Solution Structure of AF-6 PDZ Domain and Its Interaction with the C-terminal Peptides from Neurexin and Bcr*§

Heyue Zhou, Yingqi Xu, Yuedong Yang, Anding Huang, Jihui Wu‡, and Yunyu Shi§

From the Hefei National Laboratory for Physical Sciences at Microscale, School of Life Science, University of Science and Technology of China, Hefei, Anhui 230026, People’s Republic of China

AF-6 is a key molecule essential for structure organization of cell-cell junction of polarized epithelia. It belongs to a novel cell-cell adhesion system. The AF-6 PDZ domain mediates interactions by binding to a specific amino acid sequence in target proteins. Here we report the solution structure of the AF-6 PDZ domain determined by NMR. Previously, the AF-6 PDZ domain was considered to be a class II PDZ domain. However, we found that a unique hydrophilic amino acid, Gln, at position αB makes the αB/βB groove of the AF-6 PDZ domain significantly different from that of the canonical class II PDZ domain. The AF-6 PDZ domain does not have the second hydrophobic binding pocket, and the N-terminal end of αB is closer to βB. Using BIAcore and NMR chemical shift perturbation experiments, we have studied the binding characteristics of the PDZ domain to the C-terminal peptide of Neurexin, KKKD-KEYYV, and that of Bcr, KRQSLFSTEVE. The C-terminal peptide of Neurexin is a class II ligand, whereas that of Bcr is a class I ligand. The dissociation constants of these ligands were 4.08 × 10⁻⁷ and 2.23 × 10⁻⁶ M, respectively. Each of the four C-terminal positions in Neurexin and Bcr may contribute to the interaction. The three-dimensional models of the AF-6 PDZ-Neurexin C-terminal peptide complex and the AF-6 PDZ-Bcr C-terminal peptide complex were built up by molecular dynamics simulations. Unlike the canonical class II PDZ domain, Ala⁷⁴ at αB5 rather than the residue at αB1 makes direct hydrophobic contact with the side chain of Tyr at the -2 position of the ligand.

The human AF-6 gene was initially discovered as a fusion partner of the ALL-1 gene in acute lymphoblastic leukemia caused by a chromosomal translocation, t(6;11) (1). Later on, it was found that AF-6 co-localizes with tight junctions (2) and adhesion junctions (3) and is involved in connecting the junctional complexes with the cortical actin cytoskeleton. It belongs to a novel cell-cell adhesion system, which is composed of nectin and afadin (homolog molecule of AF-6 in mouse) (4, 5). It is a key molecule essential for structure organization of cell-cell junction of polarized epithelia during embryogenesis (6). This function was demonstrated by the phenotype of AF-6-deficient mice, which died because of disorganization of cell-cell junction and defects in the polarity of neuroepithelial cells (6, 7).

AF-6 is a putative effector of Ras in modulation of intercellular junctions. It competes with Raf in binding Ras and down-regulates Ras-mediated mitogen-activated protein kinase/extracellular signal-regulated kinase kinase activation (9). Moreover, AF-6 can control integrin-mediated cell adhesion by regulating Rap1 activation through the specific recruitment of Rap1GTP and SPA-1 (10).

AF-6 is a multidomain protein. It contains two Ras-binding domains (11), a forkhead-associated domain (12), and a class V myosin homology region, DIL (13), in the N-terminal part and a PDZ domain and a proline-rich region in the C-terminal part. PDZ (PSD-95, discs-large, and ZO-1) domains are structurally conserved modules consisting of 80–90 residues (14–18). The predominant function of PDZ domains is to recognize the extreme C termini of other proteins. Numerous structural and biochemical studies have demonstrated that the C-terminal peptide ligand always binds to the PDZ domain by fitting into a hydrophobic groove between a β-strand and an α-helix (19, 20).

The PDZ domain plays an important role in AF-6 function. Many interactions of AF-6 with other proteins, such as junctional adhesion molecule (21), the poliovirus receptor-related protein (22), several members of the Eph receptor family of receptor tyrosine kinases (23, 24), SPA-1 (10), Neurexin (23), and Bcr (9), are in a PDZ domain-dependent manner. Here we pay special attention to the interaction of AF-6 with Neurexin and Bcr. Neurexin is a neuronal cell surface protein. It can mediate the assembly of presynaptic terminals (25). At least three neurexin genes, designated NRXN I–III, are expressed in mammals. Thousands of neurexin isoforms are generated from these three genes by usage of alternative promoters and alternative splicing (26). They share identical transmembrane regions and cytoplasmic C-terminal domains but have variable extracellular domains on the cell surface. Neurexin can bind to many PDZ domain-containing proteins in a PDZ domain-dependent manner (23, 27, 28).

Bcr is well known as part of the chimeric fusion protein Bcr-Abl that arises by the Philadelphia chromosome that was found in 95% of patients with chronic myeloid leukemia (29). The normal cellular Bcr gene encodes a 160-kDa phosphoprotein associated with serine/threonine kinase activity. Bcr can induce down-regulation of Ras signaling via binding of the...
AF-6 PDZ domain, which increases the affinity of AF-6 for activated Ras (9).

Despite many biological progresses, major questions regarding the structural basis of interaction between AF-6 and Bcr/Neurexin remain unanswered. For instance, what is the specificity of the PDZ domain of AF-6, and how does it bind to Bcr or Neurexin? Detailed knowledge of its structure is required to better understand all these questions. We determined the solution structure of the AF-6 PDZ domain and characterized the interaction of the AF-6 PDZ domain with the C-terminal peptides of Bcr and Neurexin. Previously, the PDZ domain of AF-6 was classified as the class II PDZ domain (30). Our data revealed that both peptides can bind to the a/b/β grove of AF-6 PDZ domain, and the a/b/β grove of the AF-6 PDZ domain significantly differs from that of the canonical class II PDZ domain.

EXPERIMENTAL PROCEDURES

Expression and Purification of AF-6 PDZ Domain—The human AF-6 PDZ domain (amino acid residues 987–1078) was polymerase chain reaction-amplified from the human brain cDNA library with specific primers. The amplified DNA fragment was inserted into the plasmid PET22b (+) (Novagen). The recombinant plasmids harboring the respective target genes were transformed into Escherichia coli BL21(DE3) host cells for large scale protein production. Uniformly 15N- and 13C/15N-labeled proteins were prepared by growing bacteria in SV40 medium using 15NCl (0.5 g/liter) and 13C-glucose (2.5 g/liter) as stable isotope sources. Recombinant PDZ domain fragment was purified using HiTrap chelating column (Amersham Biosciences) chromatography. The purified recombinant protein contained an N-terminal Met and a C-terminal His tag (-LEHHHHHH) carried over from the cloning vector.

Peptide Synthesis—The C-terminal peptides of Neurexin and Bcr, KKKDKEKEYV and KQKSLFSTEV, were synthesized using conventional Fmoc (N-(9-fluorenyl)methoxycarbonyl) solid phase peptide synthesis protocols and cleaved off the resin with 25% triisopropylsilane and 2.5% H2O in trifluoroacetic acid. The peptides were purified by high pressure liquid chromatography (C18 column, 50 μ×250 mm) using a linear gradient of 15–30% acetonitrile containing 0.1% trifluoroacetic acid. The final products were lyophilized and identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

Surface Plasmon Resonance—Association and dissociation of the AF-6 PDZ domain with the C-terminal peptides of Neurexin and Bcr were measured with a BIAcore® 3000 instrument (BIAcore AB, Uppsala, Sweden). The C-terminal peptides of Neurexin and Bcr were coupled to a carboxymethyl-dextran CM5 sensor chip with an amine coupling kit containing 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, N-hydroxysuccinimide, and ethanolamine. After the chip was activated by 60 μl of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-hydroxysuccinimide mixture, the peptide was coupled to the CM5 sensor surface using about 60 μl of peptide (1 μM) in immobilization buffer (10 mM acetate and 150 mM NaCl (pH 5.5) containing 0.005% surfactant P20. 20 mM HCl were used to regenerate the chip. For fitting of the binding kinetics, BIAevaluation version 4.0 software (BIAcore AB) was applied, and the 1:1 Langmuir binding model was chosen.

NMR Structure Determination of AF-6 PDZ Domain—Both the purified 15N-labeled and 13N/15N-labeled proteins were dissolved to a final concentration of 2 mg in 50 μl of 50 mM phosphate buffer (pH 5.9) containing 1 mM EDTA in 90% H2O, 10% D2O. All of the NMR spectra were acquired at 25 °C on a Bruker DMX 500 spectrometer with self-shielded z axis gradients.

The 1H, 13C, and 15N resonances of the AF-6 PDZ domain were assigned by standard methods using triple resonance experiments and 13N/15N-separated TOCSY and NOESY experiments. All of the backbone resonances were assigned, with the exception of the N- and C-terminal residues and the 15N resonance of proline. More than 95% of the side chain 1H resonances were assigned. A total of 1564 experimentally derived distance and 15N-separated TOCSY and NOESY assignments. Side chain resonance assignments were obtained from 13N TOCSY-HSQC, H(C)(C)OHN-TOCSY, (H)C(O)NH-TOCSY, HCCH-TOCSY, 13C NOESY-HSQC, and 15N NOESY-HSQC.

The -NH2 side chains of Asn and Gln residues were assigned by a three-dimensional 13N-separated NOESY experiment with the 15N-labeled protein dissolved in H2O. The 13C/15N-separated NOESY and 13C-separated NOESY were collected with mixing times of 80 and 130 ms, respectively. The 15N-labeled sample was lyophilized and then redisolved in 99.96% D2O, which was followed immediately with HSQC experiments to monitor the disappearance of NH signal at 295 K. All of the NMR spectra were processed with NMRPipe and analyzed using Sparky 3.

NMR Titration—Titrations of AF-6 PDZ domain with peptidic ligands were performed by adding solid aliquots of the peptides to 250 μM solutions of the 15N-labeled protein. The 1H and 13N resonance variations were followed at 25 °C by collecting HSQC experiments. Combined chemical shift perturbation was calculated using the following equation,

$$\Delta \delta_{\text{exp}} = \sqrt{(\Delta \delta_{\text{H}})^2 + (\Delta \delta_{\text{C}})^2}$$

with a scaling factor (\alpha_\text{H}) of 0.17.

Structure Calculations—Approximate upper interproton distance restraints were from two NOESY spectra (13N-separated NOESY and 15N-separated NOESY). Nuclear Overhauser effects were grouped into four distance ranges: 3.0, 4.0, 5.0, and 6.0 according to their relative intensity. The chemical shift index was calculated for four types of nuclei: Cα, Cβ, C', and Hα. The derived secondary structure based on the consensus chemical shift index were converted into restraints on \phi and \psi angles. Hydrogen bond restraints were obtained by identifying the slow exchange amide protons located in regular secondary structures. Structures were calculated using the program CNS v1.1. The calculated structures were analyzed by the programs PROCHECK and MOLMOL.

Molecular Modeling of AF-6 PDZ Domain with C-terminal Pentapeptide of Neurexin or Bcr—To build up models of the AF-6 PDZ domain with the C-terminal peptide of the Neurexin or Bcr complex, we used a crystal structure of the complex of PDZ2 of Syntenin with a Syndecan-4 peptide (Protein Data Bank code 1oby) as a template. Structure alignment was performed using a CE (31) algorithm followed by molecular replacement. Molecular dynamics (MD) simulations were performed to optimize the models. First, 500 steps of steepest descent energy minimization followed by 300 ps of MD simulation at 300 K was carried out, with all heavy atoms of the protein and the ligand peptide backbone restrained to their respective starting positions by a harmonic potential, with a restraint force constant of 5.0E3 KJ/mol nm2. Then another 500-ps MD simulation was performed with position restraints applied to the heavy atoms of the residues out of the binding region including residues 13–24, 37–41, and 65–82. In the next step, the entire system was optimized by 500 ps of MD simulation without any restraints. Finally, the resulting configuration was minimized through stepwise simulated annealing; in a 400-ps simulation the temperature of the system was gradually reduced from 300 to 100 K. The GROMOS96 package (32) was used to perform these simulations with an implicit generalized-Born/solvent-accessible surface solvent model (33) was used to perform the simulations. The quality of the resulting models was checked using the program PROCHECK.

Coordinates—The coordinates of the structure of PDZ domain of AF-6 have been deposited in the Protein Data Bank (code 1T2M). Coordinates of the modeled PDZ-peptide complex are available from the authors on request.

RESULTS AND DISCUSSION

NMR Structure Determination of AF-6 PDZ Domain—The 1H, 13C, and 15N resonances of the AF-6 PDZ domain were assigned by standard methods using triple resonance experiments and 13N/15N-separated TOCSY and NOESY experiments. All of the backbone resonances were assigned, with the exception of the N- and C-terminal residues and the 15N resonance of proline. More than 95% of the side chain 1H resonances were assigned. A total of 1564 experimentally derived distance and dihedral angle restraints were utilized in the structure calculations (Table 1). Fig. 1A shows a stereoview of the superposition of a family of 20 lowest energy NMR structures. The 20 structures were selected from 200 accepted structures by requiring no nuclear Overhauser effect violation greater than 0.5Å and no dihedral angle violations greater than 5°.

1 The abbreviations used are: HSQC, heteronuclear single quantum correlation; NOESY, nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; MD, molecular dynamics; PSD-95, postsynaptic density-95.
As in other PDZ domains, a deep binding groove for substrates is critically determined by the interaction of the first residue of the disc-large protein PDZ3 (30). A hydrophilic amino acid at the position of αB1 makes AF-6 PDZ domain quite different from the canonical class II PDZ domain, as exemplified by the hCASK PDZ domain. We analyzed the structure features of this residue as well as the αB/βB groove in detail, hoping to gain insight into the target binding specificity of this PDZ domain. Fig. 2 compares the conformation of the αB/βB groove of the AF-6 PDZ domain with that of the hCASK PDZ domain and PSD-95 PDZ3, respectively. It is noteworthy that the αB/βB groove of the AF-6 PDZ domain is more similar to that of PSD-95 PDZ3, in which a His takes the αB1 position.

In the AF-6 PDZ domain, the side chain of Gln27 projects toward the αB/βB groove as the side chain of His does in the PSD-95 PDZ3. At the same time we noted that the position of βB5 is occupied by a small hydrophobic amino acid, Ala23. In the class II PDZ domain, a hydrophobic residue was also found at this position. But the side chain of this residue reaches to the αB/βB groove and forms a hydrophobic pocket with the residue at position αB1, whereas in the class I PDZ domain a highly conserved Gly is found at this position. In the structure of PSD-95 PDZ3, the presence of this Gly creates a turn of the peptide backbone away from the ligand. Because of the small size and different orientation of the side chain of Ala23, the N-terminal end of αB in the PDZ domain is closer to βB with respect to the αB helix in the hCASK PDZ domain. Fig. 3 shows a comparison of the surface representation of a αB/βB groove of the PDZ domains of AF-6, hCASK, and PSD-95 PDZ3. We found that the surface structure of the αB/βB groove of AF-6 PDZ domain is more similar to that of PSD-95 PDZ3 and does not have the second hydrophobic binding pocket, which exists in the hCASK PDZ domain to accommodate the hydrophobic or aromatic residue at the −2 position of ligand. We therefore deduce that the binding model of the AF-6 PDZ domain is different from that of the canonical class II PDZ, although the AF-6 PDZ domain was classified as class II.

Furthermore, the βA/βB connecting loop contains highly con-
served Gly-Leu-Gly-Phe sequences (GLGF repeats) in the class I and class III PDZ domains. These amino acids turn out to play an important functional role in binding the C-terminal carboxylate group of the peptide, and therefore the loop is referred to as the carboxylate-binding loop. In the AF-6 PDZ domain, the carboxylate-binding loop is replaced by the sequence GMGL. Despite the difference in sequence, the side chains of both the second and fourth amino acids in the GMGL motif are pointed toward the interior of the protein and are invariantly hydrophobic.

Although the structure of the αB/βB grooves of the AF-6 PDZ domain and the canonical third domain of PSD-95 are similar, differences are apparent. The AF-6 PDZ domain has a longer βB/βC loop with four extra residues, whereas it has one residue fewer in the βA/βB loop.

**C Termini of Neurexin and Bcr Directly Bind to AF-6 PDZ Domain**—It is controversial whether Neurexin can interact with the AF-6 PDZ domain (22, 23). To determine the dissociation constant of the AF-6 PDZ domain with the C-terminal peptides of Neurexin and Bcr, surface plasmon resonance measurements were carried out. Both peptides were coupled to a carboxymethyl-dextran CM5 sensor chip with an amine coupling kit. Binding was observed upon injection of different concentrations of the AF-6 PDZ domain (Supplemental Fig. 2). The plateau values reached after completion of the association reaction were analyzed by a Langmuir-binding isotherm. The dissociation constants of 4.08 \( \times 10^{-7} \) and 2.23 \( \times 10^{-6} \) M were obtained for Neurexin and Bcr, respectively. Furthermore, the peptide with the sequence KKNKDKEYV does not bind to the PDZ (data not shown). These results reveal that AF-6 can interact with the C-terminal peptide of Neurexin \textit{in vitro}, and the binding requires a C-terminal Val. This is consistent with the result that most of the known ligands of the AF-6 PDZ

**Fig. 1.** Structure of the AF-6 PDZ domain determined by NMR spectroscopy. **A**, stereoview of the final 20 lowest energy structures of the AF-6 PDZ domain, superimposed using backbone atoms (N, Ca, and C'). **B**, one representative structure of the AF-6 PDZ domain with the secondary structure element highlighted. The secondary structure elements are labeled following the scheme used in the crystal structure of PSD-95 PDZ3. Both **A** and **B** were generated using MOLMOL. **C**, amino acid sequence alignment of selected PDZ domains. The secondary structure of the AF-6 PDZ domain determined from this work is also included at the top of the sequence.
domain have a valine at the C terminus.

**Neurexin Binds to AF-6 PDZ Domain via aβββ Groove—**

Titration of the AF-6 PDZ domain with the C-terminal peptide of Neurexin led to intermediate exchange spectra in which the PDZ amide signals were extensively broadened at substoichiometric peptide concentrations. Titration was extended to a peptide:protein molar ratio of 8:1 that was sufficient to obtain narrow lines in the spectra of complexes except for Met17 (Supplemental Fig. 3A). The addition of the peptide leads to small chemical changes scattered over the whole protein that are typical of unspecific interactions together with larger chemical shift changes indicative of specific interactions on complex formation.

**FIG. 2. Comparison of the aβββ groove conformation.** A, AF-6 PDZ domain (red) and hCASK PDZ domain (green). B, AF-6 PDZ domain (red) and PSD95 PDZ3 (green). The program PyMOL was used to generate the figure.

**FIG. 3. Surface representation of the aβββ groove of PDZ domain.** A and B, AF-6 PDZ domain with C-terminal penta-peptide of Neurexin (KEYYV) and Bcr (FSTEV), respectively. C, hCASK PDZ domain. D, PSD-95 PDZ3. The hydrophobic amino acid residues are drawn in yellow, the positively charged residues are shown in blue, the negatively charged residues are shown in red, and the uncharged polar residues are shown in gray. The orientations of the molecules are the same as shown in Fig. 2. The program PyMOL was used to generate the figure.

Those residues experiencing the largest changes in chemical shift clustered in the C terminus of the carboxylate-binding loop and the β strand, such as from Gly18 to Ala22 (Fig. 4A and Supplemental Fig. 4A). Additionally, the hydrophobic pocket lined by Met17, Leu19, Ile21, Met77, and a highly conserved positively charged residue, Lys13, which functions to stabilize the side chain of V0 (Val at the C terminus of the ligand) and the negative charge of the carboxyl group of target peptide are found in the PDZ domain. These indicate that the Neurexin-derived peptide extends in the groove between the β strand and the αβ helix and antiparallels with βB as an additional strand, as most PDZ-binding ligands do.

As mentioned above, the position of aB1 is a unique hydrophilic residue, Gln, and the N terminus of aB is closer to βB with respect to the αB helix in the hCASK PDZ domain. We could not image the PDZ domain interacting with the −2 amino acid of the substrate peptide in the same mode as the hCASK PDZ domain. The results of titration show that Ala74 located in the αB5 is significantly perturbed. In the class I PDZ domain the position of residue Ala74 is occupied by a hydrophobic residue, whereas in hCASK it is a hydrophilic residue, Gln. It was reported that a hydrophobic residue at the αB5 position could supersede the role of the conserved hydrophobic residue at the αB1 position in the class II PDZ domain (35). We therefore postulate that the aromatic ring of Tyr at the −2 position in Neurexin make hydrophobic contacts with Ala74. At the same time, in Neurexin-derived peptide a tyrosine is located at the −1 position. Titration of Neurexin peptide causes a significant perturbation of resonance of Ser38 located in βC, which perhaps indicates that the side chain of tyrosine reaches across strand βB and inserts between the side chains of Ser38 and Val10.

In the study by Songyang et al. (30), a Glu is preferred at the −3 position of ligand for the AF-6 PDZ domain, implying a direct contribution to the specificity and affinity of interaction.
Structure analysis indicated the presence of a proximal basic residue at position β5 (Lys37). The side chain of Lys37 projects to strand βB (Fig. 3A) and can be involved in Coulombic interaction with Glu3 of the C-terminal peptide of Neurexin.

Bcr Binds to AF-6 PDZ Domain Also via aβB Groove—The peptide derived from the C terminus of Bcr was used to study its interaction with the AF-6 PDZ domain by NMR. The titration of the Bcr-derived peptide leads to a single set of resonance representing a population-weighted average of the bound and free forms of the PDZ domain (Supplemental Fig. 3B), which is characteristic of fast exchange. The resonance of Met17 disappeared when the titration began and never emerged again. The results of NMR chemical shift perturbation experiments are consistent with the BIACORE data. They reveal that the association and dissociation of the AF-6 PDZ domain with the C-terminal peptide of Neurexin and Bcr. In the diagram, the peaks of the free AF-6 PDZ domain are labeled Q70HE21 and Q70HE22, and the peaks of PDZ domain in the presence of 1:8 molar ratios of peptide are labeled Q70HE21# and Q70HE22#.

Notably, Ser81 and Ala45, which were distal from the peptide-
binding site, were affected by titration of both peptides. It is reported that proteins contain evolutionarily conserved “sparse networks” that physically link functionally distant sites in protein (39). Based on the statistical couplings (8), residues located in helix αA might be dynamically coupled to peptide binding. This is consistent with the result of perturbation. We presume that Ala45 may form a continuous van der Waals’ surface with Leu23 and be dynamically coupled to peptide binding. Ser31 is located in the ββ/αβ loop. In the glutamate receptor-interacting protein 1 PDZ6, the corresponding residue is Gly743, which exhibited the largest positional shift upon peptide (ATRTYRSC) binding by providing geometrical freedom to accommodate the side chain of Tyrβ (35). One possible explanation for the chemical shift change of Ser31 is that it may provide the conformational adaptation of helix αB for different ligands. This may indicate that the AF-6 PDZ domain is a very flexible interaction domain, with the potential to bind to different target sequences. Further more, Ala23 at the end of βB is affected by the addition of both kinds of peptides. It might be the result of the hydrogen bond formed between the O atom of Gln70 and the amide nitrogen atom of Ala23.

The Long ββ/αβ Loop Seems Not Involved in Recognition between AF-6 and Neurexin or Bcr—The ββ/αβ loop is highly variable in length and character among PDZ domains. The AF-6 PDZ domain has a long ββ/αβ loop that is four residues longer than that of PSD-95 PDZ2 but two residues shorter than that of PSD-95 PDZ2. Many extended ββ/αβ loops of the PDZ domain are involved in ligand interaction (34, 36). Importantly, the ββ/αβ loop of the PSD-95 PDZ2 contributes directly to the interaction with the neuronal nitric-oxide synthase PDZ domain by an unusual β-finger. Recently, it is reported that the AF-6 PDZ domain can interact with SPA-1 via an internal PDZ ligand motif (10). In our results, no resonances of residues in the ββ/αβ loop were perturbed. The ββ/αβ loop of the AF-6 PDZ domain seems not involved in interacting with Bcr or Neurexin.

Molecular Modeling of the AF-6 PDZ Domain—Peptide Complex—By MD simulations, we got the three-dimensional models of AF-6 PDZ-Neurexin and AF-6 PDZ-Bcr complexes (Fig. 3, A and B). Both of our models can well explain the results of the chemical shift perturbation experiment. The binding model of AF-6 PDZ-Neurexin is different from that of hCASK PDZ-ligand complex, in which a hydrophobic amino acid residue at αB1 makes a direct hydrophobic contact with the side chain of the Tyr at the −2 position of the ligand (16). In summary, although the AF-6 PDZ domain was classified as a class II PDZ domain, a unique hydrophilic amino acid, Gln70, at position αB1 makes the αB/βB groove of the AF-6 PDZ domain significantly different from that of the other class II PDZ domain. Both the Neurexin- and Bcr-derived peptides bind to αB/βB groove of the AF-6 PDZ domain, and each of the four C-terminal positions in both peptides may contribute to the specificity and affinity of the interaction. The structure of the AF-6 PDZ domain and the structural basis for the interaction of the AF-6 PDZ domain with Neurexin and Bcr represent an essential step in elucidating the interaction mediated by the PDZ domain of AF-6. Further work will try to obtain stable complexes of the AF-6 PDZ domain with ligand and to determine their high resolution three-dimensional structures, which will add to our growing knowledge of the biological functions of AF-6.