Origins of Peptide Selectivity and Phosphoinositide Binding Revealed by Structures of Disabled-1 PTB Domain Complexes

Peggy C. Stolt,1,4 Hyesung Jeon,1,4 Hyun Kyu Song,2 Joachim Herz,3 Michael J. Eck,2 and Stephen C. Blacklow1,4*
1Department of Pathology
Brigham and Women’s Hospital and Harvard Medical School
75 Francis Street
Boston, Massachusetts 02115
2Department of Cancer Biology
Dana Farber Cancer Institute and Department of Biological Chemistry
and Molecular Pharmacology
Harvard Medical School
44 Binney Street
Boston, Massachusetts 02115
3Department of Molecular Genetics
University of Texas Southwestern Medical Center
Dallas, Texas 75390

Summary

Formation of the mammalian six-layered neocortex depends on a signaling pathway that involves Reelin, the very low-density lipoprotein receptor, the apolipoprotein E receptor-2 (ApoER2), and the adaptor protein Disabled-1 (Dab1). The 1.5 Å crystal structure of a complex between the Dab1 phosphotyrosine binding (PTB) domain and a 14-residue peptide from the ApoER2 tail explains the unusual preference of Dab1 for unphosphorylated tyrosine within the NPxY motif of the peptide. Crystals of the complex soaked with the phosphoinositide PI-4,5P2 (PI) show that PI binds to conserved basic residues on the PTB domain opposite the peptide binding groove. This finding explains how the Dab1 PTB domain can simultaneously bind PI and the ApoER2 tail. Recruitment of the Dab1 PTB domain to PI-rich regions of the plasma membrane may facilitate association with the Reelin receptor cytoplasmic tails to transduce a critical positional cue to migrating neurons.

Introduction

The mammalian Disabled-1 (Dab1) gene is one of two known mammalian orthologs of Drosophila Disabled, a gene required for proper development of the fly central nervous system [1, 2]. Genetic and biochemical studies of Dab1 in mice and in humans have shown that it is required for the migration of neurons to their proper positions during embryonic development. Targeted or naturally occurring disruption of Dab1 in mice causes abnormal neuronal positioning in the cerebral cortex and the hippocampus, as well as cerebellar dysplasia [3, 4]. The Dab1 null phenotype is identical to that of the ataxic reeler mouse, which results from a naturally occurring null mutation in the reelin gene [5]. The connection between Reelin, a secreted protein found in the extracellular matrix, and Dab1, a cytosolic adaptor protein, was revealed when double-knockout mice lacking two closely related proteins of the low-density lipoprotein (LDL) receptor gene family, ApoER2 and VLDLR, were shown to be phenotypically indistinguishable from reeler- and dab1-deficient mice [6]. Studies showing that Reelin binds to the ectodomains of ApoER2 and VLDLR [7, 8] and that Dab1 binds to the cytoplasmic tails of these receptors [9] provide further compelling evidence that Reelin, the lipoprotein receptors, and Dab1 are part of a common signaling pathway.

A phosphotyrosine binding (PTB) domain at the N terminus of Dab1 mediates binding to the cytoplasmic tails of the lipoprotein receptors [9, 10]. This domain is required for normal Dab1 function and is shared among the three known isoforms of Dab1 [11]. All isoforms also include a tyrosine-rich sequence immediately C-terminal to the PTB domain, with variations resulting from alternative splicing occurring downstream (Figure 1A).

Though PTB is an acronym for “phosphotyrosine binding” [12–14], PTB domains exhibit remarkable plasticity in their ability to recognize different peptide motifs. In Dab1, the PTB domain selectively recognizes peptides with the consensus sequence φxNPxY (where φ represents F or Y, x represents relaxed amino acid selectivity, and Y is designated the “0” position on the basis of prior convention). In contrast to most other PTB domains that bind tyrosine-based motifs either with preference for phosphotyrosine (at the 0 position) or indifference to the phosphorylation state of the tyrosine, the PTB domain of Dab1 favors unphosphorylated tyrosine over phosphotyrosine by almost two orders of magnitude [15]. In accordance with this observed selectivity, the tyrosine of the NPxY motif of each receptor is believed to be unphosphorylated at rest, and whether receptor activity is regulated by phosphorylation on the NPxY tyrosine is not yet known.

PTB domains structurally resemble pleckstrin homology (PH) domains, which bind to a variety of different phospholipids with varying specificity [16–18]. Indeed, previous studies have shown that the Dab1 PTB domain binds phosphoinositides (Pis) [15], with selectivity for phosphatidylinositol-4,5-bisphosphate (PI-4,5P2), although the significance of PI-4,5P2 binding by Dab1 has not yet been elucidated. Binding of PI-4,5P2 can occur in the absence of peptide and does not prevent peptide binding in competition experiments, suggesting the existence of a distinct PI-4,5P2 binding site [15]. The other mammalian homolog of Drosophila Dab, Dab2, which seems to participate in mediating endocytosis of different LDL receptor-related proteins, also selectively binds PI-4,5P2 at a site that does not prevent peptide binding [19], suggesting that a distinct PI binding site has been

Key words: ApoER2; Disabled; lipoprotein receptor; phosphoinositide; Reelin signaling; X-ray crystallography

*Correspondence: sblacklow@rics.bwh.harvard.edu
1 These authors contributed equally to this work.
To understand the basis for selective recognition of peptides with an unphosphorylated tyrosine at the 0 position, we determined the structure of the Dab1 PTB domain in complex with a 14-residue peptide from the ApoER2 cytoplasmic tail. The structure of the complex, determined at 1.5 Å resolution, indicates how this PTB domain selectively binds peptides with the \( \phi X N P X Y \) consensus sequence and why unphosphorylated tyrosine is preferred. The structure of the PTB domain-peptide complex also reveals the presence of a basic patch on the surface of the PTB domain on the face opposite the peptide binding groove; by soaking PTB-peptide cocrystals in PI-4,5P₂, we also determined the site of PI-4,5P₂ binding within this patch in a ternary complex.

**Results and Discussion**

Primary sequence alignment and secondary structure prediction indicated that the region of homology between Dab1 and known PTB domains extends from residue 23 to 174. The construct chosen for production of the Dab1 PTB domain in bacteria spanned residues 20–175 (Dab1 PTB domain hereafter) to ensure inclusion of the entire domain with minimal flexibility at either terminus (Figure 1A).

Because this Dab1 PTB domain polypeptide differs at both termini from other previously studied Dab1 constructs [9, 15], we first verified that our purified Dab1 PTB domain associates with peptides derived from the cytoplasmic tails of ApoER2 and VLDLR in a pull-down assay. When glutathione beads are loaded with GST fusion proteins displaying either the ApoER2 or VLDLR peptide, the Dab1 PTB domain is indeed recovered with the beads, whereas it is not pulled down by GST alone (Figure 1B).

The dissociation constants of different Dab1 PTB domain-peptide complexes were then measured directly by isothermal titration calorimetry (ITC) and/or fluorescence polarization. The \( K_d \) of the complex between the ApoER2 14-mer (Ac-TKSMNFDNPVYRKT-CONH₂) and the PTB domain, measured by ITC, is \( 1.5 \pm 0.3 \mu M \) (Figure 1C), an affinity comparable to that previously measured by fluorescence for binding of a 17-residue APP peptide to a different Dab1 PTB domain-GST fusion construct [15] and within the range seen for complexes of peptides with PTB domains from other proteins [20–22]. Further analysis of the ITC data indicates that the binding of the ApoER2 peptide is enthalpy driven, as seen for the binding of peptides to the PTB domains of IRS1 and SNT-1 [21, 23] and in contrast to binding of peptides to the Shc PTB domain [23]. Competition binding experiments by fluorescence polarization show that a 10-mer ApoER2 peptide, from which the four N-terminal residues have been deleted, binds the PTB domain of Dab1 with an affinity indistinguishable from that of the 14-mer ApoER2 peptide (data not shown).

**Crystalization and Overview of Structure**

To understand the basis for recognition of the 14-mer by the Dab1 PTB domain, we solved the crystal structure.
Figure 2. Electron Density, Overview of Structure, and Sequence Alignment

(A) Representative electron density (contoured at 1σ after refinement) from the residues surrounding the NPVY motif of the peptide in the Dab1 PTB domain-peptide complex. Peptide residues, yellow; protein residues, green.

(B) Ribbon representation of the Dab1 PTB domain in stereo colored according to secondary structure, with bound peptide rendered in ball and stick form.

(C) Structure-based alignment of the Dab1 PTB domain with other PTB domains and a representative PH domain. Secondary structure elements are colored as in (B). Residues in the Dab1 PTB domain that interact with the ApoER2 peptide are colored cyan (hydrogen bonds), green (hydrophobic interactions), or yellow (both). Residues of the Dab1 PTB domain and the PLCγ PH domain that contact PI-4,5P2 are enclosed in blue boxes, as are the aligned residues of the Dab2 and ARH sequences. Numbers above the alignment refer to residue positions in Dab1. Residue positions for the other proteins are shown to the right of the aligned sequences.

of this complex by multiwavelength anomalous dispersion (MAD) to 1.5 Å resolution (Figure 2A; Table 1). The phase information was derived from selenomethionine-substituted Dab1 harboring an L87M mutation because the two consecutive native methionines of the Dab1 PTB domain (M65 and M66) alone failed to provide enough phasing power to solve the structure.

The Dab1 PTB domain exhibits a canonical PTB fold, in which seven central β strands form two antiparallel, near-orthogonal β sheets, capped by a long C-terminal
α helix (Figure 2B). Even though all PTB domains for which structures have been solved share a low level of sequence identity, structure-based alignment reveals that this core topological framework is universally conserved (Figure 2C). Unique insertions building upon this main, like the PTB domains of Shc, X11, and Numb [20, 24, 25], contains an insertion between strands 5 strand to add another strand as well as a long α helix, explaining the preference for Phe or Tyr at this position [15]. The carboxylate of the adjacent Asp at 6 to Asp forms an extra hydrogen bond between the hydrogen atoms of its NH2 group and β that forms an extra β strand as well as a long α helix (Figures 2B and 2C). The PTB domain of Dab1 includes a short N-terminal α helix also seen in the corresponding regions of the Shc and Numb PTB domains.

**Peptide Conformation**

The Dab1 PTB domain binds the ApoER2 peptide in a hydrophobic groove formed by the β strand and the C-terminal helix in a mode like that seen in the Shc-, IRS-1-, X11-, and Numb-peptide complexes [20, 22, 24, 26]. In the Dab1-β peptide complex, the four N-terminal residues of the peptide (positions –7 to –10; Figure 3A) are disordered, explaining why a 10-mer lacking these residues has comparable affinity for the Dab1 PTB domain. Asn –6 to Asp –4 of the peptide adopt an extended conformation, forming backbone hydrogen bonds with residues in the β strand to add another strand to the C-terminal β sheet of the PTB domain; the adjacent NPxY motif forms a type I β turn (Figures 3A and 3B). These global conformational features of the bound peptide are also seen in most other structures of peptides bound to PTB domains.

The Dab1 PTB domain contacts the side chains of the bound peptide at a number of sites (Figures 3A and 3B). Phe –5 of the peptide packs tightly into a hydrophobic groove created by Ile151, Leu154, the α helix also seen in the corresponding forms a saltbridge with the guanidino group of Arg56. The Dab1 PTB domain binds the ApoER2 peptide in a hydrophobic groove formed by the β strand and the C-terminal helix, explaining the preference for Phe or Tyr at this position [15]. The carboxylate of the adjacent Asp at 6 to Asp forms an extra hydrogen bond between the hydrogen atoms of its NH2 group and main, like the PTB domains of Shc, X11, and Numb [20, 24, 25], contains an insertion between strands 5 strand to add another strand as well as a long α helix, explaining the preference for Phe or Tyr at this position [15]. The carboxylate of the adjacent Asp at 6 to Asp forms an extra hydrogen bond between the hydrogen atoms of its NH2 group and α helix also seen in the corresponding regions of the Shc and Numb PTB domains.

**Table 1. Crystallographic Data Collection and Refinement**

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<th>Peak</th>
<th>Edge</th>
<th>PI-4,5P2 Soaked</th>
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<tr>
<td><strong>Number of atoms (main chain/side chain/all chains, residues)</strong></td>
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<td>41/48/89, 5–14</td>
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**Values in parentheses are for reflections in the highest resolution bin.**

| **Figure of merit** | 0.779 / 0.852 |
| **Rmerge (%)** | 5.2 (23.5) | 4.2 (13.6) | 4.9 (9.0) | 4.1 (9.4) | 4.7 (17.0) |
| **Completeness (%)** | 99.5 (98.9) | 94.1 (68.9) | 96.9 (81.1) | 96.9 (80.9) | 98.0 (97.5) |
| **Unique reflections** | 24,472 | 11,884 |
| **Number of reflections** | 24,472 | 11,884 |
| **Rwork/Rfree (%)** | 0.222/0.245 | 0.204/0.253 |
| **Number of atoms (main chain/side chain/all chains, residues)** | 609/592/1201, 23–174 | 609/592/1201, 23–174 |
| **Water** | 28.76 | 36.96 |
| **Peptide** | 41/48/89, 5–14 | 41/48/89, 5–14 |
| **Phosphate** | 5 | 48 |
| **Average B value (Å²)** | 1.12 | 1.08 |
| **RMS bond angles (°)** | 1201, 23–174 | 1201, 23–174 |

**Figure of merit = [ΣP(α)(1−P(α))] ΣP(α), where P(α) is the phase probability distribution and α is the phase.**

**Rmerge and Rwork = Σ|Fo| − |Fc| Σ|Fo| for the working set and test set (10%) of reflections.**
Figure 3. Dab1 PTB Domain-Peptide Contacts
(A) ApoER2 peptide sequence with schematic indicating contacts between the peptide and the Dab1 PTB domain. Hydrogen bonds, dotted cyan lines; hydrophobic contacts, green.
(B) Stereo representation of contacts. For the peptide, side chains are yellow, and residue labels are black. For the PTB domain, side chains are cyan, and residue labels are purple. A transparent surface of the PTB domain is shown in white, and the PTB domain backbone is illustrated with ribbons. Hydrogen bonds, dashed blue lines.

Asn 6 to Tyr 0 of the central NPxY sequence is nearly identical to that of the corresponding region of the APP peptide bound to the X11 PTB domain [20]. The APP peptide in its conformation from the X11 cocystal structure readily superimposes onto the ApoER2 peptide bound to Dab1 (Figure 4A, top panel). This superposition shows that the PTB domain of Dab1 can accommodate the APP peptide with Tyr 0 in a similar position and without steric clashes elsewhere, consistent with the known ability of an overlapping APP peptide to bind to the PTB domain of Dab1 with low micromolar affinity [15]. A distinct feature of the Dab1 PTB domain is its strong preference for unphosphorylated Tyr at the 0 position. Instead features a much shorter helix that connects strands β4 and β5 and β6 with β7 (Figures 3B and 4A), which closely approach the tyrosine side chain and should sterically exclude the bulkier pTyr. This discrimination highlights a key difference between Dab1 and X11 because X11 does not readily distinguish between Tyr and pTyr [20]. Selection against pTyr by Dab1 is established by a cleft created by the short loops connecting β4 with β5 and β6 with β7 (Figures 3B and 4A), which closely approach the tyrosine side chain and should sterically exclude the bulkier pTyr. This cleft is capped at one end by the loop connecting β1 with the central helix. The tight turn connecting strands β6 and β7 of the mDab1 PTB domain packs against this cap. This arrangement results in an H bond between the tyrosine hydroxyl and the backbone carbonyl of Gly131 (Figures 3 and 4A, bottom panel) and also places the His136 imidazole ring within van der Waals contact of the tyrosine hydroxyl. The presence of this H bond also explains the observed strong preference for Tyr over Phe at the 0 position [15]. In contrast, the corresponding loop connecting β6 with β7 is different in other PTB domains of known structure (Figure 2C). In particular, the same loop in X11 is much longer and disordered in the crystal, and the β6 strand in X11 does not extend as far toward the Tyr of the peptide. The mobility of these loops may enable X11 to bind both Tyr and pTyr sequences, while the limited flexibility of the short loops combined with the closer approach of the β6 strand in Dab1 only accommodates Tyr (Figures 3B and 4A). Whereas the long α2 helix of Dab1 packs against the β1, β6, and β7 strands, holding the β6-β7 loop in place and constraining the Tyr 0 binding pocket, IRS-1 lacks β1 and the long α2 helix and instead features a much shorter helix that connects strands β1 and β2, but does not approach the Tyr 0 binding pocket, allowing adequate space for the phosphate group (Figure 4B, top panel). Finally, the β6-β7 loop of IRS-1 also contains a bulge imposed by a GPG sequence (residues 232–234) at its C-terminal end [26]. The two glycine residues of the GPG bulge make backbone H bonds to residues on either side of Arg227, and the proline buttresses the aliphatic part of the Arg 227 side chain. These interactions situate Arg227 in position to form a salt bridge with the pTyr phosphate group and, together with Arg212, create the positively charged pocket dictating the preference for pTyr in IRS-1 (Figure 4B).

Of note, a homology model of a complex between the ICAP-1α PTB domain and a β1 integrin peptide also predicts that this PTB domain should prefer to bind peptides with unphosphorylated Tyr within the NPxY motif. In that model, which is of high structural quality...
Figure 4. Comparison of the Dab1 PTB Domain-ApoER2 Complex with Other PTB Complexes

(A) Top panel, superposition of the APP peptide onto the Dab1 PTB domain-ApoER2 peptide complex. The molecular surface representation of the Dab1 PTB domain is colored by electrostatic potential (−15 kT/e [red] to +15 kT/e [blue]). The ApoER2 (gray) and APP (green) peptides are rendered in stick form, and the lysine side chain of the ApoER2 peptide has been removed for clarity. Bottom panel, close-up view of the boxed region highlighting the residues interacting with the Tyr 0 residue of the ApoER2 peptide. The protein backbone is shown as a ribbon, while selected peptide (yellow) and protein (cyan) side chains are in stick form. Hydrogen bonds, dashed blue lines.

(B) The IRS-1 PTB domain-pTyr peptide complex. Top panel, the molecular surface representation of the IRS-1 PTB domain [26] is colored by electrostatic potential (as in [A]) to illustrate the basic pocket responsible for phosphotyrosine binding. The bound insulin receptor peptide is rendered in stick form. Bottom panel, close-up view of the boxed region to highlight the residues interacting with the pTyr of the insulin receptor peptide. Colored as in (A), except that the protein side chains are green.

and has a backbone rmsd of 1.05 Å to our X-ray structure for all alignable residues (460 atoms), the ICAP-1α PTB domain also has a short loop connecting β4 with β5, and the β6-β7 loop is predicted to project an Ile side chain toward the Tyr of the peptide, thereby discriminating between Tyr and pTyr [27].

A Mechanism for Membrane Recruitment

The selective binding of PI-4,5P₂ by both Dab1 and Dab2 may direct recruitment of these proteins to the membrane [15, 19]. PI-4,5P₂ binding may control mechanisms of transport of Dab family proteins within cells and/or their localization within specific membrane compartments, like lipid rafts. In addition, in ways not yet understood, PI-4,5P₂ binding may also participate in integrating the binding of the ApoER2 cytoplasmic tail with other events in signaling, for example, by facilitating kinase-catalyzed tyrosine phosphorylation of Dab1 or by coupling of the receptor-Dab1 complex to downstream signaling pathways [28, 29].

How does Dab1 bind PI-4,5P₂? The structure of the PTB domain-peptide complex reveals a distinct region of positive electrostatic surface potential on the surface of the PTB domain opposite the peptide binding groove. This patch results from a coalescence of basic side chains derived from the residues in the loops connecting adjacent secondary structural elements. To test whether this basic patch encompasses the PI-4,5P₂ binding site and concurrently determine how PI-4,5P₂ binds to the PTB domain, we soaked PTB-peptide cocrystals in mother liquor containing PI-4,5P₂.

Crystals soaked with PI-4,5P₂ show strong density for the phosphates in the 4 and 5 positions, with additional contiguous density from the inositol ring and 1-phosphate groups. The observed density is best modeled by the binding of PI-4,5P₂ in either of two orientations (see Experimental Procedures), which are related to each other by a 180° rotation around an axis bisecting the 4- and 5-phosphate groups and perpendicular to the C4-C5 bond of the inositol ring (Figure 5). The primary difference between these two orientations is defined by the position of the 1-phosphate group because PI-4,5P₂ is pseudosymmetric around the rotation axis. The preferred conformation (by a 70:30 ratio, as estimated by occupancy refinement; see Experimental Procedures) situates the 1-phosphate close to Arg76, and we therefore discuss interactions of the PI-4,5P₂ with the PTB domain using that orientation.

In this model, the phosphates of PI-4,5P₂ interact with six of the residues that make up the conserved basic
Figure 5. Electron Density of PI-4,5P2 Bound to the Dab1 PTB Domain

Bound PI-4,5P2 was modeled in two orientations related by a 180° axis of rotation. PI-4,5P2 in the preferred orientation, based on occupancy refinement (see Experimental Procedures), CPK colors; the orientation with the lower occupancy, orange. Orange mesh, Fo - Fc map after refinement of a model with PI-4,5P2 in the less occupied orientation, contoured at 2.5σ. Note the large regions of residual electron density overlapping the positions of the 1-phosphate and the 2-hydroxyl in the preferred orientation. Green mesh, Fo - Fc map after refinement of a model with PI-4,5P2 in the preferred orientation, contoured at 1σ. Note the residual region of electron density overlapping the position of the 1-phosphate in the other orientation. Blue mesh, 2Fo - Fc map, contoured at 1σ, of a composite model including refined coordinates of both PI-4,5P2 orientations weighted by occupancy. Protein side chains interacting with bound PI-4,5P2 are indicated.

region on the Dab1 PTB domain surface (Figures 2C and 5). Two 4-phosphate oxygens are coordinated by the guanidino group of Arg124 from the N-terminal end of β6 and the ε-amino group of Lys142 from the C-terminal end of β7. His81 coordinates one of the 5-phosphate oxygen atoms, and the ε-amino groups of Lys45 and Lys82 are in position to coordinate terminal oxygen atoms from both the 4- and 5-phosphates. The side chain of Arg76 is also close enough to the 1-phosphate of the preferred orientation to form a salt bridge to one of the terminal oxygen atoms (Figure 5).

The residues that comprise the phosphoinositide binding site are conserved among a subset of PTB domain-containing proteins homologous to Dab1 (Figure 6). All of the residues that contact PI-4,5P2 are strictly conserved in the sequence of Dab2, which also binds PI-4,5P2 and otherwise harbors only ~60% identity to Dab1 in its PTB domain [30]. A Dab1-based homology

Figure 6. The PI-4,5P2 Binding Site Is Shared by Dab1, Dab2, and PLCδ, but Not by X11 and Shc

(A) The Dab1 PTB domain, oriented with the peptide binding groove at the base and the N-terminal residues of the bound peptide projecting toward the viewer. The PI-4,5P2 binding site is on the top face, opposite the peptide binding groove. Dab1-based homology models of the Dab2 (B) and the ARH (C) PTB domains show that the location of the positively charged PI-4,5P2 binding site is conserved (arrows).

(D) The PH domain from PLCδ bound to PI-4,5P2 [33]. The basic residues that form the PI-4,5P2 binding site in PLCδ structurally align with the amino acids that create the PI-4,5P2 binding site in Dab1. The X11 (E) and Shc (F) PTB domains lack the basic residues that make up the PI-4,5P2 binding site. All molecular surface representations were generated by the program GRASP [46] and shaded by electrostatic potential (~15 kT/e [red] to +15 kT/e [blue]).
model of the Dab2 PTB domain also predicts the presence of a comparable basic region at the same location (Figure 6B), further suggesting that the conservation of the basic patch is important for the function of both proteins. In addition, these basic residues are also conserved in the ARH protein (Figure 2C), another PTB domain-containing protein that shares the ability to bind both the cytoplasmic tails of LDL receptor family members and PI-4,5P$_2$ [31, 32]. A homology model of ARH based on the Dab1 structure shows that these residues would similarly form a basic PI binding surface (Figure 6C), suggesting that membrane recruitment may be a general feature of interactions between LDL receptor family members and their adaptor proteins. Finally, the PI-4,5P$_2$ binding site of a representative PH domain, the one from phospholipase C$_6$, [33] consists of basic residues in loops connecting secondary structural elements, which also align structurally with the residues used by Dab1 (Figures 2C and 6D), suggesting a potential evolutionary relationship between Dab1 and the PI binding PH domains.

The basic patch is a distinguishing feature of the Dab1-like subset of PTB domains because the corresponding residues are not conserved in other PTB domains of known structure, like those of the X11 and Shc proteins (Figures 6E and 6F). In such proteins, there may either be no need for membrane recruitment (although the Shc PTB domain may bind weakly to PI-4P and PI-4,5P$_2$ at a site that appears to compete with peptide binding using residues distinct from the ones identified here [24, 34, 35]) or this function may be fulfilled by another domain of the protein. For example, in IRS-1, an adjacent PH domain binds to PIs to bring IRS-1 to the membrane [36].

The PTB domain of Dab1 combines the peptide binding features of typical PTB domains with the PI binding properties characteristic of most PH domains (Figure 7). This combination of membrane recruitment and peptide binding by a single domain suggests that these activities are functionally coupled. Membrane recruitment of the Dab1 PTB domain by the basic region may help to orient the ligand binding groove with respect to the ApoER2 peptide, which lies only ~10 residues from the trans-membrane sequence. Selectivity of the PTB domain for PI-4,5P$_2$ may serve to concentrate the Dab1 signaling complex in membrane patches that are highly enriched in this phospholipid, thus facilitating coupling to, and activation of, PI-3 Kinase, a component of a downstream branch of the Reelin signaling pathway [28].

**Biological Implications**

Control of neuronal migration during development depends on a signaling pathway that includes the secreted protein Reelin, the ApoER2 and VLDLR cell surface receptors, and the adaptor protein Dab1. Dab1 binds to an NPxY-containing motif in the cytoplasmic tails of ApoER2 and VLDLR via its N-terminal PTB domain. Both Dab-1 and the related protein Dab-2 also bind PI-4,5P$_2$, suggesting that the activity of Dab-1 may also depend on or be regulated by recruitment to PI-enriched membrane compartments.

![Figure 7. A Model for the Arrangement of the Dab1-ApoER2 Complex at the Membrane Surface](image)

To understand the basis for selective recognition of peptides with an unphosphorylated tyrosine at the 0 position and to identify potential sites for PI binding, we determined the structure of the Dab1 PTB domain in complex with a 14-residue peptide from the ApoER2 cytoplasmic tail. The structure of the Dab1 PTB domain-peptide complex explains why the Dab1 PTB domain strongly prefers peptide ligands in which the Tyr of the NPxY motif is not phosphorylated. Although it is not known whether the NPxY motif of either lipprotein receptor tail undergoes phosphorylation, the preference for unphosphorylated tyrosine shows that it would be possible to regulate downstream signaling by modulating the tyrosine phosphorylation state of the receptor tails.

In the ternary complex of the Dab1 PTB domain, peptide, and PI-4,5P$_2$, the PI-4,5P$_2$ binds to the large basic patch on the face of Dab1 opposite the peptide binding groove. These studies provide the first structural characterization of a hybrid PTB domain to which both peptide and PI-4,5P$_2$ have been simultaneously bound and provide support for models in which Dab1 function is enabled or regulated by PI binding. Control of membrane localization by PI binding may regulate Dab1 activity by facilitating downstream events that accompany peptide binding, such as tyrosine phosphorylation by mem-
brane-associated kinases. In addition, the ability of Dab1 to bind to PI 
may itself be diminished when the tyrosine residues adjacent to the PTB 
domain are phosphorylated upon activation of a Reelin signal [7, 37, 38]. Insights 
from our structures of Dab1 PTB-ApoER2 peptide complexes should open up 
new several lines of investigation to clarify the role of PI binding by Dab1 in living 
cells.

Experimental Procedures

Cloning, Protein Expression, and Purification 
of the Dab1 PTB Domain

The sequence-encoding the PTB domain of Dab1 (residues 20-175) 
was amplified by PCR from a full-length cDNA clone (kindly provided 
by Brian Howell). The PCR product was introduced into the pDEST51 
vector downstream of glutathione S-transferase (GST) without 
the use of restriction sites by recombinatorial Gateway cloning (Invitrogen); 
a tobacco etch virus (TEV) protease cleavage site was introduced 
between GST and the Dab1 PTB domain during cloning. 
The L87M mutation, which enabled incorporation of seleno-
methionine into the protein for structure determination by MAD, was 
carried out by PCR mutagenesis with a QuickChange site-directed 
mutagenesis kit (Stratagene). Native and selenomethionine-labeled 
fusion proteins were expressed in E. coli BL21(DE3) and in E. coli 
B834(DE3) seleno-methionine-autotrophic cells, respectively, by induction 
with IPTG (0.4 mM). For purification of native and selenomethionine-
labeled proteins, cells were lysed either by sonication or with 
French press in 20 mM NaH2PO4, buffer (pH 6.8) containing 150 mM 
NaCl, 20% sucrose, 1 mM EDTA, 5-10 mM DTT, 200 μM PMSF, 1 
μg/ml aprotinin, and 0.5 μg/ml leupeptin (buffer A). After each GST-
Dab1 fusion protein was captured on glutathione-Sepharose beads 
(Pharmacia), the PTB domain was released from bound GST by 
cleavage with His6-tagged TEV protease. After removal of TEV prote-
ase from solution with Ni-NTA agarose (Qiagen), the native and 
selenomethionine-labeled proteins were then purified to apparent 
homogeneity by anion exchange followed by gel filtration chroma-
tography. For crystalllography, purified proteins were concentrated 
and dialyzed into 50 mM Tris buffer (pH 6.8) containing 50 mM NaCl 
and 5 mM DTT. For binding studies and biophysical measurements, 
the protein was dialyzed exhaustively against a solution of 10 mM 
NaH2PO4, buffer (pH 6.8) containing 150 mM NaCl and 5 mM DTT.

GST Pull-Down

The cytoplasmic tails of ApoER2 (ApoER2-C) and VLDLR (VLDLR-C) 
were expressed as GST fusion proteins with a modified version of 
the pGEX-4T-1 vector (Pharmacia) that harbors a TEV protease 
site before the polylinker. The GST-fusion proteins, as well as GST alone, 
were expressed in E. coli BL21(DE3) cells by induction with IPTG. 
Cell were lysed by sonication in buffer A. The lysates were gently 
agitated together with glutathione-Sepharose beads (Pharmacia) to 
permit binding of the GST fusion proteins. After three washes with 
10 mM NaH2PO4, (pH 7.5), 150 mM NaCl, 5 mM DTT, and 0.5% NP-
40 (buffer B), glutathione-Sepharose beads loaded either with GST-
ApoER2-C, GST-VLDLR-C, or GST alone (10 μl) were gently rocked 
in the presence of the purified Dab1 PTB domain (10 μM) in buffer 
B (1.0 ml) for 2 hr at 4°C. The beads were recovered by centrifugation, 
washed with buffer B, and resuspended in 50 μl of SDS-PAGE loading buffer. 
After boiling for 10 min, bound proteins were separated by SDS-PAGE 
with a 15% gel and were visualized by staining with 0.1% Coomassie brilliant blue.

Binding Measurements

The affinities of the purified Dab1 PTB domain for 10- 
and 14-residue Apo-ER2 tail peptides were measured by isothermal titration calo-
rimetry (ITC) and/or by fluorescence polarization. For ITC measure-
ments, a 14-residue synthetic peptide from the ApoER2 cytoplasmic 
tail (Research Genetics; acetyl-TKSMFNPDVPYRK-amide) was pu-
ried by reversed-phase HPLC for titration into a solution of the 
Dab1 PTB domain. A stock solution of the tail peptide (170 μl) was added 
in 7.5 μl increments to a 20 μM solution of Dab1 at 25°C in 
50 mM Tris (pH 6.8) containing 150 mM NaCl and 0.2 mM DTT. To 
calculate a dissociation constant, we plotted data and performed 
curve fitting with the program Origin 5.0. For fluorescence polariza-
tion measurements, a fluorescent derivative of the 14-residue 
ApoER2 peptide was prepared by conjugation of 5-iodoacetami-
dofluorescein to acetyl-CGGTKSMFNPDVPYRK-amide (the 14-
residue tail peptide extended with a CYY N-terminal linker) through 
a thioether linkage. After HPLC purification, the peptide was dis-
solved in 20 mM NaH2PO4, (pH 6.8), 150 mM NaCl, and 0.2 mM DTT, 
and its concentration was determined by measuring the absorbance 
of fluorescein at 492 nm. Measurements of fluorescence anisotropy 
were carried out on an SLM/AMINCO AB2 luminescence spectro-
photometer fitted with polarizers (Thermospectronic) at 29°C in 20 
mM NaH2PO4 buffer (pH 6.8) containing 150 mM NaCl and 0.2 mM 
DTT. Aliquots of the Dab1 PTB domain (up to a final concentration 
of 5 μM) were titrated into a solution of 50 mM fluorescent peptide, 
and the polarization value was measured. The binding curve was 
fitted to a single site model to determine the dissociation constant. For competition measurements of fluorescence polarization with the 
unconjugated 14-residue peptide and the shorter 10-residue tail 
peptide (acetyl-NFDNPYRK-amide), aliquots of unconjugated peptide were added to a solution of 50 mM fluorescent peptide in 
the presence of 500 nM Dab1 PTB domain.

Protein Crystallization

The purified PTB domain of mDab1 was concentrated to 15-25 mg/ 
ml in 50 mM Tris buffer (pH 6.8) containing 50 mM NaCl and 5 mM 
DTT. Complexes of the PTB domain with the ApoER2 14-residue 
peptide were then prepared by mixing the peptide in a 1:1–1:1.5 
molar ratio with protein. Initial crystals grew from 2 μl hanging drops 
(1 μl well solution mixed with 1 μl concentrated protein solution) 
for 5 weeks at room temperature in 0.1 M HEPES (pH 7.5), 20% 
PEG4000, and 10% isopropanol. Crystals were obtained in 2-3 days 
by mixing 2 μl of protein/peptide solution with 2 μl of 0.1 M HEPES 
(pH 7.5), 34%–36% PEG8000, and 5% ethanol over a well solution 
of 0.1 M HEPES (pH 7.5) and 34%–36% PEG8000 and by macroseeding 
24 hr after the drops were set. Selenomethionine-derivatized 
crystals grew in 7–10 days under the same conditions. The crystals 
belong to the space group P212121 with cell dimensions of a = 
36.25 Å, b = 45.72 Å, and c = 90.12 Å.

Data Collection and Structure Determination

All diffraction data were collected at 100 K after slow transfer of the 
crystals into mother liquor to which 5% PEG4000 had been added for 
cryoprotection. A native data set to 1.5 Å was collected at the 
Cornell High Energy Synchrotron Source (Cornell University) with 
the F1 beamline with an ADSC Quantum4 CCD detector. A second 
data set was also collected to 1.8 Å with a selenomethionine 
crystal from the L87M protein with a rotating anode source (Rigaku 
RU-200EH) with a mar300 Image Plate Detector (mar research). Oscilla-
tion images were indexed and integrated with DENOZO [39], and 
the intensities were scaled and merged with the program SCALEPACK 
[38]. The same L87M crystal was used to collect diffraction data 
to 2.0 Å at three wavelengths for MAD phasing at the National 
Synchrotron Light Source (Brookhaven National Laboratory) with 
the X12C beamline with a B4 CCD detector. MAD data were pro-
sessed with the HKL2000 program suite [39]. The scaled intensity 
data from the three MAD wavelengths were input into the program 
SOLVE [40] to locate heavy atom sites. Two selenium sites were 
located, and the phases generated by SOLVE were refined with 
RESOLVE [40] and the program DM in the CCP4 program suite [41]. 
With the 1.5 Å native data set and the program ARP/wARP [42], 
the phases were further improved, and an atomic model (~90% 
complete) was built in an automatic fashion. The model was com-
pleted and manually refined with the program O [43], and simulated 
annealing, energy minimization, and B factor refinement were 
performed with CNS [44]. Further refinement included the addition 
of water molecules as well as a phosphate ion to the model and 
then further cycles of CNS refinement.

PI-45P, Soaking and Ternary Complex 
Structure Determination

Native crystals of the Dab1 PTB domain-ApoER2 peptide complex 
were soaked for 14 days in 50 μl of mother liquor containing 0.5 

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mM PI-4,5P2 (Sigma). A 1.9 Å data set was collected on one of these crystals with a rotating anode source (Rigaku RU-200EBH) with a mar300 Image Plate Detector (mar research). Data were processed with DENZO and SCALEPACK as above. The amplitudes from this data set and the phases from the Dab1 PTB domain-peptide complex structure were used to generate an electron density map of the ternary complex. The map showed strong density for the phosphates in the 4 and 5 positions. The density for the inositol ring and the 1-phosphate group indicated that the PI-4,5P2 adopts either of two possible orientations in the soaked crystals (see text). Occupancy refinement of these two alternate conformations assigned an occupancy of 0.7 to the preferred orientation, in which the 1-phosphate approaches within H bonding distance of the Arg76 guanidino group, and an occupancy of 0.3 to the other orientation. Simulated annealing, energy minimization, and B factor refinement were performed with CNS for both of the models. The two sets of refined PI-4,5P2 coordinates were then combined into a final model with weighted occupancy, from which the 2Fo − Fc map of Figure 5 was generated.

**Homology Modeling and Molecular Graphics**

The models of the PTB domain from murine Disabled-2 (Dab2) and of human ARH1 were prepared with the program Modeller [45]. Figures were prepared with the programs GRASP [46], MOLSCRIPT [47], and PyMol (DeLano Scientific; http://www.pymol.org).

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**References**

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Accession Numbers

Coordinates for the PTB domain-ApoER2 complex have been deposited in the Protein Data Bank under accession number 1NTV; coordinates for the complex with bound PI-4,5P2 have been deposited in the Protein Data Bank under accession number 1NU2.