Structure of a Numb PTB domain–peptide complex suggests a basis for diverse binding specificity

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The phosphotyrosine-binding (PTB) domain of Numb, a protein involved in asymmetric cell division, has recently been shown to bind to the adapter protein Lnx through an LDNPAY sequence, to the Numb-associated kinase (Nak) through a sequence that does not contain an NPXY motif and to GP(p)Y-containing peptides obtained from library screening. We show here that these diverse peptide sequences bind with comparable affinities to the Numb PTB domain at a common binding site on the surface of the protein. The NMR structure of the Numb PTB domain in complex with a GPpY-containing peptide reveals a novel mechanism of binding with the peptide in a helical turn that does not hydrogen bond to the PTB domain β-sheet. These results suggest that PTB domains can potentially have multiple modes of peptide recognition and provide a structural basis from which the multiple functions of the Numb PTB domain during asymmetric cell division could arise.

The PTB domain (also known as the PI domain) was first described in the context of the Shc and insulin receptor substrate-1 (IRS-1) proteins as a phosphopeptide binding module that recognizes phosphotyrosine (pTyr) residues within the β-turn-forming sequence NPXpY[1–6]. While these PTB domains are similar to SH2 domains in the sense that high affinity peptide binding depends on ligand phosphorylation, their structures and modes of phosphopeptide recognition are quite different (reviewed in ref. 7). In particular, phosphopeptide binding and specificity depend on residues N-terminal to the pTyr, which form an extended strand that hydrogen bonds to the β5 strand of the PTB domain, in effect adding an additional strand to an antiparallel β-sheet[8,9]. The pTyr residue in the NPXpY motif interacts with variable basic residues that are at least partially solvent exposed on the surface of the PTB domain[10,11]. This is in contrast to SH2 domains, which employ a buried and invariant Arg residue for pTyr recognition[12].

It is now appreciated that pTyr is not required for all PTB domain interactions, as demonstrated by data from the X11 and the C-terminal FE65 PTB domains. The PTB domain of the neuronal protein X11 binds to an NPTY site in the cytoplasmic domain of the β-amyloid precursor protein (β-APP) in a phosphorylation-independent manner. Indeed, the Tyr residue in the NPTY motif is not critical for binding as its replacement with Ala results in no significant loss of binding affinity[13,14]. While the PTB domain of another neuron-specific protein, FE65, binds an overlapping site on the β-APP protein, high affinity binding requires the presence of an extra 28 residues flanking the NPTY site and, unlike the X11 PTB domain, does not depend on the Asn residue of the NPTY motif[13,15].

The dNumb protein, and its mammalian homologs, contain an N-terminal sequence with significant identity to the Shc PTB domain[16]. Numb is one of several proteins that act as intrinsic cellular determinants of asymmetric cell division, originally identified through their role in development of the external sensory organs of the *Drosophila* peripheral nervous system[17,18]. The external sensory organs are composed of two outer (hair and socket) cells, and two inner (sheath and neuron) cells, that are formed from a single sensory organ precursor (SOP) cell. The SOP cell divides asymmetrically to give rise to the IIA and IIB cells; the IIA cell subsequently divides again to yield hair and socket cells, while the IIB cell also undergoes a further asymmetric division to give sheath and neuron cells[19]. During prophase in a dividing SOP cell, membrane-associated Numb protein is localized to a cortical crescent overlying a single spindle pole and at telophase is preferentially distributed into one of the two daughters, which as a consequence adopts a IIB cell fate[17,20,21]. Two mammalian homologs of dNumb have been identified, mNumb and Nbl, that are able to rescue the phenotype of Numb-deficient flies, and are likely important in asymmetric division of mammalian cells[22–25].

At least two functions have been ascribed to the dNumb PTB domain. First, it appears necessary for the asymmetric segregation of Numb in the procephalic neurogenic region of the ectoderm[26]. In addition, the PTB domain is required to specify the fate of the SOP cell descendants, possibly by inhibiting an extrinsic cue delivered by the Notch receptor[27,28]. In addition to having multiple potential biological functions, the Numb PTB domain may bind several distinct peptide motifs. The dNumb PTB domain interacts with the Numb-associated kinase (Nak), a neg-

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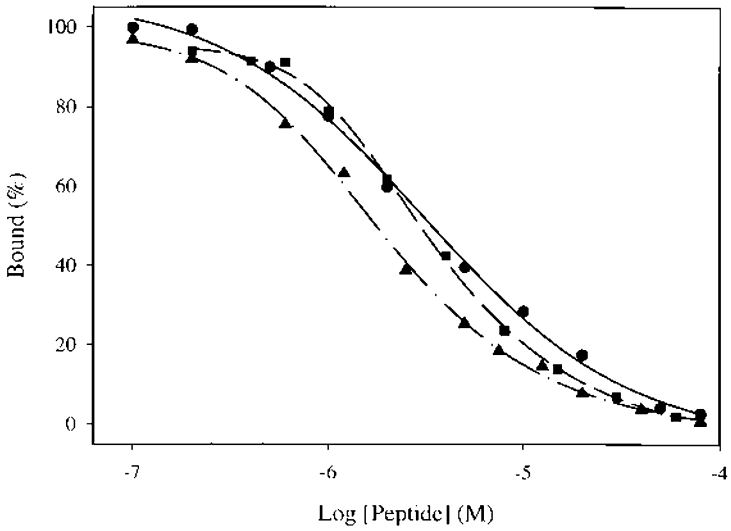

Fig. 1 Competition binding data for unlabeled peptides with a complex of the dNumb PTB domain and fluorescein-labeled Nak peptide. Percentages of the dNumb PTB domain bound to the fluorescent peptides are plotted against the log of competing peptide concentration. Curves represent the best fit to the data assuming a single site binding mode (Nak, circles and solid line; GPpY, triangle and dash-dotted line; Lnx, square and dashed line). Independent experiments produced IC50 values with less than 2% deviation from the reported values (see text).

ative regulator of Numb function, through an 11-residue motif that lacks an NPXY motif and indeed has no Tyr residue[29]. The Numb PTB domain also binds GP(p)Y-containing peptides isolated by screening a degenerate peptide library, in a fashion that is enhanced by Tyr phosphorylation[30]. Furthermore, the PTB domain of the mammalian Numb protein binds Lnx, an adapter protein that possesses four PDZ domains and a ring finger motif, through an LDNPAY sequence[31]. Here, we have investigated the structural mechanism through which the Numb PTB domain can recognize a diverse series of peptide motifs and thus potentially perform multiple functions in the control of asymmetric cell division.

Binding of Numb PTB domain to various peptides

To investigate how the Numb PTB domain recognizes diverse peptides that share no apparent sequence identity, we synthesized peptides representing its binding sites in the Lnx and Nak proteins respectively[29,31], and a GPpY-containing peptide derived from library screening[30] (Table 1). Two of the peptides in Table 1 (GPpY and Nak) do not contain sequence motifs of the sort normally recognized by other PTB domains. Fluorescence polarization[30] binding studies employing fluorescein-labeled versions of the peptides showed that all three peptides are able to bind to the dNumb PTB domain (expressed as a GST fusion protein) with K_d values ranging from 0.5 μM for the GPpY peptide to 1.8 μM for the Lnx and Nak peptides (Table 1). The control GST alone did not show appreciable binding to any of the peptides even at 1 mM concentration of the protein. Although the GPpY peptide bound more tightly to the dNumb PTB domain, the differences in binding affinity among the peptides are not large. As the GPpY peptide is the only one in the group that contains a pTyr in its sequence, one would expect that the phosphate group may contribute to the binding affinity. In fact, we have previously shown that the affinity of a non-phosphorylated version of the peptide is several-fold lower than that of the GPpY peptide[30]. Interestingly, phosphorylation of the Tyr residue in the Lnx peptide decreased its binding affinity. Moreover, substitution of Tyr by Phe in the NPAY motif produced a peptide that retains the full binding affinity of the Lnx peptide to the dNumb PTB domain[31]. Since the Nak peptide contains no Tyr in its sequence, it is clear that the dNumb PTB domain does not require a Tyr for binding.

The highest sequence identity between dNumb and its recently identified mammalian homolog, the 66,000 M_r mNumb[22,23], is in the PTB domain (70%). To test if the binding specificity observed for the dNumb PTB domain is preserved in its mammalian counterpart, we also examined the interaction of the mNumb PTB domain with the same set of peptides. All three peptides studied displayed relatively high affinities for the mNumb PTB domain (Table 1), with K_d values ranging from 1.3–3.9 μM for the corresponding peptide–PTB domain complexes. Although the affinities of the peptides for the mNumb PTB domain are slightly lower than for the dNumb PTB domain, the K_d values obtained are comparable to those reported for the IRS-1 PTB domain with an NPApY-containing peptide derived from the IL-4 receptor[9] and for the X11 PTB domain with an NPTY-containing peptide derived from the β-APP protein[14].

Competition binding studies were also performed to test if the peptide–Numb PTB domain interactions are specific, as well as to examine if one peptide can inhibit binding of the other peptides. Fluorescence polarization methods were used to generate competition binding curves for the GPpY, Lnx and Nak peptides with the dNumb PTB domain (Fig. 1). Fluorescein labeled Nak peptide in complex with the PTB domain could be displaced by any one of the three unlabeled peptides with IC50 values within a narrow range (from 1.7 μM for the GPpY peptide to 3.4 μM for

Table 1 Peptide binding affinities to the Numb PTB domain

		K_d (μM)[1]	
Peptide[2]	Sequence	dNumb PTB	mNumb PTB
GPpY	Ac-AYIGPpYL-OH	0.53 ± 0.04	1.34 ± 0.23
Lnx (residues 180–193)	H_2N-EPGLDNPAYTSSVE-OH	1.79 ± 0.54	3.89 ± 0.24
Nak (residues 1,437–1,447)	Ac-GFSNMSFEDFP-OH	1.78 ± 0.62	3.76 ± 0.13

[1]The equilibrium dissociation constants (K_d) were determined by fluorescence polarization[30], and are averages of three independent experiments (see Methods).
[2]Peptides, except for the Lnx peptide, were blocked at the N-terminus with an acetyl group. For fluorescein labeling, a GGK extension was added to the C-termini of the GPpY and the Nak peptides with the ε-H_2N of the lysine side chain providing the attachment site for fluorescein. A fluorescein moiety was coupled directly to the N-terminal amino group of the Lnx peptide to produce the labeled peptide.

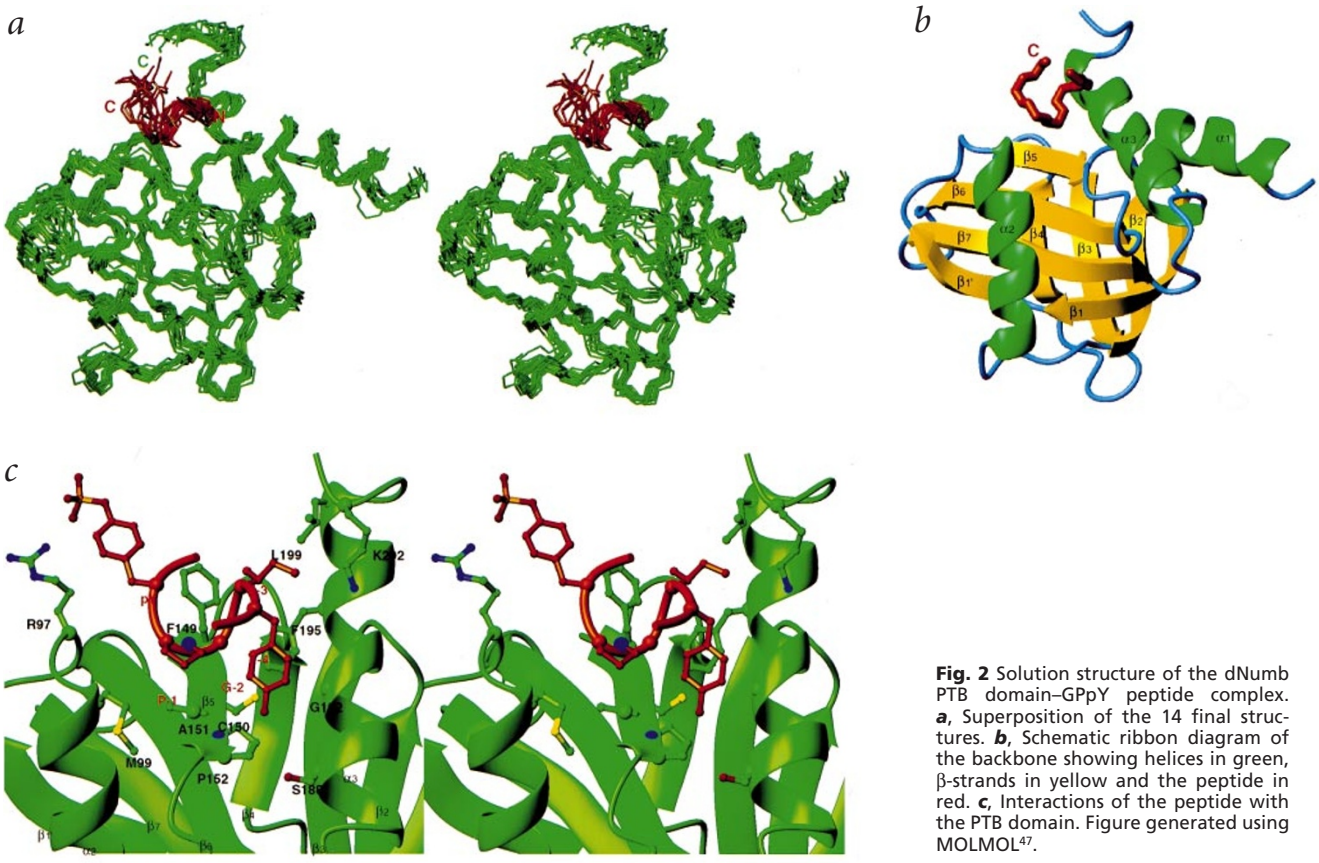

Fig. 2 Solution structure of the dNumb PTB domain–GPpY peptide complex. ***a***, Superposition of the 14 final structures. ***b***, Schematic ribbon diagram of the backbone showing helices in green, β-strands in yellow and the peptide in red. ***c***, Interactions of the peptide with the PTB domain. Figure generated using MOLMOL[47].

the Nak peptide when 4.0 μM of GST-PTB domain was used, calculation not shown). Similar results were obtained for competition of peptides with complexes between the dNumb PTB domain and fluorescein labeled GPpY and Lnx peptides. These results indicate that the observed interactions between the peptides and the dNumb PTB domain are specific and that the peptides likely bind overlapping sites in the PTB domain.

Structure of dNumb PTB–GPpY complex

To gain a more detailed understanding of the nature of dNumb PTB domain–peptide interactions, we have determined the NMR structure of the dNumb PTB domain in complex with the GPpY peptide, which displays a slightly higher affinity than the Lnx and Nak peptides for the isolated dNumb PTB domain. As well, significant line-broadening was observed for the PTB domain complex with the Lnx peptide, while the Nak peptide only became available for NMR studies recently[29]. The overall correlation time for molecular tumbling of the complex is 13.5 ns (C.Z, S.J.F.V. and L.E.K., unpublished results), larger than expected given the molecular weight (~18,700 M_r) and leading to broader NMR resonances and lower signal-to-noise in NMR spectra than for other proteins of this size. The significant overlap of many methyl resonances of the PTB domain also resulted in a large degree of ambiguity in assignment of NOE-based restraints to specific proton pairs. A new NOESY experiment[32] providing significantly improved resolution for methyl resonances of valine and isoleucines was utilized, but preliminary attempts to define the structure using standard protocols were still unsatisfactory. Application of ARIA[33], a recently described approach that performs automatic assignment of NOEs based on chemical shifts, allowed the current ensemble of structures to be calculated (Fig. 2*a*; Table 2).

The resulting structure of the dNumb PTB domain in complex with the GPpY peptide (Fig. 2*b*) reveals a topology similar to that of the human Shc[8] and X11[14] PTB domains to which dNumb has 22% and 18% sequence identities respectively, from structure-based alignments[34] (Fig. 3). The structure of the IRS-1 PTB domain[9,10] is also very similar but lacks two N-terminal α-helices found in the Numb PTB domain (α1 and α2). All share a common PH domain fold[8] with a central β-sandwich and a C-terminal α-helix (α3). The first of the two sheets in the β-sandwich is composed of the N-terminal portion of β1, β2, β3 and β4 and the second contains the C-terminal portion of β1, β1', β7, β6 and β5, using nomenclature from previous PTB domain structures[14]. The residues at the N-terminal end of the α2 helix in the dNumb PTB domain as well as residues from β5 and the α3 helix are involved in peptide interactions. The first and last residues of the GPpY peptide are disordered and the pTyr is directed into solution in most of the structures in the ensemble. An aromatic-polar interaction of the pTyr with Arg 97 is likely since substitution of Arg 97 with Ser reduces the affinity of both the GPpY peptide and a non-phosphorylated version of the peptide by a factor of two (data not shown) and one member of the ensemble shows close contacts between the pTyr and Arg 97

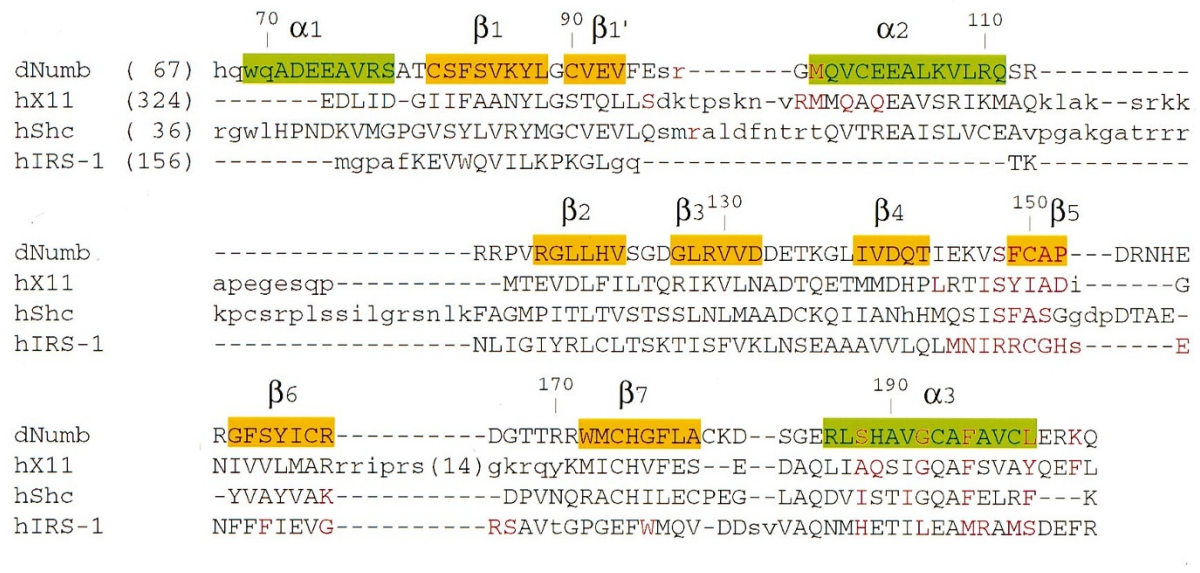

Fig. 3 Structure-based alignment[34] of sequences of the dNumb, X11[14], Shc[8] and IRS-1[10] PTB domains, with residues of the dNumb PTB domain highlighted in green for helices and yellow for β-strands. Residues in the PTB domains that have direct contacts to their target peptides are highlighted in red.

(Fig. 2*c*). The binding is mediated primarily by hydrophobic interactions (Fig. 2*c*) involving residues of α2 (Met 99), β5 (Phe 149, Cys 150, Ala 151, Pro 152) and α3 (Ser 188, Gly 192, Phe 195, Leu 199, Lys 202) of the PTB domain and the Tyr -4, Ile -3, Gly -2 and Pro -1 peptide residues, numbered relative to the pTyr(0). The same residues of the peptide have been identified as critical for high affinity interaction in our screening and alanine substitution binding studies[30].

In agreement with the structure, mutation of key residues of the PTB domain having peptide contacts (Met 99 and Cys 150 to Ala and Ala 151 to Gly) resulted in significantly reduced binding affinity for the GPpY peptide (data not shown). In particular, a Cys 150 to Ala mutation resulted in a 90% loss of affinity, compared with the wild type PTB domain. However, mutations of Ser 148 and Arg 171 (corresponding to Ser 151 and Arg 175 in the Shc PTB domain where they were shown to be important for pTyr recognition[15]) had negligible effects on the dNumb PTB domain–GPpY interaction, indicating that these residues are not critical for dNumb binding.

Yaich and coworkers have recently generated several dNumb mutants with mutations in the PTB domain[35]. These mutations, Ser 148 to Ala, Phe 149 to Leu, Arg 171 to Gln and Phe 195 to Val, correspond to sites in the Shc PTB domain shown to be important for ligand binding. Overexpression of the mutant protein in transgenic flies indicated that while residues such as Phe 195, and possibly Phe 149 as well, are important for dNumb function, residues Ser 148 and Arg 171 appear not to be required. Note that the two aromatic residues, Phe 195 and Phe 149, form part of the hydrophobic pocket for the binding of Ile -4 of the GPpY peptide in the dNumb PTB domain–GPpY complex. These data are in excellent agreement with our structural and *in vitro* binding studies.

Comparison with other PTB domains

An extremely interesting finding is that while the structure of the dNumb PTB domain is very similar to that of the Shc[8] and X11[14] PTB domains and many of the residues involved in binding are analogous (Fig. 3), the mode of peptide recognition is significantly different. In the case of the Shc, X11 and IRS-1 PTB domains, peptide recognition involves β-sheet hydrogen-bonding interactions of the target peptide with a strand of the β-sheet of the PTB domain (β5) and a β-turn immediately N-terminal to the peptide Tyr or aromatic residue[8–10,14]. The Asn -3 and the β-turn conformation have been considered the most important determinants for PTB domain binding[8,14]. The backbone dihedral angles of the GPpY peptide in complex with the dNumb PTB domain are in helical turn conformations rather than typical type I β-turn conformations observed in other PTB domain–peptide structures. The helical turn is formed by the core residues Tyr-Ile-Gly-Pro-pTyr of the peptide which also mediate interactions with the PTB domain. The turn itself is not localized to the same site on the surface of the PTB domain as for the β-turns in peptide targets of Shc, X11 or IRS-1 PTB domains; superposition of the structures places the Cα position of the Pro -1, the base of the turn in the GPpY peptide, 11–14 Å removed from that of the corresponding Cα of the -1 residue with respect to the (p)Tyr of peptide targets for the Shc, X11 and IRS-1 PTB domain complexes (Fig. 4). Furthermore, the GPpY peptide does not form an extension of the β-sheet structure by hydrogen-bonding to β5 as in the other PTB domain structures, although residues of β5 do interact extensively with the peptide. The surface area buried upon binding of the peptide is ~900 Å^2 which compares to 1,500, 1,400 and 1,900 Å^2 for Shc, IRS-1 and X11 PTB domain peptide complexes respectively. Note that in the Shc and IRS-1 structures, the pTyr is involved in a strong electrostatic interaction and is at least partly solvent inaccessible, while in the dNumb the phosphate of the pTyr is almost completely solvent exposed (Fig. 4*a–d*).

Similarity of interface for three peptides

The observed competition of the binding of the Nak, Lnx and GPpY peptides for the dNumb PTB domain suggests that they

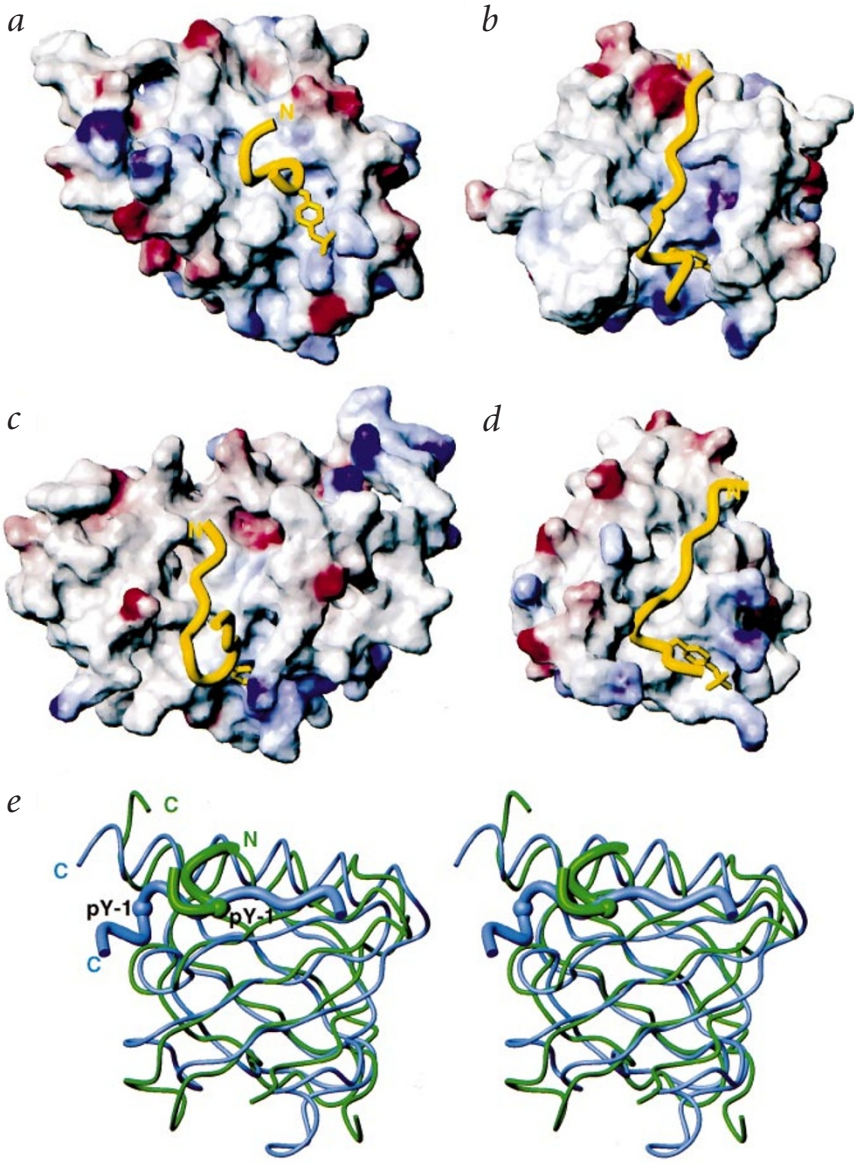

Fig. 4 ***a*–*d***, Surface representations (using MOLMOL[47]) of PTB domains in complex with target peptides (in yellow), with blue representing positive and red negative electrostatic potential. ***a***, dNumb PTB domain–GPpY peptide complex. ***b***, X11 PTB domain–βAPP peptide complex[14]. ***c***, Shc PTB domain–TrkA peptide complex[8]. ***d***, IRS-1 PTB domain–IR peptide complex[10]. Note that the orientations of the PTB domains are the same, based on structural superposition. ***e***, Superposition of schematic representations of the backbone of the structures of the dNumb (green) and IRS-1[10] (blue) PTB domains (thin tubes) in complex with target peptides (thick tubes). Only structurally homologous residues of the PTB domains are shown. Locations of the Cα atoms of the residues corresponding to the -1 position relative to the pTyr in the two peptides are indicated.

may interact with similar or overlapping sites in the protein. To verify this, we titrated each peptide into an NMR sample of the dNumb PTB domain and analyzed the changes in chemical shifts of resonances in the protein in response to peptide binding. Rather than assigning the free PTB domain and the complexes with the Lnx and Nak peptides, we have adopted a minimal chemical shift difference approach[36] in order to establish which residues are most likely involved in binding. In this method, the differences in ^{15}N and ^{1}H chemical shifts between a given correlation in the assigned spectrum of the dNumb PTB domain–GPpY complex (Fig. 5*a*) and the closest crosspeak (i) in an unassigned spectrum, $\Delta = \{[\delta_{15N}(GPpY) - \delta_{15N}(i)]^2 + [\delta_{HN}(GPpY) - \delta_{HN}(i)]^2\}^{1/2}$, is calculated. The approach is conservative since it will always provide the actual value or an underestimate of the chemical shift difference. Many of the residues with the largest minimal shift differences between the free PTB domain and the GPpY peptide complex are found in defined regions of the protein that have been identified in our structure as having direct contacts to the GPpY peptide (Fig. 5*b*). Since the assignments of the free PTB domain are not known, we have compared the Nak and Lnx complexes to the GPpY complex; thus the minimal shift differences observed (Fig. 5*c*,*d*) reflect changes from the GPpY peptide complex, not from the free PTB domain. Interestingly, most of the residues involved in GPpY peptide contacts in our structure also have significant minimal shift differences between the GPpY- and the Nak- or Lnx-PTB domain complexes (Fig. 5*c*,*d*). Furthermore, mutation of the critical residues interacting with the GPpY peptide such as Arg 97, Met 99, Cys 150 and Ala 151 to Ala or Gly decreased the binding of the dNumb PTB domain to all three peptides (data not shown). Thus, it appears that key residues in the dNumb PTB domain that are involved in binding to the GPpY peptide also mediate interactions with the Nak and Lnx peptides.

Discussion

Asymmetric division is a central process through which cellular diversity is generated in the development of metazoan organisms. The Numb protein, which plays an important role in such asymmetric divisions in the *Drosophila* nervous system, is conserved in evolution[22], and possesses multiple potential protein-binding modules and motifs. The *Drosophila* and mammalian Numb proteins are most closely related within an N-terminal sequence of ~135 amino acids that is similar to the PTB domain of the docking protein Shc[22,23]. We have undertaken biochemical and structural analysis of the Numb PTB domain in an effort to understand how a module originally identified through its role in tyrosine kinase signaling may be employed to control cell fate decisions.

Three peptides have been identified than can bind the PTB domains of the dNumb and mNumb proteins, with very similar affinities. Two of these peptides are derived from potential physiological binding partners (Nak and Lnx), while a third, GP(p)Y, was initially identified through screening a non-phosphorylated degenerate peptide library. Surprisingly, these three Numb PTB domain-binding peptides show no identity in their primary sequences, although they all contain potential sites for turn formation. Among these ligands only the Lnx peptide has an NPXY

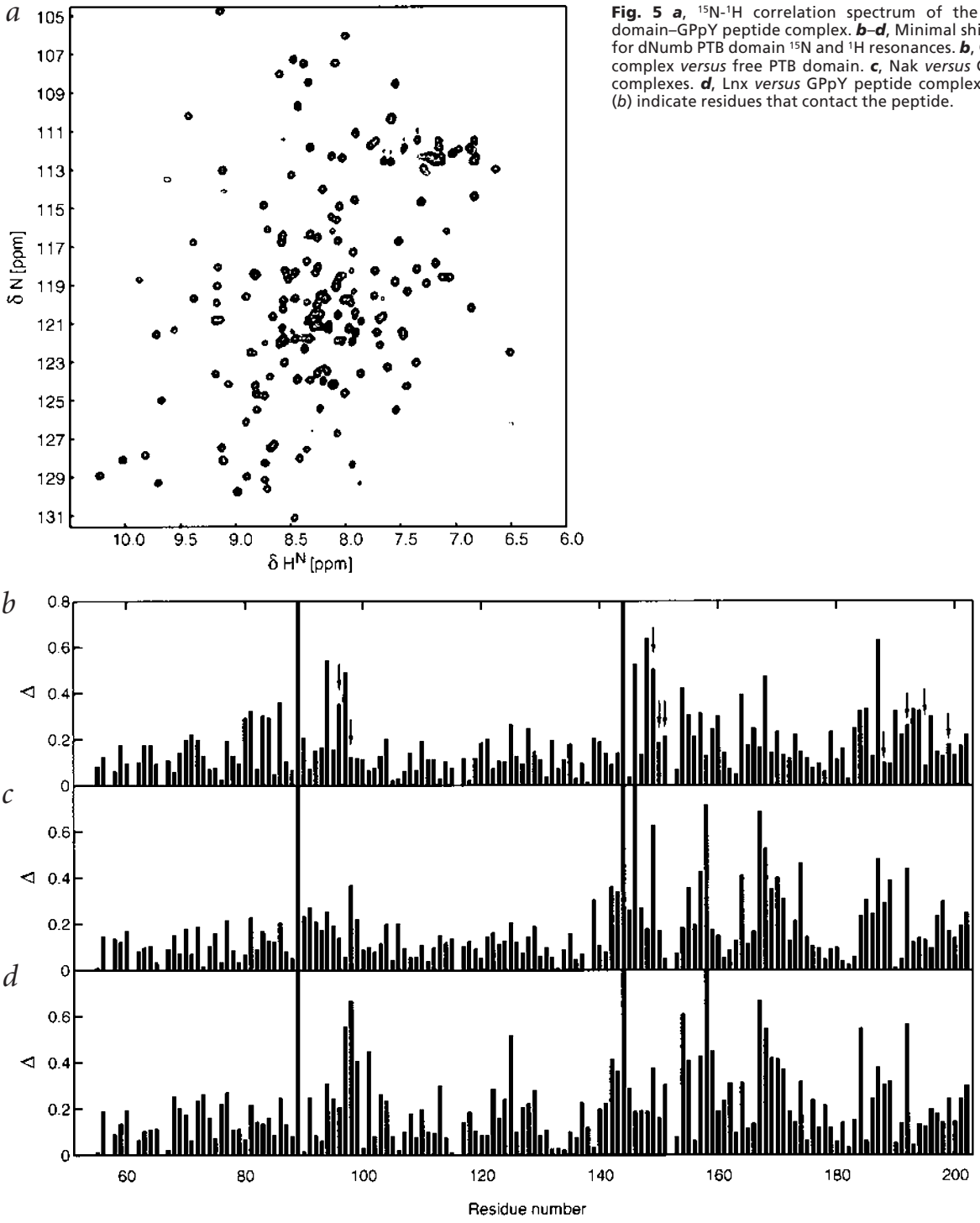

Fig. 5 ***a***, ^{15}N-^{1}H correlation spectrum of the dNumb PTB domain–GPpY peptide complex. ***b***–***d***, Minimal shift differences for dNumb PTB domain ^{15}N and ^{1}H resonances. ***b***, GPpY peptide complex *versus* free PTB domain. ***c***, Nak *versus* GPpY peptide complexes. ***d***, Lnx *versus* GPpY peptide complexes. Arrows in (*b*) indicate residues that contact the peptide.

motif; Tyr phosphorylation modestly increases binding of the GPY peptide, but decreases binding of the Lnx peptide, while the Nak peptide has no Tyr residue. These findings suggest that the Numb PTB domain has a rather wide range of potential ligands and indicate that its specificity may be defined by tertiary contacts rather than by primary sequence. The multiplicity of the Numb PTB domain ligands raises the important question of whether this module has a single site or multiple sites for peptide binding. We addressed this issue by using peptide competition and NMR titration experiments. The observation that these peptides compete effectively against one another for Numb PTB domain binding indicate that these diverse peptides interact with the same or overlapping sites on Numb. This conclusion was substantiated by NMR titration studies, in which similar amide ^{15}N and ^{1}H chemical shift change patterns were obtained for all three peptide–PTB domain complexes. Thus, three peptides with quite distinct primary sequences can bind with similar dissociation constants to related sites on the Numb PTB domain.

To investigate how the Numb PTB domain may bind such a variety of peptides, we have examined the structure of the PTB domain in complex with the highest affinity ligand, the GPpY peptide. This structure revealed a novel mode of peptide recognition that is markedly different from that used by the Shc and IRS-1 PTB domains for binding of NPXpY peptides. Although the GPpY peptide occupies the same binding groove as NPXY-containing PTB domain ligands, the dNumb PTB domain–GPpY peptide complex displays several unique features. (i) The peptide adopts a helical turn rather than a β-turn conformation. (ii) The helical turn occupies a site on the surface of the domain 11–14 Å away from the β-turn binding site observed in other PTB domain–peptide complexes. (iii) The peptide does not hydrogen bond as an additional strand of the β-sheet of the PTB domain. (iv) There exists no well-defined positively charged pocket for binding of phosphate. As a result, the pTyr residue in the peptide is completely solvent-exposed, correlating with our earlier observation that phosphorylation is not required for binding. (v) The five residues of the peptide (YIGPpY) that form the helical turn also mediate interactions with the PTB domain. The GPpY peptide is very compact and buries a significantly smaller surface area of the PTB domain than do the TrkA, IL-4 and β-APP peptides to achieve a comparable affinity. The diverse specificities of the Numb PTB domain may be explained by the large surface groove present in its structure which could readily accommodate longer peptides such as those from Nak and Lnx. We presently have no evidence that a physiological binding partner for the Numb PTB domain adopts the same structure as the GPpY peptide. However, the fact that the affinity of the Numb PTB domain for this short peptide exceeds those for the NPXY-containing Lnx peptide and the Nak peptide strongly suggests that the conformation and interactions of the GPpY peptide could represent a biologically relevant mode of binding. The compactness of the GPpY peptide also makes it an attractive candidate for designing protease-resistant peptide mimetics for elucidating the functions of the Numb PTB domain *in vivo*.

The capacity of the dNumb PTB domain to bind multiple ligands may also relate to its biological functions in cell division. The Numb protein is preferentially segregated into one daughter cell during SOP division and consequently acts as an intrinsic cell fate determinant. Asymmetric localization of Numb is mediated by an N-terminal region of the Numb protein that includes the PTB domain[26]. Furthermore, *numb* is proposed to act genetically upstream of the neurogenic gene *Notch*, and to negatively regulate *Notch* function[28]. A direct physical association involving the Numb PTB domain has been observed between dNumb and *Drosophila* Notch, as well as between mNotch and the mammalian Notch1 protein[22,28]. Interestingly, the proposed dNumb PTB domain binding regions in Notch, mapped to its RAM23 region or C-terminus[28], do not contain sequence motifs homologous to any of the three peptides used here, raising the possibility that additional Numb PTB domain ligands exist. Furthermore, the association of the Numb PTB domain with Nak has been implicated in inhibition of Numb function[29]. The apparent versatility of the Numb PTB domain may allow it to interact with multiple partners and thereby perform a variety of tasks during asymmetric cell division.

Modular binding interactions may represent an important theme in asymmetric cell division. In addition to a PTB domain, the mNumb protein has proline rich motifs that can bind SH3 domains[23] and an NPF sequence motif that can associate with the Eps homology domains of Eps15, a protein implicated in protein and vesicle trafficking[25]. Furthermore, Inscuteable[37], a protein that can determine orientation of the mitotic spindle and Numb localization in *Drosophila*[38], has multiple ankyrin repeats, a PPPPPPY motif with the potential to bind WW domains, and a C-terminal motif (ESFV) that conforms to the consensus for PDZ domain interactions[39]. In the future, it will be important to determine how these modular interactions are coordinated to control cell division.

In contrast to PTB domains, the structurally distinct SH2 domains are highly specialized for the recognition of pTyr. Specificities of binding for individual SH2 domains can in many cases be defined in terms of simple sequence patterns. The implication of our results is that not all PTB domain targets may be easily identified by amino acid sequence motifs. Understanding that PTB and other modular binding domains may bind a diverse set of targets dominated in some cases by tertiary contacts rather than primary or secondary structure is critical to avoid overlooking important biologically relevant interactions of these regulatory domains.

Table 2 Statistics for the 14 final structures of the dNumb PTB domain–GPpY peptide complex

Structural Statistics		
R.m.s. deviations from distance restraints (Å)		
All (2,903)	0.011 ± 0.001	
Unambiguous NOE-derived restraints (2,097)	0.011 ± 0.001	
PTB-PTB (1,989)	0.011 ± 0.001	
Peptide-peptide (82)	0.011 ± 0.011	
PTB-peptide (26)	0.011 ± 0.007	
Ambiguous NOE-derived restraints (760)	0.013 ± 0.002	
Hydrogen-bond restraints (46)	0.006 ± 0.002	
Deviations from idealized geometry		
Bonds (Å)	0.002 ± 0.002	
Angles (°)	0.34 ± 0.01	
Impropers (°)	0.61 ± 0.05	
PROCHECK[48] Ramachandran map analysis		
Most favored regions	55.6 ± 2.7%	
Additional allowed regions	35.3 ± 2.9%	
Generously allowed regions	7.1 ± 1.6%	
Disallowed regions	2.0 ± 0.9%	
Atomic r.m.s. deviations (Å)	Backbone	All heavy atoms
Residues 67–203 and GPpY peptide	0.69 ± 0.08	1.39 ± 0.17
Secondary structural elements and peptide	0.57 ± 0.06	1.17 ± 0.16
Secondary structural elements	0.49 ± 0.07	1.01 ± 0.08
Peptide	0.77 ± 0.19	1.72 ± 0.49
Interface A[1]	0.81 ± 0.15	1.69 ± 0.43
Interface B[2]	0.68 ± 0.14	1.06 ± 0.21

[1]PTB domain residues 97, 99, 149–152, 188, 192, 195, 199, 202 and peptide residues 1–7.
[2]PTB domain residues 99, 149–152, 188, 192, 195, 199, 202 and peptide residues 1–5.

Methods

Binding studies using fluorescence polarization. Peptide synthesis and purification were as reported[30]. The coding regions for the dNumb and mNumb PTB domains were amplified by PCR using original cDNA as templates[23,30], and subcloned into the pGEX4T2 vector (Pharmacia) for the production of GST-fusion proteins[30]. Fluorescence polarization experiments were conducted on a Beacon Fluorescence Polarization System (PanVera Co.). The labeled peptides were dissolved in a buffer containing 20 mM Tris, 100 mM NaCl, 0.1 mM EDTA, 2 mM DTT and 0.1 mM benzamidine, pH 7.5. Various amounts of purified proteins reconstituted into the same buffer were added to the labeled peptide solution, and the resulting fluorescence polarization values were recorded for the derivation of the equilibrium dissociation constants (K_d)[30]. For competition studies, 100 nM of a fluorescein-labeled peptide was incubated briefly with 4.0 μM of GST-dNumb PTB domain fusion protein, incremental concentrations of unlabeled peptides were then added to the mixture, and the resulting fluorescence polarization values were recorded. The bound percentages of the labeled peptide were calculated by comparing the fluorescence polarization values at various points to the starting value when no competing peptide was present. All measurements were carried out at 25 °C.

NMR spectroscopy. NMR samples of the isolated dNumb PTB domain (residues 58–205 with additional seven N-terminal and six C-terminal residues from the recombinant construct) were prepared according to published procedures[39]. Four different isotopic labeling schemes were employed. Sample A was uniformly $^{15}N/^{13}C$ labeled dNumb PTB domain in complex with a natural abundance peptide; sample B, used for stereospecific assignment of Leu and Val methyls[40], was uniformly $^{15}N/10\%$ ^{13}C labeled PTB domain in complex with natural abundance peptide; sample C, used to facilitate assignment of the resonances of the peptide that were significantly broadened due to dynamic processes, was a natural abundance PTB domain in complex with a peptide uniformly labeled with ^{13}C and ^{15}N (except pTyr) and synthesized by standard Fmoc-chemistry using isotopically labeled amino acids as the starting material; sample D, used for titrations with the Lnx and Nak peptide, was uniformly ^{15}N labeled.

NMR experiments were performed at 30 °C on Varian Inova or UnityPlus 500 or 600 MHz spectrometers equipped with pulsed field gradient units and triple resonance probes with actively shielded z gradients. Multidimensional, heteronuclear experiments[41] for assignment and structural analysis were acquired using pulsed field gradient sensitivity enhancement approaches[42] where appropriate and water signal was minimized by the combined use of water selective pulses and gradients[41,42]. Structural restraints for the PTB domain were obtained from 40 ms and 90 ms ^{15}N-edited NOESY experiments in H_2O (sample B), 40 ms and 90 ms ^{13}C-edited NOESY experiments in D_2O and a 100 ms Val-Cβ-NOESY[32] experiment in D_2O (sample A). Restraints for the peptide were derived from a 100 ms double-filtered NOESY (sample A) and from a 100 ms 2D CN-NOESY (sample C). Intermolecular NOEs were obtained from a 150 ms half-filtered ^{13}C-edited NOESY (sample A) in D_2O. Hydrogen bond restraints were obtained based on slowly exchanging amide protons identified in the 90 ms ^{13}C-edited NOESY, recorded in D_2O. Hydrogen bonds that included a slowly exchanging amide proton and that were observed to be in regular secondary structural elements based on medium range NOEs and analysis of low energy structures from preliminary structural calculations were utilized as restraints.

Structure calculations. NOEs derived from all NOESY experiments recorded on the PTB domain were analyzed in a conventional manner, assigning NOEs iteratively based on earlier sets of structures. Using this approach, ~1,500 NOEs were unambiguously assigned for the PTB domain. The half-filtered experiment allowed only 15 unambiguous intermolecular NOEs to be assigned. These preliminary structures were calculated using a torsion angle dynamics protocol[43,44] within the program CNS[45]. These structures were then utilized as input to ARIA[33], without data from the 40 ms ^{13}C-edited NOESY and the Val-Cβ-NOESY experiments. ARIA is a recent approach, developed to work within X-PLOR[46], that automatically assigns NOEs based on chemical shifts and calibrates NOE intensities. The resulting ARIA assignment for the half-filtered NOESY experiment is in agreement with the manual assignment. Additional intermolecular NOEs derived from the ^{13}C- and ^{15}N-edited NOESY experiments were also observed. A total of 51 intermolecular NOEs were assigned, 26 unambiguous and 25 ambiguous.

Nine iterations of the ARIA protocol were performed, reducing the toleration of violations of NOE restraints from 0.5 Å to 0 Å and reducing the ambiguous cut-off value from 0.999 to 0.85. This value determines the percentage of the NOE intensity that is accounted for by the assignment. Thus, the number of ambiguous NOEs, those that include contributions from multiple proton pairs, decreased from 2,346 in the first iteration to 760 in the final iteration, while the number of unambiguous NOEs increased from 448 to 2,097. Restraints for 23 hydrogen bonds were included in the sixth iteration and data from the 40 ms ^{13}C-edited NOESY data were introduced in the final iteration. Final minimization of the structures was performed without electrostatic or attractive van der Waals terms in the energy function. The resulting 14 members of the ensemble have no NOE violations greater than 0.3 Å.

Coordinates. The structures and restraints have been deposited in the Protein Data Bank (accession code 2nmb) and the resonance assignments in the BioMagResBank (accession number 4263).

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