Characterization of Four Mammalian Numb Protein Isoforms

IDENTIFICATION OF CYTOPLASMIC AND MEMBRANE-ASSOCIATED VARIANTS OF THE PHOSPHOTYROSINE BINDING DOMAIN* 

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Numb is a membrane-associated, phosphotyrosine binding (PTB) domain-containing protein that functions as an intrinsic determinant of cell fate during Drosophila development. We have identified four isoforms of mammalian Numb with predicted molecular masses of 65, 66, 71, and 72 kDa that are generated by alternative splicing of the Numb mRNA. The different isoforms result from the presence of two sequence inserts within the PTB domain and the central region of the protein. The endogenous expression pattern of these isoforms, examined using specific antisera, varied in different tissues and cell lines. In addition, differentiation of P19 cells with retinoic acid leads to the specific loss of expression of the 71- and 72-kDa Numb proteins, suggesting that the expression of certain forms of Numb protein is regulated in a cell type-specific manner.

Expression of Numb proteins fused to green fluorescent protein revealed that the form of the PTB domain with the alternatively spliced insert constitutively associated with the plasma membrane in polarized Madin-Darby canine kidney cells. In contrast, the isoform without the insert was cytoplasmic, suggesting that different PTB domain isoforms may regulate the subcellular localization of Numb proteins. The membrane localization may be due, in part, to differential affinity for acidic phospholipids. The distinct expression and localization patterns of the different mammalian Numb isoforms suggest that they have distinct functional properties.

The numb gene in Drosophila affects binary cell fate decisions of cells in the peripheral and central nervous system, as well as muscle cells during development (1–5). Drosophila Numb is a membrane-associated protein expressed in progenitor cells of these lineages. During cell division, Drosophila Numb asymmetrically localizes and subsequently segregates to one daughter cell, where it functions as an intrinsic determinant of cell fate (6–9). Evidence suggests that in Drosophila, Numb proteins influence cell fate by inhibiting the action of Notch by an unknown mechanism (3, 10, 12).

The mammalian homologue of Numb (mNumb)1 has been cloned (13, 14), and ectopic expression of mNumb in Drosophila produces a phenotype similar to that produced by ectopic expression of Drosophila Numb (13, 14). In the mouse embryo, Numb 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 asymmetrically localized during cell division (13), suggesting that it may have a role in cell fate decisions in the mammalian nervous system. However, mNumb is also expressed in most adult tissues, and its expression is widespread in mouse embryos, so it is likely that Numb function is not limited to neurogenesis (13, 14). Although the function of mNumb in mammals is still unclear, the identification of a conserved chicken Numb protein that is asymmetrically localized in neuroepithelial cells of the chicken telencephalon and inhibits the repression of neuronal differentiation by chicken Notch suggests that Numb function in vertebrates may be similar to its function in Drosophila (15).

Structurally, Numb resembles an adaptor or scaffold protein and is involved in bringing together multiple proteins into a functional unit or pathway. It possesses an amino-terminal phosphotyrosine binding (PTB) domain (16), a proline-rich carboxyl-terminal region (PRR) containing several putative Src homology 3 domain-binding sites (14), and an Eps15 homology (EH) domain-binding motif (17). The PTB domain was first described in the adaptor protein SHC, where it functions to mediate phosphotyrosine-dependent interactions with growth factor receptors and downstream effectors (18–22). Based on sequence similarity, PTB domains have been identified in a diverse group of proteins of which only a subset are linked to tyrosine kinase-mediated signaling (16). Functional analysis of the binding specificity of PTB domains in molecules including Numb, mDab1, X11, and FE65 has shown that high affinity ligands for these domains are not limited to phosphorylated tyrosine-containing sequences (23–28). In addition to the association with specific peptide ligands, some PTB domains, such as those of SHC and mDab-1, also have the capacity to interact with phospholipids (28, 29).

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1 The abbreviations used are: mNumb, mammalian Numb; Nbl, Numblike; PTB, phosphotyrosine binding; EH, Eps15 homology; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; PTBi, PTB domain with the alternatively spliced insert; PTBo, PTB domain without the alternatively spliced insert; PRR, proline-rich carboxyl-terminal region; PRRi, proline-rich carboxyl-terminal region with the alternatively spliced insert; PPR, proline-rich carboxyl-terminal region without the alternatively spliced insert; GFP, green fluorescent protein; MDCK, Madin-Darby canine kidney; PC, phosphatidylcholine; PS, phosphatidylserine; PCR, polymerase chain reaction; Pif4/Pip, phosphatidylinositol 4-phosphate; MES, 2-(N-morpholino)ethane-sulfonic acid.
Based on recent structure analysis, it has been suggested that the *Drosophila* Numb PTB domain can bind multiple, conformationally distinct peptide ligands (30, 31). Both of the peptide ligands of the Numb PTB domain that have been identified to date—the putative serine/threonine kinase NAK and the PDZ domain-containing protein LNX—interact with Numb in a phosphotyrosine-independent manner (23, 32). In *vivo*, the vertebrate Numb PTB domain has been reported to mediate the interaction of Numb with the intracellular domain of Notch, presumably in a phosphotyrosine-independent manner, although the specific sequences involved in this interaction have not been elucidated (13, 15).

The carboxyl-terminal region of Numb contains an EH domain-binding motif that interacts with the tyrosine kinase substrate, Eps15 (17, 33, 34). EH-domain containing proteins such as Eps15 and Eps15R are involved in processes connected with receptor-mediated endocytosis, organization of the actin cytoskeleton, and possibly other mechanisms involved in sorting molecules within the cell (35–40). The EH domain-binding motif, like the PTB domain, is conserved in vertebrate Numb, the related mammalian protein Numblike (Nbl), and the *Drosophila* Numb protein, suggesting that the interaction in the EH domain-containing proteins is functionally relevant. *In vitro*, the Src homology 3 domain of Src binds to Numb, an interaction that is likely mediated by one of the putative Src homology 3-binding motifs within the central region of the protein (PXXP (14)). Thus, Numb contains multiple regions that can mediate its interaction with other proteins.

Previous characterization of mammalian Numb protein expression suggested that there may be Numb-related gene products or modified forms of Numb, because at least two proteins of different molecular weights are detected by anti-Numb antisera. (13, 14). In addition, two different sequences of mammalian Numb have been published (13, 14, 17, 41) that differ by the presence or absence of an 11-amino acid insert within the PTB domain. Here, we describe the cloning and characterization of four different mouse Numb isoforms. Two of these represent the already described isoforms that vary in the PTB domain sequence, and the other two represent novel sequences encoding Numb proteins with an additional 49-amino acid insert in the central proline-rich region. Using isoform-specific antisera, we show that the Numb protein isoforms are differentially expressed in mouse tissues and cell lines. Furthermore, we show that the insert in the PTB domain may be an important determinant in the localization of Numb to the plasma membrane.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Numb- and Nbl-specific antisera were generated in rabbits immunized with peptides corresponding to different regions of Numb or Nbl, conjugated to KLH as follows: anti-PRRi, PVAMPVRET; anti-PRRo, PVAMPVRET; anti-PTBi, KAERK; anti-PTBo, DAVKRLKATGKK; anti-Nb-A, NPWAHAPDC; anti-PRRo, PQSPTFQGTEWGQS; anti-PTBi, KAERK; anti-PTBo, DAVKRLKATGKK; anti-Nb-A, NPWAHAPDC; anti-PRRo, PQSPTFQGTEWGQS; anti-PTBi, KAERK; anti-PTBo, DAVKRLKATGKK; and anti-Nb-A, NPWAHAPDC. The crude serum was affinity-purified using GST fusion proteins, the main-binding motif that interacts with the tyrosine kinase substrate, Eps15 (17, 33, 34). EH-domain containing proteins is functionally relevant. *In vitro*, the Src homology 3 domain of Src binds to Numb, an interaction that is likely mediated by one of the putative Src homology 3-binding motifs within the central region of the protein (PXXP (14)). Thus, Numb contains multiple regions that can mediate its interaction with other proteins.

**Cloning of Mouse Numb cDNAs**—To obtain mouse Numb cDNA, a mouse day 11 embryonic cDNA library was amplified in the following primers, AAG TTA ACA TGA ACA AAC TAC GGC; and 3′ primer, ACT GCC TAA AGT TCT ATT TCA AAT G. The PCR products were cloned into pCR2.1 (Invitrogen) and then subcloned into the expression plasmid pCDNA3.1/Zeo+ (Invitrogen). The DNA sequence of the inserts in individual plasmids was determined.

**Immunoprecipitations and Western Blotting**—Cell lysates were prepared from cultured cells (MDCK, A431, P19, NIH 3T3, or N2A) grown to 75–90% confluence on 10-cm tissue culture dishes. Following two washes with cold phosphate-buffered saline, the cells were scraped into 1 ml of lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EDTA, 10 mM sodium pyrophosphate, 100 mM NaF, 1% Triton X-100, 10% glycerol, 1 mM sodium vanadate, and protease inhibitors (EDTA, and eluted by boiling in SDS sample buffer. Proteins were separated by SDS-PAGE, transferred onto polyvinylidene difluoride membrane (Immobilon-P), and immunoblotted with primary antibodies overnight at 4 °C. Bound antibodies were visualized using horseradish peroxidase-conjugated protein A (Bio-Rad) or goat anti-mouse in conjunction with the ECL system (Amersham Pharmacia Biotech).
RESULTS

Identification of Four Isoforms of Mammalian Numb—In order to identify additional Numb mRNA species, PCR was used to amplify Numb cDNAs from a mouse embryo cDNA library, and the resulting products were sequenced. Four cDNAs were identified that differ from each other by the presence or absence of two sequence inserts encoding an 11-amino acid insert in the PTB region (denoted by PTBi), identical to that which had been previously reported in rat and human (14, 17), and a novel 49-amino acid insert within the central region of the protein adjacent to the proline-rich region (denoted by PRRI) (Fig. 1). These four different cDNA products encode proteins with predicted molecular masses of 65, 66, 71, and 72 kDa. Analysis of the mouse Numb genomic structure indicates that the 11- and 49-amino acid inserts are encoded by single exons,2 and therefore, the different cDNAs likely represent alternatively spliced mRNA species. In addition, the human Numb gene appears to have a similar genomic organization to that of the mouse gene, and a sequence encoding the central 49-amino acid insert can be found within the reported sequence of the human Numb gene (GenBank™ accession no. AF109907). This insert was not previously identified as a coding sequence presumably because the exons were identified based on comparison with the sequence of the previously published Numb cDNA.

2 M. B. French and C. J. McGlade, unpublished data.
Antibodies were generated against peptides corresponding to unique regions within the inserts (PTBi and PRRi) or regions corresponding to novel epitopes generated by the juxtaposition of amino acids adjacent to the inserts (PTBo and PRRo) (see Fig. 1). The specificity of the Numb isoform antibodies was determined by testing their ability to specifically recognize ectopically expressed Numb proteins. To this end, the four cDNA isoforms were individually cloned into the mammalian expression vector pcDNA3.1 and transiently expressed in 293T cells. Western blot analysis of total cell lysates demonstrated that anti-Nb-C recognizes all four of the Numb proteins, whereas the isoform-specific antibodies recognize only the expected protein products (Fig. 2). Antisera against the PTBi sequence recognized only the p66 and p72 proteins, anti-PTBo recognizes only the p65 and p71 forms, and anti-PRRi recognizes p71 and p72, as expected. The anti-PRRo antibody reacted most strongly with p65 and p66; however, weak cross-reactivity with p71 and p72 forms was detectable.

The panel of Numb antibodies was used to examine the expression of Numb protein isoforms in mouse tissue lysates (Fig. 3A). Fig. 3A, top panel, shows the expression of all of the Numb protein isoforms, as well as the related Nbl gene product, as determined by immunoprecipitation and Western blotting with anti-Nb-C, which recognizes all of these proteins. Fig. 3A, lower panels, represent duplicate samples that have been blotted with the isoform-specific antibodies. This experiment demonstrates that the variable pattern of Numb-C reactive proteins is related to the differential expression of the Numb protein isoforms. Proteins lacking the PTB domain insert, p65 and p71, were expressed in all tissues, whereas the isoforms with the PTB insert were largely restricted to the lung, with small amounts expressed in the brain, thymus, and embryo, and none in testis. Conversely, isoforms containing the PRR insert, p71 and p72, were primarily restricted to the testis, with very small amounts seen in lung, thymus, and embryo. Based on these results, we can confirm that p65, p66, and p71 are all expressed in vivo. However, because of the low abundance of the PRRi isoforms, particularly in brain and lung, the unequivocal identification of the 72-kDa (PTBi/PRRi) isoform could not be made.

We also examined the expression of Numb proteins in several mammalian cell lines (Fig. 3B). In these experiments, anti-Nb-A was used to examine total Numb expression (Fig. 3B, top panel) because this antibody, unlike anti-Nb-C, has minimal cross-reactivity with Nbl, which is expressed in many tissue culture cell lines (Fig. 3B, bottom panel). A variable expression pattern of all four Numb proteins was observed, and expression of p72 was confirmed in several cell lines (Fig. 3B, second panel from top). All cell lines examined expressed isoforms containing the PTB insert, and all except NIH 3T3 cells expressed isoforms containing the PRR insert. In contrast, the p65 isoform, which does not contain an insert in either region, was found predominantly in the neuroblastoma N2A cell line.

**Numb Isoforms Are Differentially Expressed during P19 Cell Differentiation—**Expression of the Numb protein isoforms containing the PRR insert (p71 and p72) was limited to mouse tissues containing actively dividing and differentiating cells (testis and embryo), whereas they are present in the majority of transformed cell lines, suggesting that expression of these isoforms may be correlated with cell proliferation or differentiation status. We investigated this further by examining Numb expression during the course of differentiation of P19 cells to postmitotic neurons (Fig. 4). Exponentially growing P19 cells except that for each immunoprecipitation, cell lysate from half of an 80% confluent plate of cells was used.

![Fig. 3. Numb isoforms are differentially expressed in mouse tissues and cell lines. A, expression of Numb in adult mouse tissue lysates. Numb was immunoprecipitated (IP) from equal amounts of mouse tissue lysate (1 mg of protein per 1 ml of lysis buffer) and Western blotted (Blot) with the indicated antibodies to determine the presence of Numb isoforms and Nbl in each. On longer exposure, an immunoreactive band was seen in embryo lysates in the anti-PTBi IP/anti-Nb-C blot and in the anti-PRRi blot. B, expression of Numb in cell lines. Experiments were carried out essentially as described in A,](image-url)
were aggregated in the presence of retinoic acid, which results in differentiation into cells that resemble postmitotic neurons, glia, and fibroblast-like cells following replating (days 6–9). Fig. 4, top panel, shows the expression of all Numb isoforms during the course of differentiation. A marked decline in the p71 and p72 isoforms (PRR insert present), as detected by anti-Nb-C, was observed, suggesting that the p71 and p72 Nb isoform expression is down-regulated during differentiation. This was confirmed by immunoprecipitation with anti-Nb-C followed by Western blotting with anti-PRRi (Fig. 4, second panel from top). Over the course of differentiation, increased expression of Nbl was detected at day 5, when the cell aggregates were replated. This is consistent with previous work suggesting that Nbl is expressed primarily in postmitotic cells of the nervous system and our own results showing the Nbl expression is restricted to the adult brain in the mouse (Ref. 43).

To determine whether the two PTB domain isoforms, in the context of the full-length protein, function to localize the endogenous Numb proteins, subcellular fractionation of MDCK cells was performed. Crude fractions of subconfluent (80%) MDCK cells were prepared, providing three fractions: P1, a low-speed pellet containing the majority of plasma membrane (as determined by the presence of the integral membrane proteins E-cadherin and Na+/K+ ATPase); P2, a high speed pellet that also contained plasma membrane; and S, a cytosolic fraction. Numb proteins were found both in the cytosol and in the plasma membrane fraction, although they were also present to some extent in the P2 fraction, although they were also present to some extent in the P1 plasma membrane containing fraction. The related Nbl PTB domain is most similar to PTBo, it can interact with unique targets localized at the cortical actin cytoskeleton. However, treatment of MDCK cells expressing GFP-NbPTBo with cytochalasin D, which has marked effects on the actin cytoskeleton, does not affect membrane localization of the PTBi domain, arguing against the former possibility (data not shown).

Both Numb PTB Domain Isoforms Bind LNX and Acidic Phospholipids—It is possible that the 11-amino acid insert modifies the PTB domain binding specificity such that, unlike PTBo, it can interact with unique targets localized at the plasma membrane and thus be retained there. Therefore, we investigated the ability of the PTBi and PTBo domains to interact with the known Numb PTB domain protein target LNX. Previously, we reported that the PTBi domain interacts...
The presence of Numb isoforms in the membrane (low speed (P1 and PTBi) and PTBo domains or full-length p67 Numb (Nb/full)) were transiently expressed in MDCK cells grown on filters and paraformaldehyde-fixed cells examined using a Leica confocal microscope. Shown are image sections (x-y) through the cell monolayer, beginning at the basolateral region (panel 1) and continuing through the apical region (panel 5).

**Fig. 6.** Confocal microscopy of polarized MDCK cells expressing GFP Numb PTB domains. GFP fusions of the Numb PTBi and PTBo domains or full-length p67 Numb (Nb/full) were transiently expressed in MDCK cells grown on filters and paraformaldehyde-fixed cells examined using a Leica confocal microscope. Shown are image sections (x-y) through the cell monolayer, beginning at the basolateral region (panel 1) and continuing through the apical region (panel 5).

![Confocal microscopy of polarized MDCK cells expressing GFP Numb PTB domains.](image)

**Fig. 7.** Identification of endogenous Numb isoforms in crude MDCK cell fractions. The presence of Numb isoforms in the membrane (low speed (P1) and high speed (P2) pellets) and cytosolic fractions (S) of MDCK cells was determined by immunoprecipitation and Western blotting with the indicated isoform-specific antibodies. Approximately 5% of the lysate used for each immunoprecipitation was Western blotted with anti-E-cadherin (bottom panel) to positively identify the plasma membrane containing fractions.

| fraction | kDa | IP x Numb-C | Blot x Numb-C |
|----------|-----|-------------|---------------|
| 87       |     |             |               |
| 87       |     |             |               |
| 87       |     |             |               |
| 120      |     |             |               |
| 87       |     |             |               |

**Fig. 7.** Identification of endogenous Numb isoforms in crude MDCK cell fractions. The presence of Numb isoforms in the membrane (low speed (P1) and high speed (P2) pellets) and cytosolic fractions (S) of MDCK cells was determined by immunoprecipitation and Western blotting with the indicated isoform-specific antibodies. Approximately 5% of the lysate used for each immunoprecipitation was Western blotted with anti-E-cadherin (bottom panel) to positively identify the plasma membrane containing fractions.

with an NPXY sequence motif in the PDZ domain-containing protein LNX (23). We examined the ability of LNX to interact with the PTBo form of the Numb PTB domain (Fig. 8A) and found that both forms of the PTB domain bind efficiently to LNX in vitro. This suggests that binding of LNX alone is not sufficient to explain the differential localization of the Numb proteins.

Another mechanism that might mediate the localization of the Numb PTBi domain to the plasma membrane, independent of peptide binding, is an interaction of the PTB domain with membrane phospholipids like that reported for the PTB domain of SHC (29, 45). Therefore, we tested the ability of the Numb PTB domains to bind acidic phospholipids. Fig. 8B shows results from experiments in which the indicated acidic phospholipids were incorporated into small, unilamellar vesicles; mixed with purified GST fusions of PTBi, PTBo, and GST alone; and then pelleted. The amount of fusion protein precipitated with the vesicles is an indication of the ability of the protein to bind the phospholipid. Both PTB domains bound to phosphatidylinositol 4, 5-bisphosphate-containing vesicles more efficiently than vesicles containing only the base lipid, 5% phosphatidylserine/95% phosphatidylcholine. However, PTBi was pelleted more efficiently than PTBo by phosphatidylinositol 4-phosphate-containing vesicles (Fig. 8B). GST alone did not bind significantly to any of the lipids tested (data not shown).

To confirm the association of the Numb PTB domains with phospholipid, a modified far Western protocol was employed (far Western) (42, 46) in which the phospholipids of interest were spotted onto polyvinylidene difluoride membrane and then blotted with GST-PTBi, GST-PTBo, or GST alone. Bound proteins were then identified using anti-GST antibodies (Fig. 7B). Both PTBi and PTBo domains exhibited relatively nonselective binding to a number of phospholipids in this assay. However, in agreement with the vesicle pull-down experiments, the Numb PTBi showed greater binding to PI(4)P than did Numb PTBo. GST alone did not bind detectably to any of the phospholipids in this assay.

**DISCUSSION**

Conserved Numb genes have been identified in many vertebrate species, including mouse, rat, chicken, and human. Previous reports of the Numb protein sequence from rat, mouse, and human revealed that there was an 11-amino acid sequence present in the rat form and one of the identified human forms that was not present in mouse Numb, even though these proteins are more than 90% identical overall (13, 14, 17). We have shown that this sequence difference is likely the result of alternative splicing of the mNumb messenger RNA and that the corresponding protein products are expressed in all of these species. Furthermore, we have identified cDNAs encoding two previously unidentified isoforms of mammalian Numb with a 49-amino acid sequence inserted in the central region of the protein. The predicted molecular masses of the four proteins encoded by the cloned cDNAs are 65, 66, 71, and 72 kDa, which likely account for the multiple anti-Numb reactive bands we and others have previously observed in a number of cell lines and tissues (13, 14, 23). The cloned cDNAs are expressed in vivo because we have identified the protein products directly in mouse tissues and in human, mouse, and rat cell lines using isoform-specific antibodies.

The expression pattern of the Numb proteins varies dramatically between different tissues and cultured cell lines. The functional relevance of this is unknown. However, it suggests that the Numb isoforms may have different functions in different cell types. Not only do the 71- and 72-kDa isoforms containing the insert in the central region of the protein (PRRi) exhibit relatively restricted expression in primarily mouse tissues, but we observed that their expression declines following retinoic acid-induced differentiation of P19 cells. This suggests that 71- and 72-kDa isoforms may have distinct functional properties related to cellular proliferation or differentiation. Additional studies on the effects of deregulated expression and investigation of the binding properties of the inserted 49-amino acid sequence may reveal the functional properties of these isoforms.

The amino-terminal region of the Numb protein contains an amino acid sequence with homology to the PTB domains of molecules such as SHC, FE65, X11, and Disabled (16). The role of these domains in mediating protein-protein interactions is well established, and therefore, this region of Numb represents a potential site for the interaction of Numb with molecules required for its specific localization or its signaling properties. Indeed structure-function analysis of the Drosophila Numb protein demonstrated that the PTB domain is essential for Numb function as a cell fate determinant (12, 25). Drosophila Numb is constitutively localized to the plasma membrane, and sequences that mediate this function include amino acids in the amino terminus, as well as part of the PTB domain (12, 44). However, this amino-terminal region is not conserved in the mammalian Numb proteins, although they have been reported to be membrane-associated (43). We have shown that mammalian Numb proteins contain either of two forms of the PTB
domain, and the presence of the 11-amino acid sequence in the PTB forms of the PTB domain promotes localization to the plasma membrane. This suggests that for some forms of mNumb, membrane association involves the PTB domain and that the mammalian and Drosophila Numb proteins are localized to the membrane by different mechanisms.

There is precedent for the role of certain PTB domains in mediating the constitutive plasma membrane localization of signaling proteins. For example, the PTB domain of SHC binds both specific phosphopeptide targets, as well as associating with phospholipids (45). Furthermore, phospholipid binding correlates with the ability of the domain to target SHC to the plasma membrane (29). It has been proposed that the binding of the SHC PTB domain to acidic phospholipids mediates the constitutive association of SHC with the membrane and facilitates the recruitment of SHC to an activated receptor, where a high affinity peptide ligand would displace the bound phospholipid. The PTB domain of mDab1 also binds phospholipids. However, in contrast to SHC, the mDab1 PTB domain appears to be able to interact simultaneously with both peptides and phospholipids (28).

The region of Numb encompassing the PTB domain insert, and the insert itself, is rich in basic residues, a property often associated with proteins that bind membrane lipids. Furthermore, specific arginine and lysine residues within the PTB domain of SHC are involved in both binding to acidic phospholipids and membrane localization (29). Therefore, we tested whether the presence of the four positively charged residues in the PTB insert could alter the folding of the domain and change its binding specificity. The structures of several PTB domains, including the PTB domain of Drosophila Numb bound to a high affinity ligand, have been solved (30, 45, 47, 48). Structure-based sequence alignments of the mammalian Numb PTB domains indicate that the insert region of Numb PTBi would reside between the α2 helix and the β2 strand. In the Drosophila Numb PTB domain structure this region is not directly involved in engaging peptide targets (30). One possibility currently being investigated is whether the insert sequence could contribute to a second binding surface on the PTBi domain and thereby promote interaction with unique targets important for membrane localization.

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