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<td>佐藤, 祐哉</td>
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Recombinant Expression and Characterization of the Basement Membrane Protein Nephronectin
（基底膜蛋白質ネフロネクチンの機能解析）

A Doctoral Dissertation
Presented to Osaka University

2009

Yuya Sato
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Abbreviations

BSA: bovine serum albumin
DMEM: Dulbecco’s modified eagle’s medium
ECM: extracellular matrix
EGF: epidermal growth factor
FGF: fibroblast growth factor
GAG: glycosaminoglycan
GDNF: glial cell line-derived neurotrophic factor
kDa: kilo Dalton
mAb: monoclonal antibody
MAM: meprin-A5 protein-receptor protein tyrosine phosphatase μ
MAPK: mitogen-activated protein kinase
MIDAS: metal ion-dependent adhesion site
PBS: phosphate-buffered saline
PE: phosphatydilethanolamine
PI3K: phosphatidylinositol 3-kinase
PMSF: phenylmethylsulfonyl fluoride
RGD: arginine-glycine-aspartic acid
SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBS: tris-buffered saline
TGF: transforming growth factor
General introduction

*Multicellular organisms and Extracellular Matrix*

Multicellular animals are composed of numerous cells (about 60 trillions of cells in human) forming specialized tissues and organs. The life of an animal is maintained by complicated and exquisite functions of organs, which consist of a variety of tissues. In vertebrates, tissues can be grouped into four morphologically distinct components, *i.e.*, nerve, muscle, epithelial and connective tissues, among which the epithelial and connective tissues are two majorities of the organization (Fig. 1). Individual tissues are ensembles of various types of cells that together execute specific functions. However, cells are not the only components to form tissues. Extracellular matrices (ECMs) also constitute the tissues to fill in the spaces among cells and to sculpture the tissue architectures. The ECM is evolutionarily ancient and present in all phyla of metazoans (Reichardt, 1999). The ECM had been defined morphologically as extracellular material visible as fibrils or sheets in the electron microscope, but now it is defined as more broadly to include almost all secreted molecules that are immobilized outside cells (Reichardt, 1999).

The ECM is localized at the extracellular space of tissues, that is, basement membranes and the interstitial matrices. The basement membrane is a sheet-like structure constituted of ECM proteins, separating the epithelium from the connective tissues. Thus, the basement membrane is the only ECM directly associated with epithelial cells that play essential roles in individual organs (Fig. 1). The connective tissues are composed of
Fig. 1 A cross-sectional view of the wall of the intestine. This long tube-like organ (upper panel) is constructed from epithelial tissue (red), connective tissue (green), and muscle organ (yellow). Each tissue represents an organized assembly of cells held together by cell-cell adhesions, extracellular matrix or both (middle panel). Basement membrane is localized between epithelial and connective tissues (lower panel). (Middle and lower panels are cited from Alberts B. et al., Molecular Biology of the Cell, Fourth Edition, p.1066 and p.1090, respectively, 2002, Garland Science)
vast majority of ECM molecules with sparsely scattered cells, and involved in the structure and support of the organizations. Formerly, the ECM had been thought to function merely as materials filling the spaces between cells. However, as the investigations proceeded, the ECM has been shown to play pivotal roles in cell survival, proliferation, and migration, and to be involved in diverse biological processes including organogenesis, thus regarded as an essential factor to regulate behaviors of the cells (Reichardt, 1999). The functions of the ECMs are now summarized as follows; 1) providing the mechanical integrity, rigidity, and elasticity for tissues to endure the force or impact of the surroundings; 2) providing adhesive substrates for cells to transmit adhesion signals that are essential for normal cells to survive; and 3) sequestering soluble factors, such as growth factors and morphogens, to help regulate the spatial and temporal properties of signals conveyed by these factors.

**Vertebrate ECMs**

Vertebrates possess various tissues that differ in their structures, functions, rigidity, and so on. Therefore, the ECMs exhibit clear diversity to be optimized for each tissue. Thus, the matrix can become calcified to form the rock-hard structures of bones or teeth, constitute the transparent matrix of the cornea, or adopt the rope-like organization that gives tendons their enormous tensile strength. To constitute such diverse structures, multiple combinations of ECMs should be used as to which individual tissues necessitate for their conditions. The ECMs are mainly composed of two classes of molecules: (1) polysaccharide chains of the class called glycosaminoglycans (GAGs), which are usually
found covalently linked to protein in the form of proteoglycans, and (2) fibrous proteins, including collagen, fibronectin and laminin, which have both structural and adhesive functions.

**The GAGs and proteoglycans**

GAGs are unbranched polysaccharide chains composed of repeating disaccharides comprising a uronic acid and an amino sugar, which in most cases are sulfated (Fig. 2). Because of the presence of carboxylate groups in uronic acids and sulfate groups, GAGs are the most anionic molecules produced by animal cells. The differences in the kinds of the sugars, the types of linkage between sugars, and the number and location of sulfate groups give rise to four main groups of GAGs: (1) hyaluronic acid (also called as hyaluronan), (2) chondroitin sulfate, (3) heparan sulfate/heparin, and (4) keratan sulfate. Chondroitin sulfates are subdivided into five types; chondroitin sulfate-A, -B (commonly called as dermatan sulfate), -C, -D, and -E, by the differences in the uronic acid types (iduronic acid or glucuronic acid) or the number and location of the sulfate groups (Fig. 2 B–F).

Except for hyaluronic acid, all GAGs are found covalently attached to core protein. Such proteins with covalently attached GAGs are called proteoglycans. Proteoglycans are a set of ubiquitous proteins found on cell surface and in ECMs, and are defined by a common type of post-translational modification by GAGs (Lander, 1999). Some of the proteoglycans are summarized in Table 1.

Proteoglycans are thought to have a major role in chemical signaling between cells.
Fig. 2 The repeating disaccharide sequences of various GAGs. The chemical structures of the repeating disaccharide sequences of hyaluronic acid (A), chondroitin sulfate-A (B), dermatan sulfate (C), chondroitin sulfate-C (D), chondroitin sulfate-D (E), chondroitin sulfate-E (F), heparan sulfate (G), and keratan sulfate (H) are shown. Sulfate groups are represented by red letters. Note that the sulfate groups and uronic acids are absent in hyaluronic acid and keratan sulfate, respectively.

They bind to various growth factors and other ECM proteins through their GAG chains, and enhance their signaling activities. For example, the heparan sulfate chains of
proteoglycans bind to fibroblast growth factors (FGFs), which stimulate various types of cells to proliferate. The interaction of heparan sulfate chains with FGFs, as well as with FGF receptors, dimerizes the FGF receptors, thereby activating them on the cell surface (Harmer, 2006). Cell surface heparan sulfate proteoglycans, particularly syndecans, have been shown to serve as co-receptors for heparin-binding ECM proteins, including fibronectin, and help regulate focal adhesion formation and actin re-organization of cells (Couchman, 2003)

**Table 1. Some common proteoglycans** (This table is cited from Alberts B. et al., *Molecular Biology of the Cell, Fourth Edition*, p. 1097, 2002, Garland Science, with minor modifications.)

<table>
<thead>
<tr>
<th>Proteoglycan</th>
<th>Approximate molecular weight of core protein</th>
<th>Type of GAG chains</th>
<th>Number of GAG chains</th>
<th>Location</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggrecan</td>
<td>210,000</td>
<td>chondroitin sulfate + keratan sulfate</td>
<td>~130</td>
<td>cartilage</td>
<td>mechanical support; forms large aggregates with hyaluronic acid binds transforming growth factor-β (TGF-β)</td>
</tr>
<tr>
<td>Betaglycan</td>
<td>36,000</td>
<td>chondroitin sulfate</td>
<td>1</td>
<td>cell surface and matrix widespread in connective tissues</td>
<td>binds to type I collagen fibrils and TGF-β</td>
</tr>
<tr>
<td>Decorin</td>
<td>40,000</td>
<td>chondroitin sulfate</td>
<td>1</td>
<td>widespread in connective tissues</td>
<td></td>
</tr>
<tr>
<td>Perlecan</td>
<td>600,000</td>
<td>heparan sulfate</td>
<td>2~15</td>
<td>basal laminae</td>
<td>structural and filtering function in basal lamina cell adhesion; binds FGF and other growth factors</td>
</tr>
<tr>
<td>Syndecans</td>
<td>20,000</td>
<td>chondroitin sulfate + heparan sulfate</td>
<td>1~3</td>
<td>cell surface</td>
<td>formation of neuromuscular junctions</td>
</tr>
<tr>
<td>Agrin</td>
<td>230,000</td>
<td>heparan sulfate</td>
<td>3</td>
<td>basal laminae</td>
<td></td>
</tr>
</tbody>
</table>

*The ECM proteins in connective tissues*

The connective tissues contain abundant ECM protein with sparsely scattered cells, typically fibroblasts. Many of the ECM proteins in the connective tissues form fibers, such as collagen and elastic fibers, to provide elasticity and rigidity of the tissues.
Fig. 3 The structure of a typical collagen molecule. A, a model of a part of a single collagen chain (left) and a triple-helix-formed three chains (right). One chain is composed of a series of Gly-Xaa-Yaa residues, so that the Gly can occupy the interior of the triple helix. B, a schematic model of the domains of a fibrillar procollagen molecule. (These figures are cited from; A, Alberts B. et al., Molecular Biology of the Cell, Fourth Edition, p.1097, 2002, Garland Science, and B, Kreis T. et al., Guidebook to the Extracellular Matrix, Anchor, and Adhesion Proteins, Second Edition, p.384, 1999, Oxford University Press.)

Representatives of such ECM proteins are collagens.

The collagens are the most abundant proteins in our body (about 25% of total proteins) secreted mainly by fibroblasts to form collagen fibers, contributing to the structural integrity of the ECMs. Collagen has characteristic three-residue repeats, Gly-Xaa-Yaa, in its primary structure, which results in a stable triple-helical conformation with the glycine residues at the core of the helix (Fig. 3). The triple-helical conformation provides collagens with rigid, rod-like structures. To date, 20 types of collagen family proteins have been identified, which can be divided into seven groups of collagens including fibrillar, fibrillar associated, short chain, basement membrane, multiplexin and membrane-associated ones, by their structures and functions (Table 2).

Some ECM proteins in connective tissues harbor strong cell-adhesive activity to
<table>
<thead>
<tr>
<th>Groups</th>
<th>Type</th>
<th>Tissue distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fibrillar</strong></td>
<td>I</td>
<td>bone, skin, tendons, ligaments, cornea, internal organs (accounts for 90% of body collagen)</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>cartilage, vertebral disc, notochord, vitreous humor of the eye</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>skin, blood vessels, internal organs</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>as for type I</td>
</tr>
<tr>
<td></td>
<td>XI</td>
<td>as for type II</td>
</tr>
<tr>
<td><strong>Fibril-associated</strong></td>
<td>IX</td>
<td>cartilage</td>
</tr>
<tr>
<td></td>
<td>XII</td>
<td>tendons, ligaments, some other tissues</td>
</tr>
<tr>
<td></td>
<td>XVI</td>
<td>heart, kidney, intestine, ovary, testis, eye, arterial walls</td>
</tr>
<tr>
<td></td>
<td>XIX</td>
<td>brain, eye, testis (ubiquitously expressed in embryo but less in adult)</td>
</tr>
<tr>
<td></td>
<td>XX</td>
<td>cornea</td>
</tr>
<tr>
<td><strong>Short chain</strong></td>
<td>VIII</td>
<td>capillary blood vessels, dermis around hair follicles, perichondrium, connective tissue surrounding nerve bundles</td>
</tr>
<tr>
<td></td>
<td>X</td>
<td>bone</td>
</tr>
<tr>
<td><strong>Basement membrane</strong></td>
<td>IV</td>
<td>basal lamina</td>
</tr>
<tr>
<td><strong>Multiplexin</strong></td>
<td>XV</td>
<td>smooth muscles in blood vessels, beneath stratified squamous epithelia, kidney, testis, ovary, intestine</td>
</tr>
<tr>
<td></td>
<td>XVIII</td>
<td>basal lamina around blood vessels</td>
</tr>
<tr>
<td><strong>Membrane-associated</strong></td>
<td>XIII</td>
<td>eye, epidermis, hair follicles, endomysium</td>
</tr>
<tr>
<td></td>
<td>XVII</td>
<td>hemidesmosomes</td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td>VI</td>
<td>Tendon</td>
</tr>
<tr>
<td></td>
<td>VII</td>
<td>beneath stratified squamous epithelia</td>
</tr>
</tbody>
</table>

contribute to cell spreading and migration. Fibronectin is one of the representative ECM proteins that have strong cell-adhesive activities. Fibronectin is a high molecular-weight glycoprotein found in many extracellular matrices and in blood plasma of all vertebrates. Fibronectin is secreted as a dimer of two subunits held together by a pair of disulphide bonds near their C-termini (Fig. 4). Each subunit of fibronectin is made up largely of repeating modules of three types, 12 x type I, 2 x type II, and at least 15 x type III repeats (Fig. 4A), giving rise to many functional domains such as fibrin-binding, collagen-binding, heparin-binding, and cell-adhesive domains. Among these functional domains, the structure and function of cell-adhesive domain has been extensively...
investigated. The cell adhesive activity of fibronectin has been shown to be reproduced by tripeptide sequence, Arg-Gly-Asp (RGD), located on the 10th fibronectin type III repeat at the center of fibronectin molecule (Pierschbacher and Ruoslahti, 1984). The RGD motif is positioned at the top of the loop protruding from the molecular surface of fibronectin (Leahy et al., 1996; Copie et al., 1998; Fig. 4C). In addition to the RGD motif, residues located on the 9th type III repeat, so called synergy site, has been shown to enhance cell adhesive activity of fibronectin (see page 28).

Fig. 4 Structure of fibronectin. A, module structure of one fibronectin subunit composed of three types of repeat. At three positions (EDA, EDB, and IIICS) alternative splicing produces variants in structure. The two cell-binding sites are recognized by different integrin receptors, i.e., α5β1 or α4β1 integrin. B, electron micrographs of individual fibronectin dimers shadowed with platinum; red arrows mark the C-termini. C, The 3D structure of two type III repeats as determined by X-ray crystallography (PDB-ID: 1FNF). (B is cited from Alberts B. et al., Molecular Biology of the Cell, Fourth Edition, p.1097, 2002, Garland Science)
The ECM proteins in basement membranes

Basement membrane is a flexible, thin (40-120 nm thick) sheet-like structure of specialized ECMs that underlie all epithelial cell sheets and tubes (Fig. 5). They also surround individual muscle cells, fat cells, and Schwann cells. Thus, all parenchymal cells, which play major roles of individual tissues and organs, directly attach to the basement membranes. The basement membranes play roles not only in contributing to the integrity of tissues but also in transmitting signals to attached cells for their survival, proliferation, polarity, differentiation, and migration. In the kidney glomerulus, a basement membrane lies between epithelium and endothelium, and filters blood to

Fig. 5 The structure of basement membranes. A, three ways in which basement membranes are organized. Basement membranes (yellow) underlie epithelia, surround muscle cells, and are interposed between two cell sheets in the kidney glomerulus. B, scanning electron micrograph of the basement membrane in the cornea of a chick embryo. Some of the epithelial cells have been removed to expose the basement membrane. A network of collagen fibrils in the underlying connective tissue interacts with the lower face of the membrane. (B is cited from Alberts B. et al., Molecular Biology of the Cell, Fourth Edition, p.1106, 2002, Garland Science)
produce urine. Not only higher triploblastic animals, but also lower diploblastic animals, such as comb jellies (*Ctenophore*) and hydra (*Cnidaria*), possess basement membranes (Dewel, 2000), and therefore the basement membranes are thought to be prototypic ECMs.

Basement membranes are primarily composed of four types of proteins, namely laminins, type IV collagens, nidogens (also called as entactin) and perlecan, although their precise composition varies from tissue to tissue and even from region to region in a continuous basement membrane. Laminin is a family of heterotrimeric cruciform glycoprotein composed of α, β, and γ chains (Fig. 6). Combinations of five α, three β, and three γ chains give rise to at least 12 laminin isoforms, most of which are capable of forming network structures by self-association through their N-terminal globular domains. The requirement of laminins in embryogenesis was clearly shown by homozygous mutation of the laminin-γ1 chain, a component of most laminin heterotrimers. These mice die at peri-implantation stage due to failure of endoderm

**Fig. 6 The structure of laminin.**

differentiation, underscoring the critical importance of laminin in mice development (Smyth et al., 1999; Li et al., 2003). Type IV collagen has a more flexible structure than the fibrillar collagens. Their triple-stranded helix is interrupted in 26 regions, allowing the type IV collagen to make multiple bends. Type IV collagens also form individual network structures by self-association. Nidogens and perlecan interact with both laminin and type IV collagens to connect laminin- and type IV collagen-networks, thus forming high order meshwork structures of the basement membranes (Fig. 7). Since these four proteins exist in all mature basement membranes of our body, these proteins are fundamental and thought as constitutive basement membrane proteins (Timpl and Brown, 1996).

Not only the four basement membrane proteins, *i.e.*, laminin, type IV collagen,
nidogen, and perlecan, but also many other proteins contribute to form basement membranes \textit{in vivo} (Manabe et al., 2008). These proteins differ from the constitutive basement membrane proteins in their expression patterns. While the constitutive basement membrane proteins are expressed throughout the basement membranes in our body, expression and localization patterns of the other proteins are restricted in tissue-specific and developmentally regulated manners. Those spatiotemporally regulated proteins may determine specific functions of the basement membranes of individual tissues and organs. Many spatiotemporally regulated basement membrane proteins are abundantly expressed in developmental stages but decreased or diminished in adult tissues, implying critical roles of these basement membrane proteins in tissue morphogenesis. Some of the spatiotemporally regulated basement membrane proteins are summarized in Table 3.

### Table 3. Representatives of spatiotemporally regulated basement membrane proteins

<table>
<thead>
<tr>
<th>Name</th>
<th>Tissue distributions*</th>
<th>Phenotypes of knockout mice</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nephronecctin</td>
<td>kidney, lung, hair follicle, intestine, tooth germ, and so on kidney agenesis or hypoplasia</td>
<td>Brandenberger et al., 2001; Linton et al., 2007</td>
<td></td>
</tr>
<tr>
<td>MAEG</td>
<td>skin and hair follicle skin, hair follicle, kidney, and lung not reported</td>
<td>Osada et al., 2005</td>
<td></td>
</tr>
<tr>
<td>Fras1</td>
<td>skin, hair follicle, kidney, and lung Fraser syndrome (kidney agenesis, cryptophthalmos, syndactyly)</td>
<td>McGregor et al., 2003; Vrontou et al., 2003</td>
<td></td>
</tr>
<tr>
<td>QBRICK/Frem1</td>
<td>skin, hair follicle, kidney, and lung Fraser syndrome</td>
<td>Smyth et al., 2004; Kiyozumi et al., 2005; 2006</td>
<td></td>
</tr>
<tr>
<td>Frem2</td>
<td>skin, hair follicle, kidney, and lung Fraser syndrome</td>
<td>Jadeja et al., 2005; Timmer et al., 2005</td>
<td></td>
</tr>
</tbody>
</table>

* major distribution patterns in mice at embryonic day 16.5
While much progress has been made in the studies of the constitutive basement membrane proteins, the studies of spatiotemporally regulated basement membrane proteins are just beginning as those proteins have been identified over the last decade. Nephronectin is one of the novel spatiotemporally regulated proteins found in only vertebrates (Brandenberger et al., 2001; Morimura et al., 2001). Nephronectin consists of N-terminal five epidermal growth factor (EGF)-like repeats, a central linker segment containing an RGD cell adhesion motif, and a C-terminal MAM domain (Fig. 8A). The alternative splicing gives rise to long- and short-isoforms, although the functional differences between them are still unclear. The RGD motif in the central linker segment of nephronectin is critical to interact with the cell surface receptor integrin (Brandenberger et al., 2001). Nephronectin is unique in its distribution patterns in vivo. In mouse embryos, nephronectin is predominantly located at basement membranes in kidneys, lungs, and hair follicles, where it is implicated in epithelial-mesenchymal interaction (Fig. 8B-D), suggesting that nephronectin may work as a mediator for epithelial-mesenchymal interactions (Brandenberger et al., 2001). Mice with homozygous null mutation of the nephronectin gene exhibited the absence of the interaction between ureteric bud epithelium and metanephric mesenchyme, resulting in severe kidney agenesis or hypoplasia due to the failure of glial cell-line derived neurotrophic factor (GDNF) expression of metanephric mesenchyme (Fig. 8E-G; Linton et al., 2007). Moreover, recent findings suggest that nephronectin is implicated in cell proliferation and cancer progression. Expression of nephronectin has been shown to precede proliferation of tubular epithelium in the recovery phase of acute tubular
distribution patterns of nephronectin in embryonic day 16.5 mice. B, localization of nephronectin in kidney. Strong signals for nephronectin are found at the basement membranes, especially stalk (arrow) and developing glomeruli (arrowhead). C, nephronectin distributions in lung. Strong signals are found at bronchial basement membranes (arrowheads). D, nephronectin distributions in skin and hair follicle. Nephronectin localizes at basement membranes of epidermis (arrows) and the tip of hair follicle (arrowheads). An asterisk represents the hair follicle. E-G, urogenital phenotypes of wild type (E) and nephronectin−/− (F and G) mice. Mice with homozygous null mutation of nephronectin exhibited unilateral kidney agenesis and hypoplasia (F), or bilateral kidney agenesis (G). Asterisks represent the primordium of kidneys. Ki, kidney; Ad, adrenal gland; Ur, ureter; Ut, uterus; Bl, bladder; DA, dorsal aorta. (E-G are cited from Linton et al., 2007)
necrosis (Cheng et al., 2008). Loss of nephronectin expression has been shown to promote tumor progression in malignant melanoma, but to inhibit metastasis of breast cancer to lung, bone, and kidney (Eckhardt et al., 2005; Kuphal et al., 2008). Despite the physiological importance of nephronectin, especially in kidney morphogenesis, most of these findings are based on the data of gene expression or tissue distribution patterns of nephronectin, and therefore, little is known about the molecular characteristics of nephronectin since full-length nephronectin has not been purified yet.

To clarify physiological functions, basement membrane proteins should be isolated and biochemically characterized. However, the basement membrane proteins interact with other ECM proteins and/or self-associate to form huge ECM structures in vivo. Moreover, the ECM proteins are frequently cross-linked by tissue transglutaminase (Griffin et al., 2002), making it difficult to purify them. Engelbreth-Holm-Swarm (EHS) tumor was a superb source of basement membrane proteins, since the tumor produces an amorphous matrix composed of abundant basement membrane proteins including laminin, nidogen, type IV collagen, and perlecan (Kleinman et al., 1982), thus enabling them to be purified (Yurchenco and O'Rear, 1994). Conditioned media of certain cell lines was also good sources to purify several laminin isoforms (Kikkawa et al., 1998; Fujiwara et al., 2001). While the sources described above enable many of constitutive basement membrane proteins to be purified, most of spatiotemporally regulated basement membrane proteins have not been purified as intact proteins because the sources containing the spatiotemporally regulated basement membrane proteins are restricted. In the present study, to overcome this problem, I established recombinant expression
systems of the spatiotemporally regulated proteins (Chapter I). I expressed and purified recombinant full-length and domain-deleted mutants of nephronectin, and characterized functions of each domain of nephronectin by using these recombinant proteins.

The receptors for ECMs

To gain information from ECMs, receptor proteins are essential for cells to recognize the ECMs and to transmit signals into cells. Indeed, individual cells express a variety of ECM receptor proteins that are inherent to the cell types. A typical receptor protein is composed of three functional domains; an extracellular domain that interacts with ECM proteins, a transmembrane domain that are rich in hydrophobic amino acid residues to be anchored into lipid bilayer of plasma membranes, and an intracellular domain that interacts with various intracellular proteins such as adaptor proteins or kinases. The ECM receptors convert the information of ECMs to intracellular signals including phosphorylation of intracellular proteins and rearrangement of cytoskeletons, thereby

<table>
<thead>
<tr>
<th>Protein/family name</th>
<th>Number of isoforms*</th>
<th>Protein type</th>
<th>Ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>Integrin</td>
<td>24</td>
<td>heterodimeric glycoprotein</td>
<td>most of cell adhesive ECM proteins</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glycoprotein</td>
<td>laminin</td>
</tr>
<tr>
<td>Dystroglycan</td>
<td>1</td>
<td>heparan sulfate/chondroitin sulfate proteoglycan</td>
<td>fibronectin, laminin, collagen etc. (heparin-binding ECM proteins)</td>
</tr>
<tr>
<td>Syndecan</td>
<td>4</td>
<td>heparan sulfate/chondroitin sulfate proteoglycan</td>
<td>collagen (type II, V, and IV), and laminin</td>
</tr>
<tr>
<td>NG2</td>
<td>1</td>
<td>chondroitin sulfate proteoglycan</td>
<td></td>
</tr>
<tr>
<td>CD44/CSPG</td>
<td>1</td>
<td>Chondroitin sulfate proteoglycan</td>
<td>type IV collagen and fibrin/fibrinogen</td>
</tr>
</tbody>
</table>

* isoforms existing in mammals
regulating cell behaviors such as survival, proliferation, differentiation, and migration. Table 4 shows typical receptor proteins for ECMS. Among these ECM receptor proteins, an integrin family has critical roles in recognition of ECMS.

**The integrins : a family of receptor proteins for ECMS**

The integrins form a large family of receptor proteins for cell adhesion to ECMS and, in vertebrates, also plays important roles in cell-cell adhesion (Hynes, 2002). They interact with various ligands including ECM proteins and receptor proteins expressed on the cell surface via a divalent cation-dependent manner (Mould et al., 1995). By integrating the information of extracellular proteins and the intracellular signaling cascades, integrins play mandatory roles in embryonic development, the maintenance of tissue architectures, immune responses, hemostasis, and many human diseases including cancer (Hynes, 1992; 2002). Integrins are found in all multicellular animals, including fruit fly, nematode, and even sponge (Muller, 1997; Johnson et al., 2009), indicating that the family evolved relatively early in the history of metazoans.

Integrins are composed of two non-covalently associated subunits, termed α and β. Amino acid sequences and electron microscopic observations of purified integrin heterodimers revealed that integrins consist of a large extracellular domain with a globular head region and two long tails, each containing a transmembrane domain and a cytoplasmic domain (Nermut et al., 1988; Fig. 9A). In mammals, 18 α and 8 β subunits have been indentified, and the combinations of these subunits give rise to at least 24 distinct integrin heterodimers, among which 18 integrin isoforms serve as receptors for
ECM proteins (Fig. 9B). The ligand-binding specificities of integrins are mainly determined by their α subunits. The integrins containing α1, α2, α10, and α11 subunits bind to collagens, while those containing α3, α6, and α7 are receptors for laminins. α5, α8, αIIb, and αV-containing integrins recognize the tripeptide sequence consisting of Arg-Gly-Asp in the ECM ligands. α4 and α9-containing integrins have been shown to interact with the sequences comprised of aliphatic residues adjacent to acidic residues, such as Leu-Asp-Val or Ile-Asp-Gly (Komoriya et al., 1991; Schneider et al., 1998; Yokosaki et al., 1998). The binding specificities of individual integrins show that each

![Fig. 9 The combinations of the integrins. A, a schematic model of integrin heterodimer. Integrins are αβ heterodimers; each subunit crosses the membrane once, with most of each polypeptide in the extracellular space and two short cytoplasmic domains. TM, transmembrane domain. B, The mammalian subunits and their αβ associations; 8 β subunits can assort with 18 α subunits to form 24 distinct integrins. These can be considered in several subfamilies based on evolutionary relationship, ligand-binding specificity and, in the case of β2 and β7 integrins, restricted expression on leucocytes. All but leukocyte-specific integrins are receptors for ECM proteins. The collagen-, LDV/IDG-binding, and leuocoyte-specific integrins are restricted to chordates, as are subunits β2-β8.](image-url)
<table>
<thead>
<tr>
<th>Subunits</th>
<th>Lethality</th>
<th>Phenotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1</td>
<td>viable, fertile</td>
<td>No immediately obvious developmental defects, reduced tumor vascularization, reduced mammary gland branching</td>
</tr>
<tr>
<td>α2</td>
<td>viable, fertile</td>
<td>Few immediately obvious developmental defects, delayed platelet aggregation and reduced binding to monomeric collagen, reduced mammary gland branching</td>
</tr>
<tr>
<td>α3</td>
<td>perinatal lethal</td>
<td>Kidney tubule defects, reduced branching morphogenesis in lungs, mild skin blistering, lamination defects in neocortex</td>
</tr>
<tr>
<td>α4</td>
<td>E11/14 lethal</td>
<td>Defects in placenta (chorioallantoic fusion defect) and heart (epicardium, coronary vessels)</td>
</tr>
<tr>
<td>α5</td>
<td>E10-11 lethal</td>
<td>Defects in mesoderm (posterior somites) and vascular development, neural crest apoptosis</td>
</tr>
<tr>
<td>α6</td>
<td>perinatal lethal</td>
<td>Severe skin blistering, other epithelial tissues also defective. Lamination defects in cortex and retina.</td>
</tr>
<tr>
<td>α7</td>
<td>viable, fertile</td>
<td>Muscular dystrophy, defective myotendinous junctions</td>
</tr>
<tr>
<td>α8</td>
<td>perinatal lethal</td>
<td>Smaller or absent kidneys, inner hair cell defects</td>
</tr>
<tr>
<td>α9</td>
<td>viable</td>
<td>Die within 10 days of birth, chylothorax due to lymphatic duct defect</td>
</tr>
<tr>
<td>α10</td>
<td>viable, fertile</td>
<td>Growth retardation of the long bones due to moderate dysfunction of growth plate chondrocytes,</td>
</tr>
<tr>
<td>α11</td>
<td>viable, fertile</td>
<td>Dwarfism with increased mortality due to severely defective incisors</td>
</tr>
<tr>
<td>αV</td>
<td>E10/perinatal lethal</td>
<td>Two classes: embryonic lethality due to placental defects, perinatal lethality with cerebral vascular defects probably due to neuroepithelial defects, cleft palate.</td>
</tr>
<tr>
<td>αIIb</td>
<td>viable, fertile</td>
<td>Hemorrhage, no platelet aggregation</td>
</tr>
<tr>
<td>αL</td>
<td>viable, fertile</td>
<td>Impaired leukocyte recruitment</td>
</tr>
<tr>
<td>αM</td>
<td>viable, fertile</td>
<td>Defective phagocytosis and apoptosis of neutrophils, mast cell development defects, adipose accumulation</td>
</tr>
<tr>
<td>αX</td>
<td>viable, fertile</td>
<td>Decreased longevity and survival rate upon infection with pneumococci,</td>
</tr>
<tr>
<td>αD</td>
<td>viable, fertile</td>
<td>No immediately obvious developmental defects, reduced T cell response and phenotypic changes after induction by Staphylococcal enterotoxin</td>
</tr>
<tr>
<td>αE</td>
<td>viable, fertile</td>
<td>Greatly reduced numbers of intraepithelial lymphocytes</td>
</tr>
<tr>
<td>β1</td>
<td>E6.5 lethal</td>
<td>Peri-implantation lethality, inner cell mass deteriorates, embryos fail to gastrulate</td>
</tr>
<tr>
<td>β2</td>
<td>viable, fertile</td>
<td>Leukocytosis, impaired inflammatory responses, skin infections, T cell proliferation defects</td>
</tr>
<tr>
<td>β3</td>
<td>viable, fertile</td>
<td>Hemorrhage, no platelet aggregation, osteosclerosis, hypervascularisation of tumors</td>
</tr>
<tr>
<td>β4</td>
<td>Perinatal lethal</td>
<td>Severe skin blistering, other epithelial tissues also defective</td>
</tr>
<tr>
<td>β5</td>
<td>viable, fertile</td>
<td>No immediately obvious developmental defects</td>
</tr>
<tr>
<td>β6</td>
<td>viable, fertile</td>
<td>Inflammation in skin and airways, impaired lung fibrosis, all probably due to failure to activate TGF-β</td>
</tr>
<tr>
<td>β7</td>
<td>Viable</td>
<td>Deficits in gut-associated lymphocytes-no Peyer’s patches, reduced intraepithelial lymphocytes</td>
</tr>
<tr>
<td>β8</td>
<td>E10/perinatal lethal</td>
<td>Two classes: embryonic lethality due to placental defects, perinatal lethality with cerebral vascular defects probably due to neuroepithelial defects</td>
</tr>
</tbody>
</table>
integrin has a specific, non-redundant function. This is most clearly shown by the phenotypes of knockout mice (Table 5). To date, genes for all integrin α and β subunits have been knocked out, and each phenotype is distinct, reflecting the different roles among the various integrins.

The integrin α8 subunit was originally identified in chick nerves, and assembled exclusively with β1 subunit (Bossy et al., 1991). Based on the homology of αV and α5 subunits and the ligand-binding specificity, the α8β1 integrin is categorized into one of the RGD-recognizing integrins (Fig. 9B). The initial works on α8 integrin, based on tissue distribution pattern and in vitro data, suggested a role in neurite outgrowth (Bossy et al., 1991; Muller et al., 1995; Varnum-Finney et al., 1995). Mice deficient in α8 integrin expression, however, displayed unexpected phenotypes. The mice showed severe kidney agenesis or hypoplasia,

![Kidney phenotype in α8-deficient mice on the day of birth.](image)

Fig. 10 Kidney phenotype in α8-deficient mice on the day of birth. The urogenital tract was dissected from wild type (A) or α8-deficient (B-D) mice. Mice deficient in α8 integrin expression showed bilateral kidney agenesis (B) or unilateral kidney agenesis and hypoplasia (C). (D) High magnification view of a kidney rudiment (asterisk, boxed in C). ad, adrenal glands; k, kidneys; u, ureter; b, bladder, t, testis; da, dorsal aorta; uh, uterine horns. Scale bars, 100 µm. (These figures are cited from Müller et al., 1997)
possibly due to the defect of the epithelial-mesenchymal interaction between ureteric bud epithelium and metanephric mesenchyme (Muller et al., 1997; Fig. 10). Since the α8β1 integrin is expressed in metanephric mesenchyme and accumulates at the basement membrane underlying the ureteric bud epithelium (Fig. 11), it has been thought that the α8β1 integrin is necessary for the metanephric mesenchyme to receive signals from ureteric bud epithelium essential for kidney morphogenesis. To date, the α8β1 integrin has been shown to interact with various RGD-containing ECMs, including fibronectin, vitronectin, nephronectin, MAEG, and QBRICK/Frem1 (Muller et al., 1995; Schnapp et al., 1995; Brandenberger et al., 2001; Morimura et al., 2001; Kiyozumi et al., 2005; Osada et al., 2005). Among these ECM proteins, nephronectin is believed to be a physiological ligand for α8β1 integrin in the

**Fig. 11 Localization patterns of the integrin α8 subunit in the developing kidney.** Sections of E12.5 mice embryos were stained with antibodies against the extracellular domain of the integrin α8 subunit (red) and nephronectin (green). Nuclei were visualized by staining of Hoechst 33342 (blue). Arrowheads indicate the accumulation of mesenchymal integrin α8 subunit at the basement membranes of ureteric bud, where the basement membrane protein nephronectin deposits. Scale bar, 50 µm.
developing kidney, because the distribution patterns and the phenotypes of gene knockout mice of nephronectin are similar to those of the integrin α8 subunit
(Brandenberger et al., 2001; Linton et al., 2007; Figs. 8, 10, and 11). However, it is still unclear why other ligands for the α8β1 integrin, such as fibronectin, failed to compensate the loss of nephronectin expression.

The mechanisms of ligand recognition by integrins

The discovery that opened the door to the study of integrin biochemistry was the identification of the cell adhesive sequence from fibronectin. The adhesive motif was initially discovered by Pierschbacher and Ruoslahti (1984); the adhesive capacity of fibronectin was mimicked by a small synthetic peptide containing the RGD sequence. Subsequently, several other peptide ligands or residues critical for integrin interactions have been identified, as shown in Table 6. Thus, αIIbβ3 integrin binds to the C-terminal peptide of γ chain of fibrinogen, containing KQAGDV sequence, in addition to the RGD motif (Calvete et al., 1992; Springer et al., 2008). The collagen-binding integrin α2β1 has been shown to interact with the sequence comprised of GFOGER (where “O” represents hydroxyproline) (Knight et al., 1998). It is noteworthy that all of these peptide ligands have an acidic residue, i.e., aspartate or glutamate. In nearly all instances, this residue is essential for function, suggesting that the acidic residue of the ligand is a conserved binding residue throughout the integrin family (Smith, 1994). Although peptide ligands have not been identified in the laminin-binding integrins, several studies
demonstrated that the Glu residue at the third position from the C-termini of the laminin \( \gamma \) chains is critically important in the recognition of laminins by integrins (Ido et al., 2007; 2008).

### Table 6. Integrin recognition sequences

<table>
<thead>
<tr>
<th>Integrin</th>
<th>Representative adhesive ligand</th>
<th>Peptide sequence</th>
<th>Additional sequences required for affinity modulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha 2 \beta 1 )</td>
<td>Collagen</td>
<td>GFOGER</td>
<td></td>
</tr>
<tr>
<td>( \alpha 4 \beta 1 )</td>
<td>FN, VCAM-1</td>
<td>EILDV</td>
<td></td>
</tr>
<tr>
<td>( \alpha 9 \beta 1 )</td>
<td>Tenascin, VCAM-1</td>
<td>EIDG</td>
<td></td>
</tr>
<tr>
<td>( \alpha V \beta 1 )</td>
<td>FN, VN</td>
<td>RGD</td>
<td></td>
</tr>
<tr>
<td>( \alpha V \beta 3 )</td>
<td>FN, VN, Fbg, OP</td>
<td>RGD</td>
<td></td>
</tr>
<tr>
<td>( \alpha V \beta 5 )</td>
<td>FN, VN</td>
<td>RGD</td>
<td></td>
</tr>
<tr>
<td>( \alpha V \beta 6 )</td>
<td>FN, LAP-TGF( \beta )</td>
<td>RGD</td>
<td>LXXL/1 (immediately after the RGD motif)</td>
</tr>
<tr>
<td>( \alpha 5 \beta 1 )</td>
<td>FN</td>
<td>RGD</td>
<td>PHSRN and other basic residues</td>
</tr>
<tr>
<td>( \alpha I I b \beta 3 )</td>
<td>Fbg, FN, VN</td>
<td>RGD, KQAGDV</td>
<td></td>
</tr>
<tr>
<td>( \alpha 8 \beta 1 )</td>
<td>FN, VN, OP, nephronectin</td>
<td>RGD</td>
<td></td>
</tr>
<tr>
<td>( \alpha M \beta 2 )</td>
<td>Fbg, C3bi</td>
<td>QKRLDG( \beta )</td>
<td></td>
</tr>
<tr>
<td>( \alpha 4 \beta 7 )</td>
<td>FN, VCAM-1, MAdCAM</td>
<td>EILDV</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations used are; FN, fibronectin; VCAM, vascular cell adhesion molecule; VN, vitronectin; Fbg, fibrinogen; OP, osteopontin; LAP-TGF\( \beta \), latency-associated peptide of transforming growth factor \( \beta \); MAdCAM, mucosal addressin cell adhesion molecule. The critical acidic residues are shown in **bold**.

The elucidation of the mechanisms of ligand recognition by integrins is greatly progressed by determining the crystal structure of \( \alpha V \beta 3 \) (Xiong et al., 2001; 2002; Fig. 12A) and \( \alpha I I b \beta 3 \) integrins (Xiao et al., 2004; Springer et al., 2008). The structures of integrins complexed with cyclic RGD peptide and ligand-mimetic compounds revealed that the arginine side chain (or a basic moiety) of the RGD motif fits into a groove of the \( \beta \)-propeller domain of integrin \( \alpha \) subunits, whereas the carboxylate group of the aspartate
side chain coordinates the divalent cation at the metal ion-dependent adhesion site (designated MIDAS) (Fig. 12B and C). It is conceivable that the coordination of the carboxylate group to the divalent cation is essential for all integrins to interact with ligands, because all of the peptide ligands identified thus far have had at least one acidic residue (aspartate or glutamate; see Table 6) and, more importantly, all of the integrin-ligand interfaces analyzed thus far revealed that the divalent cation at the MIDAS serves as the central anchor point of ligands for integrins (Takagi, 2007). Despite that the acidic residues of ligands for all integrins are conserved and prerequisite, arginine residues are not conserved except for those for RGD-binding integrins. For example, α4β1- and α9β1-targeted peptide ligands and compounds bear an aliphatic moiety adjacent to the acidic residue (Mould et al., 1991; Schneider et al., 1998; Liu et al., 2006; Peng et al., 2006). These residues adjacent to the acidic moiety that coordinates the divalent cation at the MIDAS may determine the specificities of ligand recognition by integrins, as the arginine side chain of the RGD peptide forms a salt bridge to the β-propeller of α subunit, which predominantly determines the ligand-binding specificities.

Although the specificities of ligand recognition by integrins are primarily determined by the short amino acid motifs, some residues outside the motifs can define the binding specificities as well as affinities toward individual integrins (Ruoslahti, 1996; Takagi, 2004). Those affinity-modulating sequences are extensively investigated in RGD-binding integrins, as shown in Table 6. The α5β1 integrin is well known for its potency to bind specifically to fibronectin among the many RGD-containing ECM proteins. The binding
Fig. 12 Structure of the extracellular segment of αVβ3 integrin. A, a ribbon drawing of crystallized αVβ3 integrin. Integrin αV and β3 subunits are shown in blue and red, respectively. Integrin αV subunit is composed of an N-terminal β-propeller domain, a thigh domain, and two calf domains, whereas β3 subunit consists of a βA domain, a hybrid domain, a PSI (plexins, semaphorins, and integrins) domain, four EGF-like repeats, and a β terminal domain (βTD). B, surface representation of the ligand-binding site of αVβ3 integrin, with the cyclic RGD-containing peptide shown as ball-and-stick model. The peptide ligand is bound at the β-propeller-βA domain interface. The Mn$^{2+}$ ions at MIDAS and adjacent to MIDAS (ADMIDAS) are shown as a cyan and a violet spheres, respectively. The carbon, nitrogen, and oxygen atoms of cyclic RGD peptide are shown in yellow, blue, and red, respectively. C, interactions between ligand and integrin. The peptide and residues interacting with the ligand or with Mn$^{2+}$ ions are shown in ball-and-stick representation. Hydrogen bonds and salt bridges are represented with dotted lines. (A is cited from Xiong, et al., 2001; B and C are cited from Xiong, et al., 2002.)
specificity of $\alpha_5\beta_1$ integrin is determined not only by the RGD motif in the 10th type III repeat, but also by the PHSRN sequence within the preceding 9th type III repeat, the latter serving as a so-called “synergistic binding site” (Aota et al., 1994; Redick et al., 2000; see Fig. 4). The $\alpha_V\beta_6$ integrin has also been shown to require both the RGD motif and the LXXL/I sequence for its high-affinity binding to its ligands, e.g., latency-associated peptide of TGF\(\beta\) and coat protein of foot-and-mouth disease virus (DiCara et al., 2007). In addition to the RGD-binding integrins, several laminin-binding integrins has been shown to recognize the C-terminal 20 amino acid residues of laminin $\beta_2$ chain, thereby enhancing its binding affinities to laminins containing $\beta_2$ chain (Taniguchi et al., 2009). The mechanisms by which these additional residues increase the binding affinities to individual integrins are still poorly understood. However, it is conceivable that these residues serve as auxiliary binding sites to integrins or exert a fine-tuning effect on the active conformation of the short amino acid motifs, such as the RGD motif, primarily required for the interactions.

The binding specificities of individual integrins provide insights into not only the physiological ligands for but also the physiological functions of each integrin. As shown above, however, the information about amino acid sequences of each integrin provides, if any, the types of the integrin (i.e., RGD- or laminin-type) but is incapable of determining the physiological ligands, since the additional residues in the ligands, such as the synergy site of fibronectin, facilitate the interaction of individual integrins with specific ligands. Therefore, it is necessary that the ligand-binding specificities of individual integrins should be tested one by one, with appropriate systems to assess. About a decade ago, the
binding specificities of integrins were mainly determined by inhibition assay of cell-adhesion activities to ligands with function-blocking antibodies against individual integrins (Kikkawa et al., 2000; Fujiwara et al., 2001). These cell-based assays are hard to analyze the affinities of integrin-ligand interactions, because individual cells express a series of integrins that bind to the same ligand with a different affinity. The binding assay with purified recombinant integrin is suitable to overcome these problems. For example, purified recombinant α5β1 integrin enables us to analyze the kinetics of the real-time interaction of integrin α5β1 with fibronectin (Takagi et al., 2003). A series of purified laminin-binding integrins provide the comprehensive survey of binding affinities as well as specificities of individual integrins to a series of laminin isoforms (Nishiuchi et al., 2006). These studies strongly demonstrate that recombinant integrins are powerful tools to determine ligand-binding specificities of the integrins.

Based on the backgrounds described above, I tried to clarify the following questions:

1. What is the role of individual domains of nephronectin? Are there any proteins/molecules bound to individual domains of nephronectin?
2. Does α8β1 integrin, one of the known receptors for nephronectin, selectively interact with nephronectin? If so, what determines the binding selectivity of nephronectin to α8β1 integrin?

To solve the first question, I expressed and purified recombinant nephronectin and its deletion mutants, then examined their binding activities toward a panel of GAG chains
and α8β1 integrin. In this study, I found that nephronectin strongly binds to heparin/heparan sulfate and chondroitin sulfate-E. Domain deletion mutants revealed that nephronectin mainly interact with heparin/heparan sulfate and chondroitin sulfate-E through the MAM domain and the EGF-like repeats, respectively. Although the central linker segment of nephronectin has no binding activity to GAG chains I tested, the linker segment harbors potent activity to interact with recombinant α8β1 integrin. These results indicated, for the first time, that nephronectin interacts with heparan and chondroitin sulfate proteoglycans through its MAM domain and EGF-like repeats, respectively, while the linker segment serves as a recognition site for α8β1 integrin. It is also the first case that distinct domains of one protein independently interact with GAGs, one to heparin/heparan sulfate and the other to chondroitin sulfate-E. These results are shown in Chapter I.

To solve the second question, I examined the binding activities of recombinant α8β1 integrin to a panel of RGD-containing ECM proteins and deletion mutants of nephronectin as well as full-length nephronectin. While the central linker segment as well as full-length nephronectin possesses potent activities to bind to α8β1 integrin, other RGD-containing ECM proteins, known to interact with α8β1, exhibited only marginal activities. A variety of deletion mutants of the linker segment of nephronectin revealed that the linker segment harbors two motifs required for potent interaction with α8β1 integrin, one containing the RGD motif and the other serving as an auxiliary binding site. The details are shown in Chapter II.
Chapter I

Characterization of Domain Functions of Nephronectin
I-1 Summary

Nephronectin is a novel basement membrane protein mainly expressed in embryonic kidney, and is shown to be involved in kidney development. The known receptor for nephronectin is α8β1 integrin, which is also implicated in the epithelial-mesenchymal interaction in the developing kidney. Despite the importance of nephronectin as a regulator of kidney morphogenesis along with α8β1 integrin, little is known about molecular properties of nephronectin. In the present study, I produced and purified a recombinant nephronectin and its deletion mutants using a mammalian expression system to elucidate the domain functions of nephronectin, with special reference to their binding abilities towards a panel of GAGs. Among the GAGs tested, nephronectin strongly bound to heparin and chondroitin sulfate-E and moderately to heparan sulfate. Using the deletion mutants of nephronectin, I found that nephronectin mainly bound to heparin/heparan sulfate through the MAM domain, whereas to chondroitin sulfate-E through the EGF-like repeats. Although the central linker segment exhibited no GAG-binding activity, it was capable of strong binding to α8β1 integrin with an activity comparable to that of full-length nephronectin. These results suggest that N-terminal five EGF-like repeats and C-terminal MAM domain interact with chondroitin sulfate and heparan sulfate proteoglycans, respectively, while the central linker segment is responsible for potent interaction of nephronectin with α8β1 integrin.
I-2 Introduction

The interactions of cells with ECM proteins are critically important in regulation of cell behaviors such as survival, proliferation, migration, and differentiation (Roskelley et al., 1995). These interactions occur through the cell surface receptors such as integrins and membrane-bound proteoglycans, thereby transmitting signals into cells. In epithelial cells, the ECM proteins directly attached to cells are located in the basement membrane. The basement membrane is composed mainly of laminins, type IV collagens, nidogens, and perlecan (Timpl and Brown, 1996). In addition to these proteins, basement membranes contain proteins expressed in developmental- and tissue-specific manners. These restricted proteins are thought to be involved in tissue- and organ-specific morphogenesis.

Nephronectin is one of the tissue-specific and developmentally restricted basement membrane proteins. It is composed of N-terminal five EGF-like repeats, a central linker segment containing RGD cell adhesive motif, and a C-terminal MAM domain (Brandenberger et al., 2001; Morimura et al., 2001). In developing kidney, nephronectin is selectively expressed in ureteric bud epithelial cells and located in the basement membranes under these cells (Brandenberger et al., 2001). The known receptor for nephronectin is integrin α8β1, which is expressed in metanephric mesenchymes in developing kidney and has been shown to play a crucial role in epithelial-mesenchymal interaction during early steps of kidney morphogenesis (Muller et al., 1997). Nephronectin interacts with α8β1 integrin through the RGD motif residing in the central
linker segment of nephronectin. Mice deficient in nephronectin or α8 integrin expression were associated with a transient reduction in GDNF expression in the metanephric mesenchyme (Linton et al., 2007), exhibiting severe kidney agenesis or hypoplasia. The phenotypes of the mice deficient in nephronectin or α8 integrin expression lead to the hypothesis that the interaction between α8β1 integrin and nephronectin is critical for the expression of GDNF. Despite the importance of nephronectin as a regulator of kidney morphogenesis along with integrin α8β1, little is known about molecular properties of nephronectin. The functions of domains other than the RGD-containing linker segment in nephronectin, *i.e.*, N-terminal five EGF-like repeats and C-terminal MAM domain, remain unexplored.

Proteoglycans, consisting of a protein core coupled to variably sulfated GAG chains, function as mediators of interactions between growth factors and their receptors through association of the GAG chain (Perrimon and Bernfield, 2000). The GAG chains of proteoglycans also interact with extracellular matrices as well as growth factors and their receptors, playing mandatory roles in storing and concentrating growth factors to regulate activation of growth factor receptors efficiently (Lander, 1999). In kidney morphogenesis, proteoglycans, especially heparan sulfate proteoglycans, are thought to play mandatory roles in generating a concentration gradient of growth factors and morphogens, such as FGFs (Allen et al., 2001; Clayton et al., 2001; Qiao et al., 2001), members of the TGF-β superfamily (Santos and Nigam, 1993; Sakurai and Nigam, 1997), GDNF (Barnett et al., 2002), pleiotrophin (Sakurai et al., 2001), BMPs (Grisaru et al., 2001; Bush et al., 2004), Wnts (Kispert et al., 1996), and hedgehog (The et al., 1999),
thereby modulating their biological activities. A role of heparan sulfate proteoglycans in kidney development has also been investigated with mice homogeneously mutated in heparan sulfate 2-sulfotransferase gene that encodes an enzyme responsible for sulfation of uronic acid residues of heparan sulfate; these mutated mice show severe kidney agenesis (Bullock et al., 1998). Moreover, inhibition of sulfated GAG syntheses also results in impaired ureteric bud branching in *ex vivo* cultures of kidney primordia (Steer et al., 2004).

In the present study, I produced and purified recombinant mouse full-length nephronectin and its deletion mutants to examine its binding to GAG chains. Nephronectin binds to highly sulfated GAGs including heparin, heparan sulfate, and chondroitin sulfate-E. This heparin/heparan sulfate-binding activity is mainly due to in the MAM domain, while EGF-like repeats of nephronectin bind to chondroitin sulfate-E. In addition, I found that the central linker segment of nephronectin strongly interacts with α8β1 integrin, raising a possibility that α8β1 integrin and cell surface heparan and/or chondroitin sulfate proteoglycans cooperatively transmit signals responsible for organogenesis. These results suggest that nephronectin interacts with heparan sulfate and chondroitin sulfate proteoglycans through its MAM domain and EGF-like repeats, respectively.
I-3 Materials and Methods

Materials and reagents—Human plasma fibronectin and vitronectin were purified from outdated human plasma by gelatin- and heparin-affinity chromatography, respectively, as described previously (Sekiguchi et al., 1983; Yatohgo et al., 1988). Human fibrinogen was purchased from Enzyme Research Laboratories Inc. (South Bend, IN). Antibodies against FLAG and 5xHis tags were obtained from Sigma (Saint Louis, MO) and QIAGEN (Hilden, Germany), respectively. HRP-conjugated donkey and goat anti-mouse IgG antibodies were obtained from Jackson ImmunoResearch laboratories Inc. (West Grove, PA) and American Qualex International Inc. (San Clemente, CA), respectively. Heparin-Sepharose beads were purchased from Amersham (Piscataway, NJ). Phosphatidylethanolamine-conjugated GAGs (PE-GAGs) were prepared as described previously (Sugiura et al., 1993). The mAb against human integrin \( \alpha 8 \) subunit was produced by fusion of Sp2 mouse myeloma cells with spleen cells from mice immunized with recombinant soluble integrin \( \alpha 8\beta 1 \), and was selected by positive reactivity with recombinant integrin \( \alpha 8\beta 1 \) and negative reactivity with recombinant integrin \( \alpha 5\beta 1 \). The mAb 10A8 was capable of binding to integrin \( \alpha 8 \) subunit on immunoblots under both reducing and non-reducing conditions.

Expression vectors—Mouse nephronectin cDNA was obtained from Dr. Yoshihide Hayashizaki (Riken, Japan). The cDNA was subcloned into pFLAG-CMV vector (Invitrogen) in frame to FLAG tag at the 3’ end. A cDNA fragment encoding nephronectin deleted for the MAM domain (NN-\( \Delta \)MAM) was amplified by PCR with a
pair of primers 5'-GAATTGAGATCCCGGGACGC-3' (forward) and 5'-GTCGACGTCGTCATTCATTCCTC-3' (reverse) using the full-length nephronectin cDNA as a template. To express other deletion mutants, a cDNA fragment encoding a mouse nephronectin 5'-NTR and signal peptide (from –61 to 69 base pair) was amplified by PCR with a pair of primers 5'-GAATTGAGATCCCGGGACGC-3' (forward) and 5'-GGGCCCGGTACAGTCGTCCTTTACTTCCTC-3' (reverse) using full-length nephronectin cDNA as a template. This fragment was fused in frame to the cDNAs encoding the EGF-like repeat-deleted mutant of nephronectin (abbreviated ΔEGF), the RGD-containing linker region (linker), or the MAM domain. The cDNAs encoding the RGD region and the MAM domain were amplified by PCR with pairs of primers 5'-GGGCCCAAGTCATGATTGAAC-3' (forward, named primer A) and 5'-GTCGACGTCGTCCTTTACTTCCTC-3' (reverse), and 5'-GGGCCCGGTATTCTCATACACAGC-3' (forward) and 5'-GTCGACGTCGACCTCTTTTCAAG-3' (reverse, named primer B), respectively. The cDNA encoding ΔEGF was amplified by PCR with a pair of the primer A (forward) and the primer B (reverse) using full-length nephronectin cDNA as a template. After verified by DNA sequencing, each PCR-amplified cDNA was subcloned to pFLAG-CMV vector in frame to FLAG-tag at 3' ends.

An expression vector for GST fusion protein of the RGD-containing linker segment was prepared as follows: A cDNA encoding NN-linker was amplified by PCR with a pair of primers 5'-GAATTCCCCAAAAGTCATGATTGAACCT-3' (forward) and 5'-GTCGACTCATGTCGTCCTTTTACTTCCTC-3' (reverse) using E13.5 mouse embryo
cDNA as a template. The PCR product was subcloned into pBlueScriptII SK+ vector (Stratagene, La Jolla, CA), digested with EcoRI and SalI and inserted into the corresponding restriction sites of the pGEX 4T-1 expression vector (GE Healthcare). An expression vector for PRGDV sequence directly conjugated to GST was prepared as follows. A pair of primers 5’-TCCCCAGAATTCCGGAAGTACAAGCGCCCGCCCCAGAGGAGATG-3’ (forward) and 5’- GCGTGTACGACTCAAGTCGCGCTCACATCTCCTCTGGGGGC-3’ (reverse) was directly hybridized and amplified by PCR. The PCR product was digested with EcoRI and SalI, then inserted into corresponding restriction sites of pGEX4T-1 vector.

Expression vector for EGF-like repeats of nephronectin with the N-terminal 70 kDa domain of human fibronectin (FN70K), designated FN70K-EGF, was prepared as follows: The expression vector pFLAG-FN70K was prepared as described previously (Ido et al., 2004). A cDNA encoding the five EGF-like repeats of nephronectin was amplified by PCR with a pair of primers 5’-TGAGATCTGACTTCGACGGGAGGTGGC-3’ (forward) and 5’-ATCGATATACACACAGTTCAGTCCATCCCC-3’ (reverse) using the full-length nephronectin cDNA as a template. After verified by DNA sequencing, the PCR-amplified cDNA was subcloned between FN70K and FLAG tag in pFLAG-FN70K vector in frame to FN70K at 5’ ends and FLAG-tag at 3’ ends.

A cDNA encoding human integrin α8 was amplified by PCR with a pair of primers 5’-AAGGAAGCTTCCACCATGTGCAGCCGGAGGAGGCAAGC-3’ (forward) and 5’-AATCACGACTGCCTCAGGGGTCTTTGTCATTGG-3’ (reverse) using human fetus cDNA (Clontech) as a template. The PCR-amplified cDNA was subcloned to
pBlueScript II SK vector and verified by DNA sequencing. An α8 integrin subunit expression vector that was truncated before the transmembrane domain and fused to the ‘ACID’ α-helical coiled-coil peptide with a FLAG-tag was prepared as described previously (Nishiuchi et al., 2006; Ido et al., 2007). Expression vector for a truncated soluble β1 integrin subunit with a “BASE” peptide was generously provided by Dr. Junichi Takagi (Institute for Protein Research, Osaka University) (Takagi et al., 2001).

Expression and purification of recombinant proteins—To purify recombinant nephronectin, its mutants and soluble α8β1 integrin, Freestyle™ 293-F cells were transiently transfected with each plasmid using Freestyle™ 293 Expression system (Invitrogen) according to manufacturer’s instructions. The conditioned media were collected 48-72 h after transfection, and centrifuged to remove cells and debris. To purify nephronectin and its mutants, EDTA (5 mM), PMSF (1 mM), and sodium azide (0.02%) were added to the conditioned media. The media were incubated with anti-FLAG M2 affinity beads (SIGMA), and then the beads were transferred into an empty column and washed with PBS. Bound proteins were eluted with 100 µg/ml FLAG peptide dissolved in PBS. Purified proteins were dialysed against 2 mM CAPS (N-cyclohexyl-3-aminopropanesulfonic acid) buffer (pH 11.4) containing 0.5 mM EDTA and used for the following assays. Determination of protein concentration was carried out by immunoblotting with anti-FLAG antibody.

To purify recombinant soluble α8β1 integrin, PMSF (1 mM) and sodium azide (0.02%) were added into the conditioned media. The media were incubated with nickel-nitrilotriacetic acid-agarose beads (QIAGEN), followed by washes with
Tris-buffered saline (TBS). Bound proteins were eluted with TBS containing 200 mM imidazole. The eluted fractions were applied to anti-FLAG M2 affinity beads, and the bound proteins were eluted with 100 µg/ml FLAG peptide and dialyzed against TBS.

**Heparin binding assay**—Three pmol of nephronectin and its mutants were diluted with 1 ml of phosphate buffer (pH 7.4) containing 1 mg/ml bovine serum albumin (BSA), 0.1% TritonX-100 and the indicated concentrations of NaCl (binding buffer), and then incubated with 15 µl of Heparin-Sepharose beads (50% slurry in PBS) for 12 h at 4 °C. After centrifugation, the beads were washed three times with 1 ml of binding buffer without BSA. Bound proteins were eluted from the beads by being boiled in 30 µl of SDS-gel loading buffer containing 6% of β-mercaptoethanol for 5 min. The eluted proteins were subjected to SDS-PAGE and visualized by immunoblotting with anti-FLAG M2 antibody. As a negative control, the same procedures were carried out with Sepharose beads.

**GAG binding assay**—The GAG binding assay was performed as described previously (Kozaki et al., 2003; Furutani et al., 2005). Briefly, the Maxisorp 96-well plate was coated with 20 µg/ml of PE-GAGs. After blocking with PBS containing 1% skim milk, plate was incubated with 20 nM of nephronectin and its mutants for 1 h at room temperature. The amounts of nephronectin and its mutants bound to the wells not coated with PE-GAGs were taken as negative controls, and subtracted as backgrounds. Bound proteins were quantified by enzyme-linked immunosorbent assay using anti-FLAG M2 mAb and HRP-conjugated goat anti-mouse IgG.

**Integrin binding assay**—Integrin binding assays were performed as described
previously (Nishiuchi et al., 2006). Briefly, microtiter plates were coated with the indicated substrates at 10 nM, blocked with 1% BSA, and incubated with integrins in the presence of 1 mM MnCl₂. The amounts of the integrins bound in the presence of 10 mM EDTA were taken as negative controls, and subtracted as backgrounds. The plates were washed with TBS containing 1 mM MnCl₂, 0.1% BSA, and 0.02% Tween-20 with or without 10 mM EDTA, and then the bound integrins were quantified by an enzyme-linked immunosorbent assay using the biotinylated rabbit anti-Velcro (ACID/BASE coiled-coil) antibody and HRP-conjugated streptavidin.
Results

Purification of Nephronectin and its Deletion Mutants—Nephronectin consists of five EGF-like repeats, a linker segment containing the RGD cell-adhesive motif, and a MAM domain (Fig. I-1A; Brandenberger et al., 2001; Morimura et al., 2001). To elucidate the functions of these regions of nephronectin, I constructed expression vectors for full-length nephronectin, the N-terminal region containing five-EGF-like repeats (abbreviated NN-EGF), the RGD-linker segment (NN-linker), the C-terminal MAM domain (NN-MAM), and deletion mutants lacking the N-terminal five-EGF-like repeats (NN-ΔEGF) or the C-terminal MAM domain (NN-ΔMAM). These constructs were fused with a FLAG-tag at their C termini to facilitate affinity purification of these recombinant proteins (Fig. I-1A, NN-EGF not shown). When these vectors were transfected into 293-T cells (human embryonic kidney cells stably expressing the large T-antigen of SV40), all recombinant proteins except for NN-EGF were expressed and well secreted into the conditioned media. NN-EGF was expressed in the 293-T cells, but not secreted (data not shown). Although the predicted molecular masses of full-length nephronectin, NN-ΔMAM, NN-ΔEGF, NN-RGD, and NN-MAM are 62 kD, 47 kD, 37 kD, 15 kD, and 18 kD, respectively, molecular masses of the secreted proteins were larger on SDS-PAGE under reducing conditions than their predicted sizes (Fig. I-1B). Because the central linker segment has a feature of mucin-like region, which contains a lot of serines, threonines, and prolines, thus the segment is predicted to be highly O-glycosylated on serine and threonine residues (Brandenberger et al., 2001), resulting in the higher
molecular masses of the recombinant proteins expressed here.

**Fig. 1-1.** Purification of recombinant nephronectin and its deletion mutants. *A*, schematic diagrams of full-length nephronectin (NN) and its deletion mutants. *Closed* and *open* arrowheads indicate the position of *N*-glycosilation sites and the RGD-cell adhesion motifs, respectively. *Black squares*, signal peptides; *gray pentagons*, EGF-like repeats; *open squares*, MAM domains; *hatched hexagons*, FLAG tags. *B*, purified full-length nephronectin and its deletion mutant were subjected to SDS-PAGE on 7-15% gradient gels under reducing conditions, followed by CBB staining (*left panel*) and immunoblotting with anti-FLAG mAb (*right panel*). The positions of molecular size markers are shown on the left of each panel.

*Nephronectin Binds to Heparin through the C-terminal MAM Domain*—It is well known that a lot of ECM proteins possess activities to bind to heparin/heparan sulfate, and thus these heparin-binding ECMs are thought to interact with heparan sulfate proteoglycans *in vivo*. The interaction of the ECMs with cell-surface heparan sulfate proteoglycans regulates actin re-organization signals together with integrins (Couchman, 2003). Furthermore, the ECMs also bind to basement membrane-type heparan sulfate proteoglycans to regulate spatial distributions of the ECMs (Bezakova and Ruegg, 2003; Iozzo, 2005). To examine the activity of nephronectin to bind to heparin, a kind of heparan sulfate, I adopted pull-down assays of nephronectin using heparin-Sepharose
beads. Nephronectin was precipitated with the heparin-Sepharose beads under physiological ionic strength conditions (i.e., 0.135 M NaCl) but not with control Sepharose beads (Fig. I-2), indicating that nephronectin possesses an activity to interact with heparin in vivo. Since heparin has been shown to interact with proteins through electrostatic interaction (Capila and Linhardt, 2002), the interaction of heparin with nephronectin was examined in higher ionic strength conditions (0.3 – 0.6 M NaCl). Although nephronectin binds to heparin-Sepharose beads even in the presence of 0.3 M NaCl, the interactions was not observed in the presence of 0.6 M NaCl, ensuring that nephronectin binds to heparin through electrostatic interactions. The interaction was also abolished in the presence of 2 mg/ml heparin, confirming the specificity of interaction between nephronectin and heparin.

To identify the region responsible for the heparin-binding activity of nephronectin, the deletion mutants of nephronectin depicted in Fig. I-1A were employed, and their heparin-binding activities were assessed (Fig. I-2). Both NN-ΔEGF, a mutant deleted of five EGF-like repeats, and NN-MAM, a mutant comprising only the C-terminal MAM domain, were capable of binding to heparin. Their binding manners were similar to that of full-length nephronectin; the interactions were disrupted in the presence of 0.6 M NaCl or 2 mg/ml of heparin. In contrast, both NN-ΔMAM, a mutant deleted of C-terminal MAM domain, and NN-linker, a mutant consisting only a central linker segment, were not precipitated with heparin-Sepharose even at a physiological NaCl concentration. Thus, these results indicate that the MAM domain harbors the activity to bind to heparin.
The Binding Activities of Nephronecit in Sulfated GAG Chains—Since heparin is a type of the sulfated GAG chains, next I tried to examine the binding activities of nephronecit to a series of sulfated GAG chains including heparin. To assess the binding activities of nephronecit to GAG chains, I used phosphatidylethanolamine-conjugated GAG chains (PE-GAGs), since lipid moieties facilitate absorption of GAGs onto hydrophobic plastic surfaces so that the binding activities of nephronecit to GAGs can be examined by solid phase assays. Nephronecit was highly active in binding to heparin and chondroitin sulfate-E and moderately active in binding to heparan sulfate (Fig. I-3A), but only marginal, if any, to other GAGs including chondroitin sulfate-A, -C, -D, dermatan sulfate, and hyauronic acid. The binding of nephronecit to heparin, heparan sulfate and chondroitin sulfate-E was observed under physiological ionic conditions, endorsing its physiological relevance.
To further define the binding domains of nephronectin to GAGs, the deletion mutants of nephronectin were also assessed for their activities to bind to PE-GAGs (Fig. I-3A). Both NN-ΔEGF and NN-MAM bind strongly to heparin and moderately to heparan sulfate. NN-linker had no activities to bind to heparin and heparan sulfate, ensuring the prerequisite role of the MAM domain in heparin-binding activity. However, NN-ΔMAM also showed moderate binding activity to heparin, even though the binding activities of NN-ΔMAM were significantly reduced in comparison with that of full-length nephronectin. Since NN-linker showed no activity to bind to heparin, this result suggests that five EGF-like repeats has potentials to bind to heparin. The apparent discrepancy in heparin-binding activity of the EGF-like repeats between pull-down assays and solid phase binding assays may be due to differences in the sensitivities between these assays. In contrast to heparin-binding activities, NN-ΔMAM showed potent activity to bind to chondroitin sulfate-E, and this binding activity was comparable to that of full-length nephronectin. NN-ΔEGF and NN-MAM, on the other hand, exhibited a moderate loss of binding activity to chondroitin sulfate-E. Since NN-linker exhibited no binding activity toward chondroitin sulfate-E, these results suggest that the EGF-like repeats primarily interact with chondroitin sulfate-E, although the MAM domain may have moderate activity to bind to chondroitin sulfate-E chain.

To directly clarify the roles of the EGF-like repeats in chondroitin sulfate-E interaction, I tried to purify the EGF-like repeats. As mentioned above, the N-terminal five EGF-like repeats of nephronectin was hard to purify since it was not secreted from 293-T cells. To overcome this difficulty, the EGF-like repeats were coupled to the N-terminal 70 kDa
Fig. 1-3. The binding activities of full-length nephronectin and its deletion mutants toward PE-conjugated GAG chains.
A, Ninety-six well plates were coated with 20 µg/ml of PE-GAGs and assessed for their activities to bind to 20 nM of full-length nephronectin and its deletion mutants, as described in “Experimental Procedures”. The results were expressed as the means ± S.E. of triplicate determinations. CS, chondroitin sulfate; DS, dermatan sulfate; HS, heparan sulfate; HA, hyauronic acid; Hep, heparin. B, The 96-well plates were coated with CS-E and assessed the binding activities to 4 nM (maximal concentration) of five EGF-like repeats conjugated to N-terminal 70 kDa domain of fibronectin (FN70K-EGF). The results shown were expressed as the means ± S.E. of triplicate determinations.
domain of fibronectin (FN70K) to facilitate secretion of recombinant proteins in mammalian expression systems (Matsuyama et al., 1994; Ido et al., 2004). Thus, purified EGF-like repeats conjugated to FN70K (FN70K-EGF) and control FN70K were assessed for their capabilities to bind to chondroitin sulfate-E (Fig. I-3B). As expected, FN70K-EGF was capable of binding toward chondroitin sulfate-E, while control FN70K exhibited no binding activity. These results, taken together, indicate that nephronectin predominantly interacts with chondroitin sulfate-E and heparin/heparan sulfate through N-terminal five EGF-like repeats and C-terminal MAM domain, respectively.

The Central Linker Segment of Nephronectin Harbors the Activity to Bind to α8β1 Integrin—Since nephronectin has been shown to serve as a ligand for α8β1 integrin (Brandenberger et al., 2001; Morimura et al., 2001), I wondered that the central linker segment of nephronectin, which possesses an RGD cell adhesion motif and showed no capability to interact with GAG chains, is involved in the interaction with α8β1 integrin. To examine this possibility, I purified recombinant α8β1 integrin, in which the extracellular domains of the individual α and β subunits were fused to “ACID” and “BASE” α-helical coiled-coil peptides, respectively, with an interchain disulfide bond (Takagi et al., 2001; Nishiuchi et al., 2006; Fig. I-4A). FLAG and 6xHis tags were fused to the C-termini of the ACID and BASE peptides, respectively, to facilitate purification of the recombinant integrin. The authenticity of purified α8β1 integrin was confirmed by SDS-PAGE and immunoblotting (Fig. I-4B). As expected, the recombinant α8β1 integrin gave a single band at ~210 kDa region under non-reducing conditions, confirming that
Fig. I-4. Purification of recombinant α8β1 integrin. A, A schematic diagram of a recombinant α8β1 integrin. B, SDS-PAGE and immunoblot analyses of purified α8β1 integrin. The α8β1 integrin were electrophoresed in 12% (for immunoblotting with anti-FLAG mAb under reducing conditions) or 6% (for other analyses) SDS-polyacrylamide gels under non-reducing (left panels) or reducing (center and right panels) conditions. Proteins were visualized by silver staining (silver) or immunoblotting with antibodies specific for the FLAG tag, 5xHis tag, and extracellular domain of α8 integrin (10A8). The positions of molecular size markers are shown in the left of each panel. HC, heavy chain; LC, light chain.

the α and β subunits were covalently linked through disulfide bond. After reduction with 2-mercaptoethanol, the integrin heterodimers were resolved into their individual subunits, separated into three bands with the ~130 kDa (α8 heavy chain), ~120 kDa (β1), and ~27 kDa (α8 light chain).

The activity of nephronectin to bind to α8β1 integrin was assessed with the purified recombinant α8β1 integrin (Fig. I-5A). To compare the binding capabilities of α8β1 integrin to other RGD-containing ECM molecules, fibronectin, vitronectin, and fibrinogen, were simultaneously assessed for their activities to bind to α8β1 integrin. Among the RGD-containing ECM proteins I tested, nephronectin was extremely potent
in the binding to α8β1 integrin, although other RGD-containing ECMs including fibronectin, vitronectin, and fibrinogen were less active in binding to α8β1 integrin (Fig. I-5A). These results indicate that nephronectin harbors the binding site for α8β1 integrin.

Since the RGD motif is situated within the linker segment connecting the N-terminal EGF-like repeats and the C-terminal MAM domain, I examined whether the RGD-containing linker segment could recapitulate the high affinity binding to α8β1 integrin (Fig. I-5B). Not only full-length nephronectin but also the linker segment bound strongly to α8β1 integrin, while the MAM domain of nephronectin had no activity to bind to α8β1 integrin, indicating that the linker segment of nephronectin is responsible for potent binding to α8β1 integrin. Because the linker segment of nephronectin is modified with a lot of O-glycans, I hypothesized that either the O-linked glycans or the amino acid sequences within the linker segment are responsible for the potent interaction with α8β1 integrin. To this end, I expressed the linker segment as a GST-fusion protein in bacteria and assessed its activity to bind to α8β1 integrin. A GST protein with an extension of the PRGDV sequence of nephronectin was also prepared and assessed for its binding to α8β1. The GST fusion protein of the linker segment was also capable of potent binding to α8β1 integrin (Fig. I-5B), thus indicating that the O-glycans have nothing to do with the interaction with α8β1 integrin. On the other hand, the GST protein containing the PRGDV sequence of nephronectin was significantly less potent in the binding to α8β1 integrin, suggesting that the amino acid sequence within the linker segment, besides the RGD motif, is required for strong binding of nephronectin to α8β1 integrin. These results, taken together, strongly suggest that the central linker segment of
nephronectin harbors the α8β1 integrin binding capability of nephronectin.

Fig. 1-5. The binding activities of the α8β1 integrin to RGD-containing ECM proteins and the central linker segment of nephronectin. Ninety-six well microtiter plates were coated with 10 nM of RGD-containing ECM proteins (A) or deletion mutants of nephronectin (B) indicated at the bottom of each panel, and then incubated with 10 nM of α8β1 integrin in the presence of 1 mM Mn^{2+}. The bound integrins were quantified as described in “Experimental Procedures”. The amounts of the integrins bound in the presence of 10 mM EDTA were taken as negative controls, and subtracted as backgrounds. The results are expressed as the means ± S.E. of triplicate determinations. NN, nephronectin; FN, fibronectin; VN, vitronectin; Fbg, fibrinogen.
I-5 Discussion

In the previous studies, nephronectin has been analyzed in its expression/distribution patterns and cell adhesive/integrin-binding activities (Brandenberger et al., 2001; Morimura et al., 2001). However, the analyses of nephronectin were carried out mainly using the bacterially expressed proteins from which the N-terminal five EGF-like repeats were deleted. Here, I purified full-length nephronectin, for the first time, and its deletion mutants using mammary expression systems, and then I investigated the functions of the individual domains of nephronectin with respect to their capabilities to bind to GAG chains and α8β1 integrin. My results indicated that nephronectin interacts with chondroitin sulfate-E and heparin/heparan sulfate chains through its N-terminal EGF-like repeats and C-terminal MAM domain, respectively, whereas the central linker segment containing the RGD motif is responsible for exerting high-affinity interaction of nephronectin with α8β1 integrin. Therefore, these results suggest that nephronectin binds to chondroitin sulfate and heparan sulfate proteoglycans through its N-terminal and C-terminal domains, respectively, and to α8β1 integrin through its central linker segment (Fig. I-6). Previously, Morimura et al. reported that deletion of the MAM domain of nephronectin resulted in reduced tendency of nephronectin to localize at cell surface, while substitution of RGE for the RGD motif, which reduces the activity of nephronectin to bind to integrins, did not affect the localization of nephronectin at the cell surface (Morimura et al., 2001). This observation indicates that nephronectin interacts with cells through the MAM domain, thus supporting the idea that the MAM domain of
Fig. 1-6. A schematic model of domain functions of nephronectin in vivo. Nephronectin (NN) interacts with chondroitin sulfate and heparan sulfate proteoglycans, localized at cell surface or ECMs, through its N-terminal five EGF-like repeats and C-terminal MAM domain, respectively. The central linker segment containing the RGD motif harbors potent activity to bind to α8β1 integrin. Since the chondroitin sulfate/heparan sulfate proteoglycans are abundantly expressed in ECMs and cell surfaces in developing kidney, it is plausible that NN bind to ECM-type proteoglycans to deposit onto ECMs/basement membranes, and/or to cell-surface proteoglycans to transmit appropriate signals into cells collaborating with α8β1 integrin. Blue spheres, chondroitin sulfates; pink spheres, heparan sulfates.

nephronectin interacts with cell surface heparan sulfate proteoglycans.

Nephronectin is primarily composed of three functional domains, that is, N-terminal
five EGF-like repeats, central linker segment containing an RGD cell adhesion motif, and C-terminal MAM domain. Among these, the MAM domain, consisting of ~170 amino acid residues with characteristic hydrophobic and four cysteine residues, is found in several transmembrane and ECM proteins (Beckmann and Bork, 1993). MAM domains have been shown to mediate protein-protein interactions, especially in homophilic/heterophilic interactions. Thus, meprins, transmembrane metalloproteases possessing a MAM domain, have been shown to form meprin oligomers through homophilic interaction between MAM domains (Ishmael et al., 2001; Ishmael et al., 2005). Neuropilin-1 and -2 have also been shown to form homo- and hetero-oligomers through an interaction involving the MAM domain (Chen et al., 1998). Moreover, the MAM domain of receptor protein tyrosine phosphatase µ has been shown to contribute to cell-cell adhesion through homophilic interaction between the MAM domains (Brady-Kalnay et al., 1993; Zondag et al., 1995; Cismasiu et al., 2004). Relevant to these observations, the purified MAM domain of nephronectin seems to form a dimer, since the purified MAM domain gave two bands with ~20 and ~40 kDa, possibly the MAM domain monomer and dimer, respectively, on SDS-PAGE (Fig. I-1B; page 48). Therefore, nephronectin may also interact each other to form homo-oligomers through its MAM domain.

The MAM domain of nephronectin differs from other MAM domains in the isoelectric points. The calculated isoelectric points of the MAM domains except for nephronectin are between 4.3 and 6.7, indicating that most of the MAM domains are negatively charged at the physiological pH (Table I-1). The MAM domain of nephronectin, however,
has an isoelectric point of 10.5, indicating that the MAM domain of nephronectin possesses much more basic residues than the other MAM domains (Table I-1). The property of the MAM domain of nephronectin, i.e., abundance in positive charges, may cause the interaction with highly sulfated GAG chains, including heparin/heparan sulfate and chondroitin sulfate-E, which are abundant in negative charges.

Table I-1.

**Calculated isoelectric points (pl) of the MAM domains**

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Calculated pl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nephronectin</td>
<td>10.5</td>
</tr>
<tr>
<td>Meprin-a</td>
<td>5.9</td>
</tr>
<tr>
<td>Meprin-b</td>
<td>5.2</td>
</tr>
<tr>
<td>Neuropilin-1</td>
<td>6.7</td>
</tr>
<tr>
<td>Neuropilin-2</td>
<td>4.9</td>
</tr>
<tr>
<td><strong>RPTP</strong></td>
<td></td>
</tr>
<tr>
<td><strong>µ</strong></td>
<td>5.1</td>
</tr>
<tr>
<td><strong>κ</strong></td>
<td>4.3</td>
</tr>
<tr>
<td>MAEG</td>
<td>4.9</td>
</tr>
</tbody>
</table>

a, all protein sequences are derived from mice  
b, pl calculations were performed by EMBL WWW Gateway to Isoelectric Point Service  
(http://www.embl-heidelberg.de/cgi/pi-wrapper.pl)  
c, RPTP, Receptor Protein Tyrosine Phosphatase

Although nephronectin binds to heparin/heparan sulfate through the MAM domain, the physiological roles of the interaction of nephronectin with heparin/heparan sulfate are still unclear. One possible role could be that the interaction with heparan sulfate proteoglycans enables nephronectin to be deposited onto the basement membranes (Fig. I-6). It should be noted that most of ECM-type heparan sulfate proteoglycans are localized at the basement membranes. For example, perlecan is a constitutive basement membrane protein present in all mature basement membranes (Timpl and Brown, 1996).
Agrin is also found ubiquitously in basement membranes (Manabe et al., 2008). Type XVIII collagen is located at most of the basement membranes in various organs and blood vessels (Miosge et al., 2003). Indeed, it has been reported that nephronectin is localized at basement membranes of various organs including developing kidney (Brandenberger et al., 2001). Thus, nephronectin may determine its localization at the basement membranes through the interaction of its MAM domain with the heparan sulfate proteoglycans.

The interaction of nephronectin with basement membrane-type heparan sulfate proteoglycans may also contribute to spatiotemporal distributions of multiple soluble factors. So far, multiple soluble factors, including GDNF, Wnt, BMPs and FGFs, have been shown to interact with heparan sulfate proteoglycans through their GAG chains (Kispert et al., 1996; Allen et al., 2001; Clayton et al., 2001; Grisaru et al., 2001; Qiao et al., 2001; Barnett et al., 2002). This interaction are thought to be involved in generating a concentration gradient of the soluble factors (Lander, 1999). As mentioned above, most of ECM-type heparan sulfate proteoglycans are located at the basement membranes, thus most of soluble factors expressed by cells should be sequestered by basement membranes. Since nephronectin is also located at the basement membranes and has potentials to bind to heparan sulfate proteoglycans, the interaction of nephronectin with heparan sulfates may dissociate and/or insulate the soluble factors from heparan sulfates, thereby allowing the factors to be diffused in connective tissues and mesenchymes.

Another possible explanation for the roles of heparin binding activities of nephronectin could be to regulate physiological functions of nephronectin, such as signal transduction
through α8β1 integrin. Heparan sulfates have been shown to regulate physiological functions and activities of proteins possessing heparin-binding properties. For example, the interaction between FGF and FGFR necessitates heparan sulfate to enhance the stability of FGF-FGFR complex by cross-linking ligand and receptor (Pellegrini et al., 2000; Schlessinger et al., 2000). With respect to ECM proteins, it has been shown that both the heparin- and α5β1 integrin-binding domains of fibronectin are required for stress fiber formation of the cells adhering to fibronectin. In this case, the heparin-binding domain of fibronectin probably interacts with the heparan sulfate chains of syndecan-4 on cell surface (Yoneda et al., 1995; Bloom et al., 1999; Saoncella et al., 1999). Similarly, both heparin- and α5β1 integrin-binding domains of fibrillin-1 are required for stress fiber formation of the cells adhering to fibrillin-1 (Bax et al., 2007). Since nephronectin is one of the ECM proteins possessing integrin- and heparin-binding activities, these observations give rise to a possibility that nephronectin may interact with both α8β1 integrin and cell surface heparan sulfate proteoglycans, cooperatively transmitting signals to exert various effects such as reorganization of actin cytoskeletons.

α8β1 integrin, identified as an RGD-recognizing integrin, has been shown to serve as a receptor for various RGD motif-containing ECM ligands including fibronectin, vitronectin, nephronectin, and osteopontin (Schnapp et al., 1995; Denda et al., 1998b; Brandenberger et al., 2001; Morimura et al., 2001). In my experiments, however, recombinant α8β1 integrin selectively bound to nephronectin, suggesting that nephronectin is a preferential ligand for α8β1 integrin. One might argue that the selective recognition of nephronectin by α8β1 integrin may be an artifact due to the deletion of the
transmembrane/cytoplasmic domain and forced dimerization of integrins. However, the feasibility of this strategy for the expression of recombinant integrins has previously been demonstrated for many integrins including \( \alpha_3 \beta_1, \alpha_4 \beta_1, \alpha_5 \beta_1, \alpha_6 \beta_1, \) and \( \alpha V \beta 3 \) (Lu et al., 2001; Takagi et al., 2001; 2002a; 2002b; Nishiuchi et al., 2006). The activities of recombinant integrins have been shown to principally recapitulate that of endogeneously expressed integrins on the cell surface or that of purified integrins from tissue extracts. Therefore, the specific interaction of recombinant \( \alpha_8 \beta_1 \) integrin with nephronectin should also reflect the activity of \( \alpha_8 \beta_1 \) integrin in vivo.

The central linker segment of nephronectin can replicate the high-affinity binding activity of nephronectin to \( \alpha_8 \beta_1 \) integrin, irrespective of the cells used for expression of the linker segment, i.e., bacterial or mammary cells. However, PRGDV peptide fused with GST at the C-terminus had only marginal, if any, activity to bind to \( \alpha_8 \beta_1 \) integrin. Since the GST-fusion protein of linker segment is expressed without modification of \( O \)-linked sugar chains, these results strongly suggest that the linker segment of nephronectin possesses particular amino acid sequences specifically recognized by \( \alpha_8 \beta_1 \) integrin. This situation quite resembles that of interaction of fibronectin with \( \alpha_5 \beta_1 \) integrin. Although \( \alpha_5 \beta_1 \) integrin binds to fibronectin in an RGD-dependent manner, the RGD motif itself is significantly less active in binding to \( \alpha_5 \beta_1 \) integrin than intact fibronectin. \( \alpha_5 \beta_1 \) integrin has been shown to recognize not only the RGD motif but also synergistic binding site in the vicinity of the RGD motif (Aota et al., 1994; Redick et al., 2000). Therefore, these observations raise a possibility that \( \alpha_8 \beta_1 \) integrin may recognize not only the RGD motif but also the non-RGD residues within the central linker segment.
In Chapter II, I describe the results of my investigation of this hypothesis.

In the present study, I investigated the domain functions of nephronectin, *i.e.*, the functions of N-terminal five EGF-like repeats, the central linker segment containing an RGD motif, and C-terminal MAM domain. I found that nephronectin binds to chondroitin sulfate-E and heparin/heparan sulfate through its N-terminal EGF-like repeats and C-terminal MAM domain, respectively. Furthermore, I found that the central linker segment of nephronectin is a region selectively recognized by α8β1 integrin. These results suggest that nephronectin interacts with proteoglycans as well as α8β1 integrin. Thus, nephronectin may play crucial roles in kidney morphogenesis, providing a “bridge” between the functions of α8β1 integrin and GAG chains including heparan sulfate and chondroitin sulfate. I hope that my observations in this research provide new insights into the functions of integrins and proteoglycans in kidney morphogenesis.
Chapter II

Molecular Basis of the Recognition of Nephronectin by Integrin $\alpha 8\beta 1$
II-1 Summery

Integrin α8β1 interacts with a variety of Arg-Gly-Asp (RGD)-containing ligands in the ECM. Here, I examined the binding activities of α8β1 integrin toward a panel of RGD-containing ligands. Integrin α8β1 bound specifically to nephronectin with an apparent dissociation constant of 0.28±0.01 nM, but showed only marginal affinities for fibronectin and other RGD-containing ligands. The high-affinity binding to α8β1 integrin was fully reproduced with a recombinant nephronectin fragment derived from the RGD-containing central linker segment. A series of deletion mutants of the recombinant fragment identified the LFEIFEIER sequence on the C-terminal side of the RGD motif as an auxiliary site required for high-affinity binding to α8β1 integrin. Alanine scanning mutagenesis within the LFEIFEIER sequence defined the EIE sequence as a critical motif ensuring the high-affinity integrin-ligand interaction. Although a synthetic LFEIFEIER peptide failed to inhibit the binding of α8β1 integrin to nephronectin, a longer peptide containing both the RGD motif and the LFEIFEIER sequence was strongly inhibitory, and was ~2,000-fold more potent than a peptide containing only the RGD motif. Furthermore, trans-complementation assays using recombinant fragments containing either the RGD motif or LFEIFEIER sequence revealed a clear synergism in the binding to α8β1 integrin. Taken together, these results indicate that the specific high-affinity binding of nephronectin to α8β1 integrin is achieved by bipartite interaction of the integrin with the RGD motif and LFEIFEIER sequence, with the latter serving as a synergy site that greatly potentiates the RGD-driven
integrin-ligand interaction but has only marginal activity to secure the interaction by itself.
II-2 Introductions

Integrins are a family of adhesion receptors that interact with a variety of extracellular ligands, typically cell-adhesive proteins in the ECM. They play mandatory roles in embryonic development and the maintenance of tissue architectures by providing essential links between cells and the ECM (Hynes, 1992). Integrins are composed of two non-covalently associated subunits, termed α and β. In mammals, 18 α and 8 β subunits have been identified, and combinations of these subunits give rise to at least 24 distinct integrin heterodimers. Based on their ligand-binding specificities, ECM-binding integrins are classified into three groups, namely laminin-, collagen- and RGD-binding integrins (Hynes, 2002; Takagi, 2007), of which the RGD-binding integrins have been most extensively investigated. The RGD-binding integrins include α5β1, α8β1, αIIbβ3 and αV-containing integrins, and have been shown to interact with a variety of ECM ligands, such as fibronectin and vitronectin, with distinct binding specificities.

The α8 integrin subunit was originally identified in chick nerves (Bossy et al., 1991). Integrin α8β1 is expressed in the metanephric mesenchyme and plays a crucial role in epithelial-mesenchymal interactions during the early stages of kidney morphogenesis. Disruption of the α8 gene in mice was found to be associated with severe defects in kidney morphogenesis (Muller et al., 1997) and stereocilia development (Littlewood Evans and Muller, 2000). To date, α8β1 integrin has been shown to bind to fibronectin, vitronectin, osteopontin, latency-associated peptide of transforming growth factor (TGF)-β1, tenasin-W and nephronecin (Muller et al., 1995; Schnapp et al., 1995;
Denda et al., 1998b; Brandenberger et al., 2001; Morimura et al., 2001; Lu et al., 2002; Scherberich et al., 2004), among which nephronectin is believed to be an \( \alpha 8\beta 1 \) integrin ligand involved in kidney development (Brandenberger et al., 2001).

Nephronectin is one of the basement membrane proteins whose expression and localization patterns are restricted in a tissue-specific and developmentally regulated manner (Brandenberger et al., 2001; Morimura et al., 2001). Nephronectin consists of five EGF-like repeats, a linker segment containing the RGD cell-adhesive motif (designated RGD-linker) and a MAM domain. Although the physiological functions of nephronectin remain only poorly understood, it is thought to play a role in epithelial-mesenchymal interactions through binding to \( \alpha 8\beta 1 \) integrin, thereby transmitting signals from the epithelium to the mesenchyme across the basement membrane (Brandenberger et al., 2001). Recently, mice deficient in nephronectin expression were produced by homologous recombination (Linton et al., 2007). The nephronectin-deficient mice frequently displayed kidney agenesis, a phenotype reminiscent of \( \alpha 8 \) integrin-knockout mice (Linton et al., 2007), despite the fact that other RGD-containing ligands, including fibronectin and osteopontin, were expressed in the embryonic kidneys (Ekblom, 1981; Denda et al., 1998b). The failure of the other RGD-containing ligands to compensate for the deficiency of nephronectin in the developing kidneys suggests that nephronectin is an indispensable \( \alpha 8\beta 1 \) ligand that plays a mandatory role in epithelial-mesenchymal interactions during kidney development.

Although ligand recognition by RGD-binding integrins is primarily determined by the RGD motif in the ligands, it is the residues outside the RGD motif that define the
binding specificities and affinities toward individual integrins (Ruoslanti, 1996; Takagi, 2004). For example, α5β1 integrin specifically binds to fibronectin among the many RGD-containing ligands, and requires not only the RGD motif in the tenth type III repeat but also the so-called ‘synergy site’ within the preceding ninth type III repeat for fibronectin recognition (Aota et al., 1994). Recently, DiCara et al. (DiCara et al., 2007) demonstrated that the high-affinity binding of αVβ6 integrin to its natural ligands, e.g., foot-and-mouth disease virus, requires the RGD motif immediately followed by a Leu-Xaa-Xaa-Leu/Ile sequence, which forms a helix to align the two conserved hydrophobic residues along the length of the helix. Given the presence of many naturally occurring RGD-containing ligands, it is conceivable that the specificities of the RGD-binding integrins are dictated by the sequences flanking the RGD motif or those in neighboring domains that come into close proximity with the RGD motif in the intact ligand proteins. However, the preferences of α8β1 integrin for RGD-containing ligands and how it secures its high-affinity binding toward its preferred ligands remain unknown.

In the present study, I investigated the binding specificities of α8β1 integrin toward deletion mutants of the linker segment of nephronectin. My data reveal that a LFEIFEIER sequence on the C-terminal side of its RGD motif serves as a synergy site to ensure the specific high-affinity binding of nephronectin to α8β1 integrin.
II-3  Materials and Methods

Cells and Reagents—HT1080 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum. HT1080 cells were transfected with an expression vector for human full-length α8 integrin (described below). The cells were passaged at 24 h after transfection and maintained in medium containing 0.8 mg/ml G418 to select stable transfectants. The transfectants were subjected to selection by cell adhesion to a nephronectin-coated substratum as follows. The cells were seeded onto 6-well plates (Falcon) that had been coated with 10 nM nephronectin and blocked with phosphate-buffered saline (PBS) containing 10 mg/ml of BSA, and allowed to adhere for 30 min at 37°C. After removal of non-adherent cells using serum-free medium, the remaining cells were detached with PBS containing 0.025% trypsin and 1 mM EDTA, plated on 6-well plates and grown to confluence. The resulting cells (designated HT1080-A8 cells) were cloned by limiting dilution and used for cell adhesion assays. K562 human erythroleukemic cells stably transfected with a chicken α8 integrin cDNA (KA8 cells) were kindly provided by Dr. Louis F. Reichardt (University of California, San Francisco, CA) and maintained as described previously (Muller et al., 1995).

Human plasma fibronectin and vitronectin were purified from outdated human plasma by gelatin- and heparin-affinity chromatography, respectively, as described previously (Sekiguchi et al., 1983; Yatohgo et al., 1988). Human fibrinogen was purchased from Enzyme Research Laboratories Inc. (South Bend, IN). A polyclonal
antibody against actin and a horseradish peroxidase (HRP)-conjugated monoclonal antibody (mAb) against FLAG tag were obtained from Sigma (St. Louis, MO). An HRP-conjugated polyclonal antibody against glutathione S-transferase (GST) was purchased from GE Healthcare (Piscataway, NJ). HRP-conjugated peanut agglutinin (PNA) was purchased from Seikagaku Co. (Tokyo, Japan). mAbs against the human integrin α8 subunit were produced by fusion of Sp2 mouse myeloma cells with spleen cells from mice immunized with recombinant soluble integrin α8β1, and selected by both positive reactivity for recombinant integrin α8β1 and negative reactivity for recombinant integrin α5β1. mAb 10A8 was capable of binding to the denatured integrin α8 chain on immunoblots under reducing and non-reducing conditions. mAb 7A5 was capable of binding to the non-denatured integrin α8 chain, and was used for flow cytometry. Synthetic peptides were purchased from Biologica Co. (Nagoya, Japan) and dissolved into 100% dimethyl sulfoxide (DMSO).

Expression Vectors—A mouse nephronectin cDNA was obtained from Dr. Yoshihide Hayashizaki (Riken, Yokohama, Japan). The cDNA was subcloned into the pFLAG-CMV vector (Invitrogen, Carlsbad, CA) in-frame with a FLAG tag at the 3' end. For expression of a panel of nephronectin deletion mutants in mammalian cells, a cDNA fragment encoding a mouse nephronectin 5'-non-translated region and the signal peptide (nucleotides –61 to 69) was amplified by PCR with the primer set 5'-GAATTCGAGATCCCGGGACGC-3' (forward) and 5'-GGGCCCCGTCGAAGTCCGCGACGC-3' (reverse) using the full-length nephronectin cDNA as a template. This fragment was digested with EcoRI and ApaI, and fused
in-frame with cDNAs encoding the RGD-linker segment or the MAM domain. The cDNAs encoding the RGD-linker and the MAM domain were amplified by PCR with the primer sets 5′-GGGCCAAAGTCATGATTGAAC-3′ (forward) and 5′-GTCGACGTCGTCCTTTACTTCCCTC-3′ (reverse), and 5′-GGGCCCGGTATTCTCATACACAGC-3′ (forward) and 5′-GTCGACGCAGCGACCTCTTTCAAG-3′ (reverse), respectively. After verification by DNA sequencing, each PCR-amplified cDNA was digested with ApaI and SalI, and inserted into the EcoRI and SalI sites of the pFLAG-CMV vector together with the cDNA encoding the 5′-non-translated region and signal peptide.

An expression vector for the RGD-linker as a GST fusion protein was prepared as follows. A cDNA encoding the RGD-linker was amplified by PCR with the primer set 5′-GAATTCGCCAAAGTCATGATTGAAC-3′ (forward) and 5′-GTCGACTCAGTCGTCCTTTACTTCCCTC-3′ (reverse) using an E13.5 mouse embryo cDNA as a template. The PCR product was subcloned into the pBlueScriptII SK+ vector (Stratagene, La Jolla, CA), digested with EcoRI and SalI and inserted into the corresponding restriction sites of the pGEX 4T-1 expression vector (GE Healthcare) (designated pGEX-RGD-linker). cDNAs encoding a series of deletion mutants and substitution mutants of the RGD-linker region were amplified by PCR using pGEX-RGD-linker encoding the GST-RGD-linker fusion protein as a template. A list of the primer sequences used for PCR is available upon request. The PCR products were digested with EcoRI and SalI and inserted into the corresponding restriction sites of pGEX 4T-1.
A cDNA encoding the human integrin α8 subunit was amplified by PCR with the primer set 5’-AAGGAAGCTTCCACCATGTCGCCCGGGGCCAGCCGCGG-3’ (forward) and 5’-AATCACTCGAGTGCTCAGGGGTCTTGTCATTGG-3’ (reverse) using a human fetal cDNA (Clontech, Palo Alto, CA) as a template. The PCR-amplified cDNA was subcloned into pBlueScript II SK+ and verified by DNA sequencing. A cDNA encoding the intact α8 integrin subunit was prepared by addition of a stop codon and insertion into the pcDNA 3.1 vector (Invitrogen). An expression vector for an integrin α8 subunit that was truncated before the transmembrane domain and fused to the ‘ACID’ α-helical coiled-coil peptide with a FLAG tag was prepared as described previously (Nishiuchi et al., 2006; Ido et al., 2007). Expression vectors for recombinant soluble αV, β1 and β3 integrin subunits were previously described (Takagi et al., 2001; Takagi et al., 2002b).

**Expression and Purification of Recombinant Proteins**—For purification of recombinant nephronecin, its mutants and integrins, Freestyle™ 293-F cells were transiently transfected with individual plasmids using a Freestyle™ 293 Expression system (Invitrogen) according to the manufacturer’s instructions. Conditioned media were collected at 48-72 h after transfection and centrifuged to remove cells and debris. For purification of nephronecin and its mutants, EDTA (5 mM), phenylmethylsulfonyl fluoride (PMSF; 1 mM) and sodium azide (0.02%) were added to the conditioned media. The media were then incubated with anti-FLAG M2 affinity beads (Sigma) overnight with gentle agitation at 4°C. The beads were transferred into an empty column and washed with PBS. Bound proteins were eluted with 100 µg/ml FLAG peptide dissolved
in PBS. The purified proteins were dialyzed against 2 mM CAPS (N-cyclohexyl-3-aminopropane sulfonic acid) buffer (pH 11.4) containing 0.5 mM EDTA, and used in the following assays. For purification of recombinant integrins, PMSF (1 mM) and sodium azide (0.02%) were added to the conditioned media. The media were then incubated with nickel-nitrilotriacetic acid-agarose beads (Qiagen, Valencia, CA), followed by washing with Tris-buffered saline (TBS). Bound proteins were eluted with TBS containing 200 mM imidazole. The eluted proteins were applied to anti-FLAG M2 affinity beads, and the bound proteins were eluted with 100 μg/ml FLAG peptide and dialyzed against TBS.

GST fusion proteins were induced in *Escherichia coli* by overnight incubation with 0.1 mM isopropyl-β-D-thiogalactopyranoside at 25°C. The cells were then lysed by sonication, and the supernatants were passed over a glutathione Sepharose 4B column (GE Healthcare). Bound proteins were eluted with 50 mM Tris-HCl (pH 8.0) containing 10 mM glutathione, and then dialyzed against 2 mM CAPS buffer containing 0.5 mM EDTA. Protein concentrations were determined by the Bradford method, except for those of the nephronectin deletion mutants expressed in 293-F cells, which were determined by immunoblotting with an anti-FLAG antibody owing to the very low reactivity of the highly glycosylated RGD-linker with the Bradford reagent.

**SDS-PAGE and Western Blotting**—SDS-PAGE was carried out according to Laemmli (Laemmli, 1970) using 8%, 12% or 7-15% gradient gels. Separated proteins were visualized by Coomassie Brilliant Blue (CBB) staining or transferred onto polyvinylidene difluoride (for immunoblotting) or nitrocellulose (for lectin blotting) membranes. The
membranes were probed with antibodies (for immunoblotting) or PNA (for lectin blotting), followed by visualization with an ECL detection kit (GE Healthcare).

**Gel Filtration**—Purified nephronectin (500 µl) was loaded on a Superose 6 gel filtration column (10 x 300 mm; GE Healthcare) equilibrated in PBS. Fractions were collected at a flow rate of 0.2 ml/min and monitored for their absorbance at 280 nm. The column was calibrated with the following molecular mass standards: chymotrypsinogen, 25 kDa; ovalbumin, 43 kDa; catalase, 232 kDa; ferritin, 440 kDa; thyroglobulin, 669 kDa.

**Integrin Binding Assay**—Integrin binding assays were performed as described previously (Nishiuchi et al., 2006). Briefly, microtiter plates were coated with various substrate proteins (10 nM) overnight at 4°C, and then blocked with 10 mg/ml BSA. In trans-complementation assays, the plates were coated with the first substrate protein, washed with PBS, coated with the second substrate proteins overnight at 4°C and then blocked with BSA. The plates were incubated with integrins in the presence of 1 mM MnCl₂ with or without 10 mM EDTA. In some experiments, integrins were incubated on the plates in the presence of 1 mM CaCl₂ and 1 mM MgCl₂ to see the effects of divalent cations or in the presence of synthetic peptides at various concentrations to evaluate their inhibitory activities. The plates were washed with TBS containing 1 mM MnCl₂, 0.1% BSA and 0.02% Tween-20 with or without 10 mM EDTA, followed by quantification of bound integrins by an enzyme-linked immunosorbent assay using a biotinylated rabbit anti-Velcro (ACID/BASE coiled-coil) antibody and HRP-conjugated streptavidin. Dissociation constants were calculated by saturation binding assays as described
previously (Nishiuchi et al., 2003).

**Flow Cytometry**—Flow cytometric analyses were performed as described previously (Gu et al., 2001). Suspended cells were incubated with anti-integrin mAbs for 30 min at 4°C. After washing with PBS, the cells were incubated with an Alexa Fluor® 488-labeled secondary antibody (Molecular Probes, Eugene, OR) for 30 min at 4°C and subjected to flow cytometric analysis using a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

**Cell Adhesion Assays**—Microtiter plates were coated overnight at 4°C with substrate proteins diluted to various concentrations in PBS in a volume of 50 µl/well. The coating efficiencies of GST fusion proteins were determined by their reactivities with an anti-GST polyclonal antibody. The plates were blocked with blocking medium (serum-free DMEM containing 10 mg/ml BSA and 10 mM HEPES, pH 7.5) for 1 h at 37°C. HT1080-A8 cells resuspended in blocking medium were plated at 4.0 × 10⁴ cells/well and incubated for 30 min at 37°C in a humidified atmosphere of 5% CO₂ in air. For cell adhesion inhibition assays, cells were preincubated with synthetic peptides in blocking medium at room temperature for 10 min prior to plating. After removal of non-adherent cells by washing with blocking medium, the attached cells were fixed with 3.7% formaldehyde, washed three times with PBS and stained for 10 min with 0.1% toluidine blue in PBS. After lysis in 1% SDS, the attached cells were quantified by their absorption at 595 nm.
II-4 Results

α8β1 Integrin Preferentially Binds to Nephronection—To examine the binding specificities of α8β1 integrin toward nephronection and other RGD-containing ECM ligands, we expressed and purified recombinant nephronection using a mammalian expression system. A FLAG tag was added to the C-terminus of nephronection to facilitate affinity purification of the recombinant protein. The authenticity of the purified nephronection was verified by SDS-PAGE and immunoblotting against the FLAG tag (Fig. II-1A). Purified nephronection gave a broad band at ~120 kDa under reducing conditions, which was significantly larger than the predicted protein size (61 kDa). The apparent discrepancy was probably caused by modification with a large number of O-linked sugar chains, which has been predicted to occur at the mucin-like region within the central linker segment (Brandenberger et al., 2001; Morimura et al., 2001). The recombinant nephronection gave a less prominent band at ~120 kDa upon immunoblotting with an anti-FLAG mAb under non-reducing conditions (Fig. II-1A), possibly due to the occurrence of disulfide-bonded multimers that did not enter the resolving gel. Multimer formation by nephronection was further confirmed by gel filtration chromatography using a Superose 6 column. Nephronection eluted at a position slightly after the void volume (Fig. II-1B). The second major peak that eluted at 21 ml was identified by N-terminal amino acid sequencing as the FLAG peptide used for elution of recombinant nephronection from the immunoaffinity column (data not shown). Multimer formation by recombinant nephronection was further confirmed by native PAGE (data not shown).
Fig. II-1. Expression and purification of recombinant nephronectin. A, 0.5 µg (for CBB staining) or 0.1 µg (for immunoblotting with a mAb against FLAG tag) of purified nephronectin was subjected to SDS-PAGE in 8% gels under reducing (left) and non-reducing (right) conditions. The positions of molecular size markers are shown in the left margin. The arrow points to the interface between the stacking and resolving gels. B, 500 µl of purified nephronectin was applied to a Superose 6 column immediately after elution from an anti-FLAG affinity column. Fractions were collected at 1 ml/tube and monitored for their absorbance at 280 nm. The elution positions of molecular size markers are indicated by arrows. The asterisk points to the peak arising from the FLAG peptide used for elution of nephronectin from the anti-FLAG affinity column.

Purified recombinant nephronectin was subjected to integrin binding assays along with other RGD-containing proteins including fibronectin, vitronectin and fibrinogen. The assays were performed in the presence of 1 mM Mn²⁺ to fully activate integrins. Although α8β1 integrin was previously reported to bind to fibronectin and vitronectin (Muller et al., 1995; Schnapp et al., 1995), the binding activity of α8β1 integrin toward nephronectin far exceeded those toward fibronectin, vitronectin and fibrinogen (Fig. II-2A). Saturation binding assays revealed that α8β1 integrin bound to nephronectin with a dissociation constant of 0.28 ± 0.01 nM, which was approximately two orders of
Fig. II-2. Binding activities of integrins toward nephronectin and other RGD-containing proteins. A, 96-well microtiter plates were coated with various RGD-containing proteins (10 nM) and then incubated with α8β1 integrin (10 nM) in the presence of 1 mM Mn$^{2+}$. FN, fibronectin; VN, vitronectin; Fbg, fibrinogen; NN, nephronectin. Bound integrins were quantified as described in the Experimental Procedures. The amounts of integrin bound in the presence of 10 mM EDTA were taken as negative controls, and subtracted as backgrounds. The results represent the means of duplicate determinations. B, titration curves of recombinant α8β1 integrin bound to fibronectin (diamonds), vitronectin (squares), fibrinogen (triangles) and nephronectin (circles). Microtiter plates coated with individual proteins (10 nM) were incubated with increasing concentrations of α8β1 in the presence of 1 mM Mn$^{2+}$, followed by quantification of the bound integrins in the Experimental Procedures. The results represent the means of duplicate determinations. C-E, microtiter plates were coated with substrates as in A, and incubated with 10 nM of α5β1 (C), αIIbβ3 (D), and αVβ3 (E) integrins. Bound integrins were quantified as described in the Experimental Procedures.
magnitude lower than that for fibronectin (Fig. II-2B). Vitronectin and fibrinogen exhibited only marginal binding activities, even at the highest \( \alpha_8 \beta_1 \) integrin concentration examined. The low binding affinities of \( \alpha_8 \beta_1 \) integrin toward fibronectin, vitronectin and fibrinogen were not due to inactivation of these adhesive proteins because they retained their abilities to bind to their specific integrin receptors, namely \( \alpha_5 \beta_1 \), \( \alpha_V \beta_3 \) and \( \alpha_{IIb} \beta_3 \), respectively (Fig. II-2C-E). Taken together, these results demonstrate that nephronectin is a preferred ligand for \( \alpha_8 \beta_1 \) integrin among RGD-containing proteins known to bind to \( \alpha_8 \beta_1 \) integrin.

**The RGD-Linker Segment of Nephronectin Harbors the \( \alpha_8 \beta_1 \) Binding Activity**—Since the RGD motif is situated within the linker segment connecting the N-terminal EGF-like repeats and the C-terminal MAM domain, we examined whether the RGD-linker segment could recapitulate the high-affinity binding to \( \alpha_8 \beta_1 \) integrin. To this end, we expressed the RGD-linker in mammalian cells or as a GST fusion protein in bacteria (Fig. II-3A). We also expressed the C-terminal MAM domain in mammalian cells and a GST fusion protein with a C-terminal extension of the PRGDV sequence of nephronectin in bacteria as controls. The RGD-linker expressed in 293-F cells gave a broad band migrating at 35-60 kDa upon SDS-PAGE, while the RGD-linker expressed as a GST fusion protein in bacteria gave a sharp band at 50 kDa (Fig. II-3B). The apparent heterogeneity of the RGD-linker expressed in mammalian cells was probably caused by extensive \( O \)-linked glycosylation because the RGD-linker was strongly reactive with PNA, a lectin that recognizes mucin-type \( O \)-linked sugar chains (Fig. II-3B). The
Fig. II-3. Expression and purification of nephronectin fragments. A, schematic diagrams of full-length nephronectin (NN) and its fragments. RGD-linker and RGD-linker (GST), the central RGD-containing linker segments expressed in mammalian and bacterial expression systems, respectively; PRGDV, a short RGD-containing peptide modeled after nephronectin and expressed as a GST fusion protein (see Fig. II-4A for the peptide sequence). The arrowheads indicate the positions of the RGD motif. B, purified recombinant proteins were analyzed by SDS-PAGE in 7-15% gradient (left and center panels) and 12% (right panels) gels, followed by CBB staining, immunoblotting with an anti-FLAG mAb or lectin blotting with PNA. The quantities of proteins loaded were: 0.5 µg (for CBB staining) and 0.1 µg (for blotting with anti-FLAG and PNA) in the left and center panels; 1 µg in the right panel.

recombinant MAM domain, with a predicted mass of 17 kDa, gave a major band migrating at ~20 kDa under both reducing and non-reducing conditions (Fig. II-3B). Additional bands were detected at 40 and 60 kDa by immunoblotting with an anti-FLAG mAb under non-reducing conditions, indicating that the MAM domain tends to form dimers and trimers that are resistant to dissociation under the denaturing conditions used, i.e., boiling in the presence of SDS.

The purified RGD-linkers were assayed for their binding activities towards α8β1
integrin (Fig. II-4). Not only the full-length nephronectin but also the RGD-linkers, irrespective of the cells used for recombinant protein expression, bound strongly to α8β1 integrin, whereas the MAM domain did not show any binding activity. The titration curves of the RGD-linkers were essentially the same as that of full-length nephronectin (Fig. II-4), yielding dissociation constants of 0.2-0.3 nM (Table II-1). Furthermore, the PRGDV peptide expressed at the C-terminus of GST had only marginal, if any, binding activity toward α8β1 integrin, even though it contained the RGD motif. Since the GST-fused PRGDV peptide retained the ability to bind to αVβ3 integrin (see Fig. II-5D), these results indicate that the RGD motif is necessary but not sufficient for binding of nephronectin to α8β1 integrin. We also performed saturation binding assays in the presence of 1 mM Ca$^{2+}$ and Mg$^{2+}$, instead of 1 mM Mn$^{2+}$, and confirmed that the activities of the RGD-linkers to bind to α8β1 integrin were the same as that of full-length nephronectin (data not shown). These results indicate that the linker segment is sufficient

Fig. II-4. Binding activities of α8β1 integrin to nephronectin and its fragments. Titration curves of α8β1 integrin bound to full-length nephronectin (NN, closed squares), the RGD-linker segments expressed in 293-F cells (RGD-linker, closed triangles) and E. coli (RGD-linker (GST), open triangles), the MAM domain (MAM, closed diamonds) and the PRGDV peptide expressed as a GST-fusion protein in E. coli. (PRGDV (GST), open circles). The assays were performed as described in the legend to Fig. II-2B. The results represent the means of duplicate determinations.
for the high-affinity recognition of nephronectin by α8β1 integrin. However, the PRGDV peptide modeled after nephronectin was barely active in binding to α8β1 integrin, even at the highest α8β1 integrin concentration tested, suggesting that in addition to the RGD motif, the linker segment contains a region that potentiates the binding activity of nephronectin to α8β1 integrin.

**TABLE II-1. Dissociation constants of α8β1 integrin toward nephronectin and its deletion mutants**

<table>
<thead>
<tr>
<th>Ligands</th>
<th>Kd (nM)(^a)</th>
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<tr>
<td>NN</td>
<td>0.28±0.01</td>
</tr>
<tr>
<td>RGD-linker</td>
<td>0.23±0.05</td>
</tr>
<tr>
<td>MAM</td>
<td>ND (-)(^b)</td>
</tr>
<tr>
<td>RGD-linker (GST)</td>
<td>0.22±0.04</td>
</tr>
<tr>
<td>PRGDV (GST)</td>
<td>ND(^c)</td>
</tr>
<tr>
<td>LS/366-414</td>
<td>0.32±0.03</td>
</tr>
<tr>
<td>LS/378-414</td>
<td>0.33±0.02</td>
</tr>
<tr>
<td>LS/366-393</td>
<td>ND(^c)</td>
</tr>
<tr>
<td>LS/378-407</td>
<td>0.33±0.01</td>
</tr>
<tr>
<td>LS/378-403</td>
<td>0.44±0.04</td>
</tr>
<tr>
<td>LS/378-401</td>
<td>7.1±0.6</td>
</tr>
<tr>
<td>LS/378-399</td>
<td>13±1</td>
</tr>
<tr>
<td>LS/378-393</td>
<td>ND(^c)</td>
</tr>
<tr>
<td>LS/381-407</td>
<td>0.42±0.08</td>
</tr>
<tr>
<td>LS/391-407</td>
<td>ND(^c)</td>
</tr>
<tr>
<td>LS/395-407</td>
<td>ND(^c)</td>
</tr>
</tbody>
</table>

\(^a\)Values represent the means ± SD of three independent experiments.

\(^b\)The dissociation constant was not determined owing to the absence of significant binding.

\(^c\)The dissociation constants were not determined owing to only partial saturation at the highest integrin concentration examined.

**Identification of the Region within the Linker Segment that Warrants High-affinity Binding to α8β1 Integrin**—To explore the region within the linker segment that potentiates the RGD-dependent binding of nephronectin to α8β1 integrin,
we produced a series of RGD-linker deletion mutants as GST fusion proteins (Fig. II-5A). Since the linker segment expressed as a GST fusion protein was fully competent in high-affinity binding to α8β1 integrin and devoid of the mucin-like sugar chains that have been predicted to attach to the N-terminal two-thirds of the linker segment (based on Net-O-Glyc; http://www.cbs.dtu.dk/services/NetOGlyc/ (Hansen et al., 1998)), we first produced a deletion mutant comprising the C-terminal one-third of the linker segment, designated linker segment (LS)/366-414. LS/366-414 bound avidly to α8β1 integrin with a dissociation constant of 0.32 ± 0.03 nM, comparable to that of the full-length RGD-linker (Fig. II-5C and Table II-1). Given the retention of the full α8β1 binding activity within LS/366-414, we employed this fragment as a template for the production of a series of deletion mutants (Fig. II-5A). The authenticities of the resulting mutant proteins were verified by SDS-PAGE (Fig. II-5B).

The purified mutant proteins were assayed for their binding activities toward α8β1 integrin. LS/378-414, a mutant protein lacking the N-terminal 12 amino acid residues of LS/366-414, was capable of binding to α8β1 integrin with a comparable potency to that of LS/366-414 (Fig. II-5C and Table II-1). On the other hand, LS/366-393, a deletion mutant lacking the C-terminal 21 amino acid residues, was barely active in binding to α8β1 integrin, although the RGD motif remained intact in this mutant. These results indicate that the C-terminal 21 residues (394-414) are required for the high-affinity binding of nephronectin to α8β1 integrin. To further narrow down the residues required for the high-affinity binding to α8β1 integrin, we produced a series of C-terminal deletion mutants of LS/378-414, i.e., LS/378-407, LS/378-403, LS/378-401, LS/378-399
and LS/378-393 (Fig. II-5A). Among these C-terminal deletion mutants, LS/378-407 and LS/378-403 retained integrin binding activities comparable to that of LS/378-414, while LS/378-401, LS/378-399 and LS/378-393 exhibited stepwise reductions in their integrin binding activities, resulting in an almost complete loss of the activity after deletion of residues 394-414 (Fig. II-5C and Table II-1). We also assessed the binding activities of α8β1 integrin to the C-terminal deletion mutants of the RGD-linker in the presence of 1 mM Ca$^{2+}$ and Mg$^{2+}$, instead of 1 mM Mn$^{2+}$. The stepwise reductions of the binding activities of α8β1 integrin to LS/378-401, LS/378-399 and LS/378-393 were reproduced in the assays performed in the presence of Ca$^{2+}$ and Mg$^{2+}$, although the binding activities of the mutants were less pronounced than those attained in the presence of Mn$^{2+}$ (data not shown). Since LS/378-403 retained almost full activity, these results indicate that residues 394-403 (DLFEIFEIER) are involved in the high-affinity binding to α8β1 integrin.

The RGD motif has been shown to be a prerequisite for the integrin binding activity of nephronectin (Brandenberger et al., 2001; Morimura et al., 2001). To confirm the critical role of the RGD motif, we produced a series of N-terminal deletion mutants of LS/378-407, i.e., LS/381-407, LS/391-407 and LS/395-407. Among these mutants, LS/391-407 and LS/395-407, both lacking the RGD motif but retaining the (D)LFEIFEIER sequence, were barely active in binding to α8β1 integrin, whereas LS/381-407, an N-terminal deletion mutant possessing both the RGD motif and the DLFEIFEIER sequence, was fully active in binding to α8β1 integrin (Fig. II-5C). Taken together, these results indicate that both the RGD motif and the DLFEIFEIER sequence
Fig. II-5. Binding activities of α8β1 integrin toward deletion mutants of the RGD-linker segment. A, amino acid sequences of deletion mutants of the RGD-linker segment. The mutant proteins were expressed in bacteria as GST fusion proteins. The RGD motif is indicated in **bold**. The vector-derived sequences are shown in *italics*. B, SDS-PAGE profiles of the mutant proteins (1 μg/lane) stained with CBB. The positions of molecular size markers are shown in the left margin. C and D, binding activities of α8β1 (C) and αVβ3 (D) integrins to the mutant proteins. Microtiter plates were coated with the individual mutant proteins (10 nM) and incubated with the integrins at the indicated concentrations. Bound integrins were quantified as described in the Experimental Procedures. The results represent the means of duplicate determinations.
at 10 residues to the C-terminal side of the RGD motif are required for the high-affinity binding of nephronectin to α8β1 integrin.

In contrast to the clear dependence of α8β1 integrin on both the RGD motif and the DLFEIFEIER sequence for binding to nephronectin, the binding activities of the mutant fragments to αVβ3 integrin, another integrin known to bind to nephronectin (Brandenberger et al., 2001), remained almost unchanged regardless of the presence or absence of the DLFEIFEIER sequence, as long as the fragments contained the RGD motif (Fig. II-5D). Therefore, the DLFEIFEIER sequence is involved in nephronectin recognition by α8β1 integrin but not αVβ3 integrin.

**Identification of Critical Amino Acid Residues in the DLFEIFEIER Sequence**—To further define the amino acid residues in the DLFEIFEIER sequence that are critical for the high-affinity binding to α8β1 integrin, we produced a series of alanine substitution mutants of LS/378-403, in which individual amino acid residues of the DLFEIFEIER sequence were substituted with alanine (Fig. II-6A), except for Asp-394 that is not conserved in nephronectin among different species (see Fig. II-13). Although the L395A, E397A, I398A and R403A substitutions did not cause any detectable decreases in the integrin binding activity of LS/378-403, the E400A, I401A and E402A mutants exhibited moderate reductions (Fig. II-6B), resulting in 2-, 3- and 5-fold decreases in their integrin binding affinities, respectively, compared with that of LS/378-403 (Table II-2). Small, yet reproducible, decreases were also observed for the F396A and F399A mutants.

Given the involvement of two glutamic acid residues (Glu-400 and Glu-402) in
nephronectin recognition by $\alpha 8\beta 1$ integrin, we substituted both of these Glu residues with alanine. The resulting double-substitution mutant showed a significant decrease in the $\alpha 8\beta 1$ binding activity, although no such additive effects were found when E397A substitution was combined with E400A or E402A substitution. Therefore, the marked

Fig. II-6. Effects of alanine substitutions within the LFEIFEIER sequence on the integrin binding activity. A, amino acid sequences of LS/378-403 mutants with alanine substitutions. The mutant proteins are designated by the substituted residues. B and C, binding activities of LS/378-403 and its alanine-substituted mutants toward $\alpha 8\beta 1$ (B) and $\alpha V\beta 3$ integrins. Microtiter plates were coated with the alanine-substituted mutants and incubated with integrins (1 or 3 nM) in the presence of 1 mM Mn$^{2+}$. Bound integrins were quantified as described in the Experimental Procedures. The results represent the means of duplicate determinations.
loss of α8β1 binding activity upon the E400/402A double substitutions was not simply due to an extra loss of negative charges but due to the involvement of both residues in binding to α8β1 integrin. Despite the substantial impact of the E400/402A double mutations on binding to α8β1 integrin, these mutations did not compromise the binding affinity of LS/378-403 toward αVβ3 integrin (Fig. II-6C), underscoring a role of Glu-400/Glu-402 in the high-affinity binding of nephronectin to α8β1 integrin. Given that I401A substitution also caused a moderate decrease in the α8β1 integrin binding activity, these results raised the possibility that the EIE sequence serves as an auxiliary recognition site within nephronectin that directly interacts with α8β1 integrin in concert with the RGD motif.

**TABLE II-2. Dissociation constants of α8β1 integrin toward LS/378-403 after alanine scanning mutagenesis**

<table>
<thead>
<tr>
<th>Ligands</th>
<th>Kd (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS/378-403</td>
<td>0.44±0.04</td>
</tr>
<tr>
<td>L395A</td>
<td>0.44±0.08</td>
</tr>
<tr>
<td>F396A</td>
<td>0.84±0.22</td>
</tr>
<tr>
<td>E397A</td>
<td>0.42±0.06</td>
</tr>
<tr>
<td>I398A</td>
<td>0.38±0.04</td>
</tr>
<tr>
<td>F399A</td>
<td>0.72±0.12</td>
</tr>
<tr>
<td>E400A</td>
<td>0.95±0.12</td>
</tr>
<tr>
<td>I401A</td>
<td>1.3±0.2</td>
</tr>
<tr>
<td>E402A</td>
<td>2.5±0.4</td>
</tr>
<tr>
<td>R403A</td>
<td>0.51±0.08</td>
</tr>
<tr>
<td>E397/400A</td>
<td>0.81±0.23</td>
</tr>
<tr>
<td>E397/402A</td>
<td>4.4±0.6</td>
</tr>
<tr>
<td>E400/402A</td>
<td>ND⁶</td>
</tr>
</tbody>
</table>

²Values represent the means ± SD of three independent experiments.

⁶The dissociation constant was not determined owing to only partial saturation at the highest integrin concentration examined.
**Effects of the LFEIFEIER Peptide on the Interaction of α8β1 Integrid with Nephronectin**—To address the role of the LFEIFEIER sequence as one of the bipartite α8β1 integrin recognition sites in nephronectin, we examined whether a synthetic peptide modeled after the sequence was able to inhibit the interaction of nephronectin with α8β1 integrin. We synthesized a 23-mer peptide encompassing the region from the RGD motif to the LFEIFEIER sequence and its mutant forms with RGD→RGE and EIE→AIA substitutions (Fig. II-7A), and examined their effects on the binding of nephronectin to α8β1 integrin (Fig. II-7B). The 23-mer peptide strongly inhibited the binding of α8β1 integrin to nephronectin with an IC50 of ~0.6 nM (Fig. II-7B and Table II-7).

**Fig. II-7. Inhibition of α8β1 integrin binding to nephronectin by synthetic peptides.** A, amino acid sequences of the synthetic peptides tested. The RGD motifs are shown in bold. B, α8β1 integrin (1 nM) was incubated on microtiter plates coated with full-length nephronectin (10 nM) in the presence of increasing concentrations of synthetic peptides. To prevent precipitation of the peptides, the integrin binding assays were performed in the presence of 10% DMSO. The amounts of bound α8β1 integrin are shown as percentages relative to the control, in which α8β1 integrin was incubated on nephronectin-coated plates in the presence of 10% DMSO. The results represent the means of duplicate determinations. Closed diamonds, 23 AA (23-mer containing both the RGD motif and the LFEIFEIER sequence); closed squares, 23 AA-AIA (23-mer with the E400/402A double mutation); closed triangles, 23 AA-RGE (23-mer with the RGD → RGE mutation); closed circles, NN-RGD; open diamonds, NN-RGE; open squares, NN-EIE; open circles, NN-RGD plus an equal amount of NN-EIE.
TABLE II-3. Inhibition of α8β1-nephronectin interaction by synthetic peptides

<table>
<thead>
<tr>
<th>Peptides</th>
<th>IC50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>23 AA</td>
<td>0.63±0.14</td>
</tr>
<tr>
<td>23 AA-AIA</td>
<td>7.0±0.9</td>
</tr>
<tr>
<td>23 AA-RGE</td>
<td>650±50</td>
</tr>
<tr>
<td>NN-RGD</td>
<td>1200±200</td>
</tr>
<tr>
<td>NN-RGE</td>
<td>NDb</td>
</tr>
<tr>
<td>NN-EIE</td>
<td>NDb</td>
</tr>
<tr>
<td>NN-RGD + NN-EIE</td>
<td>1300±500</td>
</tr>
</tbody>
</table>

*Determined based on the data in Fig. 5B. The values represent the means ± SD of three independent determinations.

| NDb Not determined. |

II-3). Since this IC50 value was comparable to the dissociation constant of α8β1 integrin for nephronectin, the 23-mer peptide appeared to be fully competent in the high-affinity binding to α8β1 integrin. Substitution of the EIE motif with AIA resulted in an ~10-fold decrease in the potency of the peptide to inhibit the integrin-nephronectin interaction, while substitution of the RGD motif with RGE resulted in an ~1000-fold decrease. These results indicate that, although both the RGD and EIE motifs are involved in the binding of α8β1 integrin to nephronectin, the contribution of the RGD motif is much greater than that of the EIE motif in securing the association of the synthetic peptide with α8β1 integrin. We also synthesized two shorter peptides harboring the RGD or EIE motif (Fig. II-7A), and assessed their potencies to inhibit the binding of α8β1 integrin to nephronectin. The PRGDVFIP peptide containing only the RGD motif was moderately inhibitory toward the binding with an IC50 of 1.2 µM, ~2000-fold less potent than the 23-mer peptide. On the other hand, the LFEIFEIER peptide containing only the EIE motif was barely inhibitory, in good agreement with the dominant contribution of the RGD motif over the EIE motif in the association of the 23-mer peptide with α8β1
integrin. The LFEIFEIER peptide did not potentiate the inhibitory activity of the PRGDVFIP peptide when mixed with an equimolar amount of the RGD-containing peptide, suggesting that the RGD and EIE motifs need to be aligned in tandem within the same polypeptide to exert their potency to competitively inhibit the binding of α8β1 integrin to nephronectin.

**Trans-complementation of Activity between the RGD and EIE Motifs**—To further explore the role of the EIE motif in the high-affinity binding of nephronectin to α8β1 integrin, we employed *trans*-complementation assays (Obara et al., 1988) using GST fusion proteins containing only the RGD (LS/378-393) or EIE (LS/395-407) motif. Plates were coated with LS/378-393, followed by a second coating with increasing concentrations of LS/395-407, and then subjected to integrin binding assays. Plates coated with LS/378-407 or GST were also subjected to a second coating with LS/395-407 and subsequent integrin binding assays as controls. Although LS/378-393 alone was inactive in binding to α8β1 integrin, it became active upon the second coating with increasing concentrations of LS/395-407 (Fig. II-8A). A second coating with LS/395-407 at >10 nM also endowed GST with integrin binding activity. However, the activity restored to LS/378-393 was significantly greater than the activity restored to the GST-coated plates, indicating that close juxtaposition of LS/395-407 harboring the EIE motif and LS/378-393 harboring the RGD motif synergistically potentiated their integrin binding activities. Saturation integrin binding assays demonstrated that the affinity of LS/378-393 for α8β1 integrin was significantly enhanced upon a second coating with
**Fig. II-8.** Trans-complementation assays of recombinant nephronectin fragments.

*Fig. A.* 96-well microtiter plates were coated with LS/378-407 (*closed squares*), LS/378-393 (*open squares*) and GST (*open triangles*), washed with PBS and then coated a second time with increasing concentrations of LS/395-407 lacking the RGD motif. The plates were subjected to integrin binding assays using 1 nM α8β1 integrin as described in the Experimental Procedures.  

*Fig. B.* Titration curves of α8β1 integrin bound to substrates coated with LS/378-393, followed by a second coating with LS/395-407 at 0 nM (*diamonds*), 1 nM (*squares*), 10 nM (*triangles*) and 100 nM (*circles*). Note that the affinities of α8β1 integrin for LS/378-393 are synergistically enhanced by the presence of LS/395-407.  

*Fig. C.* Microtiter plates were coated with 10 nM LS/378-393, washed with PBS and then coated a second time with increasing concentrations of LS/395-407 (*closed diamonds*), LS/395-407(E400/402A) (*closed squares*) and GST (*open triangles*). The plates were subjected to integrin binding assays using 1 nM α8β1 integrin as described in the Experimental Procedures.  

*Fig. D.* Trans-complementation activities of the RGD motif and the LFEIFEIER sequence to bind to αVβ3 integrin (1 nM) was assessed as in *Fig. A.* The requirement of the Glu-400 and Glu-402 residues was assessed by trans-complementation assays using αVβ3 integrin as probes. Note that the LFEIFEIER sequence has nothing to do with the enhancement of the interaction of αVβ3 integrin. The results represent the means of duplicate determinations.
increasing concentrations of LS/395-407 (Fig. II-8B) and reached a dissociation constant of 1.2 nM, which was comparable to that of LS/378-407 containing both the RGD and EIE motifs within the same polypeptide chain. These results support the conclusion that nephronectin has a bipartite \( \alpha_8 \beta_1 \) integrin binding site comprising the RGD and EIE motifs, of which the EIE motif plays an auxiliary role in stabilizing the complex of nephronectin and \( \alpha_8 \beta_1 \) integrin. To further confirm the importance of the EIE motif in the high-affinity binding of \( \alpha_8 \beta_1 \) integrin to nephronectin, we performed trans-complementation assays using an LS/395-407 mutant in which the two glutamic acid residues in the EIE motif were replaced with alanine, designated LS/395-407(E400/402A). The LS/395-407 mutant was incapable of potentiating the integrin binding activity of LS/378-393, even at the highest concentrations used (Fig. II-8C). No such synergism was observed in trans-complementation assays with \( \alpha V \beta 3 \) integrin (Fig. II-8D and E), consistent with the specific role of the EIE motif in selective recognition of nephronectin by \( \alpha_8 \beta_1 \) integrin.

**Involvement of the EIE Motif in \( \alpha_8 \beta_1 \) Integrin-dependent Cell Adhesion**—The role of the EIE motif in nephronectin recognition by \( \alpha_8 \beta_1 \) integrin was further assessed by cell adhesion assays. To this end, we transfected HT1080 human fibrosarcoma cells with an \( \alpha 8 \) integrin cDNA and isolated stable transfectants expressing \( \alpha 8 \) integrin. Expression of the exogenous \( \alpha 8 \) integrin in the transfectants (designated HT1080-A8 cells) was verified by flow cytometry (Fig. II-9A) and immunoblotting (Fig. II-9B). HT1080-A8 cells were readily adherent to nephronectin upon coating on substrates at >1 nM, while
Fig. II-9. Establishment of HT1080 cells stably expressing human α8 integrin. A, flow cytometric analyses of α8 integrin on HT1080 cells. Control and α8 integrin-transfected HT1080 cells (the latter designated HT1080-A8 cells) were incubated with an anti-human α8 mAb (7A5) and stained with an Alexa Fluor® 488-conjugated secondary antibody. The expressions of α8 integrin are shown as gray lines, while the black areas indicate the negative controls incubated with mouse IgG instead of an anti-α8 mAb. B, cell lysates were subjected to SDS-PAGE, followed by immunoblotting with an anti-human α8 integrin mAb (10A8) (upper panel) or anti-actin polyclonal antibody (lower panel). C, HT1080 (closed circles) and HT1080-A8 (open circles) cells were incubated at 37°C for 30 min on 96-well microtiter plates coated with increasing concentrations of nephronectin. Adherent cells were fixed and stained as described in the Experimental Procedures. The results represent the means ± SE of triplicate assays.

untransfected HT1080 cells did not adhere to nephronectin even after coating on substrates at 40 nM (Fig. II-9C), confirming that the adhesion of HT1080-A8 cells to nephronectin was dependent on α8β1 integrin.

To assess the α8β1 integrin binding activities of the RGD-linker segment and its mutant proteins on the basis of cell adhesion assays, HT1080-A8 cells were allowed to adhere to substrates coated with increasing concentrations of the linker segment and its mutants with deletions and/or amino acid substitutions. The RGD-linker was equally active to intact nephronectin in promoting the adhesion of HT1080-A8 cells, while
LS/378-403, which harbors both the RGD and EIE motifs and retains comparable \( \alpha 8\beta 1 \) integrin binding activity to that of intact nephronectin, was significantly less active than intact nephronectin and the control RGD-linker in promoting the adhesion of HT1080-A8 cells (Fig. II-10A). The difference between the cell-adhesive activities of the full-length RGD-linker and LS/378-403 could be due to the involvement of non-integrin-type adhesion receptors that possibly recognize the N-terminal region of the linker segment absent from LS/378-403. LS/378-393, which contains the RGD motif but not the EIE motif, was significantly less active than LS/378-403. A similar reduction in cell-adhesive activity was also observed with LS/378-393 in cell adhesion assays.

![Graph](image1)

**Fig. II-10. Cell-adhesive activities of recombinant nephronectin and its fragments.** A, HT1080-A8 cells were incubated at 37°C for 30 min on 96-well microtiter plates coated with increasing concentrations of nephronectin (NN, closed diamonds), the RGD-linker segment expressed as a GST fusion protein (RGD-linker (GST), closed squares), LS/378-403 (closed triangles), LS/378-393 (closed circles), LS/378-403(E400/402A) (open triangles) and GST (open circles). Adherent cells were fixed and stained as described in the Experimental Procedures. The results represent the means ± SE of triplicate assays. B, representative images of HT1080-A8 cells adhering to substrates coated with the indicated proteins. Bar = 50 \( \mu \)m.
performed in the presence of Mn$^{2+}$ (data not shown), together corroborating the importance of the EIE motif in the potent cell-adhesive activity of nephronectin. In support of the critical role of the EIE motif, alanine substitution of the two Glu residues in the EIE motif of LS/378-403 resulted in a marked reduction in the cell-adhesive activity, leading to comparable activity to that of LS/378-393.

Integrin-mediated adhesion has been shown to transduce signals that induce reorganization of the actin cytoskeleton, thereby leading to cell spreading on substrates. Cells adhering to LS/378-403 exhibited well-spread polygonal shapes at coating concentrations of >40 nM, while cells were poorly spread on LS/378-393 and LS/378-403(E400/402A) even after coating at 320 nM (Fig. II-10B). These results indicate that LS/378-403 is competent in stimulating actin cytoskeleton reorganization through binding to α8β1 integrin, and that the EIE motif within the fragment is indispensable for stimulation of integrin-mediated signaling events. Similar results were obtained in cell adhesion assays using K562 erythroleukemic cells stably transfected with chick α8 integrin (data not shown).

The importance of the EIE motif for α8β1 integrin-dependent cell adhesion to nephronectin was further addressed by cell adhesion inhibition assays using synthetic peptides. Adhesion of HT1080-A8 cells to nephronectin was strongly inhibited by the 23-mer peptide with an apparent IC50 of 3.8 µM (Fig. II-11). Substitution of the EIE motif with AIA resulted in an ~30-fold decrease in the potency of the 23-mer peptide to inhibit the cell adhesion on nephronectin, while substitution of the RGD motif to RGE resulted in an almost complete loss of the inhibitory activity. These results were in line
with those obtained in the peptide inhibition assays of direct binding of α8β1 integrin to nephronectin (Fig. II-7), underscoring the greater contribution of the RGD motif over the EIE motif in stabilizing the association of nephronectin with α8β1 integrin. Shorter peptides containing either the RGD or EIE motif did not exert inhibitory effects, even at the highest concentrations examined, consistent with the requirement for both the RGD and EIE motifs within the same peptide for efficient inhibition of the interaction between nephronectin and α8β1 integrin.

![Graph](image-url)  
**Fig.II-11. Inhibition of α8β1 integrin-mediated cell adhesion by synthetic peptides.** HT1080-A8 cells were incubated on plates coated with 3 nM nephronectin for 30 min in the presence of increasing concentrations of the following synthetic peptides: 23-mer containing both the RGD motif and LFEIFEIER sequence (23 AA, closed diamonds); 23-mer with the E400/402A double mutation (23 AA-AIA, closed squares); 23-mer with the RGD→RGE mutation (23 AA-RGE, closed triangles); PRGDVFIP (RGD, closed circles); and LFEIFEIER (EIE, open diamonds). Adherent cells were fixed and stained as described in the Experimental Procedures. Adhesion of cells in the presence of 0.5% DMSO was taken as 100%. The results represent the means ± SE of triplicate determinations.
II-5 Discussion

In the present study, I investigated the interactions of α8β1 integrin with a panel of RGD-containing proteins and found that α8β1 integrin binds strongly and preferentially to nephronectin with an ~100-fold higher affinity than those to other RGD-containing proteins including fibronectin and vitronectin. In addition to the RGD-containing proteins examined, α8β1 integrin has been shown to bind to osteopontin, the latency-associated peptide of TGF-β, tenascin-W, MAEG and QBRICK/Frem1 in RGD-dependent manners (Denda et al., 1998b; Lu et al., 2002; Kiyozumi et al., 2005; Osada et al., 2005; Scherberich et al., 2005). Although the binding affinities for these ligand proteins remain to be determined, the following observations, together with my data, indicate that nephronectin is the most preferred ligand for α8β1 integrin with the highest binding affinity. Recombinant α8β1 integrin bound to nephronectin, but not to vitronectin or osteopontin, in far-western blotting assays with tissue extracts (Brandenberger et al., 2001). Mice deficient in nephronectin expression displayed kidney agenesis or hypoplasia and were associated with a marked reduction in GDNF expression in the metanephric mesenchyme, resembling the phenotypes observed in mice deficient in α8 integrin expression (Muller et al., 1997; Linton et al., 2007). Consistent with the similarities in phenotypes between the nephronectin-deficient and α8 integrin-deficient mice, the distribution patterns of nephronectin in embryonic lungs and kidneys overlapped with those of α8β1 integrin (Muller et al., 1997; Brandenberger et al., 2001; Wagner et al., 2003; Manabe et al., 2008). Recently, my collaborators examined the
distributions of α8β1 ligands by integrin overlay assays using frozen mouse tissues and found that the signals for α8β1 ligands significantly overlapped with those for nephronectin visualized by immunohistochemistry (M. Takeichi and D. Kiyozumi, unpublished observations), further supporting the possibility that nephronectin is the physiological ligand for α8β1 integrin with the highest binding affinity among the RGD-containing proteins identified to date.

My results showed that the RGD-linker segment connecting the N-terminal five EGF-like repeats and the C-terminal MAM domain harbors full activity to bind to α8β1 integrin. Although the RGD motif in the linker segment is a prerequisite for the integrin binding activity, several lines of evidence indicate that the LFEIFEIER sequence at ~10 amino acid residues downstream of the RGD motif is required for the high-affinity binding of the linker segment to α8β1 integrin. First, deletion of the LFEIFEIER sequence from the linker segment resulted in a dramatic loss of the integrin binding activity, even though the RGD motif remained unperturbed. Second, trans-complementation assays demonstrated that almost full binding activity toward α8β1 integrin was restored to LS/378-393, a deletion mutant containing only the RGD motif, upon a second coating with LS/395-407, another deletion mutant containing only the LFEIFEIER sequence. Third, a synthetic 23-mer peptide covering the region from the RGD motif to the LFEIFEIER sequence strongly inhibited the binding of α8β1 integrin to nephronectin, while shorter peptides containing either the RGD motif or the LFEIFEIER sequence were only poorly inhibitory. Taken together, these results point to the conclusion that the LFEIFEIER sequence synergizes with the RGD motif to ensure
the high-affinity binding of nephronectin to α8β1 integrin.

Alanine scanning mutagenesis of the LFEIFEIER sequence revealed that two Glu residues within the sequence, *i.e.*, Glu-400 and Glu-402, play critical roles in the synergy of this sequence with the RGD motif. Alanine substitution of these individual Glu residues resulted in moderate decreases in the integrin binding activity of the linker segment, while double substitution of both Glu residues severely impaired the activity. In support of the critical roles of these two Glu residues, E400/402A double mutation abrogated the activity of LS/395-407, a mutant fragment containing the LFEIFEIER sequence but not the RGD motif, to potentiate the integrin binding activity of LS/378-393, a fragment containing only the RGD motif, in *trans*-complementation assays. In addition to these two Glu residues, several hydrophobic residues in the LFEIFEIER sequence may also be involved in nephronectin recognition by α8β1 integrin, since alanine substitution of Phe-396, Phe-399 and Ile-401 caused moderate reductions in the integrin binding activity of the RGD-linker segment. Recently, DiCara et al. (DiCara et al., 2007) reported that hydrophobic residues downstream of the RGD motif are involved in potentiation of the binding affinity of αVβ6 integrin toward RGD-containing oligopeptides modeled after its natural ligands, *i.e.*, the coat protein of foot-and-mouth disease virus and the latency-associated peptides of TGFβ proteins. They demonstrated that oligopeptides capable of binding to αVβ6 integrin with high affinities had an RGDLXXL or RGDLXXI motif, of which LXXL/I forms a stable helix with two hydrophobic residues (*i.e.*, Leu and Leu/Ile) exposed almost in apposition on one face of the helix, suggesting that these hydrophobic residues, together with the RGD motif,
directly interact with the ligand binding pocket of αVβ6 integrin (DiCara et al., 2007). Given the similarity between nephronectin and the natural ligands for αVβ6 integrin in the requirement for a post-RGD sequence for high-affinity integrin binding, the hydrophobic residues within the LFEIFEIER sequence may also be directly involved in ligand recognition by α8β1 integrin. Consistent with this possibility, the LFEIFEIER sequence was predicted to form a helix by PAPIA, an algorithm for protein secondary structure prediction (Akiyama et al., 1998).

The requirement for an auxiliary site for selective high-affinity recognition of RGD-containing ligands by integrins has been documented for other integrin-ligand pairs. Fibronectin has been the prototype for such ligands harboring a so-called synergy site, requiring not only the tenth type III module containing the RGD motif but also the preceding type III module (FNIII9) for its high-affinity binding to α5β1 integrin (Aota et al., 1994; Redick et al., 2000). The PHSRN sequence in the FNIII9 module was shown to be critical for the synergistic activity (Aota et al., 1994). The natural ligands for αVβ6 integrin also possess such an auxiliary site immediately on the C-terminal side of the RGD motif as described above (DiCara et al., 2007). Despite the available evidence, including the data in the present study, for the requirement of synergy sites for high-affinity binding of RGD-containing ligands to integrins, it remains to be elucidated how the synergy sites potentiate the binding affinities between integrins and their RGD-containing ligands. This is in striking contrast to the interactions of the RGD motif with integrins, since determination of the crystal structures of integrins complexed with cyclic RGD-like peptides revealed that the arginine side chain of the RGD motif fits into
a cleft in the β-propeller domain of integrin α subunits, while the aspartate side chain coordinates the divalent cation at the metal ion-dependent adhesion site (designated MIDAS) of β subunits (Xiong et al., 2002; Xiao et al., 2004). It should be noted, however, that in addition to the residues defined by the crystal structures to directly interact with the RGD motif, a group of residues located on the upper or side faces of the β-propeller domain of the α subunits have also been implicated in ligand binding by integrins. Specifically, epitope mapping of function-blocking anti-integrin antibodies as well as mutational analyses of the integrin α subunits indicated that the ligand binding specificities of α5β1 and αVβ1 integrins were dependent on residues in blades 2 and 3 of the β-propeller domain of their α subunits (Humphries et al., 2000; Mould et al., 2000; Humphries et al., 2003), of which Tyr-208 and Ile-210 in blade 3 of the integrin α5 subunit were shown to recognize the synergy site of fibronectin (Mould et al., 2003). Furthermore, mutations that disrupted ligand binding by αIIbβ3 integrin were found to be clustered on the top or side of the β-propeller domain of the αIIb subunit, and mostly located within blades 2 and 3 (Kamata et al., 2001). Similarly, the acidic clusters located on blade 3 of the β-propeller domain of the α7 subunit were shown to determine the ligand-binding specificity of α7β1 integrin (von der Mark et al., 2007). Given the involvement of the upper or side faces of the β-propeller domain, particularly those involving blades 2 and 3, in the high-affinity ligand recognition by integrins, the same regions of the β-propeller domain of the α8 subunit may be involved in recognition of the LFEIFEIER sequence, thereby ensuring the high-affinity binding of nephronectin to α8β1 integrin. It is interesting to note that the residues comprising the upper or side
loops of the β-propeller domain differ significantly among RGD-recognizing integrins. The α8 integrin subunit is unique in that it possesses evolutionarily conserved basic amino acid clusters at the loops connecting blades 1 and 2 and within blade 3 of its β-propeller domain (Fig. II-12), the latter being equivalent to the loops involved in the ligand-binding specificities of α5β1 and α7β1 integrins (Moud et al., 2003; von der Mark et al., 2007). Since two Glu residues within the LFEIFEIER sequence are required for high-affinity binding of nephronectin to α8β1 integrin and evolutionarily conserved among vertebrates (Fig. II-13), it is tempting to speculate that the cluster of basic amino acid residues in the loop either between blades 1 and 2 or within blade 3 of the α8 β-propeller domain form salt bridges with the acidic residues of the EIE motif, thereby sustaining the high-affinity recognition of nephronectin by α8β1 integrin.

Synthetic RGD-containing peptides have been widely used as probes that specifically block the interactions of RGD-binding integrins with their ligands in a variety of biochemical and cell biological assays (Pierschbacher and Ruoslahti, 1984; Akiyama and Yamada, 1985; Manabe et al., 1997). However, the RGD peptides are equally inhibitory toward all RGD-binding integrins, and therefore cannot be used as specific probes for the biological consequences of interactions between defined RGD-binding integrins and their physiological ligands. Furthermore, the absence of the synergy sequences that potentiate the integrin binding affinities of RGD-containing ligands leads to a requirement for high concentrations of RGD peptides to block the integrin-ligand interactions. For instance, RGD peptide concentrations of >1 mM are required to effectively block α5β1-mediated cell adhesion onto fibronectin-coated substrates (Pierschbacher and Ruoslahti, 1984;
Fig. II-12. Multiple sequence alignment of the β-propeller domain of RGD-binding integrin α subunits. Amino acid sequences of the β-propeller domain of human integrin α8, αV, α5, and α1b subunits were aligned by Clustal W (Thompson et al., 1994). Helices (cylinders) and strands (arrows) are predicted based on the secondary structure of αV integrin (Xiong et al., 2001). Brackets and an arc above the sequences indicate the loops located in the upper and side faces of the β-propeller, respectively. Loops that exhibit significant divergence in amino acid sequences among different α subunits are indicated by asterisks. Basic and acidic residues are boxed in black and gray, respectively.
Fig. II-13. Multiple sequence alignment of the integrin-binding site of nephronectins from different vertebrate species. Amino acid sequences in the RGD-containing linker segment of nephronectins from various vertebrate species were aligned by Clustal W (Thompson et al., 1994). The RGD and EIE motifs are highlighted in black and dark gray boxes, respectively. Two phenylalanine residues are labeled in light gray boxes.

Akiyama and Yamada, 1985; Manabe et al., 1997). In this regard, the 23-mer peptide containing both the RGD motif and the LFEIFEIER sequence fully mimics the interaction of nephronectin with α8β1 integrin and completely blocks the interaction at a concentration of 10 nM, but has no inhibitory effects on the interaction between fibronectin and α5β1 integrin (Y.S., unpublished observation). Given the remarkable potency of the 23-mer peptide in specifically blocking the nephronectin-α8β1 integrin interaction at nanomolar concentrations, the 23-mer peptide represents a promising probe for elucidating the biological functions of this integrin-ligand interaction in both physiological and pathological processes.

In summary, I have shown that nephronectin is the most preferred high-affinity ligand for α8β1 integrin and that the specificity and high-affinity binding toward the integrin are fully recapitulated by a 23-amino acid residue fragment harboring both the RGD motif and the LFEIFEIER sequence, the latter serving as an auxiliary site that ensures the specific high-affinity binding of nephronectin to α8β1 integrin in concert with the RGD
motif. Although the mechanism by which the synergy site potentiates the integrin binding affinity remains to be validated by determination of the 3D structure of the fully active RGD-linker segment at the atomic level, these results provide, for the first time, the molecular basis of the specific interaction between nephronectin and α8β1 integrin, and highlight the bipartite nature of the integrin recognition site of nephronectin.
General Discussion

To elucidate the molecular characteristics and the domain functions of nephronectin, I established recombinant expression systems to prepare and purify full-length nephronectin and its domain deletion mutants, and examined their activities to bind to a panel of GAG chains as well as α8β1 integrin, one of the known receptors for nephronectin. I demonstrated that nephronectin had potency in binding to heparin/heparan sulfate and chondroitin sulfate-E through its MAM domain and five EGF-like repeats, respectively. In addition, I tried to determine the minimal amino acid sequences of nephronectin required for potent binding to α8β1 integrin, and found that the α8β1 integrin required a bipartite binding site consisting the RGD motif and the LFEIFEIER sequence, the latter serving as an auxiliary binding site that enhances the activity to bind to α8β1 integrin. The results obtained in this study should provide new insights into the physiological functions of nephronectin.

In this chapter, I discuss the problems and prospects for purification and functional characterization of nephronectin and integrins, and integrate the descriptive data to understand how nephronectin can contribute to regulate kidney morphogenesis along with α8β1 integrin and how RGD-binding integrins selectively recognize their ligands.

Problems and Prospects for Purified Nephronectin and Integrins

To identify molecular characteristics of nephronectin, I purified nephronectin as recombinant proteins. However, there are some problems and questions with respect to
the methods of purification.

Problems in purification of nephronectin as a recombinant protein: Does recombinant nephronectin reflect physiological functions?

In this study, I purified recombinant nephronectin as an intact form. A FLAG tag was added to the C-terminus of nephronectin to facilitate affinity purification by using anti-FLAG mAb and FLAG peptide. This method allows me to purify recombinant nephronectin without denaturing processes. Although many of ECM proteins had been purified as recombinant proteins and shown to exhibit biological properties similar to native proteins, recombinant nephronectin has some problems. First, the recombinant nephronectin contains a FLAG tag at its C-terminus. Since a FLAG tag consists of many acidic residues and was added in the vicinity of the MAM domain that is rich in basic amino acid residues, it could be possible that the FLAG tag has some effects on structures of the MAM domain and/or reduces heparin-binding activities of nephronectin. Second, since nephronectin was purified by affinity chromatography with anti-FLAG mAb interacting with C-terminal FLAG tag, the purified nephronectin could contain N-terminally processed forms and intact nephronectin. Third, recombinant nephronectin should be over-glycosylated compared with native nephronectin. The apparent molecular mass of purified recombinant nephronectin expressed in 293-F cells was ~120 kDa under reducing conditions, whereas that expressed in 293-T cells was ~100 kDa (data not shown). Moreover, nephronectin in newborn kidney extracts had been shown to migrate at 70~90 kDa upon SDS-PAGE (Brandenberger et al., 2001). These apparent
discrepancies in molecular masses of nephronectin should arise from differences in cell types expressing nephronectin, since the structure and the number of oligosaccharides attached depend on the cell type, its enzymatic machinery, its developmental stage, and its nutritional or pathological state (Durand and Seta, 2000). All of these problems demonstrate that, to further analyze the authenticity of recombinant nephronectin, it is necessary to purify endogeneous nephronectin and to compare it with recombinant nephronectin. In this study, I showed that recombinant nephronectin had potency to bind to heparin and chondroitin sulfate-E and to form homo-multimers. These observations raise a possibility that the combination of chromatographies, including heparin, chondroitin sulfate-E, and gel filtration chromatographies, may enable me to purify endogenous nephronectin.

Potential problems in purification of integrins as recombinant proteins: a possibility that the C-terminal clasp diminishes the binding activity of recombinant integrins

In this study, I expressed and purified integrins as recombinant proteins. The recombinant integrins consist of extracellular regions of α and β subunits conjugated with ACID and BASE peptides, respectively, to form αβ heterodimers with an interchain disulfide bond. The feasibility of this strategy for the expression of recombinant integrins has previously been demonstrated for many integrins including α3β1, α4β1, α5β1, α6β1, and αVβ3 (Lu et al., 2001; Takagi et al., 2001; 2002a; 2002b; Nishiuchi et al., 2006). The activities of these recombinant integrins have been shown to principally reproduce those of integrins endogenously expressed on the cell surface and purified from tissue
extracts. Although the recombinant integrins appear to have activities as well as endogenous ones, they have a potential drawback: the artificial constraint at the C-termini of integrins. Whereas the N-terminal head region of integrins has been shown to make direct contacts with their ligands, the C-terminal tail region is also implicated in the interaction by regulating the activities of integrins. It is well known that integrins change their conformation according to their activation/inactivation states. An increased number of studies have established that the inactivated integrin exhibits bent, V-shape conformation with its head region faced toward plasma membranes, while ligand binding induces a large-scale conformational rearrangement in which the integrin extends with a “switch-blade” like motion and moves tail regions of α and β subunits apart from one another with a “leg spread” like motion (Takagi et al., 2002b; Luo and Springer, 2006; Luo et al., 2007). The “leg spreading” of integrin tail region has been shown to be involved in integrin activation. The membrane-proximal cytoplasmic regions of integrin α and β subunits are thought to interact with each other by forming a salt bridge, preventing integrin activation by stabilizing the low-affinity state (Hughes et al., 1996; Banno and Ginsberg, 2008). Mutations or deletions in the membrane-proximal sequences that break the salt bridge induce integrin activation (O’Toole et al., 1991; 1994). Consistently, the binding affinities of recombinant αVβ3 integrin were further increased by cleavage of the C-terminal ACID-BASE coiled-coil region, even though the integrin was activated by addition of Mn²⁺ (Takagi et al., 2002b). These data together strongly indicate not only that the interaction between the α and β membrane-proximal regions maintains the integrins in an inactivation state, but also that the C-terminal constraint
lessens the activities of recombinant integrins to bind to their ligands. Thus, there is no assurance that the activity of recombinant $\alpha_8\beta_1$ integrin used in this study is equivalent to that of endogenous one. Indeed, the results of solid-phase binding assays using recombinant $\alpha_8\beta_1$ integrin were somewhat different from those of cell adhesion assays using $\alpha_8\beta_1$ integrin expressed on cell surfaces. In solid-phase assays, the affinity of LS/378-403, which possesses both the RGD and EIE motifs, was $>10$-folds higher than that of LS/378-393, which possesses only the RGD motif (Fig. II-5 and Table II-1), whereas in cell adhesion assays the activity of LS/378-403 was only $\sim 4$-folds higher than that of LS/378-393 (Fig. II-10). Although the activities of recombinant $\alpha_8\beta_1$ integrin cannot be simply compared with those of $\alpha_8\beta_1$ integrin expressed on cells because of the differences in the assay systems, this observation again raises a possibility that the activity of recombinant $\alpha_8\beta_1$ integrin is not equivalent to those of endogenous one.

To address the problems of the differences between the recombinant and endogenous integrins, it is desirable to purify endogenous $\alpha_8\beta_1$ integrin and to compare ligand-binding activities of endogenous integrins with those of recombinant ones. So far, a variety of endogenous integrins, including $\alpha_5\beta_1$, $\alpha_V\beta_3$, $\alpha_V\beta_5$, $\alpha_{IIb}\beta_3$, $\alpha_3\beta_1$, and $\alpha_6\beta_1$, had been purified from human tissues or cell lines (Pytela et al., 1985a; 1985b; Lam et al., 1989; Smith et al., 1990; Nishiuchi et al., 2003). The ligand-mimetic RGD peptides have been used for purifying RGD-recognizing integrins under non-denaturing conditions. Based on my experiments, $\alpha_8\beta_1$ integrin preferentially binds to short amino acid sequences containing the RGD and the EIE motifs but showed only marginal affinity for that containing only the RGD motif. This result raises a possibility that a column
conjugated with peptides containing both the RGD and the EIE motifs enables me to selectively purify \( \alpha 8 \beta 1 \) integrin from tissue extracts. To verify the authenticity of recombinant \( \alpha 8 \beta 1 \) integrin used in this study, the activities of the recombinant \( \alpha 8 \beta 1 \) integrin should be compared with that of \( \alpha 8 \beta 1 \) integrin purified from tissue extracts or cell lines.

**Problems and Prospects for Functional Characterization of Nephronectin and Integrins**

To characterize the molecular functions, I mainly exploited solid-phase binding assays in which the analytes were applied to plastic plates coated with ligands. In some cases, I determined dissociation constants of the interaction from the data of solid-phase binding assays. Here, I focused on the problems of experimental systems for functional characterization of nephronectin and integrins.

*Problems with integrin binding assays in the presence of Mn\(^{2+}\)*

It is well known that integrins require bivalent cations to interact with their ligands. Bivalent cations have multiple effects on integrin functions, including modulation of ligand binding activities and specificities (Kirchhofer et al., 1990). In this study, binding activities of integrins were assessed in the presence of Mn\(^{2+}\) ion, since Mn\(^{2+}\) is known to enhance the binding affinities of integrins to their ligands. The presence of Mn\(^{2+}\) enables me to sensitively detect the bindings of integrins, and therefore to measure dissociation constants of the interaction with a variety of ligands. However, there is a problem
concerning the presence of Mn\(^{2+}\) ion. First, the concentration of Mn\(^{2+}\) ion in the assay conditions was much higher than that in the physiological conditions. The concentrations of Mn\(^{2+}\) in tissues were estimated to be in the range of 1-14 µM (Smith et al., 1994), whereas the binding assays of integrins were performed in the presence of 1 mM Mn\(^{2+}\), far exceeded the physiological concentrations. Second, manganese ions are not the ions that bind to integrins in vivo. Physiologically, Ca\(^{2+}\) and Mg\(^{2+}\) ions are more abundant than Mn\(^{2+}\), and have been shown to coordinate the divalent cation-binding sites of integrins (Xiao et al., 2004). Third, in some cases, the change of coordinating divalent cations alters the binding specificities of integrins. For example, αVβ3 integrin has been shown to interact with fibrinogen in the presence of Mn\(^{2+}\), but not in the presence of Ca\(^{2+}\) (Smith et al., 1994). The binding specificity in the presence of Mn\(^{2+}\) may be attributed to the enhancement of the binding affinity of integrins toward low-affinity ligands, since Mn\(^{2+}\) and Mg\(^{2+}\), but not Ca\(^{2+}\) ions are known to increase the binding affinities of integrins (Smith et al., 1994; Mould et al., 1995). Taken together, these observations raise a possibility that the addition of Mn\(^{2+}\) ion might cause an unfavorable effect on the binding affinity and specificity of integrins.

Despite the fact that the presence of excess Mn\(^{2+}\) may be a non-physiological condition, the binding activity of recombinant α8β1 integrin clearly depends on the presence of the EIE motif on the C-terminal side of the RGD motif, irrespective of the class of divalent cations, i.e., Ca\(^{2+}\)/Mg\(^{2+}\) or Mn\(^{2+}\). Although the activities are less prominent, the recombinant α8β1 integrin is capable of interacting with its known ligands, i.e., fibronectin and vitronectin, in the presence of Mn\(^{2+}\) ion (Schnapp et al.,
These observations strongly suggest following two points that; 1) the binding specificities of recombinant α8β1 integrin follow that of physiological α8β1 even in the presence of Mn^{2+}, and; 2) the EIE motif-dependent interaction of α8β1 integrin is physiological, not an artifact caused by the addition of non-physiological Mn^{2+} ion. The Mn^{2+} ion was also used to determine the ligand-binding specificities and affinities of laminin-binding integrins (Nishiuchi et al., 2006). The specificities of laminin-binding integrins have been shown to remain unchanged regardless of the class of divalent cations. Moreover, the binding affinities of laminins to laminin-binding integrins are in good agreement with the cell-adhesive activities of individual laminins (Fujiwara et al., 2004). Therefore, integrin-binding assays in the presence of 1 mM Mn^{2+} are considered to reflect physiological binding activities of integrins to their ligands, even though the presence of Mn^{2+} might be a non-physiological condition.

Problems in determination of dissociation constants by solid-phase binding assays

To quantitatively assess the interaction of α8β1 integrin with nephronectin, solid-phase binding assays were used to measure apparent dissociation constants for binding of α8β1 integrin to its ligands. The determination of Kd values by solid-phase assays premises that, once the formation of integrin-ligand complex reached equilibrium states, the complex is never disrupted throughout the subsequent procedures, including washing plates and incubation with anti-Velcro antibody and streptavidin. However, there is a striking contradiction. The complex formation and dissociation occur simultaneously
during the incubation of proteins with ligands. When the plates coated with ligands were washed to remove unbound integrins, the dissociation of the complex become predominant rather than the formation of the complex, therefore the condition was no longer at the equilibrium state. As a result, the integrins bound to ligand-coated plates were detected under the non-equilibrium state, thus the calculated Kd values was not the “real” dissociation constants.

Another problem for solid-phase binding assays is that the assays are able to only determine the affinity of the interactions. This means that the affinity of protein-protein interaction ignores the kinetic nature of complex formation and dissociation (Schreiber, 2002). The kinetic analyses of protein interactions provide not only the affinities but also the association and dissociation rate of the interaction. In general, the association rate is limited by diffusion of molecules, geometrical constraints (accessibilities) of the binding sites, and electrostatic forces implicated in a long-range electrostatic steering (Schreiber, 2002), therefore the rate is mainly affected by residues near, but outside, the interaction site (Clackson and Wells, 1995). On the other hand, the dissociation rate is limited by stabilities of the complex, and therefore affected by residues that are part of protein-protein interfaces (Clackson et al., 1998; Wu et al., 2002). Based on the reports described above, kinetic analyses of the interaction, together with mutational analyses, lead to elucidation of amino acid residues involved in the interaction in or outside the protein-protein interfaces. In my study, I calculated dissociation constants of the interaction of α8β1 integrin with mutant proteins of the linker segment of nephronectin, and found that the EIE motif increases the binding affinity of nephronectin to α8β1 integrin, However,
the mechanisms by which the EIE motif enhances the binding affinity remain obscure. Thus, kinetic analyses of the interactions are required to better understand how the EIE motif is involved in the interaction of α8β1 integrin with nephronectin.

**Physiological Roles of Nephronectin in Mouse Development**

In this study, I investigated molecular functions of nephronectin, and revealed the following two points: 1) nephronectin has a potency to bind to highly sulfated GAG chains (Chapter I), and: 2) nephronectin preferentially binds to α8β1 integrin in the RGD and the EIE motif-dependent manner (Chapter II). Here, I integrate the data to understand how nephronectin can contribute to regulate mouse development, especially kidney morphogenesis along with α8β1 integrin.

*The role of nephronectin in regulation of mesenchymal cells*

In epithelial tissues, basement membranes beneath the epithelium are specialized ECMs directly attached to epithelial cells. Consequently, cell-adhesive proteins in the basement membrane have been thought to regulate epithelial cell behaviors, such as survival and proliferation, through interacting with integrins expressed on the epithelium. It should be noted that, despite the localization of nephronectin in the basement membranes, several lines of evidence support the idea that nephronectin regulates mesenchymal but not epithelial cell behaviors. For example, the receptor for nephronectin is α8β1 integrin, which is predominantly expressed in mesenchymal cells of developing kidney (Brandenberger et al., 2001). The phenotypes of mice deficient in
nephronectin expression quite resemble that of α8 integrin-null mice (Muller et al., 1997; Linton et al., 2007), strongly suggesting that nephronectin binds to α8β1 integrin with high selectivity, and that physiological functions of nephronectin are closely linked to those of α8β1 integrin. Furthermore, metanephric mesenchymal cells but not ureteric bud epithelial cells failed to differentiate appropriately in the nephronectin-null mice, resulting in the failure of GDNF expression in the metanephric mesenchymes (Linton et al., 2007). It is interesting to note that the interaction of nephronectin with mesenchymal cells is possibly not restricted to kidney development. As far as my observations are concerned, nephronectin are deposited onto basement membranes in developing hair

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**Fig. D-1 Localization patterns of nephronectin and integrin α8 subunit in mice embryos.** Sections of an oral cavity at E14.5 (A) and a hair follicle at E16.5 (B) mice embryos were stained with antibodies against the extracellular domain of the integrin α8 subunit (red) and nephronectin (green). Nuclei were visualized by staining of Hoechst 33342 (blue). Mesenchymal cells surrounding the tooth germ (A) and beneath the tip of the hair follicle (B) express the integrin α8 subunit. Arrowheads indicate co-localization of nephronectin with α8 integrin. *Scale bar*, 20 µm.
follicles and tooth germs, while mesenchymal cells surrounding the hair follicles and tooth germs express α8β1 integrin, whose distribution patterns are partly overlapped with those of nephronectin (Fig. D-1). Furthermore, as shown in Figs. 11 and D-1 (pages 27 and 118, respectively), the mesenchymal cells expressing α8β1 integrin appear to be aggregated, forming mesenchymal condensations, thus the region in which nephronectin co-localizes with α8 integrin is where epithelia and mesenchymes reciprocally interact. Together with the epithelium-specific expression of nephronectin (Brandenberger et al., 2001; Tumbar et al., 2004), the observations above strongly suggest that the interaction of nephronectin with mesenchymal α8β1 integrin is a sort of epithelial-mesenchymal interactions regulating mesenchymal cell behaviors. Moreover, the observations also provide new insights into the roles of basement membranes in epithelial-mesenchymal interactions during tissue development.

Possible roles of nephronectin in epithelial-mesenchymal interaction

The accumulating evidence supports the role of nephronectin as a regulator of mesenchymal cell behaviors. Then, how can nephronectin regulate the epithelial-mesenchymal interactions? One possible explanation is that the specific, high affinity interaction of α8β1 integrin with nephronectin may anchor mesenchymal cells expressing α8β1 integrin to the mesenchymal side of basement membranes where epithelial-mesenchymal interactions take place (Fig. D-2A). Because several studies, together with my observation, have demonstrated that α8β1 integrin inhibits migration of cells adhering to RGD-containing ECM proteins even though the integrin promotes cell
A

(a) ![Diagram of epithelial and mesenchymal cells with basement membrane.]

(b) ![Diagram showing αβ1-nephronectin interactions stably anchor mesenchymes.]

(c) ![Diagram illustrating secretion of soluble factors and gene expression.]

(d) ![Diagram depicting epithelial proliferation and invagination into mesenchyme.]

- **Epithelial cell**
- **Mesenchymal cell**
- **Basement membrane**
- **Integrin αβ1**
- **Nephronectin**
- **Soluble factors**
- **Soluble factor receptors**

**Insights**

- Soluble factors are enriched at the basement membrane.
- αβ1-nephronectin interactions stably anchor mesenchymes.

**Events**

- Epithelial proliferation and invagination into mesenchyme.
- Proliferation and differentiation of mesenchyme.
Fig. D-2 Schematic models for nephronectin in epithelial-mesenchymal interaction. A, a “nephronectin-glue” model. (a) The loose mesenchyme beneath the epithelium adheres to nephronectin at the mesenchymal side of basement membranes. (b) Nephronectin stably anchors the mesenchyme through the interaction with \( \alpha_8\beta_1 \) integrin. (c) Since the basement membrane concentrates soluble factors, both the epithelium and mesenchyme receive the factors efficiently, owing to the mesenchymal adhesion to nephronectin. (d) After all, the epithelium and mesenchyme differentiate to form individual tissues. B, a “promotion of survival and proliferation by nephronectin” model. (a) Nephronectin binds to \( \alpha_8\beta_1 \) integrin expressed on the mesenchyme that transmits signals to induce epithelial cell differentiation. (b) The interaction of \( \alpha_8\beta_1 \) integrin with ligands has been shown to promote cell survival and proliferation via PI3K-PKB/Akt and MAPK pathways. (c) The adhered cells are able to survive and proliferate, thereby enhance the inductive signals. (d) After all, the cells proliferate and differentiate to form individual tissues.
adhesion to them (Bieritz et al., 2003; Zargham et al., 2007; Benoit et al., 2009; K. Morimitsu and Y. Sato, unpublished observation), the mesenchymal cells adhering to nephronectin through the α8β1 integrin may be kept staying at the adhesion site, and therefore are positioned face-to-face with the epithelium. Juxtaposition of epithelium and mesenchyme should enhance the efficiency of reciprocal signaling between them, since epithelial-mesenchymal interactions are mainly mediated by multiple soluble factors, including GDNF, Wnt, and FGFs (Schedl, 2007), which are sequestered by ECMs to form concentration gradients (Kispert et al., 1996; Allen et al., 2001; Barnett et al., 2002). Thus, the function of nephronectin in epithelial-mesenchymal interaction should be to facilitate “gluing” particular cells, i.e., α8β1-expressing mesenchymes, to regions where soluble factors secreted by epithelium are enriched.

Accumulating evidence also supports another possible explanation for the role of nephronectin in epithelial-mesenchymal interaction; nephronectin may be required for mesenchymal cell survival (Fig. D-2B). For example, the interaction of α8β1 integrin with fibronectin has been shown to stimulate the activation of phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways (Farias et al., 2005). The expression of α8 integrin subunit has been shown to prevent cells from apoptosis (Farias et al., 2005; Hartner et al., 2008; Benoit et al., 2009). The mice with homozygous null mutation of α8 integrin gene also support this explanation, since the mice exhibit degeneration of the metanephric mesenchymes at embryonic day 13.5, due to the absence of the direct interaction between the mesenchyme and ureteric bud epithelium (Muller et al., 1997). Moreover, it is of interest to note that interstitial ECM
proteins, including fibronectin and collagens, are absent from condensing mesenchyme even though these ECMs deposit in metanephric mesenchymes before the mesenchymal condensation is formed (Ekblom, 1981; Mounier et al., 1986), suggesting that, to avoid anoikis, the condensing mesenchymes have to use nephronetin as a scaffold. Thus, owing to the localization of nephronectin at the site of epithelial-mesenchymal interactions, the metanephric mesenchymes adhering to nephronectin may be able to survive and proliferate, thereby continuously secreting soluble factors, such as GDNF, for ureteric bud epithelium and thereby induce differentiation of the epithelial cells.

**Ligand-Recognition Mechanisms of Integrins that Bind to the RGD Motif**

Previous studies, as well as this study, demonstrate that the RGD-binding integrins require both the RGD motif and auxiliary site to exert selective and high-affinity binding to their physiological ligands. However, it is still unclear why and how the RGD-binding integrins require the auxiliary site to selectively interact with their ligands. Here, I discuss the possible mechanisms by which the RGD-binding integrins interact with the auxiliary binding sites of the ligands.

*The functional consequence of the auxiliary binding sites in ligand recognition by the RGD-binding integrins*

In vertebrates, 4 α and 5 β integrin subunits have been identified to form 8 types of RGD-binding integrins. Although the RGD motif primarily determines the specificities
of ligand binding to the RGD-binding integrins, it is undoubted that the auxiliary binding sites residing outside the RGD motif also define the specificities and affinities of individual integrins to their ligands (Ruoslahti, 1996; Takagi, 2004). Then, how individual RGD-binding integrins recognize the differences of the auxiliary binding site of ligands? It is worth considering that the properties of auxiliary binding sites identified so far are obviously different from each other. For example, the synergy site of fibronectin, known to enhance the binding affinity to \( \alpha 5 \beta 1 \) integrin, is comprised of PHSRN sequence and several basic residues (Aota et al., 1994; Redick et al., 2000). The auxiliary binding site to \( \alpha V \beta 6 \) integrin is the LXXL/I sequence, in which two hydrophobic residues have been shown to interact with the \( \alpha V \beta 6 \) integrin (DiCara et al., 2007). As shown in Chapter II, two acidic residues of the EIE motif are indispensable for high-affinity interaction of nephronectin with \( \alpha 8 \beta 1 \) integrin. Taken together, these observations suggest that the individual RGD-binding integrins may discriminate the specific sequences of auxiliary sites, containing acidic, basic, or hydrophobic properties, in the vicinity of the RGD motif, thereby specifically interacting with the physiological ligands of individual RGD-binding integrins.

If the physiological ligands for RGD-binding integrins possess characteristic sequences to increase the binding affinity and specificity to the individual integrins, the integrins should also possess counterparts recognizing the auxiliary binding site of the ligands. In fact, amino acid sequences in the vicinity of the ligand-binding site, which is located at upper or side faces of \( \beta \)-propeller domain of integrin \( \alpha \) subunit, show significant diversity among the RGD-binding integrins. The diversity is apparent when
Fig. D-3 Predicted structures of the head region of α8β1 integrin. A, ribbon models of β-propeller domain of α8 integrin (a, side view) and head domain of α8β1 integrin (b, upper view) are created using the crystal structure of αVβ3 integrin (PDB ID: 1jv2) as the template. The models were predicted with the SWISS-MODEL (http://swissmodel.expasy.org/; Arnold et al., 2006) and fine-tuned by energy minimization with Swiss PDB viewer. Dashed circles indicate the loops that exhibit significant divergence among different α subunits. The amino acid sequences comprising the loops are shown in upper margin. B, molecular surfaces of α8β1 (left) and α5β1 (right) integrins were generated with Chimera, colored by electrostatic potentials from -15 (red) to +15 (blue) kcal/mol·e. The viewpoints were the same as in A-(b). Dashed circles represents approximate locations of the RGD-docking site.
the amino acid sequences of the β-propeller domain of α8 integrin are compared with those of α5 integrin (Fig. D-3A). As discussed in Chapter II, the cluster of basic residues are located at the upper or side faces of α8 integrin β-propeller, whereas α5 integrin possesses the cluster of acidic residues at the equivalent sites of basic cluster in α8 integrin. The significant divergence in charge distribution patterns is evident in predicted surface electrostatic potentials of α8β1 and α5β1 integrins (Fig. D-3B). Given that the auxiliary sites of fibronectin and nephronectin are mainly comprised of basic and acidic residues, respectively, it is tempting to speculate that the acidic and basic clusters of integrin α5 and α8 subunit, respectively, interact with the auxiliary binding sites of the ligands, thereby increasing the binding specificity as well as affinity to the physiological ligands.

Possible mechanisms for the involvement of the auxiliary binding site in the interaction with the RGD-binding integrins

Although it is undoubted that the auxiliary binding sites increase the binding affinity to the individual integrins, the mechanisms by which the auxiliary sites increase the affinity are still unclear. Given that increases in the affinity of protein-protein interactions are achieved by increasing the association rate, decreasing the dissociation rate, or both of them, the auxiliary binding sites are thought to enhance the binding activities to the individual integrins through affecting the association and/or dissociation rates. Here, I propose the following two models that account for molecular mechanisms underlying the enhancement of the affinities by the auxiliary binding sites.
One possible (and simple) model for the role of the auxiliary binding sites is their direct interaction with integrins. In this model, the auxiliary site is hypothesized to reside within the binding interface of the complex, thereby decreasing the dissociation rate by stabilizing the final complex between the integrin and ligand (Fig. D-4A). Thus, the RGD tripeptide motif fits into the RGD-docking site at the top of head domain of integrins, resulting in subsequent formation of salt bridges between the auxiliary site and integrin. This model is in agreement with the structure of α5β1 integrin-fibronectin complex deduced from solution X-ray scattering (Mould et al., 2003), and easy to understand how the auxiliary sites enhance the binding affinities. Based on this model, a peptide modeled after the auxiliary binding site should partially inhibit the interaction of integrins with their ligands. However, previous studies, as well as my study, have revealed that the peptides containing only the auxiliary site is barely inhibitory (Aota et al., 1994). The reason why the peptides including the auxiliary site of the ligand failed to inhibit the interaction of integrins remains to be further elucidated.

The other possible model is that the acidic (or basic) portion of the auxiliary site and the basic (or acidic) portion of the head region of integrin stabilize the initial encounter complex between the integrin and ligand via long-range electrostatic interaction (Fig. D-4B). Therefore, the binding interface of integrin is located in the vicinity of the RGD motif, making the motif easily to bind to the RGD-docking site of integrin. In this model, the auxiliary site is hypothesized not to be part of specific protein-protein interaction but to be an “attractant” of the interaction, thereby increasing the binding affinity to integrin through affecting the association rate. This model seems to be more reasonable than the
Fig. D-4 Possible mechanisms of the bipartite recognition of ligands by RGD-binding integrins. A, a “stabilization of final complex” model. The RGD motif fits into a narrow groove between the head domains of α and β subunits of integrin (upper). Thereafter, the auxiliary binding site interacts with the β-propeller domain of α subunit (lower), stabilizing the final complex (and thereby decreasing the dissociation rate of the interaction). B, a “stabilization of initial encounter complex” model. The upper and side faces of β-propeller domain of integrin α subunit interact with the auxiliary site of ligand through long range electrostatic steering (upper), keeping the RGD motif in the vicinity of the RGD-docking site of integrin. Thus, the RGD motif easily fits into the narrow groove between the α and β subunit (middle).
former model, since in this model the peptides modeled after the auxiliary site should be barely inhibitory, consistent with the results obtained in this study (Fig. II-7; page 91). The possibility of this model was assessed using \( \alpha 5\beta 1 \) integrin and fibronectin fragment (Takagi et al., 2003). Takagi et al. demonstrated that the mutation of synergy site of fibronectin primarily affected the association rate, resulted in 26-fold decrease. By contrast, the dissociation rate exhibited only 2-fold increase in this mutant. Moreover, the observation of \( \alpha 5\beta 1 \)-fibronectin fragment complex by electron microscopy revealed that \( \alpha 5\beta 1 \) integrin did not interact with the synergy site of fibronectin, suggesting that the synergy site of fibronectin does not contribute to the stability of final \( \alpha 5\beta 1 \) integrin-fibronectin complex. Given that \( \alpha 8\beta 1 \) and \( \alpha V\beta 6 \) integrins require acidic and hydrophobic residues, respectively, to interact with their physiological ligands, it is tempting to speculate that the auxiliary sites stabilize the initial encounter complexes through electrostatic or hydrophobic interactions, thereby enhancing the affinities and providing the specificities of the individual integrins to interact with their ligands. To determine the role of auxiliary sites in the interaction with integrins, further studies, particularly analyses of three-dimensional structure of integrin-natural ligand complexes, will be required.

Studies for spatiotemporally regulated basement membrane proteins are just beginning, and their functions have been poorly known. Nephronectin is one of the spatiotemporally regulated basement membrane proteins, and has unique functions: the activities to bind to highly sulfated GAG chains and \( \alpha 8\beta 1 \) integrin. Although much progress has been made
in understanding of the molecular functions of nephronectin, there are still many questions to be answered. Namely, how the bipartite site of nephronectin is recognized by α8β1 integrin? What kinds of signaling pathways does nephronectin transmit to cells through α8β1 integrin? What are the physiological functions of nephronectin in vivo? When these questions are answered, not only the functions of nephronectin along with α8β1 integrin but also the physiological roles of spatiotemporally regulated basement membrane proteins will be better understood.
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