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Nephronectin binds to heparan sulfate proteoglycans via its MAM domain ☆

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ABSTRACT

Nephronectin is a basement membrane protein comprising five N-terminal epidermal growth factor (EGF)-like repeats, a central linker segment containing an Arg-Gly-Asp (RGD) motif and a C-terminal meprin-A5 protein-receptor protein tyrosine phosphatase μ (MAM) domain. Nephronectin has been shown to interact with α8β1 integrin through the central linker segment, but its interactions with other molecules remain to be elucidated. Here, we examined the binding of nephronectin to a panel of glycosaminoglycan (GAG) chains. Nephronectin bound strongly to heparin and chondroitin sulfate (CS)-E and moderately to heparan sulfate (HS), but failed to bind to CS-A, CS-C, CS-D, dermatan sulfate and hyaluronic acid. Deletion of the MAM domain severely impaired the binding of nephronectin to heparin but not CS-E, whereas deletion of the EGF-like repeats reduced its binding to CS-E but not heparin, suggesting that nephronectin interacts with CS-E and heparin through the EGF-like repeats and MAM domain, respectively. Consistent with these results, nephronectin bound to agrin and perlecan, which are heparan sulfate proteoglycans (HSPGs) in basement membranes, in HS-dependent manners. Site-directed mutagenesis of the MAM domain revealed that multiple basic amino acid residues in the putative loop regions were involved in the binding of the MAM domain to agrin. The binding of nephronectin to basement membrane HSPGs was further confirmed by in situ nephronectin overlay assays using mouse frozen tissue sections. Taken together, these findings indicate that nephronectin is capable of binding to HSPGs in basement membranes via the MAM domain, and thereby raise the possibility that interactions with basement membrane HSPGs may be involved in the deposition of nephronectin onto basement membranes.

1. Introduction

Basement membranes are extracellular matrix (ECM[2]) sheets present in all multicellular organisms. They serve as scaffolds for cell adhesion, sequester growth factors and provide mechanical strength to tissues and organs (Sanes, 2003; Yurchenco et al., 2004; Hynes, 2009). Basement membranes are composed of laminins, nidogens, type IV collagens, perlecan and a variety of other proteins whose expressions are regulated in developmental stage- and tissue-specific manners. The compositions of basement membranes are customized for individual cell and tissue types, thereby giving rise to distinctive extracellular microenvironments, or niches, that regulate cell fate and behavior through interactions with cell surface receptors, e.g., integrins or association with growth factors/morphogens (Manabe et al., 2008; Brizzi et al., 2012).

Nephronectin is a basement membrane protein that is mainly expressed in developing kidneys and hair follicles (Brandenberger et al., 2001; Fujiwara et al., 2011). It has been reported to be involved in kidney morphogenesis and arrector pili muscle differentiation in mice (Linton et al., 2007; Fujiwara et al., 2011), forelimb formation in *Xenopus* (Abu-Daya et al., 2011) and heart development in zebrafish (Patra et al., 2011). Nephronectin is composed of three functional domains, comprising five N-terminal EGF-like repeats, a central linker segment containing an RGD cell adhesive motif and a C-terminal MAM domain (Brandenberger et al., 2001; Morimura et al., 2001). In addition to the RGD motif, the central linker segment possesses an auxiliary binding site that is critical for binding to the cell surface receptor α8β1 integrin (Sato et al., 2009; Sanchez-Cortes and Mrksich, 2011), which plays a crucial role in epithelial–mesenchymal interactions during the early steps of kidney morphogenesis (Muller et al., 1997). In contrast to the central linker segment, the roles of the N-terminal EGF-like repeats and the C-terminal MAM domain remain poorly investigated. Previously,

Abbreviations: CBM, carbohydrate-binding module; CS, chondroitin sulfate; ECM, extracellular matrix; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; FS, Fraser syndrome; GAG, glycosaminoglycan; HRP, horseradish peroxidase; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; MAM, meprin-A5 protein-receptor protein tyrosine phosphatase μ; PE-GAG, phosphatidylethanolamine-conjugated glycosaminoglycan.

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Morimura et al. (2001) reported that deletion of the MAM domain resulted in a reduced tendency of nephronectin to localize at the cell surface, suggesting the presence of unknown receptor(s) for the MAM domain on the cell surface. The N-terminal EGF-like repeats have been shown to promote osteoblast differentiation, possibly through activation of the extracellular signal-regulated kinase (ERK) pathway (Kahai et al., 2010). These observations suggest that the EGF-like repeats and the MAM domain interact with hitherto unknown cell surface molecules, thereby regulating the physiological functions of nephronectin, including its cell-surface deposition and ERK activation.

Proteoglycans containing variably sulfated GAG chains are present in ECMs as well as on cell surfaces. They play mandatory roles in the immobilization of soluble factors and morphogens, and in the regulation of protease activities, cellular responses to growth factors, cell–cell and cell–ECM interactions and ECM assembly (Lander, 1999). To date, three HSPGs, perlecan, agrin and collagen XVIII, have been reported to be localized at basement membranes (Iozzo, 2005). These basement membrane HSPGs bind not only to a variety of heparin-binding growth factors, including fibroblast growth factors (FGFs) and members of the transforming growth factor-β superfamily (Aviezer et al., 1994; Friedl et al., 1997; Li et al., 2010; Sengle et al., 2011), but also to basement membrane proteins such as laminins and type IV collagen (Fujiwara et al., 1984; Talts et al., 1999) through their HS chains. Although the physiological relevance of the interactions of ECM proteins with HS chains remains to be elucidated, HS chains may regulate the matrix assembly and deposition of ECM proteins, including laminin-332 and fibrillin-1 (Tiedemann et al., 2001; Tsubota et al., 2005). Thus, HSPGs may contribute to the regulation of local structures and the composition of extracellular microenvironments by entrapping a variety of proteins, including soluble factors and ECM components, in the surrounding milieu.

In the present study, we show that nephronectin is capable of binding to heparin, HS and CS-E. The MAM domain is responsible for the interactions with heparin and basement membrane HSPGs, including agrin and perlecan. Nephronectin interacts with basement membranes in tissue sections, suggesting the possibility that the interactions of the MAM domain with HSPGs may be involved in the deposition of nephronectin onto basement membranes.

2. Results

2.1. Interactions of nephronectin and its deletion mutants with a panel of GAGs

To examine the binding activities of nephronectin to a panel of GAGs, we expressed and purified recombinant nephronectin (Fig. 1A and B). A FLAG tag was added to the C-terminus of nephronectin to facilitate affinity purification (Sato et al., 2009). The FLAG-tagged nephronectin was assessed for its ability to bind to phosphatidylethanolamine-conjugated GAGs (PE-GAGs) by solid-phase binding assays using an anti-FLAG monoclonal antibody (Fig. 1B). Among the PE-GAGs examined, nephronectin bound strongly to heparin and CS-E, and moderately to HS, but only marginally, if at all, to CS-A, CS-C, CS-D, dermatan sulfate and hyaluronic acid. The binding of nephronectin to heparin, HS and CS-E was detected under physiological ionic conditions, thereby endorsing its physiological relevance.

To define which domain(s) bound to GAGs, we expressed deletion mutants of nephronectin, namely ΔMAM (deletion of the MAM domain), ΔEGF (deletion of the EGF-like repeats), Linker (deletion of both the EGF-like repeats and MAM domain) and MAM (deletion of both the EGF-like repeats and linker segment), as shown in Fig. 1A. These deletion mutants were purified by affinity chromatography utilizing their C-terminal FLAG tags (Fig. 1C–F). The activities of the deletion mutants for binding to PE-GAGs were assessed by solid-phase binding assays (Fig 1C–F). ΔMAM retained the ability to bind avidly to CS-E, but was almost devoid of the ability to bind to heparin (Fig. 1C). These results indicate that the MAM domain is required for binding of nephronectin to heparin, but dispensable for its binding to CS-E. Consistent with these conclusions, ΔEGF and MAM, both of which contain the MAM domain, retained the ability to bind to heparin, while their binding to CS-E was significantly impaired (Fig. 1D and F). The central linker segment did not bind to any of the PE-GAGs examined (Fig. 1E). Since the binding of ΔMAM to CS-E was roughly comparable with that of intact nephronectin, these results suggest that the five EGF-like repeats of nephronectin are responsible for binding to CS-E, while the MAM domain is mainly responsible for the binding to heparin.

The second, fourth and fifth EGF-like repeats of nephronectin are assigned to Ca^{2+}-binding EGF-like repeats (Brandenberger et al., 2001). To examine the effects of Ca^{2+} ions on the interactions of intact nephronectin and ΔMAM with CS-E, we assessed the binding activities of these proteins to CS-E in the presence of EDTA. When incubated with CS-E in the presence of EDTA, both nephronectin and ΔMAM exhibited significant reductions in their binding activities to CS-E, resulting in 30–40% loss of the activities compared with those in the presence of 1 mM Ca^{2+} (Fig. 2A). The binding activity of nephronectin to heparin was only marginally affected by EDTA, resulting in less than 10% loss of the binding activity (Fig. 2B). ΔMAM did not bind to heparin irrespective of the presence of Ca^{2+} or EDTA. These results suggest that the Ca^{2+}-binding EGF-like repeats are partly involved in the interaction of nephronectin with CS-E, thus further corroborating the conclusion that nephronectin interacts with heparin and CS-E through distinct domains.

2.2. Nephronectin interacts with HS chains of agrin and perlecan through the MAM domain

Next, we examined whether nephronectin could bind to basement membrane HSPGs, i.e., agrin and perlecan, because both nephronectin and non-membrane bound HSPGs are localized at basement membranes (Brandenberger et al., 2001; Sarrazin et al., 2011). As expected, solid-phase binding assays demonstrated that recombinant nephronectin bound to both agrin and perlecan in dose-dependent manners (Fig. 3A). We also examined the abilities of deletion mutants of nephronectin to bind to agrin and perlecan. ΔEGF and MAM, but not ΔMAM and Linker, retained the ability to bind to agrin and perlecan (Fig. 3B). Given that the MAM domain binds avidly to heparin, these results suggest that nephronectin interacts with agrin and perlecan through their HS chains. It was noted that the binding of ΔEGF and MAM to agrin and perlecan were less pronounced than the binding of intact nephronectin, and exhibited a bias for agrin. Because intact nephronectin, but not their deletion mutants, forms multimers (Sato et al., 2009), the difference in their avidity towards agrin and perlecan may be, at least in part, because of the increased valency of the interaction between intact nephronectin and HSPGs immobilized on microtiter plates. The apparent bias for agrin may reflect the difference in the sulfation of HS chains between agrin and perlecan.

The HS-dependent interactions of nephronectin were further verified by removal of the HS chains from agrin and perlecan. The binding activities of nephronectin to agrin and perlecan were severely impaired when the HS chains were removed by treatment with heparitinase (Fig. 3C). Treatment with chondroitinase did not decrease the binding of nephronectin to these HSPGs. The binding was also inhibited by addition of an excess amount of heparin (Fig. 3C), corroborating the requirement of the HS chains for the interactions of nephronectin with agrin and perlecan.

2.3. Clusters of basic amino acid residues in the MAM domain are involved in the interactions with HSPGs

The binding sites for heparin/HS have been shown to comprise basic amino acid residues that interact electrostatically with acidic