antibodies and RGD peptides disrupt kidney morphogenesis (Rogers *et al.*, 1997), it seems likely that interactions between OPN and integrin $\alpha 8\beta 1$ may contribute to the regulation of epithelio-mesenchymal interactions during kidney development.

MATERIALS AND METHODS

Materials

Human plasma FN, the 120-kDa fragment of FN (FN120), bovine plasma VN, and the peptides GRGDSP and GRGESP were pur-

chased from Life Technologies (Gaithersburg, MD). A glutathione S-transferase-human OPN fusion protein (GST-OPN) (Hu *et al.*, 1995) was a gift from Dr. J.W. Smith (Burnham Institute, La Jolla, CA). Purified human urine OPN (Shiraga *et al.*, 1992) was a gift from Dr. J.R. Hoyer (Children's Hospital of Philadelphia, Philadelphia, PA). Recombinant mouse entactin/nidogen was a gift from Dr. R. Timpl (Max-Planck-Institut für Biochemie, Martinsried, Germany). GST-entactin fragment fusion proteins were gifts from Dr. A. E. Chung (University of Pittsburgh, Pittsburgh, PA).

Antibodies

Anti-mouse integrin $\alpha 8$ subunit antibody was prepared by immunizing rabbits with affinity-purified mouse integrin $\alpha 8^t$ monomer (Figure 1B, lane 2) expressed in COS cells. The antibody reacted specifically with integrin $\alpha 8$ in Western blot, immunoprecipitation, and immunohistochemistry of mouse tissue sections. The other antibodies were as follows: anti-rat OPN peptide (residues 291–306) antiserum OST-1 (a gift from Dr. B.B. Mukherjee, McGill University, Montreal, Quebec, Canada); anti-OPN antiserum pp69 (Nemir *et al.*, 1989); anti-GST-mouse OPN antibody (Stern *et al.*, 1995); anti-OPN monoclonal antibody (mAb) MPIIB10 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, and Johns Hopkins University, Baltimore, MD); anti-rat FN antiserum (Yang *et al.*, 1993); anti-human FN antibody (Organon Teknika, Durham, NC); anti-mouse VN antibody (Seiffert, 1996); anti-rat integrin $\beta 1$ mAb HA2/11 (Mendrick and Kelly, 1993); anti-mouse integrin $\beta 1$ mAb 9EG7 (Lenter *et al.*, 1993); anti-human integrin $\alpha v\beta 5$ mAb P1F6 (Life Technologies); anti-human integrin $\alpha 5$ mAb B1E5 (Hall *et al.*, 1990); anti-human integrin $\beta 1$ mAb A1B2 (Hall *et al.*, 1990); and anti-human placental AP mAb MIA1801 (Medix Biotech, San Carlos, CA). For some experiments, IgG fractions were prepared from antisera using protein A-Sepharose (Pharmacia LKB Biotechnology, Piscataway, NJ).

Preparation of Expression Vectors for Expression of Modified Extracellular Domains of $\alpha 8$ and $\beta 1$ Subunits

Cloning of murine integrin $\alpha 8$ cDNA and the constructs to express truncated soluble heterodimers are described elsewhere (Denda *et al.*, 1998). In brief, using mRNA purified from NIH 3T3 cells, cDNA was synthesized, and clones encoding $\alpha 8$ cDNA were amplified using primers based on the sequences of the human and chick integrin $\alpha 8$ subunits. The sequence data are available from GenBank under accession number AF041409.

To express a secreted protein, a synthetic DNA fragment encoding a chick $\alpha 8$ signal peptide was fused to the N-terminal portion of the mature mouse $\alpha 8$ subunit. A clone encoding a truncated mouse $\alpha 8$ ($\alpha 8^t$) was generated by PCR with a gene-specific 5' primer and a 3' primer encoding the C terminus of the deduced extracellular domain of mouse $\alpha 8$ plus a (His)⁶-myc tag and a stop codon (VI-WATPNVSHHHHHHGEQKLISEEDL-stop). The truncated mouse $\beta 1$ ($\beta 1^t$) was generated by introducing a stop codon after the end of extracellular domain (amino acid sequence number 728) by PCR using the mouse $\beta 1$ cDNA clone ST1 as a template. These were subcloned into pCR3 (Invitrogen, San Diego, CA). The mouse $\beta 1$ extracellular domain-AP chimera ($\beta 1$ -AP) was generated by isolating a modified pCR3- $\beta 1^t$ in which the stop codon at the end of the extracellular domain of $\beta 1^t$ was eliminated by PCR and was then ligated to an *Sna*BI (filled in with Klenow)-*Xho*I fragment containing the AP from APTag-1 (Flanagan and Leder, 1990). All PCR-generated clones were sequenced to ensure that they did not contain mutations.

Expression and Purification of Soluble Truncated Integrin Heterodimers

COS-7 cells were transiently cotransfected using LipofectAMINE Reagent (Life Technologies) with the two plasmids encoding extra-

cellular domains of the α and β subunits plus indicated C-terminal tags ($\alpha 8^t$ and $\beta 1$ -AP) to express $\alpha 8^t\beta 1$ -AP (Figure 1A). The conditioned medium was collected every 2–4 d for 1 wk. After addition of $MgCl_2$ (at a final concentration of 1 mM), phenylmethylsulfonyl fluoride (PMSF, 1 mM), and sodium azide (0.02%), the conditioned medium was filtered and concentrated 10- to 20-fold using a YM100 membrane (Amicon, Beverly, MA). After addition of Tris-Cl (pH 8.0; 20 mM), imidazole (10 mM), and PMSF (0.5 mM), the solution was incubated with Ni-NTA beads (Qiagen, Chatsworth, CA), which were transferred into an empty column and washed with 20 mM Tris-Cl, pH 7.5, 300 mM NaCl, 1 mM $MgCl_2$, 0.02% sodium azide, 20 mM imidazole, 0.5 mM PMSF. After washing, bound proteins were eluted with 20 mM Tris-Cl (pH 7.5), 50 mM NaCl, 1 mM $MgCl_2$, 0.02% sodium azide, 100 mM imidazole, 0.5 mM PMSF. To remove imidazole, $\alpha 8^t$ monomer, and nonspecifically bound contaminants of low molecular weight, the buffer of the eluate was exchanged by repeating concentration and dilution with the elution buffer without imidazole, using a Centricon 100 or a Centriplus 100 filter apparatus (Amicon, Beverly, MA). The truncated integrin $\alpha 8$ monomer ($\alpha 8^t$) was purified as above, except that a YM30 membrane (Amicon) was used to concentrate the medium from cells expressing only $\alpha 8^t$. Protein concentrations were determined by both Coomassie Plus protein assay reagent (Pierce, Rockford, IL) and silver staining of proteins after fractionation by SDS-PAGE. The purified heterodimers retained activity for at least 5 mo when stored at either 4 or $-80^\circ C$.

Histochemistry

C57Bl/6 mouse embryos were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at 7 μm , stained with antibodies, and counterstained as described (Jones *et al.*, 1994). Staining with $\alpha 8^t\beta 1$ -AP was performed as described (Cheng and Flanagan, 1994) with some modifications. Sections were blocked with 1% BSA, 25 mM Tris-Cl (pH 7.5), 100 mM NaCl; incubated for 4–12 h at room temperature with 7 $\mu g/ml$ $\alpha 8^t\beta 1$ -AP in 20 mM Tris-Cl (pH 7.5), 50 mM NaCl, 1 mM $MnCl_2$, 0.05% BSA, 0.02% sodium azide; washed with the same buffer; fixed with 60% acetone, 3% formaldehyde, 20 mM HEPES (pH 7.0); washed with 20 mM HEPES (pH 7.0), 150 mM NaCl; heated at $65^\circ C$ for 1 h in this buffer; rinsed in AP buffer (100 mM Tris-Cl [pH 9.5], 100 mM NaCl, 5 mM $MgCl_2$); and incubated at room temperature with AP substrate solution (0.33 mg/ml nitroblue tetrazolium and 0.17 mg/ml 5-bromo-4-chloro-3-indoyl-phosphate in AP buffer). Sections were counterstained with methyl green (Zymed, South San Francisco, CA) and mounted with GVA-mount (Zymed).

Far Western Blotting

Protein extracts were obtained by homogenizing mouse embryos and kidneys in ice-cold extraction buffer (50 mM Tris-Cl [pH 7.5], 50 mM octylglucoside, 20 mM NaCl, 1 mM $MgCl_2$, 1 mM sodium vanadate, 1 mM sodium molybdate, 1 mM PMSF, 10 $\mu g/ml$ leupeptin, 3 $\mu g/ml$ pepstatin A) followed by centrifugation to remove the debris. Protein concentrations were determined by Coomassie assays (Pierce). Far Western blotting was performed as described (Hildebrand *et al.*, 1995) with some modifications. Proteins were separated by electrophoresis on SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and renatured. Membranes were blocked at $4^\circ C$ with 10% BSA, 20 mM HEPES (pH 7.5), 75 mM KCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 0.05% NP40 for 1 h and then 1% BSA, 25 mM Tris-Cl (pH 7.5), 50 mM NaCl, 0.05% NP-40 for 1 h. Membranes were washed in 0.1% BSA, 25 mM Tris-Cl (pH 7.5), 50 mM NaCl, 1 mM $MnCl_2$, 0.05% NP-40, incubated with 0.3 $\mu g/ml$ $\alpha 8^t\beta 1$ -AP in the same solution for 2 h at room temperature, washed with this solution, rinsed in AP buffer containing 0.5 mM $MnCl_2$, and incubated with AP-substrate solution containing 0.5 mM $MnCl_2$. Where indicated, extracts were depleted of FN by passing the extracts three times through an FN antibody column, or extracts

were immunoprecipitated with OPN antiserum (pp69; Nemir *et al.*, 1989) and protein A beads before far Western analysis.

Solid-Phase Binding Assays, Cell Adhesion Assays, and Cell-spreading Assays

Ninety-six-well plates (Maxisorp, Nunc, Rochester, NY) were coated with substrate proteins in Tris-buffered saline (TBS; 25 mM Tris-Cl [pH 7.5], 100 mM NaCl) at $4^\circ C$ overnight, blocked with 1% BSA in TBS, and washed with TBS-Mn (1 mM $MnCl_2$ in TBS). As background control and positive control, wells were coated with 1% BSA and 10 $\mu g/ml$ FN120, respectively. $\alpha 8^t\beta 1$ -AP (5 $\mu g/ml$ in TBS-Mn) was then added to each well and incubated at room temperature for 2 h. After washing with TBS-Mn, 100 μl AP substrate (1 M diethanolamine [pH 9.8], 0.5 mM $MgCl_2$, 12 mM *p*-nitrophenyl phosphate) was added and incubated at room temperature. Integrin binding was quantified by measuring absorbance at 405 nm.

The KA8 cells ($\alpha 8$ -transfected K562 cells), K562 cells, and cell adhesion and cell-spreading assays have been described previously (Müller *et al.*, 1995).

RESULTS

Soluble Recombinant Integrin $\alpha 8\beta 1$ Heterodimers Bind FN In Vitro and In Situ

To generate soluble integrin heterodimers, we have designed truncated mouse $\alpha 8$ and mouse $\beta 1$ cDNA constructs (Figure 1A). The truncated $\alpha 8$ ($\alpha 8^t$) has (His)⁶ and c-myc epitope tags for the purpose of affinity purification and detection, respectively. Human placental AP was chosen for incorporation into the truncated $\beta 1$ -AP chimera ($\beta 1$ -AP) because it has well-characterized detection properties (Cheng and Flanagan, 1994). The two cDNA constructs ($\alpha 8^t$ and $\beta 1$ -AP) were coexpressed in COS cells, and the secreted integrin heterodimers ($\alpha 8^t\beta 1$ -AP) were purified from the conditioned medium (Figure 1B, lane 1).

The secreted $\alpha 8^t\beta 1$ -AP chimera shows similar ligand-binding properties to integrin $\alpha 8\beta 1$ expressed on the cell surface, because purified $\alpha 8^t\beta 1$ -AP interacts specifically with ECM ligands in a solid-phase binding assay; $\alpha 8^t\beta 1$ -AP bound to the known ligands (FN and VN) in an RGD-dependent manner (see Figure 5A, discussed later in this article); it did not bind to several other ECM molecules, such as laminin-1, collagen I, and collagen IV, in agreement with earlier observations that these molecules do not appear to be ligands for cell surface-expressed $\alpha 8\beta 1$ (Müller *et al.*, 1995; Denda *et al.*, 1998). Binding to each of these substrate-bound ligands was completely blocked by the inhibitory anti- $\beta 1$ mAb HA2/11 (Denda *et al.*, 1998), and binding was observed in the presence of Mn^{2+} but not detectably in the absence of Mn^{2+} . $\alpha 8\beta 1$ expressed on the cell surface of K562 cells exhibits the same requirement for Mn^{2+} (Müller *et al.*, 1995).

Purified soluble heterodimers also bound to an FN affinity column and could be used to localize FN secreted into the ECM by cultured fibroblasts, as shown by double immunofluorescence with $\alpha 8^t\beta 1$ -AP and

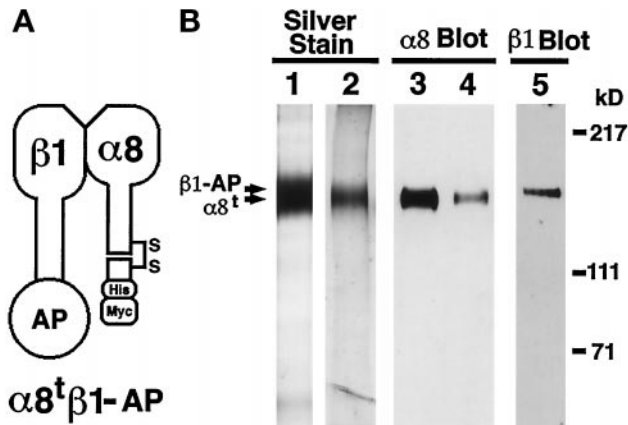


Figure 1. Purification of soluble truncated integrin $\alpha 8\beta 1$ heterodimer and $\alpha 8$ monomer. (A) Schematic representation of the soluble $\alpha 8\beta 1$ -AP heterodimer. Mature integrin $\alpha 8$ and $\beta 1$ subunits consist of extracellular, transmembrane, and cytoplasmic domains. The truncated $\alpha 8$ ($\alpha 8^t$) consists of the entire extracellular domain of mouse $\alpha 8$ cDNA with c-terminal (His)⁶ and c-myc epitope tags. The $\beta 1$ -AP chimera ($\beta 1$ -AP) consists of the entire extracellular domain of $\beta 1$ fused in frame to human placental AP. $\alpha 8\beta 1$ -AP is a heterodimer of $\alpha 8^t$ and $\beta 1$ -AP. (B) The purified $\alpha 8\beta 1$ -AP heterodimer (lanes 1, 3, and 5) and $\alpha 8^t$ monomer (lanes 2 and 4) were separated by electrophoresis in 6% polyacrylamide gels in nonreducing conditions. The gels were either silver stained (lanes 1 and 2) or transferred to nitrocellulose membranes and probed with anti- $\alpha 8$ antiserum (lanes 3 and 4) or anti- $\beta 1$ mAb 9EG7 (lane 5). In lane 1, both $\alpha 8^t$ and $\beta 1$ -AP bands are within the overexposed band shown. The purified $\alpha 8^t$ monomer (lane 2) was used as an antigen to prepare the rabbit polyclonal anti-mouse $\alpha 8$ antibody.

anti-FN antibodies. Both reagents stained FN fibrils on the surface of fibroblasts (our unpublished results). The $\alpha 8\beta 1$ -AP binding was specific, because it was completely inhibited by an RGDS-containing peptide and by the anti-integrin $\beta 1$ mAb HA2/11 (our unpublished results).

Ligand Detection with $\alpha 8\beta 1$ -AP in Tissue Sections: Colocalization of an $\alpha 8\beta 1$ Ligand and OPN

Because the $\alpha 8\beta 1$ -AP chimera recognized the known ligands of this integrin, we extended the use of $\alpha 8\beta 1$ -AP to histochemistry on tissue sections to localize ligands in situ (Figures 2 and 3). On sections of embryonic day (E) 13.5 and E16.5 mouse embryos, the $\alpha 8\beta 1$ -AP bound to restricted sites, including developing rib bones (Figure 2) and kidneys (Figure 3).

Within the developing bone, $\alpha 8\beta 1$ -AP binding (staining) was confined to the bony collar (Figure 2, C and E, arrowheads), and the binding was specific, because it was inhibited by the $\beta 1$ mAb HA2/11 (our unpublished results). Binding was also inhibited by an RGDS-containing peptide (Figure 2F), whereas a control RGES peptide had no effect (Figure 2G), indicating that the binding pattern of $\alpha 8\beta 1$ -AP reflects the distribution of RGD-containing ligand(s). The $\alpha 8\beta 1$ -AP

binding pattern (Figure 2, C and E) was similar to the previously reported immunohistochemical localization of OPN, an ECM protein with an RGD cell attachment site (Mark *et al.*, 1988; Gorski *et al.*, 1990; Chen *et al.*, 1991; Stern *et al.*, 1995). We confirmed this by staining the adjacent sections with anti-OPN antibodies (Figure 2, A and D). The anti-OPN staining pattern overlapped with $\alpha 8\beta 1$ -AP staining, although the $\alpha 8\beta 1$ -AP staining was more restricted and concentrated at cell-cell junctions.

We have previously shown that the integrin $\alpha 8$ subunit is expressed in the metanephric mesenchyme with high levels in the condensing mesenchymal cells surrounding the tips of the branching ureter and in the mesenchymal cells bordering on the growing and maturing ureter epithelium (Müller *et al.*, 1997). The $\alpha 8\beta 1$ -AP chimera stained the surface of the ureter epithelium in a pattern complementary to staining with antibodies against the integrin $\alpha 8$ subunit, as described (Figure 3, A and C; Müller *et al.*, 1997). We have previously demonstrated also that the $\alpha 8\beta 1$ -AP binding is specific, because it can be inhibited by an RGDS-containing peptide but not by a control RGES peptide (Müller *et al.*, 1997). We now demonstrate by immunohistochemistry that OPN is also localized to the ureter epithelium in a pattern that overlaps with that of $\alpha 8\beta 1$ -AP staining (Figure 3, compare A and B). The staining for OPN was confined to the growing and maturing ureteric epithelium, and no staining was apparent in the surrounding mesenchymal cells. The same OPN expression pattern was observed with three different antibodies against OPN (Figure 3B and our unpublished results). This is in agreement with recent analysis of OPN expression by in situ hybridization (Rogers *et al.*, 1997). Our data suggest that $\alpha 8\beta 1$ -AP may recognize OPN in situ, and OPN is appropriately localized to mediate interactions with integrin $\alpha 8\beta 1$ in vivo in the kidney.

Ligand Detection by Far Western Blot Analysis

To characterize the biochemical nature of $\alpha 8\beta 1$ ligands in mouse embryos, extracts from E13.5 mouse embryos were analyzed by far Western blot using $\alpha 8\beta 1$ -AP as a probe. The $\alpha 8\beta 1$ -AP bound to proteins of approximate molecular masses of 240, 130, and 65–85 kDa (Figure 4, lane 1). Bands with the same molecular masses were recognized in an extract from E16.5 kidney (Müller *et al.*, 1997). The binding to each of these proteins was completely inhibited by RGDS (Figure 4, lane 2) but not by RGES peptides (our unpublished results). The sizes of some of the proteins recognized by $\alpha 8\beta 1$ -AP appeared the same as those of FN, VN, and OPN, as detected by Western blotting (Figure 4, lanes 4–6). Indeed, the 240-kDa band (Figure 4, lane 1) was identified as FN, because it could be depleted from extracts by FN-antibody affinity chro-

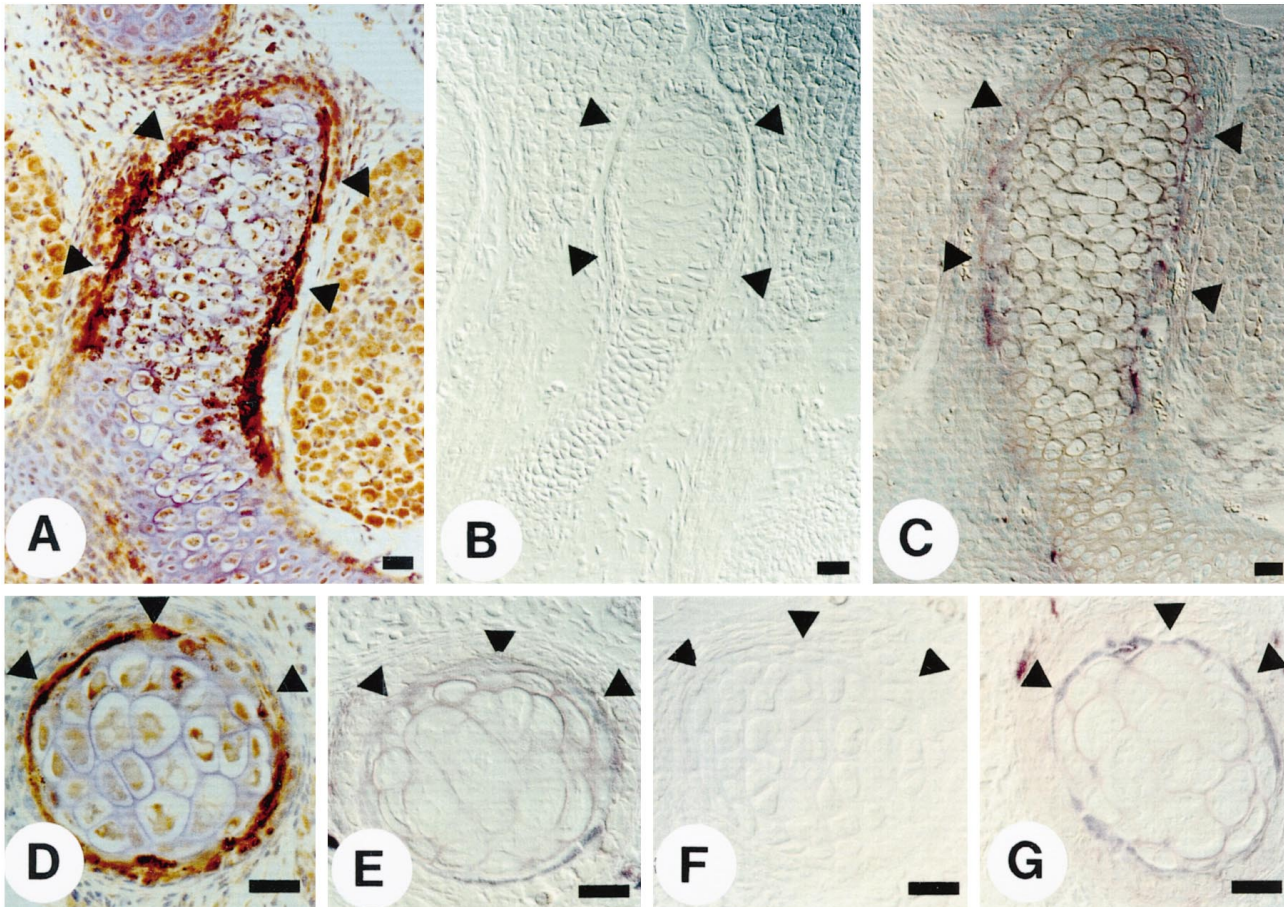


Figure 2. Colocalization of $\alpha 8 \beta 1$ -AP binding sites and OPN in the regions of bone morphogenesis. Paraffin sections of E16.5 (A–C) and E13.5 (D–G) mouse embryos were incubated with indicated reagents: (A) anti-OPN peptide antiserum; (B) control conditioned medium containing nonfused, secreted AP; (C and E) $\alpha 8 \beta 1$ -AP; (D) anti-GST-mouse OPN IgG; (F) $\alpha 8 \beta 1$ -AP in the presence of 50 $\mu\text{g}/\text{ml}$ GRGDSP peptide; and (G) $\alpha 8 \beta 1$ -AP in the presence of 50 $\mu\text{g}/\text{ml}$ GRGESP peptide. Arrowheads indicate the bony collar. Bar, 10 μm .

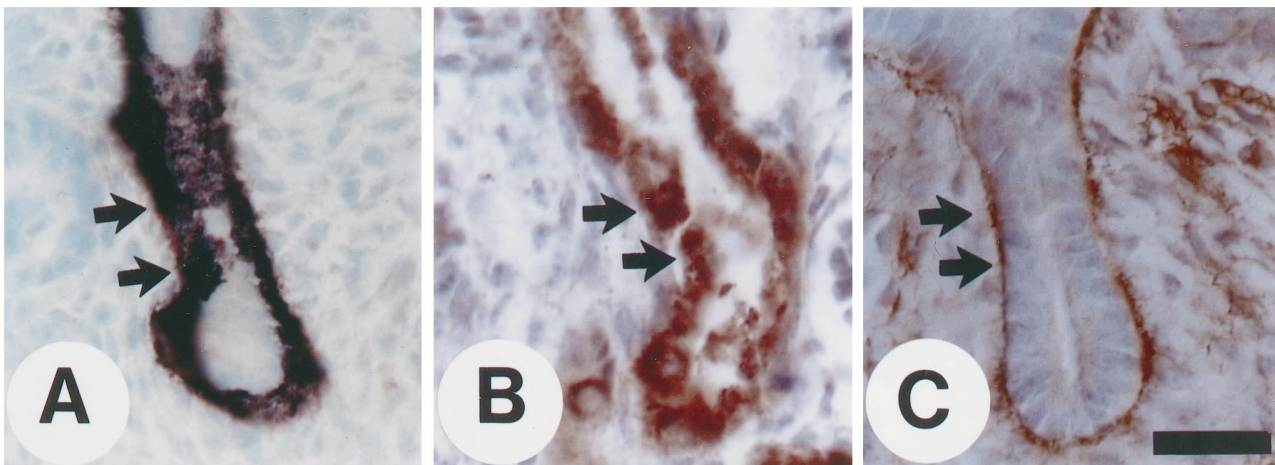


Figure 3. Colocalization of $\alpha 8 \beta 1$ -AP binding sites, OPN, and the integrin $\alpha 8$ subunit in the developing kidney. Paraffin sections (A and C) and frozen sections (B) of an E16.5 kidney were incubated with $\alpha 8 \beta 1$ -AP (A), anti-OPN peptide antiserum (B), and affinity-purified anti-mouse integrin $\alpha 8$ subunit IgG (C). The panels show a cross-section through the ureter epithelium, and the arrows point to the border between the epithelium and surrounding mesenchyme. Note that the section is at a level below the tips of the ureter; therefore, no condensing mesenchymal cells are visible. Bar, 50 μm .

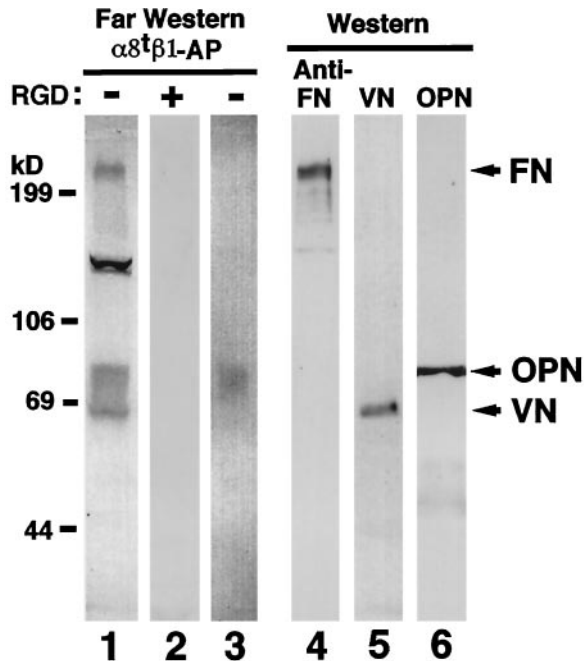


Figure 4. RGD-dependent binding of $\alpha 8\beta 1$ -AP to proteins including OPN in mouse embryo extract. Fifty micrograms of protein per lane of the extract from E13.5 embryo (lanes 1, 2, and 4–6) and immunoprecipitates from E16.5 kidney extract with OPN antiserum pp69 (lane 3) were separated electrophoretically on a 7.5% polyacrylamide gel in reducing conditions and analyzed by far Western (lanes 1–3) and Western (lanes 4–6) blotting. For far Western blotting of the E13.5 extract, membrane strips were incubated with $\alpha 8\beta 1$ -AP in the absence (lane 1) or presence (lane 2) of 50 $\mu\text{g}/\text{ml}$ GRGDSP peptide. For Western blotting, the membrane strips were probed with anti-FN IgG (lane 4), anti-VN IgG (lane 5), or anti-OPN mAb MPIIB10 (lane 6). The integrin-AP chimera bound to protein bands of 240, 130, and 65–85 kDa in the extract (lane 1) and ~80 kDa in the anti-OPN immunoprecipitates (lane 3). This 80-kDa band comigrates with the band detected by OPN Western blot (lane 6).

matography (our unpublished results). Proteins in the range of 65 kDa (Figure 4, lane 1) were purified from tissue extracts over an $\alpha 8\beta 1$ -AP affinity column and identified by microsequencing as VN (our unpublished results). We were not able to purify the proteins of ~130 and 65–85 kDa (Figure 4, lane 1) with an $\alpha 8\beta 1$ -AP column in sufficient quantities for microsequencing or blotting with OPN antibodies. In addition, the OPN antibodies that were available immunoprecipitated OPN inefficiently; so we could not immunodeplete OPN from extracts. However, using anti-OPN immunoprecipitates from the E16.5 kidney extract, a band in the expected size range for OPN was detected in far Western blots (Figure 4, lane 3), indicating that OPN in the extract can be recognized by $\alpha 8\beta 1$ -AP and that OPN may be among the proteins of 65- to 85-kDa bands detected in the E13.5 embryo and E16.5 kidney extracts. The data prompted us to determine whether there are interactions between purified

OPN and integrin $\alpha 8\beta 1$. The data presented below clearly show that OPN is a novel ligand for integrin $\alpha 8\beta 1$, and the 80-kDa protein observed in far Western blots may correspond to OPN.

OPN is a Novel Ligand for Integrin $\alpha 8\beta 1$

The colocalization and far Western studies described above suggest that OPN is an additional ligand for integrin $\alpha 8\beta 1$. To pursue this possibility, we analyzed interactions of $\alpha 8\beta 1$ -AP with purified recombinant GST-OPN and native human OPN in a solid-phase binding assay (Figure 5, A and B). The $\alpha 8\beta 1$ -AP bound to GST-OPN and native OPN (Figure 5A) and the binding was specific, as it was inhibited by an RGD-containing peptide and a function blocking mAb against the $\beta 1$ subunit (Figure 5B). Binding of $\alpha 8\beta 1$ -AP to purified, bacterially expressed GST-OPN was stronger than binding to purified human OPN; this suggests that the $\alpha 8\beta 1$ -AP binding site within the purified human OPN may have been partially masked, for example, by posttranslational modification, or that the preparation may have only been partially active (see DISCUSSION).

To confirm that OPN binds to integrin $\alpha 8\beta 1$, we performed cell adhesion assays with K562 and KA8 cells on GST-OPN and purified OPN. KA8 cells were obtained by transfecting K562 cells with an $\alpha 8$ cDNA expression vector (Müller *et al.*, 1995). KA8 cells express integrin $\alpha 8\beta 1$ and $\alpha 5\beta 1$, whereas K562 cells express only $\alpha 5\beta 1$ as major integrin receptors. KA8 and K562 cells also express low amounts of $\alpha \nu$ -containing integrins (e.g. Müller *et al.*, 1995). Interaction of both cell lines with integrin ligands such as FN has been shown to be dependent on prior activation of the integrin by activating antibodies or by Mn^{2+} (Müller *et al.*, 1995). KA8 cells but not K562 cells adhered strongly to OPN in the presence of Mn^{2+} (Figure 5C). Neither Mg^{2+} nor Ca^{2+} promoted KA8 cell adhesion to OPN in the absence of Mn^{2+} (our unpublished results). Adhesion of KA8 cells was inhibited by an RGD-containing peptide and by a function-blocking anti- $\beta 1$ mAb but not by other integrin antibodies tested (Figure 5D). In addition to adhesion, KA8 cells spread on GST-OPN and purified OPN as efficiently as on FN (Figure 6). The parent K562 cells did not spread on either substrate.

To exclude that integrin $\alpha 8\beta 1$ interacts nonspecifically with purified proteins that contain an RGD motif, we tested interactions of $\alpha 8\beta 1$ -AP and KA8 cells with purified entactin and recombinant entactin fragments that contain an RGD site (Figure 5A and our unpublished results). We did not observe any interactions, further supporting that binding to OPN was specific. We thus conclude that OPN is a ligand for the integrin $\alpha 8\beta 1$.

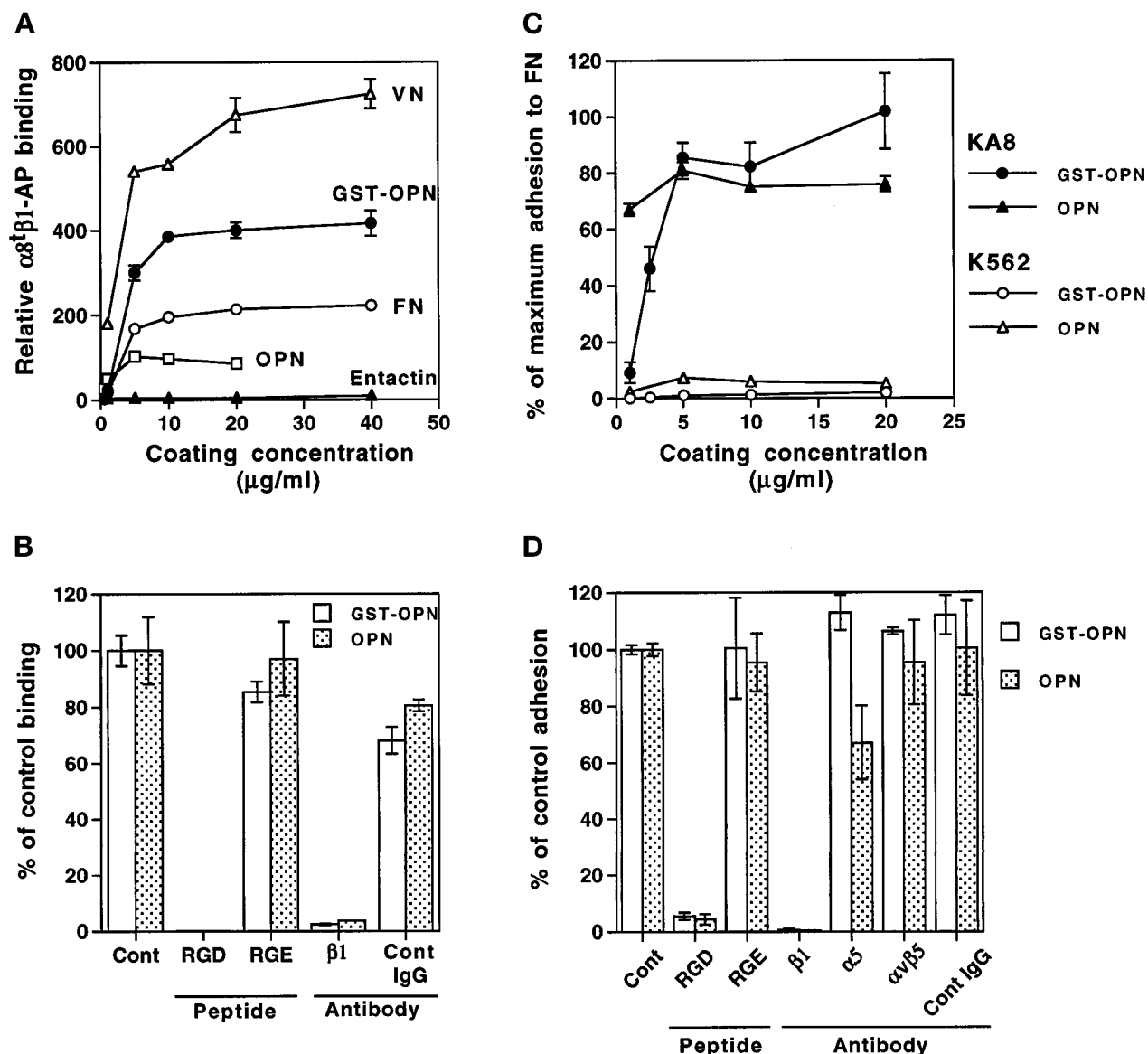


Figure 5. Binding of $\alpha 8 \beta 1$ -AP and cells expressing $\alpha 8 \beta 1$ to known ligands and to purified native OPN and recombinant GST-OPN. (A and B) Solid-phase binding assays were performed with $\alpha 8 \beta 1$ -AP in the presence of 1 mM MnCl_2 . (A) $\alpha 8 \beta 1$ -AP binding to entactin, VN, FN, native OPN, and GST-OPN. Plates were coated with indicated concentrations of purified proteins. The absorbance values were normalized using the absorbance value for binding to substrata coated with 10 $\mu\text{g/ml}$ FN120. Results demonstrate that $\alpha 8 \beta 1$ -AP binds to OPN and GST-OPN as well as to FN and VN. (B) Effects of peptides and an inhibitory anti- $\beta 1$ antibody on $\alpha 8 \beta 1$ -AP binding to native OPN and GST-OPN. Wells coated with 5 $\mu\text{g/ml}$ OPN or 10 $\mu\text{g/ml}$ GST-OPN were incubated with $\alpha 8 \beta 1$ -AP with additions indicated: (Cont) no addition; (RGD) 0.1 $\mu\text{g/ml}$ GRGDSP peptide; (RGE) 0.1 $\mu\text{g/ml}$ GRGESP peptide; ($\beta 1$) 100 $\mu\text{g/ml}$ anti-integrin $\beta 1$ mAb HA2/11; and (Cont IgG) 100 $\mu\text{g/ml}$ control IgG. The absorbance was quantified as the percentage of control binding (no addition). (C) Cell adhesion to native OPN and GST-OPN. Cell adhesion assays were carried out with KA8 and K562 cells on plates coated with indicated concentrations of OPN and GST-OPN in the presence of 1 mM MnCl_2 . The absorbance was quantified as the percentage of maximum adhesion to FN coated at 5 $\mu\text{g/ml}$. Note that only KA8 cells adhered to OPN and GST-OPN. (D) Effects of peptides and anti-integrin mAbs on KA8 cell adhesion to OPN and GST-OPN. KA8 cell adhesion assays were carried out on OPN and GST-OPN (coated at 5 $\mu\text{g/ml}$) in the presence of 1 mM MnCl_2 with additions as indicated: (Cont) no addition; (RGD) 10 $\mu\text{g/ml}$ GRGDSP peptide; (RGE) 10 $\mu\text{g/ml}$ GRGESP peptide; ($\beta 1$) anti- $\beta 1$ mAb A11B2; ($\alpha 5$) anti- $\alpha 5$ mAb B1E5; ($\alpha v \beta 5$) anti- $\alpha v \beta 5$ mAb P1F6; and (Cont IgG) 100 $\mu\text{g/ml}$ control IgG. Adhesion with no addition (Cont) was set at 100%. Note that KA8 cell adhesion to OPN and GST-OPN was RGD sensitive and was blocked by the anti- $\beta 1$ mAb. Data represent mean \pm SD of triplicate wells.

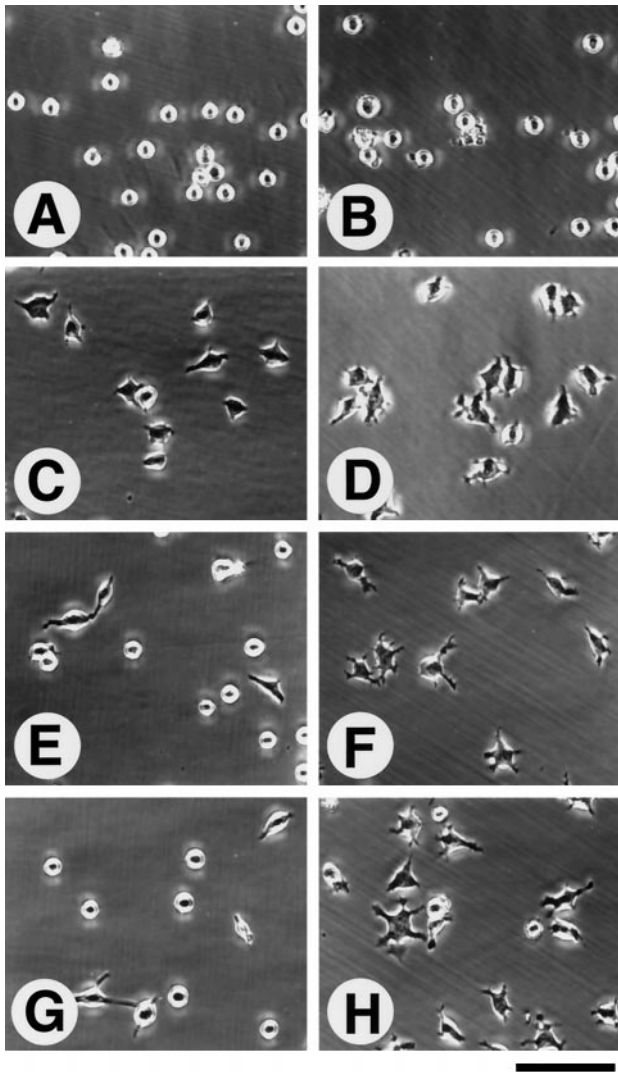


Figure 6. Cell-spreading assay. In the presence of 1 mM $MnCl_2$, K562 cells (A, C, E, and G) and KA8 cells (B, D, F, and H) were plated onto different substrata: (A and B) BSA; (C and D) FN (coated at 5 μ g/ml); (E and F) purified native OPN (5 μ g/ml); and (G and H) recombinant GST-OPN (5 μ g/ml). Note that both cell types spread on FN, but only KA8 cells spread on native OPN and recombinant GST-OPN. Bar, 100 μ m.

DISCUSSION

In the present study, we describe methodological approaches that should be of general usefulness for characterizing interactions between integrins and their ligands *in situ* and *in vitro*. With this method, we have identified OPN as a novel ligand for integrin $\alpha 8\beta 1$. We demonstrate that the $\alpha 8\beta 1$ integrin, OPN, and $\alpha 8\beta 1$ ligands show partially overlapping expression patterns within the developing kidney. This raises the possibility that developmental defects in early stages of kidney morphogenesis observed in integrin $\alpha 8\beta 1$ -

deficient mice (Müller *et al.*, 1997) may be a consequence, at least in part, of disrupted interactions between $\alpha 8\beta 1$ and OPN. In agreement, it has recently been shown that kidney morphogenesis is disrupted in organ cultures by antibodies to OPN and by RGD peptides (Rogers *et al.*, 1997), which would be expected to block interactions of integrin $\alpha 8\beta 1$ and OPN.

Our data demonstrating an interaction between the $\alpha 8\beta 1$ integrin and OPN is in apparent disagreement with previous data of Schnapp *et al.* (1995), who reported that human kidney-derived 293 cells transfected with a human $\alpha 8$ cDNA (and therefore expressing the human $\alpha 8\beta 1$ heterodimer) did not bind detectably to this ligand, although they did bind to other $\alpha 8\beta 1$ ligands. As one possible explanation, the ligand-binding properties of several integrin receptors has been shown to vary, depending on cell type (reviewed by Hynes, 1992). It is also possible that differences in assay conditions or differences between the human $\alpha 8\beta 1$ and the mouse and chick-human chimeric $\alpha 8\beta 1$ used in the present article explain these apparent discrepancies. In addition, Schnapp *et al.* (1995) performed their assay in the presence of Mg^{2+} and Ca^{2+} , although Ca^{2+} has been reported to inhibit the binding of αv integrins to OPN (Hu *et al.*, 1995). Our data show convincingly that in some cells and after purification integrin $\alpha 8\beta 1$ can interact with OPN.

Interactions of $\alpha 8\beta 1$ -AP with GST-OPN prepared by expression in bacteria were stronger than interactions with OPN purified from human urine. Surprisingly, no such difference was observed in cell adhesion assays. As one possible explanation, the binding site within native OPN but not GST-OPN may have been partially masked, preventing strong interactions between the purified integrin and native OPN. Cellular integrin $\alpha 8\beta 1$ may in contrast exhibit cooperative interactions, strengthening cell binding to native OPN. Masking of integrin binding sites in native OPN may reflect conformational differences between native OPN and GST-OPN, posttranslational modifications of the former, or both. Native OPN is known to be highly phosphorylated, which could potentially modify the affinity of integrin binding sites (Shiraga *et al.*, 1992). Nevertheless, the fact that both the purified integrin and the same integrin expressed on the surface of K562 cells bind to purified OPN and to GST-OPN argues that these interactions are specific; the inhibition of these interactions by an anti-integrin $\beta 1$ mAb and an RGD-containing peptide indicates that the interactions are mediated by the integrin ligand binding site. The possible importance of posttranslational modifications of OPN for regulating integrin interactions *in vivo* is an interesting subject for future investigation.

Soluble integrin-AP chimera should be generally useful for detecting endogenous ligands *in situ* on the surface of isolated cells and in tissue sections. Inte-

grin-AP chimeras are easy to prepare and appear to be more sensitive and specific probes of ligand receptor interactions than biotinylated or iodinated integrins (e.g., compare data in this article to data of DeFreitas *et al.*, 1995). Fusion of the C terminus of the $\beta 1$ subunit with AP does not appear to affect ligand-receptor interactions, because we have detected interactions of the chimera with the previously described ligands for this integrin. Binding of $\alpha 8^t\beta 1$ -AP requires integrin activation and is inhibited by an anti- $\beta 1$ mAb or peptides containing the RGDS sequence. The specificity of binding observed in these assays appears to be identical to the specificity observed in cell adhesion assays using K562 cells modified by transfection to express the integrin $\alpha 8\beta 1$ heterodimer. The integrin-AP chimera are also useful to identify and characterize novel integrin ligands. We show that integrin chimera can be used to map ligand distributions in situ and that potential ligands can be identified in far Western blots after biochemical fractionation. Such blots have the potential to reveal the repertoire of integrin ligands in different tissues. Combined with simple biochemical fractionation procedures, such blots can indicate whether potential ligands are associated with the ECM or cell membrane. As described in this article for FN, the identifies of candidate ligands in tissue extracts can be confirmed by immunodepletion of extracts before far Western blotting.

In our previous analysis of function of integrin $\alpha 8\beta 1$ during murine development, we have shown that this integrin is strongly expressed in the metanephric mesenchyme but not the ureteric bud or epithelial tubules within the developing kidney (Müller *et al.*, 1997). We further showed that it is critically important both for initial ingrowth of the ureteric bud and for subsequent formation of epithelial structures from the metanephric mesenchyme. We show here that histochemical staining of sections of embryonic mouse tissues with $\alpha 8^t\beta 1$ -AP reveals several interesting sites where ligands for integrin $\alpha 8\beta 1$ are concentrated, including the surface of the ureteric epithelium within the developing kidney and areas where condensing mesenchyme is forming bone. The distribution of ligands in areas of bone and kidney formation has striking similarities to the previously described distribution of OPN, and integrin-ligand interactions were inhibited completely by RGD peptides; however, we were not able to demonstrate directly that the staining pattern obtained with $\alpha 8^t\beta 1$ -AP was due to OPN. This would require an antibody that inhibits effectively interactions of integrins with the RGD site in murine OPN. We do not have such an antibody available. Consequently, it remains to be determined whether binding of this integrin chimera to sections of developing bone and kidney is mediated primarily by OPN or reflects the presence also of additional ligands. For example, in bone matrix, there are several proteins that contain

an RGD sequence such as type I collagen, thrombospondin, FN, bone sialoprotein, TN-C, VN, and OPN (Mackie and Tucker, 1992; Grzesik *et al.*, 1994). The detection of additional bands in far Western blots using this integrin suggests that there are additional, uncharacterized ligands.

In the present study we show that purified OPN binds integrin $\alpha 8\beta 1$ and that OPN protein is appropriately localized within the developing kidney to mediate essential functions of this integrin during kidney organogenesis. Rogers *et al.* (1997) have recently shown by in situ hybridization that the *OPN* gene is expressed within the developing ureter. We have shown that integrin $\alpha 8\beta 1$ is expressed in the mesenchyme surrounding the ureter and that ureter development is defective in $\alpha 8\beta 1$ -deficient mice (Müller *et al.*, 1997). Thus, OPN is expressed in cells that are defective in $\alpha 8\beta 1$ -deficient animals, suggesting that it may mediate $\alpha 8\beta 1$ functions on these cells during development. In agreement, Rogers *et al.* (1997) demonstrate that block of OPN activity with anti-OPN antibodies leads to disruption of kidney tubulogenesis in organ culture. RGD peptides that inhibit ligand-receptor interactions of several integrins, including interactions of integrin $\alpha 8\beta 1$ with OPN, lead to a similar block in kidney morphogenesis (Rogers *et al.*, 1997). This raises the possibility that the effects of RGD peptide in these organ cultures are consequences of blocking interactions of integrin $\alpha 8\beta 1$ with OPN. Surprisingly, mice carrying a targeted mutation in the *OPN* gene are viable, and a preliminary analysis suggests that kidney development progresses normally (Hogan, personal communication). This suggests that other integrin $\alpha 8\beta 1$ ligands, such as FN, TN-C, VN, or yet to be identified proteins, may substitute for the absence of OPN in *OPN* knock-out mice and may also help mediate essential functions of $\alpha 8\beta 1$ during normal kidney organogenesis.

OPN has been identified as a major protein constituent of bone- and cartilage-associated ECM, where it is expressed by osteoblasts, osteoclasts, and hypertrophic chondrocytes (Denhardt and Guo, 1993). Thus it may function in bone morphogenesis and remodeling. The importance in bone morphogenesis and remodeling of interactions of the integrin $\alpha 8\beta 1$ with OPN remains uncertain. Analysis of mice lacking integrin $\alpha 8\beta 1$ or OPN has failed to reveal any deficits in bone development (Müller, unpublished observations; Hogan, personal communication). There are several reports of $\beta 1$ integrins with unidentified α subunits expressed by developing bone or cultured osteoblasts (Clover *et al.*, 1992; Grano *et al.*, 1994). By immunoprecipitation and immunofluorescence, expression of $\alpha 8\beta 1$ has been detected in rat embryonic calvarial bone (Damsky, personal communication). Using paraffin sections, though, we have not seen prominent expression of this integrin at areas of primary bone

formation in E13.5 and older mouse embryos. On balance, these data suggest that interactions of $\alpha 8\beta 1$ with OPN will prove to modulate, but not be essential for, bone morphogenesis in some physiological circumstances.

In summary, findings in this article have identified a novel ligand for the integrin $\alpha 8\beta 1$ and suggest strongly that integrin-AP chimeras will be generally applicable for characterizing interactions of integrins with known ligands and for identifying additional receptor–ligand interactions with important physiological or developmental roles. It will be important in the future to establish whether OPN indeed mediates essential functions of integrin $\alpha 8\beta 1$ in the kidney, whether other known or unknown ligands are involved, and whether any of these ligands can substitute for lack of OPN in OPN-deficient animals.

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