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Glypican-2 binds to midkine: The role of glypican-2 in neuronal cell adhesion and neurite outgrowth

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Cell-surface heparan sulfate proteoglycans participate in molecular events that regulate cell adhesion, migration, and proliferation. The present study was performed to elucidate whether glypican-2 plays a role in interactions of neurons with midkine (MK), a heparin-binding neuroregulatory factor. MK bound to heparan sulfate chains of glypican-2 in a manner similar to syndecan-3. Microbeads coated with MK or poly-L-lysine induced clustering of glypican-2 as well as syndecan-3. Substratum-bound MK or poly-L-lysine induced cell adhesion of N2a neuroblastoma cells, while only MK promoted neurite outgrowth of these cells. Ligation of cell-surface glypican-2 with MK or an antibody against epitope-tagged glypican-2 induced cell adhesion and promoted neurite outgrowth. These results verified that cell-surface glypican-2 bound to MK and suggested that MK-glypican-2 interactions participate in neuronal cell migration and neurite outgrowth. In addition, we observed different localization of epitope-tagged glypican-2 and syndecan-3 on the surface of N2a cells; the result suggested that they may play different roles in MK-mediated neural function.

Keywords: Glypican, syndecan, midkine, HB-GAM

Abbreviations: BSA, Bovine serum albumin; DMEM, Dulbecco's modifield Eagle's medium; ECM, extracellular matrix; FCS, felal calf serum; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; GPI, glycosylphosphatidylinositol; HB-GAM, heparin-binding growth-associated molecule; ITS, insulin-transferrin-selenate; LRP, low density lipoprotein receptor related protein; PCR, polymerase chain reaction.

Introduction

Midkine (MK) is a basic, cysteine-rich polypeptide found as a product of a retinoic acid-responsive gene [1]. MK shares 45% sequence identity with heparin-binding growth-associated molecule (HB-GAM)/pleiotrophin (PTN) and their limited homology to the thrombospondin type I repeat has recently been reported [2–4]. NMR spectroscopy and site-directed mutagenesis revealed the structure of two heparin binding sites in the C-terminal half of MK molecule [5,6]. Recognition of heparin-binding domains of MK by cell-surface heparan sulfate proteoglycans is important both for neurite outgrowth and for enhancement of fibrinolytic activity [7–9].

The heparan sulfate chains at the cell surface are mostly attached to syndecan and glypican core proteins, which differ in the mode of anchoring to the membrane and domain structures [10]. Syndecans have a role in organizing the cytoskeleton by binding to extracellular matrix (ECM). The conserved cytoplasmic domain on all syndecans interacts with the PDZ domains of CASK/LIN-2 and thus might link syndecans to the actin cytoskeleton [10]. To date, syndecan-1, 3 and 4 have been shown to bind to MK [11–14].

Glypicans consist of a family of glycosylphosphatidylinositol (GPI)-anchored heparan sulfate proteoglycans. Studies of mutations in the *dally* gene, a gene encoding the *Drosophila* glypican, suggested that *dally* is a regulator of decapentaplegic and wingless signaling [15–17]. Furthermore, the human homologue of glypican-3/OCI-5 is mutated in patients with the Simpson-Golabi-Behmel syndrome, who show severe malformations and both pre- and postnatal overgrowth [18]. Targeted gene dis-

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ruption of the glypican-3 gene in mice suggested a significant relationship between bone morphogenic protein signaling and glypican-3 function [19]. Although the precise functions of other members of this family are not known, these results suggest that glypicans are involved in the control of cell growth and division, acting as coreceptors for soluble heparin-binding growth factors [20–22]. All members of the glypican family are expressed in the developing nervous system, in which glypican-1 and 2 are expressed mainly in neurons [23]. Glypican-2 gene expression changes markedly during development with highest levels of expression in the embryonic brain [24]. Furthermore, glypican-2 protein is prominently found on axon tracts when axons are actively growing, but not after axons have reached their targets [25]. These results prompted us to examine the possibility that glypican-2 may be an important heparan sulfate proteoglycan that recognizes MK, in terms of neurite outgrowth and neuronal cell migration at embryonic stages.

Materials and methods

Materials

CNBr-activated Sepharose 4B and Ni-sepharose were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Fluorescein isothiocyanate-conjugated anti-mouse IgG was from Sigma-Aldrich (St. Louis, USA). MK-Sepharose and [^{125}I]-MK was prepared as described previously [26]. The specific activity of iodinated proteins was $1.0–2.8 \times 10^7$ cpm/μg. Heparin was from Wako Pure Chemicals (Osaka, Japan). Chondroitin sulfate A, D and E were purchased from Seikagaku Kogyo (Tokyo, Japan).

Construction of expression plasmid

An expression plasmid encoding *N*-terminal FLAG-tagged glypican-2 (FLAG-GP2) was constructed as follows. A glypican-2 cDNA was amplified by PCR with primers 5-*tctaga*ggtccaggaccgggacat-3 (nt. 284–301, nucleotide numbers refer to GenBank accession No. L20468, italic nucleotides indicate a synthetic restriction enzyme site for subcloning), 5-actaagcctcctgacgctagggcgc-3 (nt. 1986–2010), and rat embryonic brain cDNA as a template. After restriction enzyme digestion, the cDNA was subcloned into pFLAGCMV-1 (Sigma) to generate pFLAG-GP2, which encoded the signal peptide of preprotrypsin, FLAG-epitope followed by glypican-2 (amino acid 17–579, amino acid numbers refer to GenBank accession No. AAA40961). To generate soluble form of C-terminal FLAG- (GP2-FLAG) or His-tagged glypican-2 (GP2-His), cDNAs encoding glypican-2 were also amplified by PCR with primers; 5-ggaaggagagcagctatgtccgcg-3 (nt. 221–243) and *ttattacttgtcgtcatcgctttgtag*tctcctcctctcactccaagacc-3) (nt. 1600–1620, italic sequence denotes the position of the FLAG epitope) or 5-ggaaggagagcagctatgtccgcg-3 (nt. 221–243) and 5-*gatcattagtggtggtgatggtggtg*agacgaccccagattcctgc-3 (nt. 1671–1651, italic sequence denotes the position of histidine-tag), respectively. After enzyme treatment, the cDNAs were subcloned into pRc/CMV (promega) to generate pGP2-FLAG and pGP2-His, respectively. To construct the soluble- GP2-FLAG/ΔGAG or membrane-anchored form of glypican-2 without glycosaminoglycan chains (GP2-FLAG/ΔGAG), two putative glycosaminoglycan-attachment serine residues (amino acid No. 498 and 500) were replaced with threonine by site-directed mutagenesis. Primers used were 5-tgaagacgccac*t*ggc*act*ggaggggac-3 (nt. 1482–1510, mutations were introduced at the positions italic) and 5-gtccccctcc*agt*gccag*t*ggcgtcttca-3 (nt. 1510–1482). To construct the transmembrane form of N-terminal FLAG-tagged syndecan-3 (FLAG-SYN3), syndecan-3 cDNA (encoding amino acid 46–442, GenBank accession No. Q64519) was amplified by PCR with primer 5-*agatct*gcaacgctggcgcaatgagaac-3 (nt. 171–190, GenBank accession No. NM011520, italic sequence denotes restriction enzyme site), 5-gtggctctctctgctaagcgta-3 (nt. 1355–1376), and mouse embryonic brain cDNA as a template. After enzyme treatment, the cDNA was subcloned into pFLAGCMV-1 to generate pFLAG-SYN3. To generate the soluble form of C-terminal FLAG-tagged syndecan-3 (SYN3-FLAG), a cDNA encoding syndecan-3 (amino acid 1-383) was amplified by PCR with primers 5-gctcgcccgcgccgcgaacaacaaaggccgccatg-3 (nt. 6–37) and 5-*ctactacttgtcatcgtcgtccttgta*ctcctgtatgctcttctgagggag-3 (nt. 1154–1186, italic sequence denotes the position of the FLAG epitope) and mouse embryonic brain cDNA as a template. After restriction enzyme digestion, the cDNA was subcloned into pRc/CMV to generate pSYN3-FLAG.

DNA transfection

Transfection and subsequent metabolic labeling were performed as previously described [27]. Briefly, cells (1×10^5/6 cm culture dish) were transiently transfected with 2 μg of indicated plasmid DNA. Forty-eight hours after the transfection, the cells were cultured in methionine/cysteine-free Dulbecco's modified Eagle's medium (DMEM) with [^{35}S]-methionine/cysteine (250 μCi/ml) (Amersham) for 3 h. Subsequently, the supernatants were collected, and used for immunoprecipitation and binding assay. Immunoprecipitation was performed by absorption of FLAG-tagged proteins to anti-FLAG M2 agarose (Sigma-Aldrich). Briefly, anti-FLAG agarose (50 μl) was mixed with 0.5 ml of cell culture medium, and was incubated overnight at 4°C with rotation. The agarose resin precipitated by centrifugation, washed three times with phosphate-buffered saline (PBS), and then subjected to SDS-PAGE.

Binding assay

MK-Sepharose (50 μl) was mixed with 2 ml of COS cell medium containing [^{35}S]-labeled protein and incubated at 4°C overnight with rotation. After three washes with 1 ml of PBS, the MK-Sepharose was packed into a small column, and

washed with 1 ml of wash buffer (10 mM of phosphate pH 7.5, 0.1% Triton X100) containing 0.2 M of NaCl.

Preparation of glypican-2 resin

COS cells transfected with pCMV/GP2-His were grown in serum-free DMEM containing 1% ITS and cultured for 48 h. The conditioned medium (500 ml) was applied to an Ni-Sepharose column (1 ml), washed with 100 ml of wash buffer containing 0.5 M of NaCl and 2 mM of imidazole, followed by washing with 50 ml of wash buffer containing 5, 10 and 20 mM imidazole. After re-equilibration in PBS, the Ni-Sepharose (GP-His resin) was stored in PBS as a 50% suspension. The resin (100 µl) was mixed with [^{125}I]-MK (20 ng) in a final volume of 1 ml and rotated for 3 h at 4°C. The resin was washed three times with 1 ml of PBS and packed in a small column. MK was eluted successively with 0.5 ml each of wash buffer containing 0.3, 0.4, 0.6, 0.8 and 1 M of NaCl. The eluate was subjected to SDS-PAGE. Radioactivity was visualized using a BAS2000 radioimage analyzer (Fuji Film, Tokyo, Japan).

Microbead binding assay

Polystyrene microbeads (10 µm in diameter, Polysciences Inc, Warrington, PA, USA) were washed with water, and aliquots of 0.3 ml of the beads were incubated in water containing MK (20 µg/ml), poly-L-lysine (20 µg/ml) or BSA (20 µg/ml) overnight at 4°C. The beads were washed twice with PBS and blocked with BSA (1 mg/ml) in PBS for 30 min at 37°C. COS-7 cells were plated at 50% confluence onto 33 mm cell culture dishes and transfected with 0.5 µg of the indicated plasmids. Twenty-four h after transfection, cells were harvested, plated on collagen-coated coverslips and grown for 12 h. Microbeads were applied to the COS-7 cells and incubated at 37°C for 1 h. Cells were then fixed with 4% paraformaldehyde and immunostaining was performed using anti-FLAG M2 mouse monoclonal antibody, followed by FITC-conjugated anti-mouse IgG antibody (Sigma-Aldrich). Fluorescence images were obtained with a confocal microscope system (Bio-Rad, MicroRadiance).

Neurite outgrowth assay

For the neurite outgrowth assay, coverslips (Thermanox Plastic Coverslips 13 mm in diameter, Nunc USA; the untreated side was used) were coated overnight with MK (10 µg/ml), poly-L-lysine (100 µg/ml) or anti-FLAG-M2 antibody (10 µg/ml) at 4°C. The coverslips were washed three times with distilled water and then blocked with DMEM containing 1% FCS for 30 min at 37°C. N2a cells were cotransfected with 0.1 µg of pEGFP and 0.9 µg of pFLAG-GP2, pFLAG-SYN3 or pCMV-FLAG. Twenty-four h after transfection, cells were harvested with PBS-0.02% EDTA treatment, and 1.5×10^4 cells were added to each well in 500 µl of DMEM containing 1.0% of B27 supplement (Invitrogen). Cells were allowed to adhere for 1 h after plating, and then the medium was removed and replaced with fresh medium. Cultures were grown for 48 h, fixed with 4% paraformaldehyde in phosphate-buffered saline, and then fluorescence images were obtained.

Results

MK binding to glypican *in vitro*

We examined glypican-2 binding to MK by affinity chromatography on MK-Sepharose. As syndecan-3 has been reported to bind to MK and HB-GAM in a heparan sulfate-dependent manner [12,14], we used soluble form of syndecan-3 with C-terminal epitope-tag (SYN3-FLAG) as a positive control for the MK binding. As shown in Figure 1A, SYN3-FLAG bound to the MK column and was eluted with 0.5 M–0.8 M NaCl. When soluble form of glypican-2 with C-terminal epitope-tag (GP2-FLAG) was applied on MK column, it bound to the column and was eluted with 0.5 M–0.8 M NaCl. This binding profile was similar to that of SYN3-FLAG. To investigate whether glypican-2 binds to MK in heparan sulfate-dependent manner, we constructed a plasmid encoding glypican-2 devoid of heparan sulfate attachment site (GP2-FLAG/ΔGAG) by site direct mutagenesis. As shown in Figure 1C, the glypican-2 core protein (GP-2-FLAG/ΔGAG) bound to MK column, but was eluted from it with 0.4 M NaCl (Figure 1C). These results clearly indicated that MK bound to glypican-2 and its binding affinity was almost comparable to that of syndecan-3 with

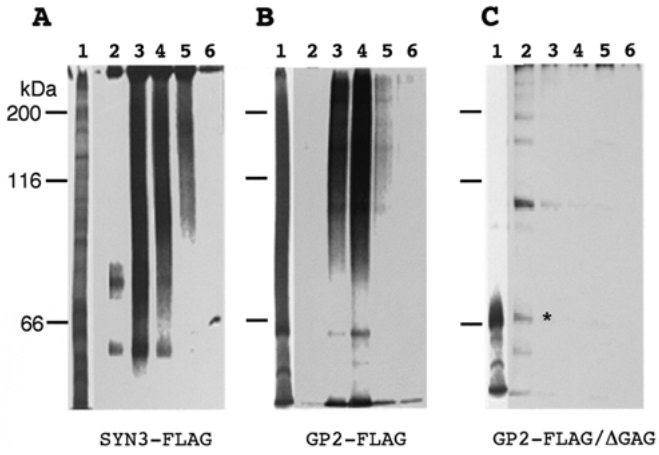

Figure 1. Binding of soluble syndecan-3 and glypican-2 to MK-column. COS-7 cells were transfected with pSYN3-FLAG (A), pGP2-FLAG (B) or pGP2-FLAG/ΔGAG (C), and labeled with [^{35}S]-methionine/cysteine. FLAG-tagged proteins were immunoprecipitated and analyzed by 10% SDS-PAGE (lane 1). SYN3-FLAG as well as GP2-FLAG was detected as broad smears in the molecular size range of 60–200 kDa, suggesting that both core proteins carry large amounts of heparan sulfate. MK-Sepharose was mixed with the COS-7 cell medium containing SYN3-FLAG, GP2-FLAG or GP2-FLAG/ΔGAG, and the bound proteins were eluted successively with wash buffer containing 0.4, 0.5, 0.6, 0.8 and 1 M NaCl (lane 2–6). Aliquots of 25 µl of each fraction were subjected to SDS-PAGE and radioactivity was visualized. Asterisk shows the position of glypican-2 core protein.

respect to NaCl elution profile. Furthermore, the binding was mainly through the heparan sulfate chain, while MK also bound to the core protein weakly.

As an alternative approach, [^{125}I]-MK binding to glypican-2 was examined. When [^{125}I]-MK was mixed with glypican-absorbed resin (GP2-His resin), it bound to and was eluted from the resin with 0.4–1.0 M NaCl (Figure 2A). [^{125}I]-MK did not bind to control resin. The binding was inhibited by the addition of an excess amount of unlabeled MK to the mixture (Figure 2B). In order to investigate whether binding between MK and glypican-2 is determined by MK glycosaminoglycan interactions with defined specificity [28,29], we analyzed the effects of various glycosaminoglycans. As shown in Figure 2C, heparin potently inhibited the binding of MK to glypican-2, and chondroitin sulfate E also suppressed the binding. Chondroitin sulfate D had a moderate effect, but chondroitin sulfate A scarcely influenced the binding. The above observation is consistent with the carbohydrate binding specificity of MK [28]; neurite outgrowth induced by MK is inhibited by heparin [8] and chondroitin sulfate E [29] as well as by heparitinase digestion [8].

Induction of glypican-2 clustering by beads coated with MK

When COS cells were transfected with a plasmid encoding N-terminal epitope-tagged glypican-2 (FLAG-GP2) or syndecan-3 (FLAG-SYN3), the two proteoglycans were found to

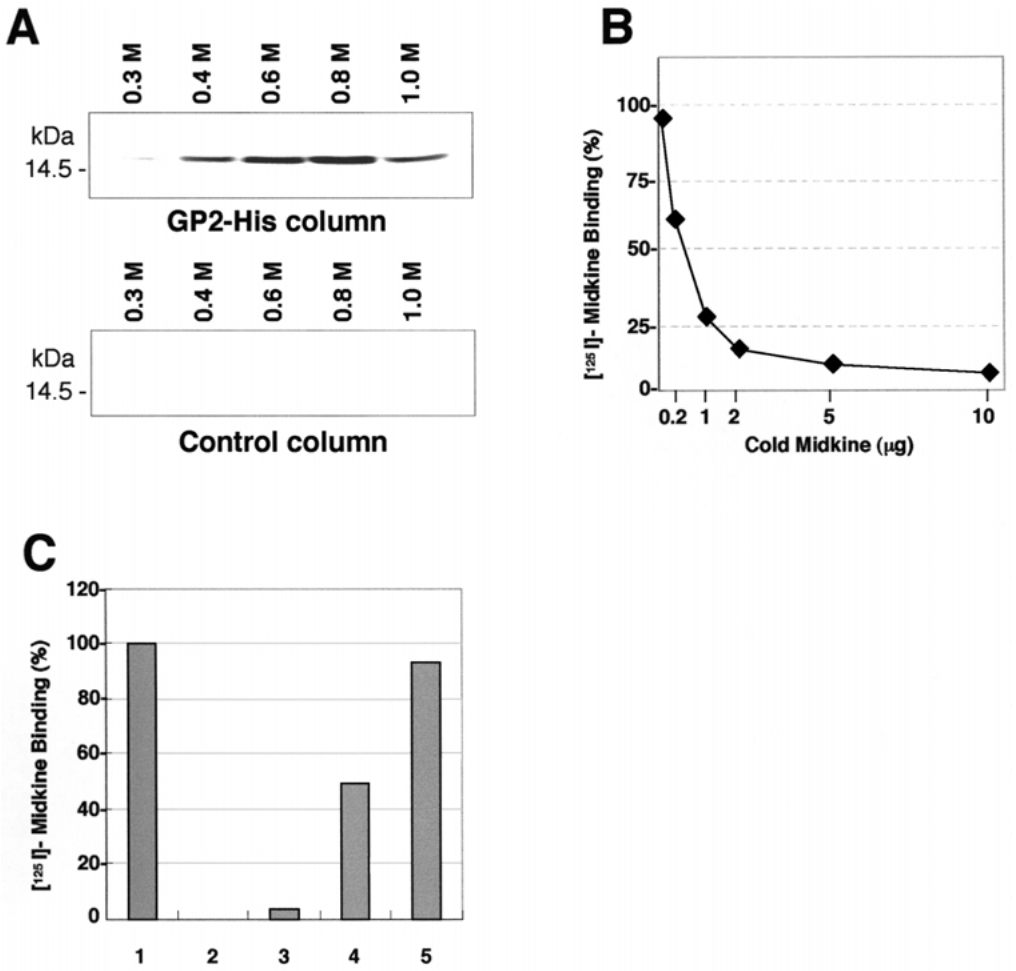

Figure 2. Binding of [^{125}I]-MK to glypican-2. (A) MK-glypican-2 binding. [^{125}I]-MK was mixed with GP2-His-Ni-Sepharose (GP2-His resin) or control resin. About 12% of the total radioactivity bound to the GP-2 His column, whereas 1.8% bound to the control column. Bound MK was eluted successively with 0.5 ml of wash buffer containing 0.3, 0.4, 0.6, 0.8 and 1 M of NaCl and analyzed by SDS-PAGE. (B) Effects of unlabeled MK on [^{125}I]-MK binding to glypican-2. GP2-His resin (100 μl) was mixed with [^{125}I]-MK (20 ng) in the presence of varying concentrations of unlabeled MK in a final volume of 1 ml. The resin was washed with PBS and bound MK was eluted with 0.5 ml of washing buffer containing 1 M NaCl. MK binding to GP2-His resin in the absence of unlabeled MK corresponded to 100% binding. (C) Influence of various glycosaminoglycans on the binding of [^{125}I]-labeled MK to glypican-2. GP2-His resin (100 μl) was mixed with [^{125}I]-MK (20 ng) and various glycosaminoglycans (1 μg) in a final volume of 1 ml. Unbound MK was washed with PBS and bound MK was eluted with 0.5 ml of wash buffer containing 1 M of NaCl. Lane 1, no inhibitor; lane 2, heparin; lane 3, chondroitin sulfate E; lane 4, chondroitin sulfate D; lane 5, chondroitin sulfate A. MK binding to GP2-His resin in the absence of inhibitors corresponded to 100% binding.

be localized on different compartments on the cell surface. Glypican-2 was located in a distinctly punctate pattern similar to that seen for other cell-surface GPI-anchored proteins (Figure 3A). Syndecan-3 was mainly localized on microspike-like structures (Figure 3F). We coated polystyrene beads with MK and applied them to COS cells to examine whether immobilized MK can bind to glypican-2 and induce clustering of the protein. As shown in Figure 3B, intense signals for glypican-2 were observed around the beads coated with MK. Clustering of syndecan-3 around the beads coated with MK was also observed (Figure 3G). Relatively weak signal around the beads were found on COS cells expressing glypican-2 without heparan sulfate (Figure 3D); the result is consistent with the finding that the core protein bound to MK with low affinity (Figure 1C) and also indicate that the clustering is mainly attained by interaction of MK with the heparan sulfate portion. Clustering of glypican-2 or syndecan-3 around the beads was also seen when beads coated with poly-L-lysine were applied (Figure 3C and H). Beads coated with bovine serum albumin (BSA) did not induce the clustering of glypican-2 or syndecan-3 (Figure 3E and I).

Midkine-Glypican-2 interaction can induce neurite outgrowth

To address the possible roles of cell-surface glypicans in cell adhesion and neurite outgrowth, the neuroblastoma cell line

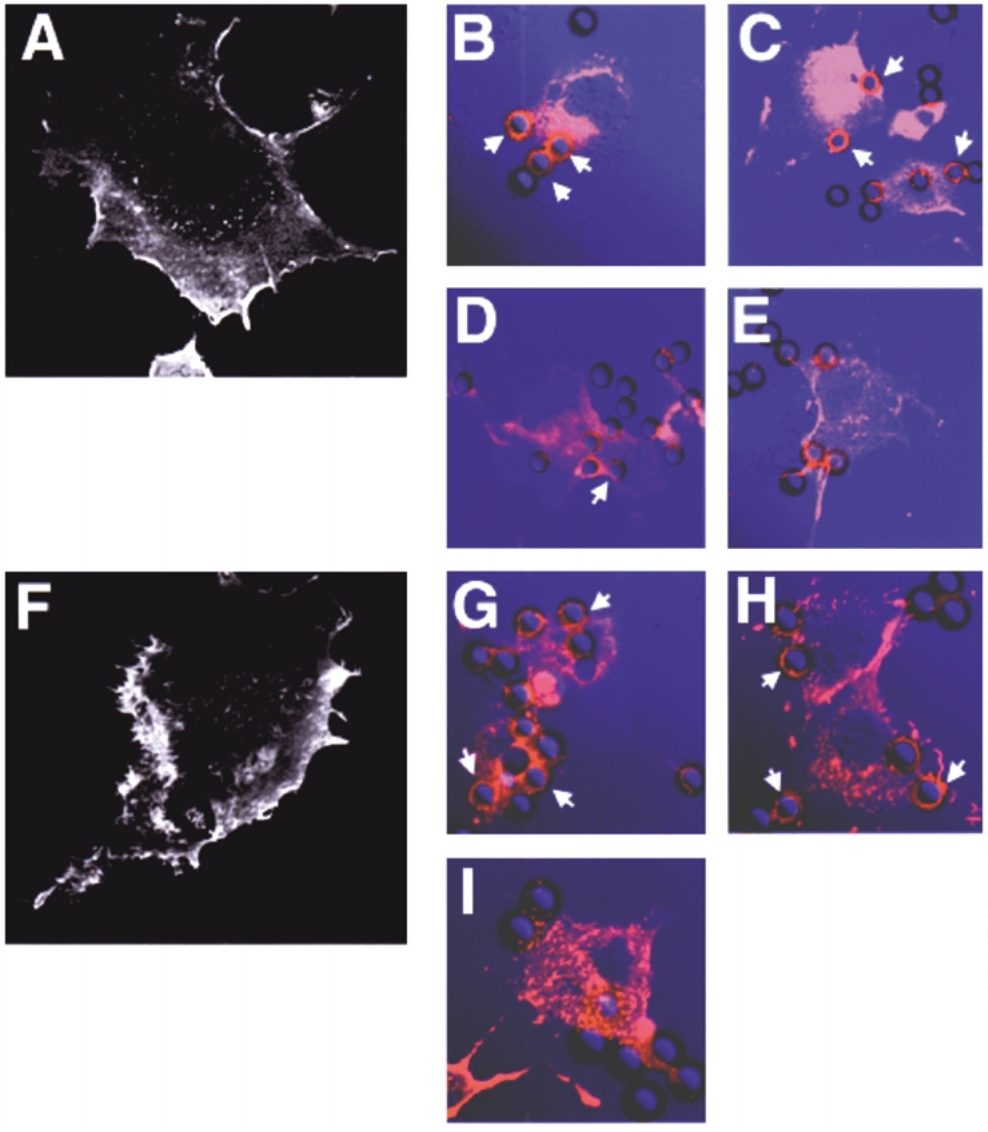

Figure 3. Microbeads coated with MK induced clustering of glypican-2. COS cells tansfected with pFLAG-GP (A–C and E), pFLAG-GP/ΔGAG (D) or pFLAG-SYN3 (F–I) were plated on collagen-coated coverslips and cultured for 18 h at 37°C prior to treatment with beads coated with MK (B, D and G), poly-L-lysine (C and H) or BSA (E and I) for 1 h. After fixation of the cells with 4% paraformaldehyde, FLAG-tagged proteins were detected by treatment with anti-FLAG M2 antibody followed by FITC-conjugated anti-mouse IgG. Signals were visualized by confocal microscopy at 400 × 1.6 magnification. White arrows indicate clustering of the FLAG-tagged proteins.

N2a was cultured on plates coated with MK, poly-L-lysine or BSA, and the cell adhesion and neurite outgrowth activity were examined. N2a cells attached to the plates coated with MK or poly-L-lysine within 1 h, but BSA exerted almost no effect (Figure 4A–C). Interestingly, the cell adhesion induced by MK or poly-L-lysine showed significant differences in ability to promote neurite outgrowth. When cells were cultured for 48 h in serum-free medium, cells adhering to the plates coated with MK extended well-developed neurites (Figure 4D). However, cells adhering to poly-L-lysine-coated plates formed cell aggregates and did not extend neurites (Figure 4E). These results indicated that temporal cell adhesion induced by nonspecific electrostatic interaction mediated by the lysine residues of poly-L-lysine is not sufficient to promote neurite outgrowth of N2a cells. When N2a cells were transfected with a plasmid encoding FLAG-GP2 or FLAG-SYN3 and cultured on plates coated with MK, the cells started to extend neurites. Overexpression of syndecan-3 induced the formation of long filopodia-like structures (Figure 4G and I). Immunocytochemical studies indicated glypican-2 immunoreactivity on cell bodies and a portion of neurites; especially strong signals were concentrated on growth cones (Figure 4F and H). Interestingly, syndecan-3 expression was found not only on growth cones but also on filopodia-like processes (Figure 4G and I).

Due to the ubiquitous expression of heparan sulfate and chondoroitin sulfate chains on the cell-surface, it is difficult to conclude that the glypican-MK interaction alone can induce cell adhesion and promote neurite outgrowth without the contribution of interactions with other proteoglycans. To clarify this point, an anti-FLAG antibody was immobilized on plates and was used as a ligand for FLAG-tagged glypican-2 by mimicking the glypican-MK interaction. When N2a cells expressing FLAG-tagged glypican-2 and green fluorescent protein (GFP) were plated on antibody-coated plates, the cells adhered strongly to the plates within a short period (Figure 5A). Similar results were seen with N2a cells expressing FLAG-tagged syndecan-3 and GFP (Figure 5B). However,

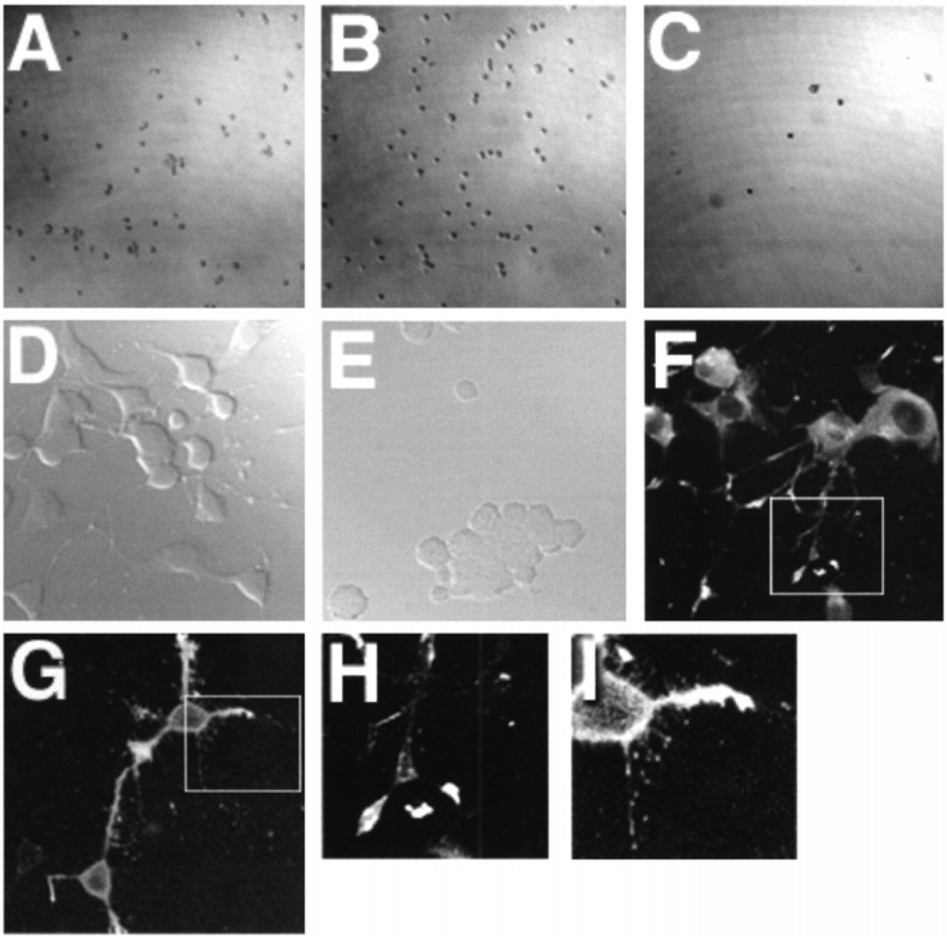

Figure 4. MK immobilized on plates induced neurite outgrowth on N2a cells. N2a cells (A–E) and N2a cells transfected with pFLAG-GP2 (F) or pFLAG-SYN3 (G) were cultured on coverslips coated with MK (A, D and F–I), poly-L-lysine (B and E) or BSA (C) and cultured for 1 h (A–C) or 24 h (D–I). After fixing the cells with 4% paraformaldehyde, FLAG-tagged proteins were detected with anti-FLAG M2 antibody followed by FITC-conjugated anti-mouse IgG. Phase-contrast (A–E) and confocal images (F–I) were taken (A–C, 100× ; D–G, 400× magnification). The boxed areas in F and G are enlarged in H and I, respectively.

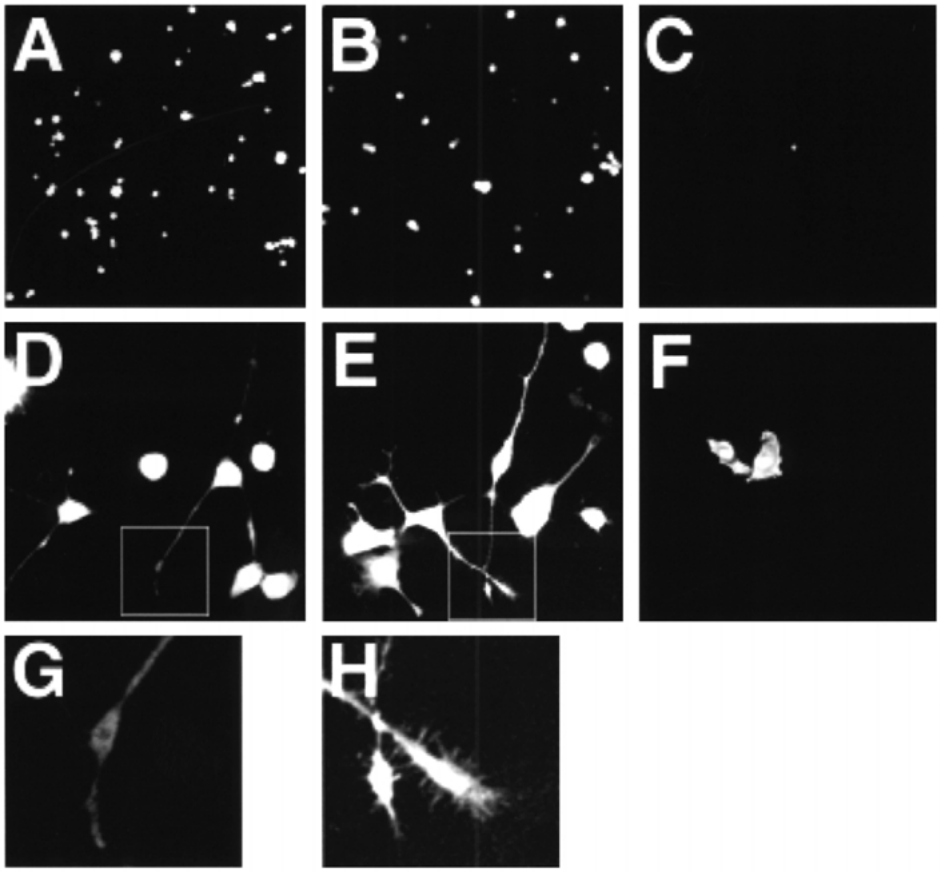

Figure 5. Mimicking of glypican-MK interaction with antibody can induced neurite outgrowth. N2a cells were cotrasnsfected with pEGFPN1 and pFLAG-GP2 (A, D and G), pFLAG-SYN (B, E and H) or pCMV-FLAG1 (C and F). Twenty-four h after transfection, cells were harvested with PBS-EDTA and were plated on anti-FLAG-coated plates. Cells were cultured with serum-free medium for 1 h (A–C) or 48 h (D–H) and confocal images of GFP fluorescence were taken (A–C, 100×; D–F, 400× magnification). The boxed areas in D and E are enlarged in G and H, respectively.

cells expressing only GFP, which did not have the FLAG-tagged proteoglycans, did not adhere to the plates (Figure 5C). When cultured for 48 h in serum-free medium, cells expressing glypican-2 or syndecan-3 developed neurites (Figure 5D and E). The neurite outgrowth activity by the antibody mimicking was not as strong as that of syndecan-3, suggesting that MK-syndecan interactions play key roles in neurite outgrowth. Interestingly, ligation of syndecan-3 to the substratum by an antibody induced the formation of filopodia-like structures on N2a cells (Figure 5E and H).

Discussion

Here, we presented evidence that glypican-2 bound to MK with intensity similar to syndecan-3, and explored the possibility that the MK-glypican interaction plays a role in neurite outgrowth. Indeed, ligation of glypican-2 with substratum by MK or an antibody was found to induce cell adhesion and neurite outgrowth of N2a neuroblastoma cells.

There are two distinct families of heparan sulfate proteoglycans on the cell-surface, syndecans and glypicans, which have different structural organizations [10]. The localization of syndecan-3 on filopodia, key players in the control of axonal growth and guidance, suggests that syndecan-3 but not glypican-2 plays a role in the adhesive interaction of filopodia and ECM. The site of glycosaminoglycan chains on the core proteins may also influence the ability to interact with ECM-bound MK. Due to the differences in the structure of the core proteins, heparan sulfate chains of syndecans are located distal from the plasma membrane but those of glypicans are close to the plasma membrane [10]. This may mean that syndecans dominate over glypicans with respect to ECM binding. It is likely that MK-syndecan interactions play key roles in neurite outgrowth and MK-glypican interactions have a further enhancing effect. This idea is supported by the strong neurite-outgrowth activity of syndecan by antibody mimicking (Figure 5). Furthermore, the different localization of epitope-tagged glypican-2 and syndecan-3 suggested that they may generally play different roles in various aspects of MK-mediated neural function, [8,28,31,32,34].

While regulation of cytoskeletal reorganization by syndecans has been the subject of detailed analysis, few studies have

established glypican-specific functions [10]. As glypicans are anchored to the highly mobile extracytoplasmic face of the plasma membrane via GPI, and their heparan sulfate chains are close to the plasma membrane, they have been considered to act as co-receptors for soluble heparin-binding proteins. Recently, GPI-anchored proteins were found to localize in microdomains, sites of signal transduction for several extracellular stimuli [30]. Although there is no direct evidence for the localization of glypicans in microdomains, the punctuate localization of glypican-2 in COS cells suggested that glypican-2 forms oligomers on the plasma membrane. Binding to MK may induce and stabilize glypican-2 oligomers, and this allows glypican-2 to interact with signaling molecules in the microdomain. MK works not only as a matrix-bound molecule promoting cell adhesion, neurite outgrowth and cell migration, but also as a soluble factor enhancing cell growth [31] and neuronal survival [32]. Thus, it is possible that glypican-2 is also involved in mediating these activities.

In addition to syndecans and glypican-2, receptor-like protein-tyrosin phosphatase ζ, has been indicated to act as a receptor for MK and HB-GAM [33,34]. The orchestrated adhesive interaction between these proteoglycans and MK or HB-GAM deposited on the ECM may transmit forces and signals and achieve neuronal cell migration and neurite outgrowth. Furthermore, low density lipoprotein receptor-related protein (LRP) has also been implicated in reception of MK signals, at least in the case of MK-induced survival of embryonic neurons [35]. The mode of interaction of cell-surface proteoglycans with transmembrane proteins including LRP in MK signaling is not well understood, and further studies are needed also for glypican-2 in this respect.

Acknowledgments

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