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The Multi-PDZ Domain Protein MUPP1 Is a Cytoplasmic Ligand for the Membrane-Spanning Proteoglycan NG2

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Abstract A yeast two-hybrid screen was employed to identify ligands for the cytoplasmic domain of the NG2 chondroitin sulfate proteoglycan. Two overlapping cDNA clones selected in the screen are identical in sequence to a DNA segment coding for the most amino-terminal of the 13 PDZ domains found in the multi-PDZ-protein MUPP1. Antibodies made against recombinant polypeptides representing these two clones (NIP-2 and NIP-7) are reactive with the same 250-kDa molecule recognized by anti-MUPP1 antibodies, confirming the presence of the NIP-2 and NIP-7 sequences in the MUPP1 protein. NIP-2 and NIP-7 GST fusion proteins effectively recognize NG2 in pull-down assays, demonstrating the ability of these polypeptide segments to interact with the intact proteoglycan. The fusion proteins fail to bind NG2 missing the C-terminal half of the cytoplasmic domain, emphasizing the role of the NG2 C-terminus in the interaction with MUPP1. The existence of an NG2/MUPP1 interaction in situ is demonstrated by the ability of NG2 antibodies to co-immunoprecipitate both NG2 and MUPP1 from detergent extracts of cells expressing the two molecules. MUPP1 may serve as a multivalent scaffold that provides a means of linking NG2 with key structural and/or signaling components in the cytoplasm. J. Cell. Biochem. 79:213–224, 2000.

Key words: NG2 proteoglycan; MUPP1 protein; PDZ modules; cytoplasmic scaffolding; transmembrane signaling; yeast two-hybrid screen

As a membrane-spanning molecule, the NG2 proteoglycan has the potential for interaction with both extracellular and cytoplasmic binding partners. Because of this we have been intrigued by the idea that NG2 might function as a signal transducing molecule, mediating communication between the extracellular and intracellular compartments of the cell. Evidence supporting this type of role for the proteoglycan is now beginning to accumulate. For example, the interaction of NG2 with type VI collagen, a specific extracellular matrix ligand for the proteoglycan [Nishiyama and Stallcup, 1993; Burg et al., 1996; Tillet et al., 1997], stimulates cell motility [Burg et al., 1997], suggesting that NG2/matrix interactions can result in activation of the cytoskeletal machinery required for cell migration. In a similar vein, NG2 and the α4β1 integrin have been proposed as coreceptors that have distinct signaling roles in mediating the spreading of melanoma cells on fibronectin-coated surfaces [Iida et al., 1995].

Both of these examples imply that NG2 might be able to impact or to interact in some way with the actin cytoskeleton, a possibility that is supported by previous results from our lab. In cells that are well spread on the substratum, NG2 is arranged on the cell surface in linear arrays that are codistributed with actin- and myosin-containing stress fibers in the cytoplasm. Both the disruption of these stress fibers and the removal of the NG2 cytoplasmic tail result in the loss of NG2 organization on

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the cell surface, suggesting that NG2 might use stress fibers as a means of anchorage [Lin et al., 1996a; Fang et al., 1999]. In migrating cells, NG2 is associated with actin-containing retraction fibers on the trailing edge of the cell [Lin et al., 1996b]. These retraction fibers have been postulated to provide a specialized mechanism for release of the trailing edge from the substratum, thus freeing the cell to migrate in the direction of the traction developed by adhesive lamellipodia on the leading edge [Mitchison, 1992; Palacek et al., 1996]. We have speculated that NG2 might influence cytoskeletal rearrangements that enhance retraction fiber release from the substratum.

Recent studies have shed more light on the involvement of NG2 in reorganization of the actin cytoskeleton. Attachment of NG2-positive cells to substrata coated with monoclonal antibodies against the proteoglycan has been shown to induce both cell spreading and cell migration [Fang et al., 1999]. Examination of these cells using rhodamine-labeled phalloidin reveals that this engagement of NG2 by the substratum triggers specific types of actin rearrangements, namely, the extension of actin-containing filopodia and lamellipodia. These processes are thought to be mediated by the rho family members cdc42 and rac, respectively [Nobes and Hall, 1995; Ridley et al., 1992], suggesting a role for NG2 in activation of these GTPases. Significantly, we find that the cytoplasmic domain of NG2 is required for these cytoskeletal rearrangements to occur [Fang et al., 1999], demonstrating that this domain of the proteoglycan is likely to be involved in activation of the cytoplasmic signaling cascades associated with cytoskeletal reorganization.

It has now become a priority for us to identify the mechanisms by which NG2 initiates these signaling processes. This includes not only the characterization of specific signaling pathways activated by engagement of the proteoglycan, but also the identification of cytoplasmic ligands for NG2 that are responsible for the apparent cytoskeletal anchorage of the proteoglycan and for the apparent ability of NG2 to trigger cytoskeletal reorganization. This report describes the use of a yeast two-hybrid screen to identify ligands for the NG2 cytoplasmic domain. One of the NG2-binding molecules identified in this screen is a previously-described cytoplasmic anchorage protein (MUPP1) that contains multiple PDZ domains but no apparent catalytic motifs [Ullmer et al., 1998; Lee et al., 1997]. These PDZ modules can interact with specific carboxy-terminal motifs present in a variety of transmembrane and intracellular proteins, and thus may allow MUPP1 to serve as a molecular scaffold for clustering of NG2 and other components into multimeric structural and signaling complexes [Saras and Heldin, 1996; Craven and Bredt, 1998].

MATERIALS AND METHODS

Yeast Two-Hybrid Selection

The DNA sequence corresponding to amino acids 2250–2325 (i.e., the cytoplasmic domain) of rat NG2 was inserted in frame into the LexA binding domain of the yeast expression vector pBTM116 [Bartel et al., 1993]. The resulting plasmid was designated as pBTMcNG2. The transcriptional activation domain for the two-hybrid selection was supplied by the pVP16 vector [Vojtek et al., 1993]. A random-primed, size-selected (350–700 nucleotides) CD1 mouse embryo (E9.5 and E10.5) cDNA library was ligated into the Not I site of pVP16. Using the lithium acetate method [Hill et al., 1991], yeast strain L40 [Vojtek and Hollenberg, 1995] was sequentially transformed with pBTMcDNA and with 350 μg of the pVP16/library plasmid DNA, and transformants were plated in synthetic THULL medium lacking tryptophan, histidine, uracil, lysine, and leucine. Transformants that grew in THULL medium were also screened for activation of the lacZ reporter gene. Of 2×10^6 transformants screened, five were β-galactosidase-positive and could grow in THULL medium. As a control, cotransformants of pBTMcNG2 and pVP16 containing human lamin C cDNA were unable to grow in THULL medium even after 10 days. Weak intrinsic activation of the His3 reporter gene by pBTMcNG2 was suppressed by the addition of 25 mM 3-amino-1,2,4-triazole to the THULL medium.

The two-hybrid system was also used to examine possible interactions between NG2-interacting clones (specifically NIP-2 and NIP-7, see Results) and the cytoplasmic domains of rat syndecan-2 [Ethell and Yamaguchi, 1999] and chicken EphB2 [Zisch et al., 1998]. For this purpose, cDNA segments coding for the cytoplasmic domains of syndecan-2 and EphB2 were inserted into the pBTM116 vector

and transformed into L40. Following subsequent transformation with the NG2-interacting clones (in pVP16), transformants were checked for growth in THULL medium.

Sequencing

Library cDNAs were amplified by polymerase chain reaction (PCR) [Saiki et al., 1988] directly from the yeast colonies, using the M13 universal primer and a pVP16-complementary primer [Vojtek et al, 1993]. PCR products with inserts of 280–570 base pairs were sequenced in both directions by ABI PRISM dye termination cycle sequencing (Perkin-Elmer, Foster City, CA). Sequences were analyzed for similarity to known nucleotide and protein sequences by BLAST searches of databases at the National Center for Biotechnology Information [Altschul et al., 1990]. Sequences for cDNA segments amplified by PCR from rat MUPP1 were also determined by the cycle-sequencing method.

Northern Blotting

Poly $(A)^+$ RNA was isolated from embryonic day 9.5–10.5 mice using a Fast Track 2.0 kit (Invitrogen, San Diego, CA). Five-microgram samples of the RNA were electrophoresed on 1% agarose gels containing 2.2 M formaldehyde. Samples were transferred to nitrocellulose filters, which were hybridized according to the method of Thomas [1980] with NIP-2 and NIP-7 cDNA probes that had been labeled with ^{32}P using a random priming kit (Amersham Pharmacia Biotech). Autoradiograms were prepared using Kodak X-Omat AR film.

GST Fusion Proteins

cDNAs for NG2-interacting proteins (NIPs) and for lamin C were subcloned in frame into the pGEX-4T-1 expression vector (Pharmacia). cDNA sequences coding for MUPP1 PDZ domains other than the first were amplified by PCR from rat MUPP1 cDNA [Ullmer et al., 1998]. These segments represent the following pairs of MUPP1 PDZ domains: 2/3 (nucleotides 943–1572), 10/11 (nucleotides 5008–5556), and 12/13 (nucleotides 5707–6345). These cDNA fragments were subcloned in frame into pGEX-2T-1, using the BamH1 and EcoR1 restriction sites. Glutathione S-transferase (GST) fusion proteins were expressed in *Escherichia coli* XL1 Blue (Stratagene, San Diego, CA). Induction with IPTG and purification of fusion proteins were carried out as previously described [Ausubel et al., 1997] using glutathione-agarose beads (Sigma, St. Louis, MO).

Cell Lines

The U251MG human astrocytoma cell line [Ponten and Westermark, 1978] was used in these studies. U251 cells transfected with cDNA for rat NG2 and the truncated NG2/t3 mutant have been described previously [Nishiyama and Stallcup, 1993; Nishiyama et al., 1995; Lin et al., 1996a, 1996b; Burg et al., 1997; Fang et al., 1999]. NG2/t3 is prematurely terminated after amino acid residue E-2276 [see Nishiyama et al., 1991a; Fang et al., 1999], resulting in deletion of the C-terminal half of the NG2 cytoplasmic domain. Cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Tissue Culture Biologicals, Tulare, CA).

Antibodies

A rabbit antibody (designated RaNG2/EC) raised against the complete recombinant extracellular domain of NG2 [see Tillet et al., 1997; Goretzki et al., 1999] was affinity purified on this same extracellular fragment and used for both immunoprecipitation and immunoblotting. For immunoblotting studies, we also utilized a rabbit antibody raised against the C-terminal half of the mouse homolog of MUPP1 (designated 9BP-1) [Lee et al., 1997]. Additional rabbit antibodies were prepared against the polypeptides encoded by the NIP-2 and NIP-7 cDNA clones selected in the yeast two-hybrid screen. These polypeptides were first prepared as GST fusion proteins, and after thrombin treatment to cleave the GST linkage, were purified by electroelution from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Rabbits were initially immunized with the purified polypeptides emulsified in complete Freund's adjuvant. Subsequent boosts were made with polypeptides emulsified in incomplete Freund's adjuvant.

Pull-Down Assays

GST fusion proteins representing lamin, NIP-2, NIP-7, and the MUPP1 PDZ pairs 2/3, 10/11, and 12/13 were prepared as described above, and purified on glutathione-agarose beads. These fusion protein-coated beads were

used as a matrix for binding NG2 in 0.2% NP40 extracts of NG2-transfected U251 and B28 cells that had been surface-labeled with ^{125}I [Nishiyama et al., 1991a; Dahlin-Huppe et al., 1997]. Beads were incubated with the ^{125}I-labelled extracts for 2 h at 4°C with constant agitation on a rotary shaker. They were then washed three times with phosphate-buffered saline (PBS) containing 0.05% NP40 and treated with 0.02 units of chondroitinase ABC (ICN, Costa Mesa, CA) for 1 h at room temperature to remove chondroitin sulfate chains from NG2. After boiling in SDS-PAGE loading buffer, the samples were subjected to SDS-PAGE analysis on 3%–20% gradient gels. Labeled components were identified by autoradiography using Kodak X-Omat AR film.

Immunoprecipitation

Immunoprecipitation of NP40-extracted components from ^{125}I-labelled cells was performed as described previously, using Protein A-Sepharose beads (Pharmacia) to isolate antigen-antibody complexes [Nishiyama et al., 1991a; Dahlin-Huppe et al., 1997]. In most cases, half of each immunoprecipitate was treated with 0.02 units of chondroitinase. Samples were then boiled in SDS-PAGE sample buffer and analyzed on 3%–20% SDS-PAGE gels, as described for the pull-down assays.

For co-immunoprecipitation experiments, unlabelled U251 cells expressing rat NG2 were extracted with 0.2% NP40 and incubated for 30 min at room temperature with affinity-purified RaNG2/EC antibody or, as a control, with Protein A-purified nonimmune rabbit immunoglobulin. Agitation with Protein A-Sepharose beads for 1 h at room temperature was then used to isolate antigen-antibody complexes, which were washed with PBS containing 0.05% NP40 and treated with 0.02 units of chondroitinase. Precipitates were divided into two equal aliquots, which were run on parallel 3%–20% SDS-PAGE gels and used for immunoblotting with antibodies against NG2 and MUPP1.

Immunoblotting

Immunoblotting of NP40 extracts and immunoprecipitated material was performed as described previously [Nishiyama et al., 1995; Grako et al., 1999; Fang et al., 1999]. Immunoreactive bands were visualized using an enhanced chemiluminescence kit (Amersham Life Sciences, Buckinghamshire, England).

RESULTS

Characterization of cDNA Clones

Two of the clones for NIPs selected in the yeast two-hybrid screen (NIP-2 and NIP-7) proved to be overlapping cDNA segments with 90% identity at the nucleic acid level to the first of the 13 PDZ domains of MUPP1, a multi-PDZ domain protein of rat origin. MUPP1 was previously identified in two independent laboratories on the basis of its ability to interact with the C-terminal PDZ-binding motif present in a serotonin (5-HT$_{2C}$) receptor [Ullmer et al., 1998] and with a PDZ-binding motif at the C-terminus of the 9ORF1 viral transforming protein [Lee et al., 1997]. The NIP-2 and NIP-7 sequences are identical to the corresponding segment of the mouse homolog of MUPP1 (Genebank database, accession number 4150878). Figure 1 compares the sequences of NIP-2 and NIP-7 with the corresponding segments of mouse and rat MUPP1.

On Northern blots of RNA extracted from embryonic day 9.5–10.5 mouse embryos, NIP-2 and NIP-7 cDNA probes recognized an mRNA of 8.5 kb (data not shown). A transcript of this size is consistent with the predicted molecular mass of the MUPP1 polypeptide (250 kDa). An 8.5-kb component has also been identified as the MUPP1 transcript in human tissues [Ullmer et al., 1998].

Identification of Proteins Containing the NIP-2 and NIP-7 Motifs

As further verification that the polypeptides encoded by the NIP-2 and NIP-7 cDNA clones are derived from the MUPP1 protein, rabbit antisera were prepared against both NIP-2 and NIP-7 and used for immunoblotting of detergent extracts from U251 cells transfected with NG2. For comparison, parallel sets of extracts were immunoblotted with a rabbit antibody against NG2 and with a rabbit antibody prepared against the 9BP-1 polypeptide, which represents the C-terminal portion of mouse MUPP1 [Lee et al., 1997]. Figure 2 shows that antibodies against NIP-7 and 9BP-1 both recognize a chondroitinase-insensitive 250-kDa band in these extracts (asterisk). A similar result was obtained with the antibody against NIP-2 (not shown). The NG2 core polypeptide appears as a slightly larger (300 kDa) band that becomes much more prevalent after treatment with chondroitinase (arrow). It is impor-

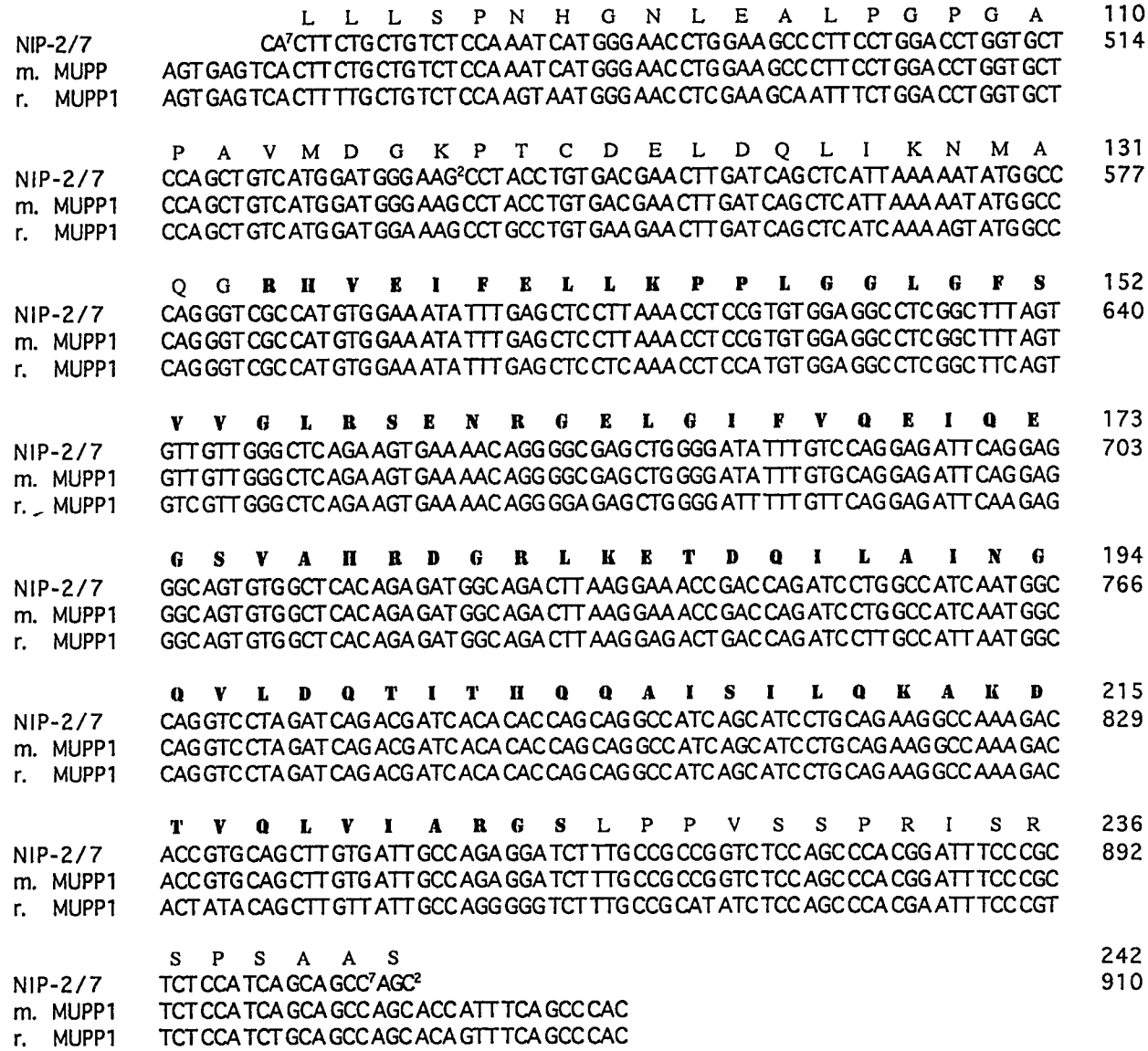

Fig. 1. Sequence homology of NIP-2 and NIP-7 with MUPP1. The nucleotide sequence of the NIP-2 and NIP-7 cDNA clones are compared with the nucleotide sequences of the appropriate segments of mouse (m) and rat (r) MUPP1. The amino acid sequence of the mouse molecule is also shown. Residues in bold type correspond to the first PDZ domain of MUPP1 (Ullmer et al, 1998). Nucleotide and amino acid numbers are listed in the righthand margin. Superscript numerals 2 and 7 mark the beginning and end of the NIP-2 and NIP-7 cDNA clones.

tant to note that the anti-NG2 antibody does not cross-react with MUPP1, and conversely, that the MUPP1 antibodies are not cross-reactive with NG2.

Binding of NG2 by MUPP1 PDZ Domain 1

The NG2 binding capability of the MUPP1 polypeptide segment encoded by the NIP-2 and NIP-7 cDNA clones was investigated through the use of a pull-down assay. GST-fusion proteins containing the NIP-2 and NIP-7 polypeptides were isolated on glutathione-agarose beads and tested for their ability to bind NG2 in detergent extracts of ^{125}I-labelled U251 cells transfected with rat NG2. Immunoprecipitation with rabbit antibodies against NG2 served as a positive control in these experiments, while GST-lamin beads served as a negative control. Figure 3 shows that both the NIP-2 and NIP-7 fusion proteins compare favorably with the NG2 antibody in terms of their ability to bind NG2 from the detergent extract. The

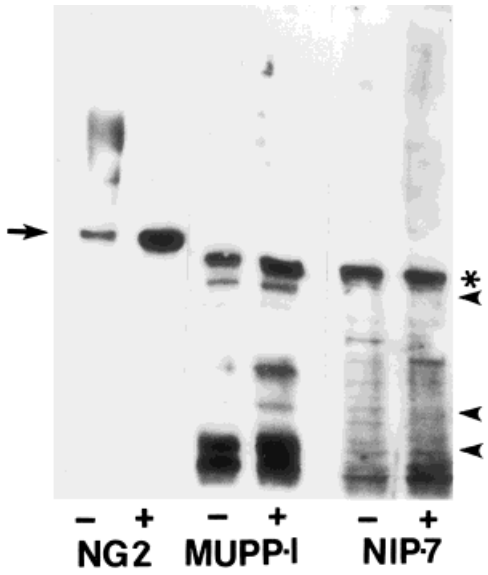

Fig. 2. Identification of molecules containing NIP-2 and NIP-7 segments. NP40 extracts of U251 cells transfected with NG2 were treated with chondroitinase ABC (+) or left untreated (−) and then electrophoresed on 3-20% SDS-PAGE gels. After transfer to Immobilon P, samples were subjected to immunoblot analysis with rabbit antibodies against NG2, MUPP1, NIP-7, and NIP-2 (not shown). The 300 kDa NG2 core protein (arrow) becomes much more prominent following removal of chondroitin sulfate chains from the intact proteoglycan. In contrast, the 250 kDa MUPP1 band, recognized by both the MUPP1 and NIP-7 antisera (asterisk), is insensitive to chondroitinase treatment. Arrowheads at right indicate positions of 200, 116, and 92 kDa molecular weight standards.

ability of chondroitinase to convert the intact proteoglycan into the characteristic 300-kDa core protein (arrow) helps to confirm the identity of this component as NG2. In contrast, the lamin-GST fusion protein does not bind a detectable quantity of NG2.

An additional characterization of the specificity of the interaction between NG2 and the NIP-2 and NIP-7 polypeptides is provided by the use of extracts from ^{125}I-labelled U251 cells transfected with the NG2/t3 deletion mutant. Although this truncated molecule is still immunoprecipitated by the NG2 antibody, it is not recognized by the NIP-2 and NIP-7 fusion proteins. This experiment demonstrates that the interaction between NG2 and the first MUPP1 PDZ domain requires the presence of the C-terminal half of the NG2 cytoplasmic domain. This is consistent with the localization of the putative PDZ binding sequence (QYWV) at the extreme C-terminus of the proteoglycan.

Selectivity of NG2 Binding to the First PDZ Domain of MUPP1

As a means of determining whether NG2 preferentially binds to the first PDZ domain of MUPP1, we compared GST fusion proteins representing PDZ domains 1 (NIP-7), 2/3, 10/11, and 12/13 for their ability to interact with NG2 in the pull-down assay. Immunoprecipitation with rabbit anti-NG2 once again served as a positive control, while beads coated with GST alone were used as the negative control. Figure 4A shows that the fusion protein containing PDZ domains 2 and 3 is comparable to PDZ domain 1 in its ability to bind full-length NG2, which once again can be recognized by its conversion to the 300-kDa core protein (arrowheads) by chondroitinase treatment. In contrast, GST-coated beads and beads coated with domains 10/11 and 12/13 fail to pull down significant amounts of NG2 in this assay. Figure 4B shows the sizes of the different fusion proteins used in this study and demonstrates that the various beads carry similar amounts of protein. Thus, the dramatic differences in NG2-binding capability between the various preparations is not due to large differences in the amount of fusion proteins carried by the glutathione-agarose beads.

Another aspect of the NG2/MUPP1 interaction is the specificity of the first MUPP1 PDZ domain for NG2 versus other PDZ-binding molecules. We used the yeast two-hybrid system to test the ability of NIP-2 and NIP-7 to interact with the cytoplasmic domains of two other PDZ-binding molecules, EphB2 and syndecan-2. In the same type of screen that showed binding of NIP-2 and NIP-7 to the cytoplasmic domain of NG2, we failed to detect interactions with EphB2 and syndecan-2 (data not shown). Thus, the first PDZ domain of MUPP1 exhibits at least some level of discrimination between NG2 and other transmembrane PDZ-binding ligands.

Co-Immunoprecipitation of NG2 and MUPP1

Evidence for an intracellular interaction between NG2 and endogenous, full-length MUPP1 is provided by experiments that show MUPP1 is co-immunoprecipitated along with NG2 from extracts of cells that express both molecules (Fig. 5). In these experiments, NP40 extracts of NG2-transfected U251 cells were subjected to immunoprecipitation with affinity-purified rabbit antibody against the NG2 ectodo-

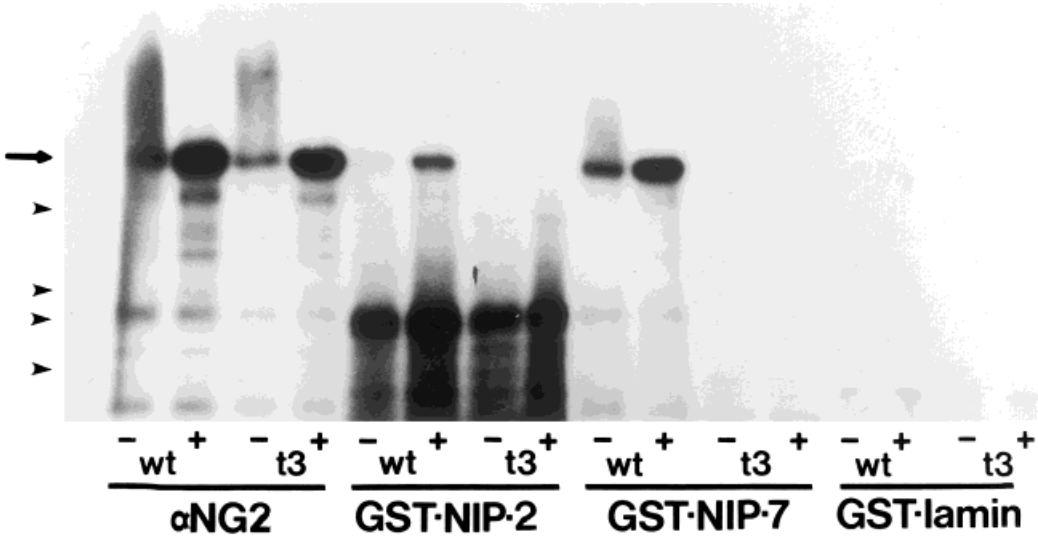

Fig. 3. Pull-down of intact NG2 by NIP-2 and NIP-7 fusion proteins. NP40 extracts of ^{125}I-labelled U251 cells expressing either wild type NG2 (wt) or the NG2/t3 mutant (t3) were incubated with glutathione-agarose beads carrying GST fusion proteins of NIP-2, NIP-7, or lamin (as a negative control). A positive control is provided by incubation of the extract with a rabbit antibody against NG2 (αNG2) followed by Protein A-Sepharose beads. Samples were treated with chondroitinase ABC (+) or left untreated (−) and analyzed by electrophoresis on 3–20% SDS-PAGE gels. Arrow marks position of 300 kDa NG2 core protein. Arrowheads indicate positions of 200, 116, 92, and 67 kDa molecular weight standards. NIP-2 and NIP-7 are very effective binders of wild type NG2, but fail to recognize NG2/t3, which is missing the C-terminal half of the cytoplasmic domain. The antibody against NG2 recognizes both the wild type and mutant NG2 species.

main. Identical aliquots of the chondroitinase-treated precipitates, as well as chondroitinase-treated samples of the crude detergent extract, were fractionated in parallel by SDS-PAGE and then immunoblotted with NG2 and 9BP-1 antibodies. These immunoblots reveal the presence of both NG2 and MUPP1 in the immunoprecipitates. Because the results in Fig. 2 show that antibodies against NG2 do not cross-react with MUPP1, these co-immunoprecipitation results are consistent with the existence of a physical association between NG2 and MUPP1. This is our best evidence that NG2 binds to full length MUPP1 in the context of the normal cellular environment.

DISCUSSION

The data from our yeast two-hybrid screen, as well as the results from pull-down assays and co-immunoprecipitation experiments demonstrate binding of the NG2 proteoglycan to the multi-PDZ domain protein MUPP1. The ability of NG2 to utilize MUPP1 as a cytoplasmic ligand is consistent with the presence of a putative PDZ-binding motif at the C-terminus of the proteoglycan. This QYWV sequence fits the general requirements of a four amino acid PDZ-binding motif in which valine occupies the final position (position 0) and tyrosine is the hydroxyl group-containing residue at position -2 [Songyang et al., 1997]. The importance of the C-terminal portion of the NG2 cytoplasmic domain for interaction with MUPP1 is illustrated by the failure of the truncated NG2/t3 mutant to yield positive results in pull-down assays using fusion proteins representing the first MUPP1 PDZ domain (Fig. 3). To define the PDZ binding motif of NG2 more precisely, we will need to perform additional experiments with NG2 mutants carrying smaller C-terminal deletions and with variants in which individual residues are mutated.

The fact that the yeast two-hybrid screen yielded two cDNA clones (NIP-2 and NIP-7) representing the first MUPP1 PDZ domain but no clones for other MUPP1 PDZ domains suggests that there could be considerable specificity for binding of NG2 to the amino-terminal portion of MUPP1. Although the NG2 binding capability of all 13 MUPP1 PDZ domains remains to be assessed in detail, this expectation is partially borne out by our comparison of the

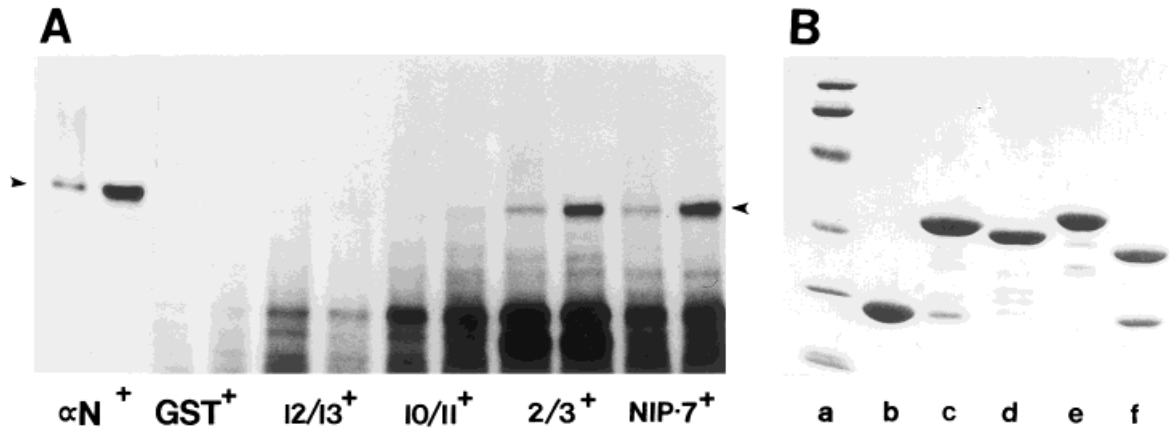

Fig. 4. Comparison of NG2 binding by different MUPP1 PDZ domains. **A.** NP40 extracts of ^{125}I-labelled U251 cells expressing wild type NG2 were incubated with glutathione-agarose beads carrying GST alone, the NIP-7 GST fusion protein, or GST fusion proteins representing MUPP1 PDZ pairs 2/3, 10/11 and 12/13. A positive control is provided by use of rabbit anti-NG2 (αN). Washed samples were analyzed by electrophoresis on 3–20% SDS-PAGE gels. Samples marked as (+) were treated with chondroitinase ABC to facilitate visualization of the 300 kDa NG2 core protein (arrowheads). NIP-7 and PDZ 2/3 are effective in pulling down full length NG2, while PDZ 10/11 and 12/13 are comparable to the GST negative control. **B.** Equal quantities of glutathione-agarose beads carrying GST (b), PDZ 10/11 (c), PDZ 12/13 (d), PDZ 2/3 (e), and NIP-7 (f) were boiled in SDS-PAGE sample buffer and electrophoresed on 3–20% SDS-PAGE gels. Staining with Coomassie Blue shows that comparable amounts of protein are carried by the various types of beads. Lane (a) contains molecular weight standards of 116, 92, 67, 45, 31, and 21 kDa.

NG2 binding capability of MUPP1 PDZ domains 2/3, 10/11, and 12/13. Although the PDZ pairs 10/11 and 12/13 fail to bind NG2 in the pull-down assay, the 2/3 pair has activity comparable to that of PDZ-1. Additional tests with NG2 deletion mutants will be required to determine whether PDZ-1 and PDZ-2/3 interact with the same segment of the NG2 C-terminus, or whether the PDZ domains recognize distinct binding sites in the proteoglycan. Nevertheless, there appears to be a significant preference of NG2 for binding to PDZ domains in the N-terminal portion of MUPP1. Other known MUPP1 ligands such as the 5-HT_{2C} receptor [Ullmer et al., 1998] and the viral 9ORF1 protein [Lee et al., 1997] appear to bind to PDZ domains in the C-terminal half of MUPP1, giving this scaffolding protein a decided polarity in its ligand binding. This property might be important for the ability of MUPP1 to cluster ligands in specific spatial patterns. The structural details of MUPP1 PDZ domains that determine their respective specificities for NG2, 9ORF1, and the 5-HT_{2C} receptor remain to be defined. An additional indication of specificity in the interaction of NG2 with the first PDZ domain of MUPP1 comes from the yeast two-hybrid data showing that our NIP-2 and NIP-7 clones fail to interact with the cytoplasmic segments of syndecan-2 and Eph2B. Thus the first MUPP1 PDZ domain has the ability to discriminate between NG2 and other potential PDZ-binding transmembrane receptors.

The interaction of NG2 with a PDZ domain-containing cytoplasmic ligand presents an interesting parallel to the case of the syndecan family of proteoglycans, which also utilize PDZ-containing molecules as cytoplasmic binding partners. Syndecans have been shown to bind via their conserved C-terminal EFYA sequence to the PDZ-containing molecules syntenin [Grootjan et al., 1997] and CASK/LIN-2 [Cohen et al., 1998; Hsueh et al., 1998]. The colocalization of CASK and syndecan-2 in neuronal synapses [Hsueh et al., 1998] suggests a role for this interaction in the development and/or stabilization of these complex structures. In support of this idea, syndecan-2 has been shown to be specifically clustered on dendritic spines and to be involved in the morphologic maturation of the spines, which serve as primary sites for the formation of excitatory synapses [Ethell and Yamaguchi, 1999]. The EFYA motif of the syndecan is required for its ability to mediate spine maturation, suggesting the involvement of the syndecan-PDZ interaction in this process.

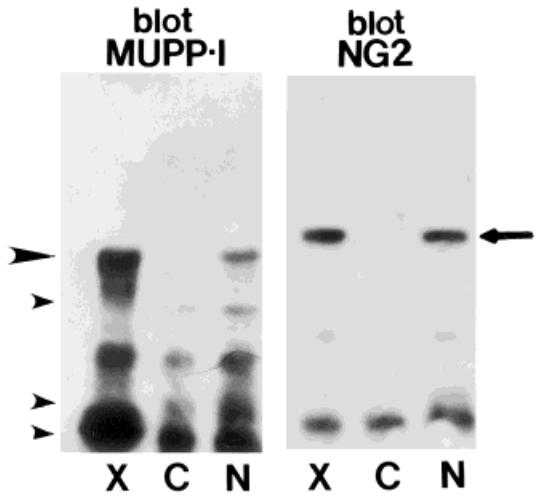

Fig. 5. Co-immunoprecipitation of NG2 and MUPP1. Chondroitinase-treated NP40 extracts of NG2-transfected U251 cells were subjected to immunoprecipitation with affinity-purified rabbit antibody against NG2 (N) or with control nonimmune immunoglobulin (C). Immunoprecipitates and samples of the crude NP40 extract (X) were run on 3%–20% SDS-PAGE gels, transferred to Immobilon P, and immunoblotted with antibodies against NG2 (blot NG2) or MUPP1 (blot MUPP1). Anti-NG2 immunoprecipitates contain both NG2 (arrow at right) and MUPP1 (large arrowhead at left). Arrowheads at left indicate positions of 200, 116, and 92 kDa molecular weight standards.

Although the functional importance of NG2 binding to MUPP1 has not been established in this type of detail, the general properties of PDZ domain-containing molecules suggest some interesting possibilities. As indicated by the foregoing set of observations on syndecans, molecules containing PDZ domains are thought to serve as cytoplasmic scaffolds for anchoring proteins or assembling them into complexes that serve either structural or signaling functions. For example, PDZ-containing proteins are postulated to be involved in localizing ion channels, cell adhesion molecules, neurotransmitter receptors, and Eph receptors and their ephrin ligands at key sub-cellular sites [Kornau et al., 1997; Ponting et al., 1997; Craven and Bredt, 1998; Hata et al., 1996; Irie et al., 1997; Torres et al., 1998]. A scaffolding function of this nature would appear to be relevant to two types of phenomena involving NG2. First, the detailed pattern of NG2 localization on the cell surface suggests an association with the actin cytoskeleton [Lin et al., 1996a, 1996b]. The mechanism for this association is unknown, but binding to MUPP1 could provide a means for linking NG2 to other molecules that have a cytoskeletal localization. Second, engagement of NG2 by the substratum induces reorganization of the actin cytoskeleton leading to changes in cell morphology and cell motility [Fang et al., 1999]. This is consistent with the ability of NG2 to activate cytoplasmic signaling cascades that control cytoskeletal dynamics [Eisenmann et al., 1999]. Binding of NG2 to MUPP1 could provide a link to these signaling pathways. In this regard it will be important to determine the relative cellular localizations of NG2 and MUPP1. Unfortunately, the existing MUPP1 antibodies do not give a clear picture of MUPP1 localization, so investigation of this aspect of the NG2/MUPP1 interaction will require the development of better reagents.

Although MUPP1 itself contains no obvious catalytic motifs, the 13 PDZ domains in the molecule provide a wealth of potential sites for bringing NG2 together with key structural and/or signaling molecules. At present only a few such MUPP1 ligands have been identified. One such ligand is the 5-HT_{2C} receptor [Ullmer et al., 1998], which is mainly expressed in the central nervous system [Molineaux et al., 1989; Mengod et al., 1990]. It is not immediately apparent how the interaction of MUPP1 with this serotonin receptor might be relevant to the function of NG2 in the brain, since the receptor and the proteoglycan are almost certainly found in different neural cell types (neurons and oligodendrocyte progenitors, respectively). However, the closely-related 5-HT_{2A} and 5-HT_{2B} receptors also contain PDZ-binding motifs similar to the one found in the 5-HT_{2C} receptor [Hoyer et al., 1994], and these isoforms are found in peripheral cell types such as vascular smooth muscle and cardiac muscle [Corson et al., 1992; Loric et al., 1992], which also express NG2 [Grako and Stallcup, 1995; Grako et al., 1999]. The 5-HT_2 serotonin receptors activate phospholipase C-β/protein kinase C signaling pathways [Foguet et al., 1992; Wainscott et al., 1993], so that interaction of NG2 with the MUPP1/5-HT_2 complex might have an impact on signaling cascades that rely on phosphatidylinositol turnover.

In addition, the PDZ-mediated interaction of MUPP1 with a second type of ligand, represented by 9ORF1 and other viral transforming proteins [Lee et al., 1997], suggests that association of NG2 with this complex could play a

role in growth control. Since 9ORF1 appears to compete with the APC tumor suppressor protein for binding to the PDZ-containing protein hDLg/SAP97 [Matsumine et al., 1996; Lee et al., 1997], it seems possible that APC might also be able to interact with the 9ORF1 binding site in MUPP1. If the APC/MUPP1 or 9ORF1/MUPP1 interactions were modulated by NG2 binding to MUPP1, this would provide a mechanism for involvement of NG2 in pathways that are relevant to cell proliferation.

We have previously suggested such a role for NG2, based on its pattern of expression during development. Although NG2 is expressed in a variety of developing tissues, a common theme in each case is the expression of the proteoglycan on immature, mitotic progenitor cells followed by its down-regulation once these progenitors undergo terminal differentiation [Stallcup and Beasley, 1987; Nishiyama et al., 1991b; Nishiyama et al., 1996; Grako and Stallcup, 1995; Levine and Nishiyama, 1996]. Moreover, NG2 expression is up-regulated in a number of neoplastic cell types, including melanomas [Real et al., 1985], glioblastomas [Schrappe et al., 1991], chondrosarcomas [Leger et al., 1994], and lymphomas [Behm et al., 1996], and some evidence has been presented that NG2 is responsible for the increased rate of proliferation of these tumor cells [Harper and Reisfeld, 1987; Burg et al., 1998]. Although the relationship between the NG2/MUPP1 complex and APC or 9ORF1-mediated processes is speculative at this point, such a model nevertheless provides a framework for testing specific hypotheses involving these molecules. The results of these experiments, coupled with identification of additional MUPP1 ligands, may shed more light on the functional importance of the NG2/MUPP1 interaction.

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