Solution Structure and Peptide Binding of the PTB Domain from the AIDA1 Postsynaptic Signaling Scaffolding Protein

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Abstract

AIDA1 links persistent chemical signaling events occurring at the neuronal synapse with global changes in gene expression. Consistent with its role as a scaffolding protein, AIDA1 is composed of several protein-protein interaction domains. Here we report the NMR structure of the carboxy terminally located phosphotyrosine binding domain (PTB) that is common to all AIDA1 splice variants. A comprehensive survey of peptides identified a consensus sequence around an NxxY motif that is shared by a number of related neuronal signaling proteins. Using peptide arrays and fluorescence based assays, we determined that the AIDA1 PTB domain binds amyloid protein precursor (APP) in a similar manner to the X11/Mint PTB domain, albeit at reduced affinity (~10 µM) that may allow AIDA1 to effectively sample APP, as well as other protein partners in a variety of cellular contexts.

Methods

Cloning, Expression and Protein Purification

A gene fragment encoding the PTB domain (aa. 1043–1195) of human AIDA1b was PGR amplified with NdeI and EcoRI restriction sites and was subsequently inserted into pET28a (Novagen). The expressed protein contained an amino terminal 6xHis tag and intervening thrombin site. Other PTB domain fragments that lacked either the N-terminal 6xHis tag or the entire affinity tag along with 16 additional unstructured residues were also as insoluble as the fragment chosen for this study. To align the
PTB domain described in this study with numerous AIDA1 isoforms. S1 in the PTB domain structure corresponds to S1045 in AIDA1b, the longest isoform. A one-liter fermentation in a minimal medium containing 1 g of 15NH4Cl and 4 g of 13C-glucose was sufficient to produce 5–10 mg of purified protein. Purification was achieved by Nickel-NTA affinity chromatography (Qiagen) and gel filtration chromatography on a S-100 HR 16/60 size exclusion column (GE Biosciences). Final buffer conditions were 20 mM Na-phosphate, pH 7.8, 0.15 M NaCl, 0.05% (w/v) NaN₃. Five single aromatic-alanine substitutions (Y6A, F16A, F24A, Y70A and Y131A) were produced from pET28-AIDA1-PTB using a service provided by Genscript (Piscataway, NJ). A 6xHis tagged PTB domain variant containing all five substitutions (PTB5M) was produced by DNA2.0 (Menlo Park, CA) by direct gene synthesis in the expression vector, pJExpress401 (T5 promoter plus kanamycin resistance). A 6xHis-tagged, APP-peptide (GYENPTYKFFE) fused to the amino terminus of the AIDA1 PTB5M mutant with an intervening thrombin site was also synthesized by DNA2.0 in pJExpress401.

Figure 1. (a) Sequence alignment of the AIDA1 PTB domain against the APP binding proteins, Dab1 [25], X11 [17] and Fe65 [20]. Five aromatic amino acids selected for alanine substitution in AIDA1 PTB domain are boxed. (b) Backbone atom superposition of top15 structures according to lowest refinement energy. (c) Strip plots of a 13C-edited NOESY spectrum at the Cβ chemical shift of each alanine substituted in the PTB5M mutant. A asterisk denotes a resonance not associated with that strip. (d) A ribbon representation of the PTB5M model highlighting the positions of the alanine substitutions. Y6A is not shown in the figure as the first 14 amino acids are unstructured and were excluded from the structure calculation.

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was determined by heating samples from 20–90°C at a concentration of 0.15 mM thereby permitting experiments to be performed but to the extent of a structure determination. Each aromatic-alanine substitution mutant was concentrated to 0.15 mM, assessed by NMR and then concentrated until increased resonance line broadening was observed or there was apparent turbidity.

**CD Spectroscopy**
Far UV circular dichroism (CD) spectra were acquired with a Jasco J-810 instrument at a protein concentration of 50 μM using a rectangular cell with a 0.1 cm path length. Spectra were recorded from 200–200 nm with a scan rate of 50 nm/min and a 1.0 nm bandwidth. A midpoint denaturation temperature (T_m) was determined by heating samples from 20–90°C at 2°C/min and monitoring ellipticity at 222 nm.

**Protein Binding Studies**
Fluorescein isothiocyanate (FITC) labeled peptides spanning portions of APP were produced and purified by CanPeptide (Montreal, QC) for fluorescence anisotropy based binding studies at 25°C using an Agilent Eclipse spectrophotometer equipped with a manual polarizer accessory. Buffer conditions were similar to those used for NMR spectroscopy. Measurements were made under identical conditions and averaged. Anisotropy was calculated from the relationship I_{parallel}/I_{perpendicular} = (I_{parallel}/2I_{perpendicular}) and normalized with the blank experiment. The equilibrium dissociation constant (K_D) was calculated by direct fitting the titration curves with a standard two-state relationship using proFit 6.2.

**Table 1.** Solubilities and thermal denaturation midpoints of the AIDA1 PTB domain and alanine substitution mutants.

| PTB domain | Tm (℃) | Solubility (mM) | Side chain exposure |
|------------|--------|----------------|--------------------|
| wild type  | 62     | 0.10           | N/A                |
| Y6A        | 65     | 0.10           | exposed            |
| F16A       | 64     | 0.20           | exposed            |
| F24A       | 64     | 0.45           | partially exposed  |
| Y70A       | 64     | 0.45           | exposed            |
| Y131A      | 64     | 0.15           | exposed            |
| SM         | 64     | 0.80           | N/A                |
| APP-PTB    | 72     | 0.20           | N/A                |
| APP-PTB5M  | 73     | 0.80           | N/A                |

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**Protein Solubility Assessment**
Since the objective of the aromatic-alanine substitutions was to improve solubility for a structure determination, 13N-HSQC spectra were used qualitatively. From experience, the wild type AIDA1 PTB domain was soluble for a least one day at room temperature at a concentration of 0.15 mM thereby permitting experiments to be performed but to the extent of a structure determination. Each aromatic-alanine substitution mutant was concentrated to 0.15 mM, assessed by NMR and then concentrated until increased resonance line broadening was observed or there was apparent turbidity.

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**Peptide Array**
A set of 12-mer peptides on a 150×100 mm cellulose membrane in a 10×30 array were synthesized using the SPOTS method [9] with an Intavis MultiPep instrument. A crude estimate of the peptide content in each spot was made by staining the array with Fast Green FCF. The array was probed with 1 μM of the solubility enhanced 6xHis-PTB5M mutant in PBST (3.2 mM sodium phosphate, 0.5 mM potassium phosphate, 1.3 mM KCl, 135 mM NaCl, 0.1% Tween-20, pH 7.4). Following blocking and incubating the array in a 1:5000 dilution of horseradish peroxidase (HRP)-conjugated 6xHis monoclonal antibodies in PBST and developing with a chemiluminescent reagent (Santa Cruz Biotechnology). A complete table of peptides is provided in Supplementary Material as Table S1.
NMR Spectroscopy

15N-edited HSQC spectra of the wild type PTB domain, mutants and protein-peptide complexes were acquired at 30 °C on a Varian 600 MHz NMR spectrometer equipped with a salt tolerant cold probe. The use of a low protein concentration (0.10–0.15 mM) permitted assessment of all protein fragments regardless of intrinsic solubility. Chemical shift assignments on a uniformly 15N, 13C labeled sample of PTB5M at 0.8 mM were obtained using a conventional heteronuclear, triple-resonance strategy that incorporated non-uniform sampling for improved resolution and sensitivity. Backbone directed experiments: HNCACB, CBCA(CO)NH, HNCO, HNCACO, side chain directed experiments: H(C)(CO)NH, C(CO)NH, and 13C/15N-edited NOESY spectra were acquired on a Bruker Avance 900 MHz spectrometer equipped with a cold probe. Side chain HCCH-TOCSY, and aromatic HB(CBCG)CD, HB(CBCGCD)CE were acquired at 600 MHz. Protein solutions contained 10% D2O with the exception of the 13C-edited NOESY dataset in which the PTB5M sample was buffer exchanged into 95% D2O before data acquisition. Datasets were processed with NMRPipe [10] or the Rowland Toolkit [11] as required and interpreted with CCpNMR Analysis 2 [12]. Chemical shift assignments of PTB5M were deposited in the BMRB with the accession code 17934.

Structure Determination

From an initial set of 500 structures calculated with CYANA 3, the top 20 structures were selected with no NOE violations >0.3 Å and no torsion angle violations <5°. This ensemble was then subjected to additional refinement in explicit solvent with a Python script (wrefine.py) supplied with XPLOR-NIH 2.30. The top 15 structures according to lowest refinement energy was deposited as an ensemble in the Protein Data Bank with the accession code 2M38. The ensemble was aligned using MOL-MOL 2K1 [13].

Structure Comparisons

Cα RMSDs and alignments between the AIDA1 PTB domain and related proteins were performed with PDBDeFold [14].

Peptide Docking Simulations

Starting from the AIDA1 PTB5M structure and APP peptide ligand placed in analogous position to that observed in the X11 PTB domain crystal structure [7], a two-stage docking simulation, at low resolution (200 structures) and then all-atoms high resolution (100 structures) was performed with FlexPepDock, part of the Rosetta 3.4 software package [15]. A low energy structure was selected for analysis.

Results

Prior to the structure determination, a molecular model of the AIDA-1 PTB domain was made with HOMA [16] using the crystal structure of the X11 PTB domain as the template [17]. Final refinement was performed with FOLDX [18]. The surface of the PTB model was scanned for exposed aromatics and compared to a sequence alignment consisting of the PTB domains from X11, Numb [19] and Fe65 [20]. Of the sixteen aromatics in the AIDA1 PTB domain, Y6, F16, F24, Y70, and Y131 were selected as candidates that were most likely to be surface-exposed (Figure 1a). While aliphatic amino acids could have been targeted as well, this decision would have added an additional layer of complexity. Thus, by selecting aromatic amino acids (Phe/Tyr/Trp considered equally), we were effectively sampling mutations under sparse conditions that still cover a wide range of surfaces.

As shown in Table 1, the calculated Tm of the mutants was comparable to the wild type PTB domain suggesting that the alanine substitutions did not destabilize the fold. Like the wild type PTB domain, the Y6A and Y131A single mutants could only be concentrated to 0.1 mM before precipitation was observed. The remaining single mutants – F16A, F24A and Y70A – could be concentrated up to 0.5 mM; however, HSQC spectra at these concentrations suffered from line broadening and missing resonances. In contrast, the PTB5M mutant was very soluble at 0.8 mM, with line widths that were comparable to the single mutants acquired at low concentration. Thus, we observed a

![Table 4. Affinities of APP-derived peptides for two solubility enhanced mutants of the AIDA1 PTB domain. ND: not done.](doi:10.1371/journal.pone.0065605.t004)
synergistic effect when multiple aromatic amino acids were
substituted with alanine.

The impact of the APP ligand on solubility was also
investigated. The APP ligand was added exogenously, as a 17-
mer peptide and endogenously, by appending the sequence to the
amino terminus of the wild type PTB domain. Tethering a peptide
ligand to a protein is a useful approach to shift binding kinetics
from biomolecular to unimolecular and ensure stoichiometric
binding. In either case, addition of the APP ligand enhanced
thermostability by 8°C but did not affect solubility. A structural
determination of the APP-bound AIDA1 PTB domain was not
pursued because there were fewer HN resonances a 15N-edited
HSQC spectrum of the bound PTB domain (Figure S1) versus the free
PTB domain (Figure 1b) suggesting that ligand and binding cleft were
severely line broadened beyond detection. While the HSQC spectra of the Y6A, F16A, F24A, Y70A and
Y131A PTB domains were all qualitatively similar in terms of
chemical shifts and line widths, the F24A mutant spectrum was
least similar to the other four mutant spectra under closer
inspection suggesting that A24 could be making more structural
contributions than the other alanine substitutions. Before the
structure was determined (an ensemble of structures is shown in
Figure 1b), we assessed the surface exposure of each aromatic-
alanine substitution by examining the NOEs observed from the
side chain methyl group. As shown in Figure 1b, only intramolecular and short range intermolecular NOEs were
observed at A6, A16 and A70, suggesting that these methyl
groups were significantly solvent-exposed. This was certainly the
case for A6 as the chemical shift assignments indicated that the
first 15 amino acids of the PTB domain were unstructured. Long-
range NOEs were observed between the methyl group of A24 in
b1 and the side chains of the adjacent b-strand (b7), specifically,
the aromatic ring of F125 and the side chain of T123. The portion
of the b-sheet in which substitution A24 resides was deemed to be
resistant to hydrogen exchange as an NOE was observed between
the methyl group of A24 and its own backbone amide despite the
protein being dissolved in D2O. Taken together, these observa-
tions suggested that A24 was the least surface exposed of the five
mutants chosen for the study. Once the structure determination
was completed (a cartoon representation is shown in Figure 1d),
these observations were confirmed and the F24A substitution
appeared to be accommodated well. A PTB domain variant
lacking the F24A substitution was not pursued because APP
binding activity was unaffected.
The structure of the AIDA1 PTB5M mutant was aided substantially from data acquired at high field. A statistical summary is provided in Table 2. Overall, and as somewhat anticipated, the structure compares favorably to the other PTB domains that bind APP (Table 3). The PTB domain family can be divided into three major classes, namely Shc-like, IRS1-like and Dab-like [21,22]. The AIDA1 PTB domain is a representative of the Dab-like class that binds non-phosphorylated-tyrosine peptides. While essentially complete chemical shift assignments were made, the α1-β2 loop spanning Q51-P62 remains unstructured and consequently dynamic due to a lack of long range NOEs observed throughout the region. The β6-β7 loop spanning K110-H116 also samples more conformations on average, supported by the observation that no resonance assignments could be attributed to N115.

Structural and biochemical investigations of the Fe65 PTB2 domain demonstrated >100-fold difference in affinity between an 11 aa. minimal sequence (K_D = 100 μM) and an amino terminally extended 32 aa. (K_D = 0.2 μM) [8,20]. One threonine (T668) in APP located in this extended region is susceptible to phosphorylation and acts as a switch that repartitions the cis and trans states of the adjacent proline (P669) that, in turn, affects the ability of Fe65 to engage its ligand. Titrations of long (APP32) and short (APP17) peptides showed no differences in binding affinity to the AIDA1 PTB domain suggesting that AIDA1, like many other PTB domains, binds an NPsY motif with a K_D of ~10 μM (Figure 2 and Table 4). As predicted from the NMR structure, a semi-solubilizing Y70A single variant or the fully-solubilizing PTB5M variant had no affect on the affinity of the AIDA1 PTB domain to APP. An APP peptide bearing a phosphorylated Y687 did not bind the AIDA1 PTB domain providing further evidence for its inclusion in the Dab-like family.

The K_D of the X11 PTB domain with a short APP peptide (14 aa., which is comparable to APP17 used in this study) is 0.3 μM, or over 100× stronger than the AIDA1 PTB domain [17]. From the perspective of the AIDA1 PTB domain, though, a lower affinity may not necessarily decrease its occupancy on APP relative to X11 and others, as the effective concentration of AIDA1 within the PSD is extremely high. Relative to X11 and others, as the effective concentration of APP located in this extended region is susceptible to phosphorylation and acts as a switch that repartitions the cis and trans states of the adjacent proline (P669) that, in turn, affects the ability of Fe65 to engage its ligand. Titrations of long (APP32) and short (APP17) peptides showed no differences in binding affinity to the AIDA1 PTB domain suggesting that AIDA1, like many other PTB domains, binds an NPsY motif with a K_D of ~10 μM (Figure 2 and Table 4). As predicted from the NMR structure, a semi-solubilizing Y70A single variant or the fully-solubilizing PTB5M variant had no affect on the affinity of the AIDA1 PTB domain to APP. An APP peptide bearing a phosphorylated Y687 did not bind the AIDA1 PTB domain providing further evidence for its inclusion in the Dab-like family.

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A 12-mer SPOT peptide array (Figures 4a and 4b) was used to survey the amino acid preference of the AIDA1 PTB domain for APP and APP-like peptides. From an initial window scan of the APP carboxy terminal cytosolic region (Figure 4c), a minimal binding sequence of YENPTYKFFE was observed that is consistent with previously described peptide titrations and docking simulations. The minimal binding sequence was then used to exhaustively survey each position in the form of an ‘alphabet array’ (exhaustive amino acid substitutions at each position in the peptide). The results, summarized in Figure 4d, present a consensus sequence of XNyX0YxΨF, where Φ is a hydrophobic amino acid and Ψ is an aromatic amino acid. Since the requirement for proline in the NPsY motif is not absolute, AIDA1 has the potential to sample NxxY motifs in receptors such as Ret that guides the development of neurons in the enteric nervous system [23]. If this is the case, a lower K_D, and consequently a higher off-rate, would permit more ‘handshaking’ or sampling of potential protein partners to occur.

Discussion

Our initial attempts at biochemical and structural studies of the AIDA1 PTB domain were precluded by poor solubility. As a result, we made five aromatic-alanine substitutions. While individual substitutions were helpful, it was the combination of all five substitutions that increased solubility to extent that an NMR structure determination was possible.

In addition to the solution structure of the AIDA1 PTB domain, we have determined that its affinity for unphosphorylated APP is moderate relative to similar APP binding proteins such as X11/Fe65 for which dissociation constants of <1 μM have been observed. This difference in affinity may be advantageous for AIDA1 to participate in signaling contexts beyond APP. From a peptide array study, we determined that the consensus sequence is less stringent NxxY versus NPsY for others in the same Dab-like class of PTB domains. Thus, at the neuronal synapse, AIDA1 could serve as a versatile collator and convenor of signaling events arising from the NMDA receptor, and possibly others.

Recent structural studies have revealed how the PTB domains of X11 [7] and Talin [24] are autoinhibited by flanking sequences. The AIDA1-APP interaction is antagonized by a short 26 aa. sequence specified by exon14 in some isoforms through an unknown mechanism [2]. The sequence itself, rich in hydrophobic amino acids, does not resemble the NPsY motif suggesting that regulation of the AIDA1 PTB domain may be occuring by non-competitive binding. Further structural and biochemical studies of AIDA1 may lead to selective modification of some neuronal signaling pathways while sparing others. Fine control of signaling pathways may be one strategy to improve preventive and anti-progression therapies of Alzheimer’s disease.

Figure 4. Amino acid preferences of the AIDA1 PTB domain for APP determined from a peptide array. A list of peptides on the array are provided in supplementary material. (a) The array probed with anti 6xHis mAb only. Positive control 6xHis peptides are identified by a +. (b) The array probed with 6xHis-AIDA1 PTB domain. (c) Sliding window peptide scan of 12-mers spanning aa. 672–697 of APP. Peptides are duplicated on the array; for example, at A3 and A18. Since peptide content per spot can vary, if a signal was observed at the exposure presented it was deemed to be interaction. (d) Results of a window scan across the APP C-terminal sequence and an exhaustive positional scan. Grey boxes indicate binding was observed, regardless of signal intensity.
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Supporting Information

Figure S1 A comparison of 15N-edited HSQC spectra from the (a) AIDA1 PTB5M protein and the (b) AIDA1 PTB5M protein with an APP binding sequence (GYENP-TYKFFE) appended to the N-terminus along with a linker sequence (TLRPPNEATALQ) derived from the native AIDA1 protein. Both protein concentrations are 0.8 mM.

Table S1 A complete list of the 12-mer peptide sequences on the APP peptide array presented in Figure 4.

Author Contributions

Conceived and designed the experiments: LWD JJK. Performed the experiments: ES RS AK MM. Analyzed the data: ES RS AK SS MM JJK LWD. Wrote the paper: LWD.