The Mammalian Numb Phosphotyrosine-binding Domain

CHARACTERIZATION OF BINDING SPECIFICITY AND IDENTIFICATION OF A NOVEL PDZ DOMAIN-CONTAINING NUMB BINDING PROTEIN, LNX

Sascha E. Dho, Sara Jacob, Cheryl D. Wolting, Michelle B. French, Larry R. Rohrschneider‡, and C. Jane McGlade§

From AMGEN Institute, Ontario Cancer Institute, Department of Medical Biophysics, University of Toronto, Toronto, Canada M5G 2C1 and the §Fred Hutchinson Cancer Research Center, Basic Sciences Division, Seattle, Washington 98109-1024

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Numb is a phosphotyrosine-binding (PTB) domain-containing protein implicated in the control of cell fate decisions during development. A modified two-hybrid screen in yeast was used to identify Numb PTB domain-interacting proteins important for Numb function. Here we report the identification of a novel protein, LNX, which interacts specifically with the Numb PTB domain. Two differentially expressed LNX messages encode overlapping proteins with predicted molecular masses of 80 kDa (LNX) and 70 kDa (LNX-b). LNX and LNX-b contain unique amino-terminal sequences and share four PDZ domains. The unique amino-terminal region of LNX includes a RING finger domain. The Numb PTB domain binding region of LNX was mapped to the sequence motif LDNPAY, found in both protein isoforms. Mutational analysis of LNX and peptide competition experiments showed that phosphorylation of the tyrosine residue within this motif was not required for binding to the Numb PTB domain. Finally, we also provide evidence that tyrosine phosphorylation of the LDNPAY sequence motif in LNX could generate a binding site for the phosphorylation-dependent binding of other PTB domain-containing proteins such as SHC. We speculate that LNX may be important for clustering PTB-containing proteins with functionally related transmembrane proteins in specific membrane compartments.

The generation of cellular diversity is achieved, in part, by asymmetric cell division in which specific molecules are segregated to one daughter cell. In Drosophila, numb was originally identified as a gene affecting binary cell fate decisions during development of the sensory organ and specific neurons of the peripheral and central nervous system (1). dNumb1 is a membrane-associated protein which is asymmetrically localized during cell divisions and subsequently segregated to one daughter cell where it functions as an intrinsic determinant of cell fate (2–6).

Mammalian Numb, a protein having homology with dNumb, was recently identified (7, 8). Ectopic expression of mNumb in Drosophila produces a similar phenotype to ectopic expression of dNumb (7, 8). The function of mNumb is unknown, but it may, in part, be involved in cortical neurogenesis because it is expressed in all layers of the developing cortical plate including the progenitor cells of the ventricular and subventricular zones. In these cells, mNumb is symmetrically distributed around the circumference of the cell and becomes asymmetrically localized during cell division (9). In addition, mNumb overexpression enhances the differentiation of P19 cells into neuronal cells (8). Thus, mNumb may have a role in cell fate decisions in the mammalian nervous system. However, mNumb is expressed in most adult tissues, and its expression is widespread in mouse embryos (8, 9) suggesting that its function is not limited to neurogenesis. Recently, a mammalian protein called Numblike (Nbl) with 76 and 63% homology to mNumb and dNumb, respectively, was described. Its expression pattern differs in comparison to mNumb and is restricted to cells of the nervous system, in particular post-mitotic neurons (9).

Signal transduction pathways, which regulate many cellular processes such as cell division and differentiation, require protein-protein interactions that often occur via conserved protein-binding domains (10). A highly conserved part of mNumb, dNumb, and Nbl exhibits strong homology to a phosphotyrosine-binding (PTB) domain first identified in the adapter protein SHC (7–9, 11–15). Several other PTB domain-containing proteins have been identified based on sequence similarity to SHC including the SHC-related gene SHC B, FE65, X11, and Drosophila disabled (12). A domain with functional properties and three-dimensional structure similar to the SHC PTB domain was also identified in insulin receptor substrate-1. The insulin receptor substrate-1 PTB domain shows no similarity at the primary amino acid level to the SHC, Numb, or Nbl PTB domains (16).

The binding specificity of PTB domains has been defined in only a limited number of examples. The SHC PTB domain, which mediates the association of SHC with tyrosine-phosphorylated growth factor receptors and downstream effectors (14, 15, 17–19), specifically recognizes phosphotyrosine within the core sequence motif, Asn-Pro-X-Tyr(P) (NPXYP, where X is any amino acid) (15, 16, 20, 21). The PTB domain of insulin receptor substrate-1 recognizes a similar sequence motif in the insulin receptor and also strictly requires phosphorylation of the tyrosine residue within the motif for binding. However, it is becoming apparent that not all PTB domain-mediated interactions
require phosphorylation of the tyrosine residue. In particular, the PTB domain of the neural protein X11 binds to an unphosphorylated NPXY peptide sequence in the β-amyloid precursor protein (βAPP) (22, 23).

The mechanism of action of Numb has not been defined although its structure suggests that it functions as an adapter protein. The conservation of the PTB domain in dNumb and mNumb suggests that this domain likely mediates important protein-protein interactions required for the asymmetric localization or function of Numb proteins. There is evidence that Numb interacts with Notch, a transmembrane receptor that mediates cell fate in many cell types (5, 24, 25). In Drosophila, genetic studies suggest that dNumb acts upstream of Notch and functions to antagonize Notch signaling, possibly by preventing Notch-induced translocation of Suppressor of Hairless to the nucleus by an unknown mechanism (24). In vitro biochemical studies suggest that the amino-terminal region of Numb interacts directly with the intracellular domain of Notch (25). In addition, interactions between dNumb and the products of the Drosophila genes miranda and hairless have also been reported although, as in the case of Notch, these interactions have not been clearly defined in vitro and have not been demonstrated in vivo (26, 27). Recently, both mNumb and Nbl were identified in a screen for proteins that interact with the EH domain of the EGFR substrate, Eps15 (28). This interaction is mediated by an EH domain-binding motif near the carboxyl terminus of both Numb and Nbl (28). This motif is also conserved in the dNumb protein suggesting that interactions with EH domain-containing proteins may also be important for Numb localization or function.

Given the role of PTB domains in protein-protein interactions, we have used the PTB domain of mNumb in a yeast two-hybrid system to identify mNumb binding proteins and elucidate a functional pathway for mNumb. Here we describe the cloning of a specific target of the mNumb PTB domain which we have termed LNX (Ligand of Numb-protein X). LNX is a unique protein containing multiple protein-protein interaction motifs including a RING finger and four PDZ domains. Furthermore, we have used LNX to define the binding specificity of the Numb PTB domain.

MATERIALS AND METHODS

Yeast Two-hybrid and cDNA Library Screening—The region of the rat Numb cDNA encoding the PTB domain was cloned into pBTM116-PDGFR kinase (19). A cDNA library was constructed from mRNA extracted from multiple staged rat embryos in pAD-GAL4 (Stratagene). The pBTM116-PDGFR kinase-NUMB-PTB plasmids and pAD-GAL4 library plasmids were used to cotransform the yeast strain L40. Six million transformants were screened, and 20 positive colonies were selected based on HIS3 and lacZ reporter gene expression. The positive clones were tested for their ability to activate transcription when introduced with the pBTM116-PDGFR kinase alone. The cDNA-containing plasmids from the remaining 10 positive clones were isolated using standard procedures. The cDNA inserts were excised, labeled with 32P by random hexamer priming, and used as probes on dot blots containing all rescued plasmids. Representative cDNA clones from each group were sequenced. Full-length LNX cDNAs were cloned by screening a 16-day mouse embryo library (Novagen), and the LNX-b cDNA was obtained by screening a mouse brain library (Stratagene) using the radiolabeled 2.5-kb partial cDNA as a probe. Positive phage were
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plaque-purified, and the cloned cDNA was sequenced. A cDNA encoding Numblike was cloned by screening a mouse brain library (Stratagene) with the Numb cDNA using low stringency hybridization.²

**Northern Blot Analysis**—Northern blots (CLONTECH) containing 2 μg of poly(A)⁺ mRNA isolated from a variety of murine tissues and embryos were probed with the 2.5-kb cDNA or a region corresponding to the RING finger domain which was radiolabeled by random hexamer priming (Amersham Pharmacia Biotech). Blots were incubated at 42 °C in a solution of 50% formamide, 4× SSPE, 1% SDS, 0.5% skim milk powder, 10% dextran sulfate, and 10 μg/ml sheared salmon sperm DNA.²²P-Radiolabeled probe was then added at 10⁶ cpm/ml, and the blot was further incubated for 16 h at 42 °C. Blots were washed twice for 10 min at room temperature in 2× SSC, 0.1% SDS, then twice at 65 °C in 0.1× SSC, 0.1% SDS, and then exposed to film. The blots were also probed with radiolabeled β-actin cDNA (CLONTECH) as an indicator of RNA loading.

**Cell Culture**—Human A431 and 293T cells and HER14 cells (3T3 TKX-1 strain [Amersham Pharmacia Biotech]) were grown in DMEM containing 10% fetal bovine serum (Life Technologies, Inc.). For experiments requiring phosphorylated EGFR, HER14 cells were grown in DMEM containing 10% fetal bovine serum (CLONTECH) as an indicator of RNA loading.

**Production of GST Fusion Proteins**—pGEX constructs were expressed in either DH5α or BL21 strain of *E. coli*. Expression of the GST fusion protein was induced with 0.1 μM isopropyl-1-thio-β-D-galactopyranoside at 37 °C for 3–5 h. The bacterial pellets were sonicated in PBS plus protease inhibitors, and 1% Triton X-100 was then added. The cleared bacterial lysate was incubated for 1 h at 4 °C with glutathione-Sepharose beads (Amersham Pharmacia Biotech) which were then washed 4 times with PBS. Where indicated, fusion proteins were eluted from beads by addition of 10 mM reduced glutathione (Sigma) in 50 mM Tris, pH 8.

To examine the effect of tyrosine phosphorylation of LNX, GST LNX-RING/N Pay mutant and WT constructs were transformed into TKX-1 strain *E. coli* (Stratagene), which contain the ELK receptor tyrosine kinase domain under the control of the tryptophan promoter. Following induction of the GST fusion protein, the bacteria were incubated a further 2 h in indole-acrylic acid-containing induction media as described in the Stratagene protocol. Phosphorylation was monitored by anti-phosphotyrosine immunoblotting.

In *Vitro Binding and Peptide Competition*—Purified protein was quantified by Coomassie staining. For GST fusion protein pull-down experiments, 5–10 μg of protein attached to beads was added to the cell lysate. Peptide competition assays were performed by preincubating the GST fusion protein with peptide (the concentrations and sequences of the peptides are indicated in the figure legends) for 1 h followed by the addition of lysate for a further 3 h at 4 °C. The samples were then washed and processed as described for immunoprecipitations.

**RESULTS**

**Identification of a Numb PTB Domain Binding Protein, LNX**—To identify specific Numb PTB domain targets, a modified version of the yeast two-hybrid screen was used which allows the detection of phosphorylation-dependent interactions (19). The PTB domain of Numb was cloned into the pBTM116-LexA-PTGDR kinase plasmid to allow the simultaneous expression of a LEXA DNA-binding domain-Numb PTB fusion protein, and the kinase domain of the PDGFR. A rat embryo library was cotransformed with pBTM116-PTGDR-Numb PTB plasmid generating 6 × 10⁶ transformants. From the primary transformants 10 clones were positive for both lacZ and HIS3 expression and interacted specifically with the Numb PTB domain (data not shown).

Subsequent analysis of the rescued cDNA encoding plasmids by dot blot hybridization indicated that 7 of the 10 clones represented the same cDNA that we have called LNX (Ligand of Numb-protein X). Sequence analysis of the largest cDNA (2.5 kb) revealed that it contained

a open reading frame encoding a novel protein. The 2.5-kb partial LNX cDNA was used to screen a 16.5-day mouse embryo library to obtain a 2.8-kb cDNA clone containing additional 5′ nucleotides including a Kozak consensus sequence, a putative initiating methionine, and sequences encoding a 728-amino acid protein with a predicted molecular mass of 80 kDa (Fig. 1A). A search of GenBank databases identified five regions within the LNX coding region that are similar to known proteins. A cysteine-rich region (amino acids 41–99) at the amino terminus of the predicted LNX protein is homologous to the RING finger domain identified in TRAF2 (30), BRCA1 (31), CART1 (32), and PAR2 (33) (Fig. 1B). Four separate regions of LNX including amino acids 276–362, 382–467, 506–593, and 636–723 are 35–40% identical at the amino acid level to numerous PDZ-containing proteins. Alignment of these regions shows that LNX contains four PDZ domains that have close similarity to the PDZ domains found in mammalian Dishevelled 2 (34), the protein tyrosine phosphatase PTP-BAS (35), and PSD-95 (36) (Fig. 1C). The region between the RING finger and the first PDZ domain includes a sequence motif LDNPAY that matches the known consensus binding motif for several PTB domains (18).

Two LNX Isoforms Are Differentially Expressed—The LNX cDNA was used to probe Northern blots containing poly(A)+ RNA from adult mouse tissues to determine its expression pattern. Two major messages of approximately 2.6 and 2.8 kb were observed in most tissues (Fig. 2A, top panel). Notably, while heart appeared to express both messages, only the smaller message was detected in brain. In kidney, skeletal muscle, and lung only the larger message was observed. Subsequent screening of a mouse brain library for LNX-related sequences yielded a second smaller cDNA which likely represents an alternatively spliced transcript. Conceptual translation of this cDNA predicts a protein of 628 amino acids in which the amino-terminal amino acids are distinct from LNX but which is 100% identical to LNX from amino acid 132 to the stop codon (Fig. 2B). Therefore, the product of the 2.6-kb message is predicted to encode a protein of 68.9 kDa lacking the RING finger motif. To test whether the different message sizes observed by Northern blotting represented these two cDNAs, a region of LNX corresponding to the RING finger region was used as a probe. Fig. 2A (middle panel) shows that the RING finger probe detected a message of 2.8 kb in heart, lung, skeletal muscle, and kidney but not in brain. In addition, only a single message was detected in heart. This suggests that the smaller message represents an alternatively spliced form of LNX (LNX-b) which encodes a protein with four PDZ domains but with no RING finger domain.

Numb PTB Domain Interacts Directly with LNX—To confirm the interaction of LNX and the mNumb PTB domain outside of the yeast two-hybrid system, the full-length LNX cDNA was cloned into a mammalian expression vector and transfected into 293T cells. Protein lysates from LNX-transfected cells were incubated with GST-PTB domain fusion proteins. The PTB domains of mNumb, dNumb, and Nbl bound to epitope-tagged LNX, whereas neither GST alone nor the SHC PTB domain bound to LNX (Fig. 3A). The ability of PTB domains to bind directly to LNX was addressed using a modified Western blotting procedure. The target PTB proteins, EGFR or HA-tagged LNX, were immunoprecipitated and separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes, which were subsequently incubated with purified GST fusion proteins in PLC lysis buffer. Both mNumb PTB and dNumb PTB (dNumb PTB) domains bound directly to immobilized LNX, whereas GST alone or a GST fusion protein containing the proline-rich car-

![Fig. 2. Expression of LNX mRNA in adult mouse tissues. A, upper panel, Northern blot analysis of LNX mRNA expression using a full-length LNX cDNA. Middle panel, the same Northern blot was stripped and reprobed with a region of the LNX cDNA corresponding to the RING finger domain to show differential expression of the longer isoform. Lower panel, the blot was stripped and reprobed with β actin. B, alignment of the amino-terminal amino acid sequence of the long and short isoforms of LNX. The unique amino-terminal sequence of LNX-b is shown. The sequence similarities are blocked. The sequences of LNX and LNX-b are identical beyond amino acid 32 in LNX-b and 132 in LNX and therefore the alignment is not shown.](http://www.jbc.org/)

![Fig. 3. Numb PTB Domain Interacts Directly with LNX. A, Western blot analysis of LNX expression in 293T cells transfected with full-length LNX cDNA. The PTB domains of mNumb, dNumb, and Nbl bound to epitope-tagged LNX, whereas neither GST alone nor the SHC PTB domain bound to LNX. B, modified Western blotting procedure. Protein lysates from LNX-transfected cells were incubated with GST-PTB domain fusion proteins. The PTB domains of mNumb, dNumb, and Nbl bound to epitope-tagged LNX, whereas neither GST alone nor the SHC PTB domain bound to LNX.](http://www.jbc.org/)

![Fig. 4. Numb PTB Domain Binds a Novel PDZ Protein, LNX. A, alignment of the amino-terminal amino acid sequence of the long and short isoforms of LNX. The unique amino-terminal sequence of LNX-b is shown. The sequence similarities are blocked. The sequences of LNX and LNX-b are identical beyond amino acid 32 in LNX-b and 132 in LNX and therefore the alignment is not shown.](http://www.jbc.org/)
A-horseradish peroxidase to visualize bound fusion protein. EGF. Each membrane was overlaid with the indicated GST fusion protein and incubated with pFLAG-LNX transfected 293T cell lysate. FLAG LNX fusion proteins encoding the PTB domains of mNumb (8) (amino acids 2–187; NbPTB); dNumb (amino acids 58–205; dNbPTB); Nb (amino acids 2–209 of mouse SHC (29); SHCPTB), or the carboxyl-terminal region of mNumb (amino acids 184–603 of mNumb (8); NbPRR) were incubated with protein lysates from A431 cells. The immobilized GST-LNX fusion proteins were washed, separated by SDS-PAGE, and analyzed by immunoblotting with anti-Numb antibodies to detect Numb proteins that had complexed with the GST-LNX fusion proteins. An amino-terminal region of LNX containing amino acids 8–210 and including the RING finger motif (GST-NDNPAY/RING) was able to bind to endogenous Numb proteins (Fig. 4B). The multiple anti-Numb-C immunoreactive bands represent different splice isoforms of Numb. Regions corresponding to the RING finger alone or the PDZ domains did not bind to Numb, suggesting that the region between the RING finger and PDZ-1, which contains the LDNPAY sequence motif, also includes the Numb-binding site. Much weaker binding of Numb proteins to a region including PDZ-1 was also detected in this experiment.

**Binding of Numb PTB to LNX Does Not Require Tyrosine Phosphorylation**—The potential Numb PTB domain binding motif in LNX contains a tyrosine residue and is a possible target for phosphorylation in vivo and therefore could regulate binding. To test the importance of phosphorylation at this site, a fusion protein containing the NPAY sequence motif was produced in bacteria in which an activated tyrosine kinase can be induced to phosphorylate the expressed GST fusion proteins. Efficient phosphorylation of GST-LNX fusion proteins following induction of the kinase was monitored by anti-phosphotyrosine immunoblotting (data not shown). Fig. 4C shows that both the phosphorylated and unphosphorylated GST-NPAY fusion proteins could precipitate Numb proteins from A431 cell lysates. Mutant GST fusion proteins were also generated in which tyrosine 188 within the LDNPAY motif was changed to alanine. This indicates that phosphorylation of this motif in LNX is not important for interaction with Numb.

To confirm the interaction of LNX and NUMB and the effect of the Y188A mutation in the context of the full-length proteins, we examined the interaction of LNX with Numb in transfected 293T cells. The full-length FLAG-tagged LNX (WT) or FLAG-tagged LNX with an alanine substitution at tyrosine 188 (Y188A) was cotransfected with Numb into 293T cells. The cotransfected cells were lysed and immunoprecipitated with anti-FLAG or anti-Numb antibodies and then immunoblotted with either anti-FLAG or anti-Numb antibodies. FLAG-tagged LNX co-precipitated with anti-Numb antibodies only when Numb was also present demonstrating that the full-length LNX binds to Numb when co-expressed in mammalian cells (Fig. 4D). This interaction was competed by incubation with a peptide spanning the PTB-interacting motif showing that this was not due to a nonspecific interaction resulting from overexpression of proteins. The interaction between LNX and Numb was abolished by a single point mutation (Y188A) in the binding region of LNX. The shorter isoform of LNX lacking the RING finger domain (LNX-b) was also immunoprecipitated with full-length Numb (data not shown).

Residues in the SHC PTB domain that are important for phosphotyrosine binding (Ser115 and Arg138) are conserved in the Numb PTB domain (Ser115 and Arg138). We predicted that if, as indicated by the results above, the interaction between Numb PTB and LNX is phosphorylation-independent, then mutation of these residues within the PTB domain should have no effect on this interaction. In agreement, we found that GST-Numb PTB mutants in which either serine 115 or arginine 138 was changed to alanine are still able to bind to transfected FLAG-LNX (Fig. 5). However, mutation of a con-

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served phenylalanine residue, which structural studies of SHC indicate mediates the interaction with the asparagine of the NPXY motif (43), abolishes Numb PTB domain binding to LNX.

Analysis of the Numb PTB Domain Binding Specificity Using Peptide Competition Assays—To define further the Numb PTB domain binding site, LNX peptides were synthesized and used to compete the binding of Numb PTB to LNX. In addition, peptides containing amino acid substitutions were used to map the essential residues within this motif. Incubation with the WT peptide (EPGLDNPAYTSSVE) completely abolished GST-LNX binding to Numb (Fig. 6) which confirmed that this sequence of LNX was sufficient for binding to Numb PTB. Single replacement of Tyr0, Asn2, and Leu25 with alanine prevented competition by the respective peptides which indicated that these residues are required for Numb PTB domain binding. As predicted, a peptide with a Phe substitution for Tyr188 competed as efficiently as the wild type peptide. Alanine substitution of glycine (26), aspartate (24), and proline (22) were intermediate in their ability to compete, whereas substitution at proline (7) and threonine (1) was without effect (Fig. 6).

We tested the specificity of this competition by examining the ability of related PTB target sequences to compete binding of FLAG-tagged LNX to GST-Numb PTB (GST-NbPTB). 293T cells were transfected with pFLAG-LNX, and the cell lysate was mixed with GST-NbPTB in the presence or absence of unphosphorylated or phosphorylated peptides corresponding to the PTB target sequence of LNX (LNX, LNX-P), phosphorylated EGFR (Tyr(P)1148), or phosphorylated TrkA (Tyr(P)490). The amount of bound FLAG-LNX was determined by immunoblotting. Both unphosphorylated and phosphorylated LNX peptides effectively prevented binding of Numb PTB domain to FLAG-LNX (Fig. 7A). Although the phosphorylated LNX peptide did not compete as efficiently in this experiment, this was not consistently observed. In contrast, EGFR Tyr(P)1148 and TrkA Tyr(P)490 peptides were unable to compete the Numb PTB interaction with FLAG-LNX. The core PTB domain binding motif of these peptides is quite similar, having a hydrophobic residue at position 25 in addition to the NPXY, which suggested binding of Numb PTB domain to LNX is selective and may involve residues outside the core motif.

Since the SHC PTB domain target sequence of the EGFR (LDNPDY) is very similar to the Numb PTB domain target of LNX (LDNPAY), we were interested in the possibility that LNX may also bind to SHC, another widely expressed adapter protein. To address this question we examined the efficacy of the unphosphorylated and phosphorylated LNX peptides to compete with EGFR binding to immobilized GST-SHC PTB, and we compared this to EGFR Tyr(P)1148 and TrkA Tyr(P)490 peptides.
In this study we describe the binding of mNumb PTB domain to a novel protein, LNX, and map the PTB domain binding site to a sequence motif, LDNPAY. The residues within this sequence essential for high affinity binding of Numb PTB to LNX are Leu\(^{5}\), Asn\(^{-3}\), and Tyr\(^{2}\). This is in agreement with the binding specificity described for other PTB domain interactions, including that of SHC PTB domain for the autophosphorylated EGFR and TrkA receptors, for which a consensus binding motif of \(\Phi XNPxY\) has been defined (where \(\Phi\) represents a hydrophobic amino acid) (15, 21, 37, 38). However, unlike SHC, binding of Numb PTB domain to LNX does not require tyrosine phosphorylation. Although replacement of the tyrosine residue with an alanine within this motif prevents the binding of LNX to Numb, replacement with phenylalanine has no effect on binding, confirming that phosphorylation is not required and suggesting that a hydrophobic residue at this position is important. Consistent with our findings, the PTB domain of X11 also binds to a phosphorylation-independent manner to a peptide containing the core motif NP\(x\)Y found in the cytoplasmic domain of \(\beta\)APP (39). We conclude that like SHC and X11 PTB domains, the Numb PTB domain binding site requires an amino acid sequence conforming to a \(\beta\) turn, with Asn and Pro residues contributing to this configuration, and additional specificity being contributed by the hydrophobic residue at position 0, represented by tyrosine in this case (40).

It is becoming increasingly clear that PTB domains exhibit a much broader specificity than for the originally described NP\(x\)Y motif and, as suggested by Yajnik et al. (41), are likely to represent a general protein interaction domain that in only some cases requires tyrosine phosphorylation. Recently the binding specificity of dNumb PTB was investigated by screening a tyrosine-oriented peptide library and shown to bind to a GP\(p\)Y motif (42). Tyrosine phosphorylation was not essential for binding; however, the phosphorylated peptide containing the GP\(p\)Y motif bound with higher affinity. We have found that the GP\(p\)Y peptide also competes the interaction of LNX with mNumb PTB domain (data not shown). This observation, in addition to our data showing that dNumb PTB domain also binds to LNX, which does not contain the GP\(p\)Y motif, suggests that the Numb PTB domain may have diverse binding specificities.

Mutational analysis of SHC implicates the conserved residues Ser\(^{151}\) and Arg\(^{172}\) within the PTB domain as being involved in the interaction with the phosphotyrosine moiety (37, 41, 43). We have found that in vitro binding to LNX is unaffected when the corresponding residues in mNumb PTB domain are mutated. In addition, it has recently been demonstrated that mutation of the same conserved residues in dNumb does not affect Numb function in vivo (4). This is further evidence that Numb is likely involved in phosphotyrosine-independent interactions.

FIG. 6. Analysis of binding specificity using peptide competition. Purified, immobilized GST-RING/NPAX fusion protein was mixed with A431 cell lysates in the absence (no pep) or presence of 10 \(\mu\)M peptides corresponding to amino acids 179–193 of LNX. The sequence of the wild type LNX peptide is indicated above (WT: EPGLDNPAYTSSVE\(^{193}\)). Mutant peptides were synthesized containing alanine (A) substitutions at residues 181 (P181A), 182 (G182A), 183 (L183A), 184 (D184A), 185 (N185A), 186 (P186A), 188 (Y188A), and 189 (T189A). A phenylalanine substitution of Tyr\(^{188}\) was also synthesized (Y188F). Bound Numb proteins were determined by Western blotting with anti-Numb-\(C\) antibody.

DISCUSSION

In this study we describe the binding of mNumb PTB domain to a novel protein, LNX, and map the PTB domain binding site to a sequence motif, LDNPAY. The residues within this sequence essential for high affinity binding of Numb PTB to LNX are Leu\(^{5}\), Asn\(^{-3}\), and Tyr\(^{2}\). This is in agreement with the binding specificity described for other PTB domain interactions, including that of SHC PTB domain for the autophosphorylated EGFR and TrkA receptors, for which a consensus binding motif of \(\Phi XNPxY\) has been defined (where \(\Phi\) represents a hydrophobic amino acid) (15, 21, 37, 38). However, unlike SHC, binding of Numb PTB domain to LNX does not require tyrosine phosphorylation. Although replacement of the tyrosine residue with an alanine within this motif prevents the binding of LNX to Numb, replacement with phenylalanine has no effect on binding, confirming that phosphorylation is not required and suggesting that a hydrophobic residue at this position is important. Consistent with our findings, the PTB domain of X11 also binds to a phosphorylation-independent manner to a peptide containing the core motif NP\(x\)Y found in the cytoplasmic domain of \(\beta\)APP (39). We conclude that like SHC and X11 PTB domains, the Numb PTB domain binding site requires an amino acid sequence conforming to a \(\beta\) turn, with Asn and Pro residues contributing to this configuration, and additional specificity being contributed by the hydrophobic residue at position 0, represented by tyrosine in this case (40).

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4 B. Margolis, personal communication.
An essential structural element for recognition of phosphopeptides by the SHC PTB domain has been shown to be a β turn generated by the NPXY sequence motif which forms a β strand antiparallel to the β sheet of the PTB domain (43). A recent three-dimensional structure of the X11 PTB domain bound to the unphosphorylated βAPP peptide revealed that the peptide recognition and binding occurs in essentially the same manner (44). This is in keeping with our findings which demonstrated that the residues which are important for the conformation of the LNX peptide are also critical for Numb PTB domain recognition. Interestingly, Zhang et al. (44) found that the unphosphorylated tyrosine residue is bound in the same conformation as the phosphorylated tyrosine bound to SHC PTB and that phosphorylation of the tyrosine residue in the βAPP peptide did not change the binding affinity.

In addition to the Numb PTB domain binding motif, LNX also possesses five other potential protein-protein interaction domains as follows: one RING finger and four PDZ domains. The RING finger is a zinc-binding cysteine-rich sequence motif found in a diverse group of unrelated proteins including CBL, BRCA1, TRAF2, and CART1 (45, 46). PDZ domains are conserved protein-protein complexes either by homo- or hetero-oligomerization interactions (45, 46). PDZ domains are conserved protein modules that were originally identified as a 90–100-amino acid repeat in several cell junction proteins including PSD-95, a synaptic protein; Drosophila Discs Large, a membrane associated guanylate kinase (MAGUK) found at septate junctions; and ZO-1, another MAGUK protein associated with tight junctions in epithelial cells. More recently, PDZ domains have been identified in a wide variety of unrelated proteins with diverse functions (47). PDZ domains from a number of proteins recognize the sequence motif (Ser/Thr)-X-Val found at the carboxyl terminus of K+ channel subunits, subunits of the NMAD receptor, and Fas, a cell surface receptor mediating a cell death pathway (48–50). However, recent evidence derived from peptide library screens indicates that PDZ domains may interact with carboxyl-terminal sequences that are very different from the (S/T)XV motif (51, 52) or sequences not situated at the carboxyl terminus (53). In addition certain PDZ domains form homophilic associations with other PDZ domains (54).

Most functional data on PDZ domains suggests that they are important for the formation of protein complexes at specialized membrane sites. For example, an interaction between Shaker type K+ channels and the PDZ-containing protein PSD-95 is required for clustering of these channels in discrete membrane patches (49). Proteins containing multiple PDZ domains also appear to be involved in assemblies signaling complexes. In Drosophila, the InaD protein, which contains five PDZ domains, functions to bring together the components of the phototransduction cascade into a preassembled signaling unit which includes the light-activated ion channels and the downstream effectors, phospholipase C-β and protein kinase C (53). Another protein with multiple PDZ domains, PAR3, has been shown to play an essential role in asymmetric cell divisions of the Caenorhabditis elegans embryo (55, 56). Given its multiple protein interaction modules, and its ability to bind an adapter molecule, Numb, we predict that LNX is similarly involved in the organization of multimolecular complexes at the cell membrane. A recent study examining asymmetric divisions in developing Drosophila proposed the involvement of an unidentified membrane anchor protein required for the asymmetric localization of Miranda, Prospero, and Numb (26). We speculate that LNX could play a role in such a complex, particularly in light of recent data suggesting that the asymmetric localization of dNumb requires the amino-terminal region including an intact PTB domain (57).

Our data suggest that phosphorylation of LNX at Tyr 188 would generate a binding site for the SHC PTB domain. Although, to date, we have not detected tyrosine phosphorylation of LNX in vivo, this raises the interesting possibility that LNX may be involved in several different multi-protein complexes depending on its phosphorylation status. SHC is an important downstream substrate of many receptor tyrosine kinases. A complex between SHC and LNX could function in the assembly of a receptor tyrosine kinase signaling complex at a specific membrane site. In C. elegans, LIN-7 encodes a small PDZ-containing protein that is required for cell junction localization and signaling activity of the receptor tyrosine kinase LET23 (58). It seems likely that PDZ proteins will also be involved in the spatial organization of receptor tyrosine kinases in mammalian cells.

In conclusion, we have identified a novel target of the mNumb PTB domain and characterized the specificity of the interaction. Our results show that the Numb PTB domain is one of a subgroup of PTB domains, which also includes the X11 PTB domain, that are capable of specific interactions with non-phosphorylated sequences. In addition, the cloning of a PDZ-containing protein as a target of the Numb PTB domain suggests that the interaction between PDZ and PTB-containing proteins may play role in asymmetric protein localization, asymmetric cell division, and cell fate signaling through the formation of multi-protein complexes. The identification of proteins that interact with the LNX PDZ domains will determine whether LNX has a role in assembling signaling complexes and may shed light on the signaling pathways that are influenced by Numb.

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