Structure of the C-terminal Phosphotyrosine Interaction Domain of Fe65L1 Complexed with the Cytoplasmic Tail of Amyloid Precursor Protein Reveals a Novel Peptide Binding Mode*

Received for publication, May 21, 2008, and in revised form, July 9, 2008 Published, JBC Papers in Press, July 23, 2008, DOI 10.1074/jbc.M803892200

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Fe65L1, a member of the Fe65 family, is an adaptor protein that interacts with the cytoplasmic domain of Alzheimer amyloid precursor protein (APP) through its C-terminal phosphotyrosine interaction/phosphotyrosine binding (PID/PTB) domain. In the present study, the solution structures of the C-terminal PID domain of mouse Fe65L1, alone and in complex with a 32-mer peptide (DAAVTPEERHLSKMQQNGYENPTYKFFEQMQN) derived from the cytoplasmic domain of APP, were determined using NMR spectroscopy. The C-terminal PID domain of Fe65L1 alone exhibits a canonical PID/PTB fold, whereas the complex structure reveals a novel mode of peptide binding. In the complex structure, the NPTY motif forms a type-I β-turn, and the residues immediately N-terminal to the NPTY motif form an antiparallel β-sheet with the β5 strand of the PID domain, the binding mode typically observed in the PID/PTB-peptide complex. On the other hand, the N-terminal region of the peptide forms a 2.5-turn α-helix and interacts extensively with the C-terminal α-helix and the peripheral regions of the PID domain, representing a novel mode of peptide binding that has not been reported previously for the PID/PTB-peptide complex. The indispensability of the N-terminal region of the peptide for the high affinity of the PID-peptide interaction is consistent with NMR titration and isothermal calorimetry data. The extensive binding features of the PID domain of Fe65L1 with the cytoplasmic domain of APP provide a framework for further understanding of the function, trafficking, and processing of APP modulated by adapter proteins.

Alzheimer disease is a neurodegenerative disorder characterized by senile plaques and neurofibrillary tangles. The predominant constituent of the amyloid is the 39–43-residue amyloid β peptide (Aβ), a proteolytic cleavage product of the amyloid precursor protein (APP) (1). APP is an integral transmembrane glycoprotein composed of a large extracellular domain, a single membrane-spanning region, and a short cytoplasmic domain (see Fig. 1A). The APP gene encodes several different isoforms of APP as a consequence of alternative splicing. The three major isoforms are APP770, APP751, and APP695, which all possess the same 47-residue cytoplasmic domain. The functional role of APP remains unclear; however, several lines of evidence suggest that APP is part of a diverse protein-protein interaction network, which is centered on the short cytoplasmic domain. The cytoplasmic domain participates in the important cellular processes of intracellular trafficking and secretion of APP and signal transduction via interactions with adapter and signaling proteins, respectively. Several proteins reportedly interact with the cytoplasmic domain of APP. These include heterotrimeric G protein (G3, 2), the 59-kDa ubiquitously expressed protein APP-BP1 (3), the neuron-specific X11 protein (4), Fe65 family proteins (4–9), mammalian Disabled (Dab) protein (10, 11), and c-Jun N-terminal protein kinase-interacting protein (JIP) (12, 13) as well as the microtubule-binding protein PAT1 (14). Some of these proteins are phosphotyrosine interaction/phosphotyrosine binding (PID/PTB) domain-containing proteins, including X11 (4), Fe65 (4–9), Dab (10, 11), and JIP (12, 13). They recognize the NPTY sequence within the cytoplasmic domain of APP.

The Fe65 family proteins, Fe65, Fe65L1, and Fe65L2, are adaptor proteins that possess three protein–protein interaction domains: one WW domain and two PID/PTB domains (see Fig. 1B) (15). The N- and C-terminal PID/PTB domains are referred...
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to as PID1 and PID2, respectively. The three protein-protein interaction domains are well conserved among the Fe65 family proteins, sharing 50–60% amino acid sequence identity, whereas most of the remaining parts of the proteins are unrelated. Among the three Fe65 family proteins, the most significant difference is their tissue distribution: Fe65 mRNA is neuron-specific (16), whereas Fe65L1 mRNA is ubiquitously expressed (7), and Fe65L2 mRNA significantly accumulates in the brain and testis (9). The Fe65 gene was originally isolated as a neuron-specific gene, and it has some characteristics of a transcription factor (16, 17). The interaction between the Fe65 family proteins and the APP cytoplasmic domain has been confirmed both in vitro (5, 7) and in vivo (8). For all three Fe65 family proteins, the C-terminal PID/PTB domain (PID2) was demonstrated to be sufficient for their binding to the cytoplasmic domain of APP (4). Furthermore phosphorylation of the tyrosine in the NPTY motif is not required (4). On the other hand, a yeast two-hybrid screening study (5) and a peptide competition experiment (8) showed that a 32-residue-long peptide, DAAVTEERHLSKMQQNGYNENPTYKFFEQMQN, located at the extreme C terminus of the cytoplasmic domain of APP, was necessary for binding PID2 of Fe65. The 32-residue-long peptide (termed APP-32mer in Fig. 1A), including the NPTY motif, is much longer than the peptide that was reported previously to be recognized by the PID/PTB domain. The YENPTY sequence is also a sorting motif, or an internalization motif, previously to be recognized by the PID/PTB domain. The YENPTY motif, is much longer than the peptide that was reported previously to be recognized by the PID/PTB domain. The YENPTY sequence is also a sorting motif, or an internalization motif, required for trafficking of APP into the endocytic pathway (18). Studies have revealed that Fe65 family proteins can alter the processing of APP by influencing APP trafficking (19–21). Fe65 has been shown to increase α-secretase-cleaved APP and Aβ production (19). Fe65L1 also promotes α-secretase-cleaved APP secretion and APP maturation (20). These regulatory activities of Fe65L1 require the binding of Fe65L1 to APP C-terminal fragments (21).

Although the interaction between APP and the Fe65 family proteins is highly significant, in terms of the biology of Alzheimer disease, little is known about the molecular basis of the interaction. Here we report the solution structure of PID2 of mouse Fe65L1 in the free form and in complex with APP-32mer and the characterization of their interaction by isothermal titration calorimetry (ITC) and NMR spectroscopy. Among the three Fe65 family proteins, we selected PID2 of Fe65L1 as a target because the mRNA encoding Fe65L1 is ubiquitously expressed. To facilitate the structure determination of the complex of PID2 of Fe65L1 and APP-32mer, we designed several different chimeric proteins in which the corresponding regions of PID2 and APP-32mer were integrated. The complex structure reported here reveals a novel peptide binding mode as compared with those of the canonical PID domains that recognize the NPX(p)Y motif ((p) indicates that tyrosine may be phosphorylated).

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of PID2 of Fe65L1**—The DNA fragment encoding PID2 of mouse Fe65L1 (amino acid residues Pro658 to Cys704; Swiss-Prot accession number Q9DBR4) was amplified via PCR from the RIKEN full-length enriched mouse cDNA library (Clone ID 1200015107) (22, 23) and was cloned into the plasmid vector pCR2.1 (Invitrogen) as a fusion with an N-terminal His tag and a tobacco etch virus protease cleavage site. The 13C,15N-labeled protein was synthesized by the cell-free protein expression system (24–26). The cell-free reaction solution was first absorbed to a TALON affinity column, which was washed with 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM NaCl, and was eluted with 20 mM Tris-HCl buffer (pH 8.0) containing 300 mM NaCl and 500 mM imidazole. The solution was then desalted on a HiLoad 26/10 Desalting column with 20 mM Tris-HCl buffer (pH 8.0) containing 300 mM NaCl. The His tag was cleaved by an incubation at 30 °C for 1 h with tobacco etch virus protease. The sample was then loaded on a TALON affinity column, which was washed with 20 mM Tris-HCl buffer (pH 8.0) containing 300 mM NaCl, and was eluted with 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA and 1 mM DTT. The fraction containing the PID domain was concentrated to 1.49 mg/ml in 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA, and protease inhibitor mixture (Complete, EDTA-free (Roche Applied Science)). For the NMR structure determination, a 0.84 mM sample of free PID2 was prepared in 20 mM deuterated Tris buffer (pH 7.0) containing 100 mM NaCl, 2 mM deuterated DTT, 0.02% NaN3, and 10% D2O, 90% H2O. The protein sample for the NMR measurements consisted of 136 amino acid residues. The first 7 amino acid residues at the N terminus (GSSGGSG) and the last 6 residues at the C terminus (SGPSSG) were derived from the linker sequence used in the expression and purification system.

**NMR Sample Preparation of the PID2 and APP-32mer Complex**—To prepare the sample of the PID2 and APP-32mer complex for NMR measurement, a solution of non-labeled APP-32mer (Toray Research Center, Tokyo, Japan), dissolved in buffer A (20 mM deuterated Tris buffer (pH 7.0) containing 100 mM NaCl, 1 mM deuterated DTT, 0.02% NaN3, and 10% D2O, 90% H2O) with pH adjustment, was gradually added to 1H-15N-labeled PID2, which had been buffer-exchanged in buffer A, until the free form of PID2 was completely converted into the peptide-bound form as confirmed by the 1H-15N HSQC spectra. The final concentration of PID2 was 0.37 mM.

**Design and Expression of the PID2-APP-32mer Chimera**—To prepare the PID2-APP-32mer chimera, the gene encoding PID2 was fused to the cDNA of APP-32mer (amino acid residues Asp739 to Asn770; Swiss-Prot accession number P12023), and the resulting gene was inserted within the plasmid vector pCR2.1 (Invitrogen), incorporating an N-terminal His tag and a tobacco etch virus cleavage site. The 13C,15N-labeled

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4 The asterisk (*) after the residue is used to indicate the residue derived from the cytoplasmic tail of APP.
Chimera I included, from the N to the C terminus, a His tag, APP-32mer, a 14-residue linker (SGSSGSSGSSGSSG), and PID2. The Chimera II construct consisted of a His tag, APP-32mer, a 23-residue linker containing a protease Factor Xa cleavage site (SGPSSGIEGRGSSGSSGSSGSSG), and PID2; the construct of Chimera III included a His tag, PID2, the 23-residue linker, and APP-32mer. The \(^{13}\)C,\(^{15}\)N-labeled chimeras were synthesized and purified as described below. After the His tag cleavage in the purification procedure, Chimera I consisted of 176 amino acid residues; both Chimeras II and III consisted of 185 amino acid residues. The first 7 amino acid residues at the N terminus (GSSGSSG) in each sample were derived from the linker sequence used in the expression and purification system.

**Purification of the PID2-APP-32mer Chimeras**—The cell-free reaction solution of each chimera was adsorbed onto a HiTrap chelating column (Amersham Biosciences), which was washed with 20 mM Tris-HCl buffer (pH 8.0) containing 1 M NaCl and 20 mM imidazole, and was eluted with 20 mM Tris-HCl buffer (pH 8.0) containing 500 mM NaCl and 500 mM imidazole. To cleave the His tag, the eluted sample was incubated with tobacco etch virus protease at 30 °C for 3 h for Chimeras I and II and overnight for Chimera III. The sample was then loaded on a HiPrep 26/10 Desalting column, which was eluted with 20 mM Tris-HCl buffer (pH 8.0) containing 300 mM NaCl and 20 mM imidazole. The fractionated dialysate was applied to a HiTrap chelating column, which was equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 300 mM NaCl and 20 mM imidazole, and was eluted with 20 mM Tris-HCl buffer (pH 8.0) containing 500 mM NaCl and 500 mM imidazole. The concentration of imidazole (500 mM final concentration). The eluted fraction of each chimera sample was concentrated in 20 mM Tris-HCl buffer (pH 8.0) containing 300 mM NaCl, 1 mM DTT, 0.5 mM EDTA, and protease inhibitor mixture (Complete Mini). Each NMR sample contained ~1.0 mM of uniformly \(^{13}\)C,\(^{15}\)N-labeled chimera in 20 mM deuterated Tris buffer (pH 7.0) containing 0.05 M NaCl.
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100 mM NaCl, 1 mM deuterated DTT, 0.02% NaN3, and 10% 2H2O, 90% 1H2O.

ITC Measurements—ITC measurements were performed on a MicroCal VP-ITC isothermal titration calorimeter (MicroCal, Inc.). HEPES buffer (20 mM HEPES buffer (pH 7.0) containing 100 mM NaCl and 1 mM DTT) was used for the ITC measurements. PID2 was buffer-exchanged in the ITC buffer, and APP-32mer was dissolved in the same buffer with pH adjustment. The concentrations of PID2 and APP-32mer used for the ITC measurement were 0.109 and 1.640 mM, respectively, as determined using UV absorbance values measured at 280 nm. A degassed sample of PID2 was kept at room temperature (25°C) and was stirred at 307 rpm in a 1.4-ml reaction cell. For each titration, 5-μl aliquots of peptide were delivered into the PID2 solution at 240-s intervals to allow complete equilibration. The final ratio of peptide to protein reached 2:1 at the end of the titration. Heat transfer was measured as a function of elapsed time. The heat of dilution, obtained by titrating the identical peptide solution into the reaction cell containing only the HEPES buffer, was subtracted prior to analysis. The corrected titration curve was fitted with a one-site model, and the thermodynamic parameters were calculated using the Origin software (version 7.0) provided by MicroCal.

Titration Experiments by NMR—NMR titration experiments were carried out by adding the unlabeled APP-32mer solution to 13C,15N-labeled PID2. In total, four NMR samples were prepared for the titration experiment. In the four NMR samples, the concentration of PID2 was maintained at 0.050 mM, and the concentration of APP-32mer was varied to generate a series of different PID2:APP-32mer molar ratios (1:0, 1:0.5, 1:1, and 1:1.5). The buffer used for the APP-32mer titration was the same as that used for the structure determination. 1H-15N HSQC spectra were measured after each titration step, and the signals were monitored by observing the changes in the chemical shifts of the amide signals in 1H-15N HSQC spectra. The weighted chemical shift change (in ppm units) of the amide proton (ΔδH) and nitrogen (ΔδN) was calculated according to the following equation: \[ \Delta \delta_{\text{total}} = \left( \delta_{\text{HSQC}} W_{\text{HSQC}}^2 + \delta_{\text{N}} W_{\text{N}}^2 \right)^{1/2} \]
where \( W_{\text{HSQC}} = 1 \) and \( W_{\text{N}} = 0.154 \) (27).

NMR Spectroscopy—The NMR data for PID2 in the free form were recorded at 298 K on Varian Inova 600- and 900-MHz spectrometers equipped with pulsed field gradient probes. The NMR data for the three chimeras, Chimera I, Chimera II, and Chimera III, and PID2 complexed with non-labeled APP-32mer were recorded at 296 K on a Bruker AVANCE 700-MHz spectrometer, which was equipped with a triple resonance cryoprobe, and on AVANCE 800- and 900-MHz spectrometers. Sequence-specific resonance assignments were made using the standard triple resonance techniques (28). For PID2 complexed with the non-labeled APP-32mer, two-dimensional [F2] 13C,15N-filtered total correlation spectroscopy and two-dimensional [F1,F2] 15N-filtered NOESY (29) were measured for the chemical shift assignments of the non-labeled APP-32mer, and two-dimensional [F2] 13C,15N-filtered NOESY (29), three-dimensional 13C,15N F1-filtered, 13C F3-edited NOESY and three-dimensional 13C,15N F1-filtered, 15N F3-edited NOESY (30) with mixing times of 80, 100, and 100 ms, respectively, were recorded for the detection of intermolecular NOEs. The filter-related spectra were measured on an 800-MHz spectrometer. All of the spectra were processed using the NMRPipe software package (31). The programs KUJIRA (32) and NMRView (33, 34) were used for visualization of the NMR spectra and chemical shift assignments.

Structure Calculations—For the structure calculations of PID2 in the free form, Chimera I, Chimera II, and Chimera III, 11N-edited NOESY and 13C-edited NOESY with 80-ms mixing times were used to determine the distance restraints. Dihedral angle restraints were derived using the program TALOS (35). The stereospecific assignments of the Val and Leu methyl groups were determined when they were distinguishable from their NOE patterns. Automated NOE cross-peak assignments and structure calculations with torsion angle dynamics were performed using the program CYANA (36–39).

For the structure calculation of PID2 in complex with the non-labeled APP-32mer, the stereospecific assignments and the dihedral angle restraints for the PID2 part were added in a way similar to that described above. The dihedral angle restraints for APP-32mer were only added in its secondary structural region by checking the corresponding NOE patterns. In addition, a total of five NOE peak lists were used in the complex structure calculation. Among the five peak lists, two were obtained from three-dimensional 13C-edited NOESY-HSQC and 15N-edited NOESY-HSQC, and three were obtained from two-dimensional [F2] 13C,15N-filtered NOESY, three-dimensional 13C,15N F1-filtered, 13C F3-edited NOESY, and three-dimensional 13C,15N F1-filtered, 15N F3-edited NOESY. Dihedral angle restraints and stereospecific assignments of the Val and Leu methyl groups were obtained in a similar manner as described above. The peak list from two-dimensional [F2] 13C,15N-filtered NOESY provided information about the intramolecular NOEs of APP-32mer and the intermolecular NOEs between PID2 and APP-32mer, whereas the peak lists from three-dimensional 13C,15N F1-filtered, 13C F3-edited NOESY and three-dimensional 13C,15N F1-filtered, 15N F3-edited NOESY provided information about the intermolecular NOEs between PID2 and APP-32mer. The cross-peaks along the F2 axis in two-dimensional [F2] 13C,15N-filtered NOESY and those along the F1 axes in three-dimensional 13C,15N F1-filtered, 13C F3-edited NOESY and three-dimensional 13C,15N F1-filtered, 15N F3-edited NOESY are the signals coming only from the non-labeled APP-32mer. Therefore, in principle, the information about the APP-32mer assignment can be integrated into the three filter-related peak lists in advance of the structure calculation. In the CYANA peak list, each line contains the following information: the peak number, the chemical shifts, the peak volume, the atom numbers that identify the atoms in the corresponding chemical shift list, and so on. The default value for the atom number in the peak list is zero, indicating a missing assignment. CYANA makes the assignment automatically for each peak according to its structure calculation algorithms. If the assignment of a certain atom is known, then an atom number, instead of zero, can be given for that atom. CYANA then starts the structure calculation using the assignments of these atoms as inputs. In the filter-related peak list, one column of the atom number is known to correspond to that of APP-32mer; therefore, instead of zero,
the atom number of APP-32mer is used. In this way, the assignment of that column will be restricted only to APP-32mer. The atom number of APP-32mer was given according to the chemical shift assignment of that atom and the chemical shift tolerance, which was set to the same value as that used in the CYANA calculation. In the end, automated NOE cross-peak assignments and structure calculations with torsion angle dynamics were performed using the program CYANA (36–39).

In the structure calculations described above, a total of 100 structures were calculated, and the 20 structures in the final calculation cycle with the lowest target function values were selected. The stereochemical quality of the structures was assessed using the program PROCHECK-NMR (40). Molecular models were generated using the programs MOLMOL (41) and PyMOL (42). The statistics of the structures, as well as the distance and torsion angle constraints used for the structure calculation, are summarized in Table 1.

**Protein Data Bank Accession Numbers**—The structures of PID2 in the free form and in the complex form and Chimera I, Chimera II, and Chimera III have been deposited in the Protein Data Bank with the accession codes 1WGU, 2ROZ, 2YT0, 2YT1, and 2YSZ, respectively.

**RESULTS**

Structure Determination of PID2 of Mouse Fe65L1 in Its Free Form—The domain boundary of PID2 of mouse Fe65L1 is defined as residues Glu587–Pro717 according to the Simple Modular Architecture Research Tool. Before the structure determination, several constructs with an elongated or short-ened N or C terminus were prepared to obtain an optimized sample (data not shown). As a result, the construct encompassing residues Pro582–Cys704 was obtained as a suitable sample with sharp and well dispersed signals in the 1H-15N HSQC spectrum. The NMR structure determination was performed for 13C15N-labeled PID2, encompassing residues Pro582–Cys704. The ensemble consisting of 20 structures with the fewest violations is shown in the wire model in Fig. 2A, and the ribbon representation is shown in Fig. 2B. As shown in Fig. 2, PID2 of Fe65L1 consists of seven β-strands (β1, Gln590–Pro601, β2, Ser629–Ala635, β3, Thr638–Ile642, Val650–Arg655, Leu659–Gly663, Phe671–Phe705, Phe682–Trp688, and Ala694–Cys704) and seven β-strands (β1, Gln590–Cys704 from PID2 and Thr743*–Phe765* from APP-32mer). The root mean square values for PID2 and APP-32mer were calculated individually and are separated by a slash (/).

The Binding of PID2 of Fe65L1 to the Cytoplasmic Domain of APP—The binding properties between PID2 of Fe65L1 and the cytoplasmic domain of APP were investigated by ITC and NMR spectroscopy. Two peptide fragments with different lengths derived from the cytoplasmic domain of APP (Fig. 1A), a 14-residue peptide APP-14mer (sequence, QNGYENPTYKFFEQ) and a 32-residue peptide APP-32mer (sequence, DAAVT-PEERHLSKMQQNYENTYKFFEQMQN), were used in the

**TABLE 1**

Summary of the conformational restraints and statistics of the final 20 best structures

|                     | PID2 | Chimera I | Chimera II | Chimera III | Complex |
|---------------------|------|-----------|------------|-------------|---------|
| NOE upper distance limits |      |           |            |             |         |
| Total               | 2485 | 3237      | 3123       | 3052        | 2538    |
| Intraresidue (| | | | | |
| Sequential (| | | | | |
| Medium range (| | | | | |
| Long range (| | | | | |
| Intermoleculara | —    | 180       | 145        | 152         | 184     |
| Torsion angle restraints | 114  | 120       | 130        | 138         | 130     |
| CYANA target function value | 0.45 | 0.16      | 0.17       | 0.22        | 0.76    |
| Distance restraint violations | 4    | 2         | 0          | 0           | 0       |
| Number >0.1 Å | 1    | 0         | 0          | 0           | 0       |
| Number >0.3 Å | 0    | 0         | 0          | 0           | 0       |
| Maximum (Å) | 0.17 | 0.20      | —          | —           | 0.27    |
| Torsion angle restraint violations | Number >5 | 0 | 0 | 0 | 0 |
| Maximum (°) | —    | —         | —          | —           | —       |
| PROCHECKb | | | | | |
| Residues in favored regions (%) | 83.5 | 86.1      | 89.2       | 84.1        | 75.0    |
| Residues in additionally allowed regions (%) | 14.8 | 13.9      | 10.8       | 15.9        | 22.7    |
| Residues in generously allowed regions (%) | 1.4  | 0.0       | 0.1        | 0.0         | 1.1     |
| Residues in disallowed regions (%) | 0.4  | 0.0       | 0.0        | 0.0         | 1.2     |
| r.m.s. deviation to the averaged coordinates | | | | | |
| All regions | 0.31 | 0.30/0.67 | 0.31/0.60 | 0.34/0.68 | 0.27/0.83 |
| Backbone atoms (Å) (PID2 APP-32mer) | 0.70 | 0.70/1.25 | 0.75/1.15 | 0.78/1.29 | 0.70/1.52 |
| Heavy atoms (Å) (PID2 APP-32mer) | 0.23 | 0.23/0.42 | 0.25/0.46 | 0.24/0.36 | 0.19/0.40 |
| Ordered regionsc | 0.55 | 0.51/0.88 | 0.56/0.89 | 0.56/0.90 | 0.56/0.81 |
| Backbone atoms (Å) (PID2 APP-32mer) | —    | —         | —          | —           | —       |
| Heavy atoms (Å) (PID2 APP-32mer) | —    | —         | —          | —           | —       |
| Maximum (Å) | —    | —         | —          | —           | —       |

a A dash (—) is inserted to indicate the lack of data. The intermolecular NOEs in each chimera represent the NOEs between the two regions corresponding to APP-32mer and PID2 of Fe65L1, respectively.
b The region for the PROCHECK calculation includes both Gln590–Cys704 from PID2 and Thr743*–Phe705* from APP-32mer.
c All regions include Gln590–Cys704 from PID2 and Thr743*–Phe765* from APP-32mer. The root mean square (r.m.s.) values for PID2 and APP-32mer were calculated individually and are separated by a slash (/).
binding study. Although APP-32mer and APP-14mer both include the NPTY motif of the cytoplasmic domain of APP, APP-32mer is known as the peptide that is required for effective binding between PID2 of Fe65 and the cytoplasmic domain of APP (8).

ITC experiments were performed to determine the thermodynamic parameters for the binding. The upper panel of Fig. 3A shows the heat of reaction for about 40 titrations of APP-32mer into the PID2 solution at 25 °C. The binding reaction is exothermic, and the integrated heats of reaction are shown in the lower panel of Fig. 3A. Assuming a 1:1 binding model, a non-linear least squares fit of the binding curve provides values for the stoichiometry of binding (n) of 1.18, the equilibrium binding constant (K_D) of 1.26 × 10^6 ± 3.47 × 10^5 M^-1 (corresponding to a dissociation constant (K_D) of 0.79 μM), and the change in enthalpy (ΔH) of −12.86 ± 0.025 kcal/mol. The free energy of binding (ΔG) and the change in entropy (ΔS) were −8.33 and −4.53 kcal/mol, respectively. The K_D of 0.79 μM, obtained in the ITC measurement, suggested strong binding between PID2 and APP-32mer. Using APP-14mer at a concentration similar to that of APP-32mer as the titrant, the exothermic nature of the protein-peptide binding reaction was also observed from the titration curve, but it was quite weak (data not shown). It was difficult to obtain the thermodynamic parameters, including K_D, from the curve fitting because the titration curve did not reach complete saturation under these conditions. Therefore, it can be inferred that the binding affinity between PID2 and APP-14mer is much weaker than that between PID2 and APP-32mer.

The peptide binding properties and the binding site were further investigated by NMR using a chemical shift perturbation analysis. A 13C, 15N-labeled PID2 sample was titrated with non-labeled APP-32mer and APP-14mer, and the residues showing chemical shift changes were monitored on 1H-15N HSQC spectra. The addition of APP-32mer to PID2 resulted in peaks disappearing and reappearing at many positions in the spectra (Fig. 3B). This observation of slow exchange on the NMR time scale is indicative of high affinity binding. In the case of APP-14mer, fewer peaks disappeared and reappeared, and at the same time, peak broadening was also observed (data not shown). This observation of intermediate to slow exchange on the NMR time scale is indicative of intermediate affinity binding. This NMR titration result is consistent with the ITC result. In Fig. 3C, the weighted chemical shift changes of the amide 1H and 15N, obtained from NMR titration experiments with APP-32mer, are plotted as a function of the residue number. The residues with large weighted chemical shift changes are mapped onto

Overall Structure of PID2 of Fe65L1 in Complex with APP-32mer—Based on its high binding affinity for PID2, APP-32mer was chosen for the complex structure study. To prepare the sample for the complex structure determination, non-labeled APP-32mer was added to 13C, 15N-labeled PID2. To facilitate the chemical shift and NOE assignments of APP-32mer, three kinds of chimeras containing the corresponding region of PID2 and APP-32mer were also designed as shown in Fig. 1C. Each chimera with amino acid residues that were uniformly 13C, 15N-labeled behaved like a single chain protein; therefore, the structure determination of the chimeric protein was performed in a conventional manner. With the help of the chemical shift assignment of the APP-32mer portion in the chimeras, the assignment of the non-labeled APP-32mer in the complex sample was readily achieved. Most of the chemical shifts of the protons in APP-32mer were assigned except for the protons from the 2 residues at the N terminus (Asp739* and Ala740*) and the 2 residues at the C terminus (Gln769* and Asn770*). The structures of the three chimeras are shown in Fig. 4, A–C, and the complex structure of PID2 with APP-32mer is shown in Fig. 4, D–F. The overall structures of the three chimeras and the complex are very similar. In all of the structures, the N-terminal part of APP-32mer (Pro744*–Gln753*) forms a 2.5-turn α-helix, the middle region of APP-32mer (Tyr757*–Glu758*) forms an antiparallel β-sheet with strand β5 of PID2, and the region Asn759*–Tyr762* of APP-32mer forms a type-1 β-turn.

Interaction of PID2 of Fe65L1 with APP-32mer—APP-32mer interacts with PID2 of Fe65L1 using its residues ranging from Glu745* to Tyr762*. A detailed view of the intermolecular contacts is given in Fig. 5, and a schematic drawing of the intermo-
molecular contacts is shown in the left panel of Fig. 6B. The NPTY motif, Asn759* to Tyr762*, forms a type-I β-turn (Fig. 5B). The amide group of Asn759* forms a hydrogen bond with the main chain carbonyl group of Ser660, whereas the side chain of Asn759* forms two hydrogen bonds with the main chains of Val656 and Leu659 (Fig. 6B). Tyr762*, whose aromatic ring stacks with the CαH9252 portion of Ser660, interacts with Pro605, Phe658, and Asp676 (Fig. 5B). Tyr757* and Glu758*, which are located immediately N-terminal to the NPTY motif, form an antiparallel β-sheet with Phe661 and Met662 on the β5 strand of PID2. In addition, the side chain of Tyr757* is surrounded by Met662, Gln701, and Cys704 of PID2, whereas the hydrophobic part of the Glu758* side chain interacts with Val668 (Fig. 5B). With regard to Gly756*, intermolecular NOEs with Phe661 and Met662 of PID2 are observed. In the N-terminal region of APP-32mer, the region from Pro744*–Gln753* forms a 2.5-turn β-helix and interacts with PID2 mainly via Leu749* and Met752* (Fig. 5B). The main intermolecular contacts involving Leu749* and Met752* are hydrophobic. Leu749* binds at a pocket lined with the hydrophobic residues Val668, Ala693, and Ala694 and the

FIGURE 3. Peptide binding studies by ITC and NMR. A, isothermal titration calorimetry data for the interaction of PID2 and APP-32mer. The upper panel shows the exothermic heat of reaction measured for the injection of APP-32mer into the PID2 sample. The lower panel shows the integrated areas under the respective peaks in the upper panel plotted against the molar ratio of APP-32mer to PID2. The solid line is the best fit to the data for a single binding site model using a non-linear least squares fit. B, overlay of 1H-15N HSQC spectra of the free (black) and APP-32mer-bound (red) forms of PID2. C, the weighted chemical shift changes obtained from NMR titration experiments are plotted as a function of the residue number. The secondary structural elements are shown at the top. D, the weighted chemical shift data are mapped onto the ribbon drawing of PID2. The residues with weighted chemical shift changes larger than 0.40 ppm are shown in red, those between 0.20 and 0.40 ppm are orange, and those smaller than 0.20 ppm or for which there are no data are gray.
hydrophobic part of Asn^692 of PID2, whereas Met^752 of APP-32mer interacts with Met^662, Gly^663, Val^664, Ser^697, and Gln^701 (Fig. 5C). Intermolecular interactions were also observed for other residues in the N-terminal region of APP-32mer. Glu^745* interacts with Ala^694, and His^748* interacts with Glu^698 and Gln^701. The hydrophobic region of the Gln^753* side chain interacts extensively with the hydrophobic residues of Val^664, Val^668, and Met^608 of PID2 (Fig. 5C). Moreover the side chain of Gln^753* forms two hydrogen bonds with the main chains of Gly^665 and Lys^666. Consequently the molecular interface covered by APP-32mer includes /H9252, /H92512, and the /H92521–/H92511, /H92524–/H92525, /H92525–/H92526, and /H92527–/H92512 loops of PID2.

After examination of the intermolecular interface, a structure comparison was made between PID2 in the free and complex forms. The fact that the structured regions of PID2 in the free form fit well with those in the complex and chimeric forms with root mean square deviation values of 0.8 and 1.4 Å for the backbone and heavy atoms, respectively, indicates that the structural changes of PID2 induced by APP-32mer binding were negligible.

Several results indicated that Thr^743* of APP has a regulatory role in the interaction with Fe65 as its phosphorylation prevents the interaction (43). On the basis of the present structural data, it still remains unclear how the phosphorylated Thr^743* prevents the interaction with Fe65, and further studies will be necessary in the future.

DISCUSSION

Fe65L1, an adaptor protein, interacts with the cytoplasmic domain of APP (6, 7). Here we performed binding assays with PID2 of Fe65L1 and the APP peptide using ITC and NMR chemical shift perturbation and then solved the structures of PID2 of Fe65L1 in the ligand-free form and in complex with APP-32mer. In addition, we described three structures of PID2-APP-32mer chimeras. These structures provided the first view of a PID/PTB domain belonging to the Fe65 family. In the complex structure, the extensive binding features, characterized by the involvement of the N-terminal region of APP-32mer, revealed a novel mode of peptide binding
that has not been observed in PID/PTB-peptide complexes. The binding site of APP-32mer, identified from the complex structure study, covers the β3 strand, the α2 helix, and the β1–α1, β4–β5, β5–β6, and β7–α2 loops of PID2.

Besides the Fe65 family proteins, some other PID/PTB domain-containing proteins also reportedly bind the cytoplasmic domain of APP, including X11, Dab, and JIP family proteins. The structures of the PID/PTB domains of X11 and Disabled proteins (Dab1 and Dab2) in complex with a short APP peptide have been solved, and the mode of protein-peptide recognition has been investigated (44, 45). In the following, a comparison of these PID/PTB-peptide complex structures, especially the protein-peptide binding features, will be discussed.

Comparison with the X11 PID/PTB APP Peptide Complex—Biochemical characterization of the interaction between X11 and APP indicated that a 14-residue peptide with the sequence encompassing the NPTY motif of APP (QNGY-ENPTYKFFEQ, residues 754*–767*) competes efficiently with the full-length APP in binding with the PID/PTB domain of X11 (4). The surface plasmon resonance binding assay gave a $K_D$ value of 0.32 μM for the PID/PTB domain of X11 and the 14-residue APP peptide (44). However, a much longer peptide sequence (residues 739*–770*) of APP is required for high affinity binding to PID2 of Fe65L1. To clarify the different modes of APP peptide recognition by the PID/PTB domains of Fe65L1 and X11, the structures of the two complexes were compared (Fig. 6A).

A schematic drawing of the protein-peptide interaction network is shown in Fig. 6B. In both complexes, the NPTY motif in the peptide forms a type-I β-turn, and the region N-terminal to the NPTY motif forms an antiparallel β-sheet with the β3 strand of each PID/PTB domain. However, Pro760*, Thr761*, Lys763*, Phe764*, and Phe765* from the APP peptide interact further...
with the C-terminal α-helix of the X11 PID/PTB domain; this
prolonged region of the helix was omitted from the Fe65L1
PID2 construct to obtain a feasible NMR sample. It may be
argued that the shorter length of the C-terminal α-helix in the
Fe65L1 PID2 construct is the reason for the fewer protein-pep-
tide contacts and hence the lower binding affinity. However,
this is probably not the critical reason. In the peptide competi-
tive binding study performed by Borg et al. (4), the 14-residue
peptide was able to compete with X11 for APP binding with
submicromolar affinity, but it had no effect on Fe65 binding
even though their Fe65 PID2 construct was 10 residues longer
at the C terminus than our Fe65L1 PID2 construct reported here.
Moreover in their site-directed mutagenesis study (4), three of the APP mutations were at positions in/around the
NPTY motif, corresponding to Tyr757*, Asn759*, and Tyr762* of
APP-32mer. The Y757*G mutation severely impaired the bind-
ing of both X11 and Fe65, whereas the Y762*A mutation had
almost no effect. In contrast, the N759*A mutation exhibited a
differential effect; it abolished the X11 binding but did not
affect the Fe65 binding. Therefore, we closely examined the
protein-peptide interaction network involving the 3 residues
(Fig. 6B). Consistent with the mutagenesis results (4), Tyr757*
forms two main chain-main chain hydrogen bonds with its side
chain involved in many van der Waals interactions, whereas
Tyr762* only makes weaker van der Waals interactions in both
PID/PTB-peptide complexes. On the other hand, Asn759* forms
one main chain-main chain hydrogen bond and two side chain-
main chain hydrogen bonds with both PID/PTB domains and
also makes van der Waals interactions in both PID/PTB-peptide complexes. On the other hand, Asn759* forms
one main chain-main chain hydrogen bond and two side chain-
main chain hydrogen bonds with both PID/PTB domains and
also makes van der Waals interactions with Cys704 in Fe65L1
PID2 and Phe779* in the X11 PID/PTB domain, respectively.
Both Cys704* of Fe65L1 and Phe779* of X11 are buried in the
hydrophobic core of the PID/PTB domains. As Phe779* is more
bulky than Cys704*, the van der Waals interaction between
Asn759* and Phe779* of X11 was evaluated as being much stron-
ger than that between Asn759* and Cys704 in PID2 of
Fe65L1. Therefore, Asn759* is again important for the stronger
binding to the Dab2 PID/PTB domain. In addition, the hydroxyl group of Tyr762* in the 9-residue peptide forms a side
chain-main chain hydrogen bond with Gly739* of Dab2 that may
also contribute somewhat to the stronger binding. In PID2 of
Fe65L1, the loop between β6 and β7 is 1 residue longer than the
corresponding region of the Dab2 PID/PTB domain where
Gly739* is located (Fig. 6C), providing a space wide enough to
accommodate a phosphorylated tyrosine. In this sense, PID2 of
Fe65L1 belongs to the kind of PID/PTB domain in which the
NPTY motif binding is independent of the tyrosine phosphor-
ylation state, like the X11 PID/PTB domain.

The crucial role of Cys704* in the interaction with APP was
demonstrated by the functional impairment of the Cys to Phe
mutation (4). It is interesting to discuss the consequences of the
Cys to Phe transition, considering that in X11 and Dab2, this
residue is Phe (Phe779* and Phe166*). In the structure of the free
Fe65L1 PID2, Cys704* fits snugly into the hydrophobic pocket
formed by Val266*, Leu259*, and Met246*, and thus the replacement of Cys704* by a bulky Phe would inevitably cause steric hindrance
with these neighboring residues. Therefore, the Cys to Phe
transition would disrupt the structure of Fe65L1 PID2, and
hence the APP binding ability would be lost. In the X11 and
Dab2 PID/PTB domains, the residues surrounding Phe779*
and Phe166* are different from those in PID2 of Fe65L1, and thus
the spaces are large enough to accommodate the bulky Phe.

**FIGURE 6. Comparison of the recognition of the cytoplasmic domain of APP by the PID/PTB domains of Fe65L1, X11, and Dab2.** A, ribbon representation of the PID/PTB domain in complex with the APP peptide. PID2 of Fe65L1 is shown in green (left), and the PID/PTB domains of X11 (middle) and Dab2 (right) are shown in light green. APP-32mer is shown in magenta, and the APP peptide in complex with X11 and Dab2 PID/PTB domains are orange. The NPTY motif in the APP peptide is shown in cyan with the tyrosine and asparagine residues depicted by stick models. B, schematic drawing of the intermolecular contacts between the APP peptide and the PID/PTB domains of Fe65L1 (left), X11 (middle), and Dab2 (right). The color scheme of the residues represented by the ovals is the same as that in A. The solid red line represents the main chain-main chain hydrogen bond, and the solid cyan line represents the main chain-side chain or the side
chain-side chain hydrogen bond. One solid line represents one hydrogen bond. The dotted line represents the van der Waals interaction. The van der Waals interaction involving Asn759* is highlighted by thick dotted lines, and the side chains of the related residues in the PID/PTB domains, Cys704*, Phe166*, and Phe779*, are shown in red stick models. The intermolecular interaction was evaluated by the program LIGPLOT (46). C, structure-based sequence alignment of the PID/PTB domains of Fe65L1, X11, and Dab2. The secondary structure elements of PID2 of Fe65L1 are shown at the top of the sequence. The β-strands and α-helices are highlighted in cyan and pink, respectively. The residues involved in the intermolecular contacts are indicated in red, and the residues involved in
main chain-main chain and other kinds of hydrogen bonds are labeled by red and cyan ovals, respectively.
Comparison with the Cytoplasmic Domain of APP in the Free Form—The solution structure of the cytoplasmic domain of APP, spanning all 47 residues, has been examined by NMR spectroscopy (47). The study showed that although the peptide does not adopt a stably folded structure, regions with transient structure exist and, when bound to its receptor, the structure changes. The features of the transient structure in the free APP-32mer include an N-terminal helix capping box formed by Val742*–Arg747*, a type-I β-turn at Asn759*–Tyr762*, and nascent helices for Asp739*–Ala741*, Ser750*–Glu758*, and Lys763*–Met768*. When APP-32mer binds PID2 of Fe65L1, Asn759*–Tyr762* remains as a type-I β-turn, and Pro744*–Gln753*, covering part of the helix capping box and the nascent helix in the free form, is folded into a 2.5-turn α-helix. Therefore, the regions of the type-I β-turn (Asn759*–Tyr762*) and the short segment of the nascent helix (Ser750*–Gln753*) are preordered and stabilized in the bound state. On the other hand, Tyr757* and Glu758*, featured as a nascent helix in the free form, form an antiparallel β-sheet with β5 of PID2 of Fe65L1, suggesting that a conformational rearrangement occurred for these 2 residues.

Binding thermodynamics studies are useful to characterize the molecular mechanism of binding, and they reflect the vari-
ous types of molecular forces that drive binding. The thermodynamic parameters obtained from ITC in this study revealed a highly favorable change in enthalpy and an unfavorable change in entropy. Although the significant folding of the peptide upon binding contributes to the loss of entropy (\(\Delta S < 0\)), additional hydrophobic and enthalpic interactions, accounting for the favorable change in enthalpy (\(\Delta H < 0\)), are gained upon binding, leading to a favorable change in the free energy (\(\Delta G < 0\)) and stable binding (47).

The Complex of PID2 of Fe65L1 and APP-32mer Shows a Novel Mode of Peptide Binding—Given their functions as adaptors or scaffolds to regulate and organize signaling networks, structural and functional studies of PID/PTB and PID/PTB-like domains and their complexes have been gaining much attention recently (48, 49). In most of the PID/PTB-peptide complexes studied, the NXP(p)Y motif, the consensus sequence recognized by PID/PTB domains, forms a type-1 \(\beta\)-turn, whereas the residues N-terminal to the NXP(p)Y motif form an antiparallel \(\beta\)-sheet with the \(\beta5\) strand of the PID/PTB domains. In addition, this common binding mode does not depend on the phosphorylation state of the peptide, although the phosphorylation state of the peptide greatly influences the protein-peptide binding affinity for some PID/PTB domains. This typical binding mode is seen, for example, in the complexes of X11 PTB and APP peptide (Fig. 7B) and IRS-1 PTB and IL-4 peptide (Fig. 7C) (50). On the other hand, some non-canonical peptide binding modes have also been reported. In the complex of the Numb PTB domain and GPpY-containing peptide (Fig. 7D), the peptide binds the protein in a helical turn conformation (51). In the complex of the SNT-1 PTB domain and the peptide derived tide binds the protein in a helical turn conformation (51). In the complex of the APP peptide (Fig. 7) and IRS-1 PTB and IL-4 peptide (Fig. 7C) (50). On the other hand, some non-canonical peptide binding modes have also been reported. In the complex of the Numb PTB domain and GPpY-containing peptide (Fig. 7D), the peptide binds the protein in a helical turn conformation (51). In the complex of the SNT-1 PTB domain and the peptide derived tide binds the protein in a helical turn conformation (51).

In conclusion, our structural data and binding studies have elucidated the molecular mechanism by which PID2 of Fe65L1 recognizes the cytoplasmic domain of APP. The peptide-peptide recognition mode is unique in that the N-terminal region of the peptide forms an \(\alpha\)-helix and interacts extensively with PID2 of Fe65L1. These studies provide a framework for better understanding of the function, trafficking, and processing of APP and may make it possible to develop new therapeutic strategies toward Alzheimer disease through the regulation of the interactions between APP and APP-binding adapter proteins.

Acknowledgments—We thank Yutaka Muto for the NMR pulse program and the spectral analysis. We also thank Noriyuki Iwasaki, Jun Yokoyama, Manami Kato, Yasuko Tomo, Ayako Hiyoshi, Kae Kikumi, Miyuki Sato, and Tomomi Ide for sample preparation and Azusa Ishii and Tomoko Nakayama for clerical assistance.

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