SOLITARY AND REPETITIVE BINDING MOTIFS FOR THE AP2 COMPLEX α-APPENDAGE IN AMPHIPHYSIN AND OTHER ACCESSORY PROTEINS

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Running Title: Classification of AP2 α-appendage-binding FxDxF and DxF/W motifs

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Adaptor protein (AP) complexes bind to transmembrane proteins destined for internalisation and to membrane lipids, so linking cargo to the accessory internalisation machinery. This machinery interacts with the appendage domains of APs, which have platform and β-sandwich subdomains, forming the binding surfaces for interacting proteins. Proteins which interact with the subdomains do so via short motifs, usually found in regions of low structural complexity of the interacting proteins. So far, up to four motifs have been identified which bind to and partially compete for at least two sites on each of the appendage domains of the AP2 complex. Motifs in individual accessory proteins, their sequential arrangement into motif-domains and partial competition for binding sites on the appendage domains coordinate the formation of endocytic complexes in a temporal and spatial manner. In this work, we examine the dominant interaction sequence in amphiphysin, a synapse-enriched accessory protein which generates membrane curvature and recruits the scission protein dynamin to the necks of coated pits, for the platform subdomain of the α-appendage. The motif domain of amphiphysin1 contains one copy of each of a DxF/W and FxDxF motif. We find that the FxDxF motif is the main determinant for the high affinity interaction with the α-adaptin appendage. We describe the optimal sequence of the FxDxF motif using thermodynamic and structural data and show how sequence variation controls the affinities of these motifs for the α-appendage.

Clathrin-mediated endocytosis is the process whereby proteins and lipids destined for internalisation from the plasma membrane are packaged into vesicles with the aid of a clathrin coat. Purified coated vesicles from brain contain three major components: clathrin, AP180 and AP2 complexes (1-3). Clathrin triskelia oligomerize to provide the scaffold around the forming vesicle (and can form similar cages in solution (4)). With its terminal domain, clathrin interacts with other endocytic proteins including the AP2 complex, AP180, epsin, disabled-2 (Dab2) and amphiphysin. These interactions are mediated via short motifs: for example, clathrin

binds to amphiphysin through motifs such as LLDLD or PWxxW (5,6). Because oligomeric clathrin presents an array of binding sites for these motifs it serves as a network hub, organizing binding partners within the lattice. The AP complexes, as well as many accessory proteins and alternative cargo adaptors such as AP180, Dab2, epsin and amphiphysin, recruit clathrin to PtdIns(4,5)P_2-rich areas in the membrane and promote its polymerization into a lattice. Due to its significant number of interaction partners, another key hub in the endocytic interactome is the AP2 complex (7-9). It consists of 4 subunits (α, β2, μ2 and σ2) and forms a stable heterotetramer in solution (10). Using electron microscopy it was shown that the AP2 complex can be subdivided into i), a trunk domain, which interacts with cargo proteins and PtdIns(4,5)P_2 and ii), two appendage domains made from the C-termini of the α and β-subunits, which interact with a large number of accessory proteins by binding to short motifs in these proteins. For example, the α-adaptin appendage binds to DxF/W, FxDxF, WxxF/W and FxxFxxL motifs (7,11-16). These can be highly clustered in motif-domains of the accessory proteins where they are also frequently found close to clathrin binding motifs. The appendage domains are connected to the trunk domain by flexible linkers, which lack a defined secondary structure in solution.

Though the AP2 appendages are only 16% identical in terms of their sequences, they are structurally very similar (12,17). We and others have previously proposed that both the α and β2 appendage domains bind to DxF/W motifs in accessory proteins (7,8,12,18). The DxF/W motifs on accessory proteins are often found in proline- rich regions. For example, rat epsin1 has 9 DPW motifs and the majority of these are found in a proline-rich stretch of 105 amino acids, a region that by CD spectroscopy has no obvious structure (19). Human Eps15 contains 15 DPF and two other DxF motifs in a stretch of 230 amino acids. These motifs may allow clustering of the AP2 complexes at the endocytic assembly zones and enhances the binding to PtdIns(4,5)P_2-containing membranes (8,20). DxF/W motifs bind to sites on the platform subdomains of the appendages, centered around a hydrophobic pocket (W840 in α; W841 in β2) (12,17) in a tight turn conformation (18). In their study, the authors also found a secondary binding site for DPW motifs on the β-sandwich subdomain of the α appendage. More importantly they found that an FEDNF peptide from amphiphysin binds to the top of the α appendage using the site around W840 but in a different conformation by inserting the first Phe residue into the hydrophobic pocket. It was therefore proposed that FxDxF is a general high affinity binding motif for the α-adaptin appendage. Recently, a fourth binding motif for the α-adaptin appendage has been identified in the proteins NECAP, stonin, synaptojanin, connecdenn and others (15,21-23). The binding motif consensus WxxF/W resembles the second clathrin binding motif PWxxW but the proline residue seems to mediate the discrimination between the binding partners.

Recently, we showed how multiple adaptin binding motifs provide an avidity effect where the overall apparent affinities for AP2 complexes will depend on the type, number and spacing of binding motifs as well as the clustering of available appendage domains (7,8). The unclustered cytoplasmic AP2 complex has single α- and β2- appendage domains whereas in the coated pit there will be many such domains from clustered AP2 complexes. However, when AP2 complexes are bound to polymerized clathrin the β2-adaptin appendage domain is predicted to be largely occupied by clathrin and steric hindrance is thought to exclude most accessory proteins. AP2 complexes at the edge of coated pits, where there is a lower concentration of clathrin, are predicted to still be free to bind the full complement of accessory proteins. The complement of accessory proteins bound to AP2 complexes in the cytosol is again proposed to be different (8). Thus, Eps15 has been shown to be restricted to the edges of a nascent coated pit as its interaction with AP2 adaptors is displaced by clathrin (24,25). A similar scenario applies for amphiphysin1 which contains an FxDxF, a DPF and a DLW motif, the latter two of which overlap with two clathrin binding motifs (Fig. 1A). As a result, amphiphysin would also be primarily localized to the edges of the invaginating pit, ending up at

the neck of the nascent vesicle. This would be a convenient way for amphiphysin to ensure that its C-terminal SH3 domain delivers dynamin to the neck of a coated pit while the N-terminal BAR domain of amphiphysin binds to PtdIns(4,5)P_2 and generates vesicle curvature in the membrane (26). The BAR domain is also responsible for the dimerization of amphiphysin which increases the efficiency of binding to the proline-rich domain in dynamin..

Mammals have 2 isoforms of amphiphysin (27) – amphiphysin1 and 2 (amph1 and 2). In here is sequence conservation of the domain in which these motifs occur. However, only the FEDNF motif is well conserved (as an FEDAF in amph2) while the DPF motif is EPL in amph2. The muscle form of amph2 (bridging integrator-1/Bin1) and amphiphysin in *Drosophila* have no DxF motifs in this region. In muscle, the protein is associated with T-tubule formation and not with clathrin/adaptor endocytosis. Accordingly, in *Drosophila* deletion of the protein gives a muscle weakness phenotype and a defect in T-tubule formation (28,29). Thus, the presence of a clathrin/adaptor-binding domain targets amphiphysin for function in clathrin-mediated endocytosis.

The selection of amphiphysin1 to study the individual contributions of different binding motifs for α-adaptin offers several advantages over other accessory proteins such as epsin1, Eps15 or AP180. First, amphiphysin is highly specific for the α-adaptin appendage and binds to other adaptins weakly (17), thereby avoiding avidity effects from interactions with other AP components. Second, amphiphysin contains single copies of the possible adaptin binding motifs which limits interference by avidity effects and makes mutagenesis straightforward. These motifs are separated by 20-30 residues which should be distant enough to avoid steric hindrance. Finally, in spite of the low number of motifs, the binding has a high affinity and amphiphysin is a major AP2 binding partner with an essential biological function. Using a combination of thermodynamic, biochemical and structural observations we show that the FxDxF motif is the main determinant for the high affinity binding to the α-adaptin appendage. Moreover, we define the optimal FxDxF motif and use these data to explain the observed binding characteristics of other FxDxF- and DxF/W- containing accessory proteins.

Experimental Procedures

Constructs and protein expression. The α-adaptin appendage domain (residues 701-938) and the appendage-plus hinge domain (653-938), the human β2-adaptin appendage domain (residues 701-937), rat amphiphysin1AB (Amph1AB: residues 1-378) and rat amphiphysin2AB (Amph2AB: residues 1-422) were expressed as N-terminal glutathione-S-transferase (GST) fusion proteins (in pGex4T2) in BL21 cells following overnight IPTG-induction at 22°C. All GST fusion proteins were purified from bacterial extracts by incubation with glutathione-sepharose beads, followed by extensive washing with 20mM HEPES, pH 7.4, 150mM NaCl, 1mM DTT. The fusion proteins were cleaved by incubation for 2 hours with thrombin, and further purified by passage over a Q-sepharose column. For isothermal titration calorimetry (ITC) experiments, the protein was additionally passed down a Superdex75 gel-filtration column and dialyzed into 100mM HEPES, pH 7.4, 50mM NaCl, 2mM DTT. GST-Amph1AB was not cleaved to prevent degradation of the protein. Myc-tagged proteins (in pCMV-myc) were used for expression in COS-7 fibroblasts. The appendage domain of human β2-adaptin (residues 701-937) was expressed in BL21 cells as an N-terminal 6xHis fusion protein (in pET-15b) and purified by passage over nickel-NTA, followed by Q-sepharose and gel filtration chromatography. Mutations were generated by PCR using overlapping primers incorporating the base pair changes.

Transfections, antibodies and cell extracts. COS-7 cells were transfected using GeneJuice (Novagen) according to the manufacturer's protocol. Over-expressed Amph1AB was detected using a polyclonal anti-Myc antibody (Cell Signalling, green in merged images). The endogenous AP2 complex was detected using a Sigma monoclonal (red in merged images). Cells

were imaged using a Bio-Rad Radiance confocal system. For extracts, two 70mm dishes of COS cells were scraped in 1ml PBS + 0.1% Tx-100, or one rat brain was homogenized in 10ml PBS + 0.1% Tx100 and cleared by centrifugation.

Pull-downs from COS-7 cell or rat brain extracts with GST-appendages. For interaction experiments, the extracts described above were incubated with 30-50µg of GST fusion protein on glutathione-sepharose beads for one hour at 4°C and then the bead bound proteins were washed 4x with 150mM NaCl, 20mM HEPES pH7.4, 2mM DTT, protease inhibitors and 0.1% Tx-100. Interaction partners were analysed by SDS-PAGE and western blotting. α and δ-adaptin and amphiphysin1 were detected using monoclonal antibodies from BD Biosciences, β1,2- and γ-adaptin were detected with monoclonal antibodies from Sigma.

Isothermal titration calorimetry. Binding of peptides and proteins to α- and β2-adaptin appendage domains was investigated by isothermal titration calorimetry (30) using a VP-ITC (MicroCal Inc., USA). All experiments were performed in 100mM HEPES, pH 7.4, 50mM NaCl, 2mM DTT at 10°C unless otherwise stated. The peptides or proteins were injected from a syringe in 40-50 steps up to a 3-4 fold molar excess. The cell contained 1.36ml protein solution and typically the ligand was added in steps of 4-8µl every 3.5min. Concentrations were chosen so that the binding partners in the cell were at least 5 fold higher than the estimated dissociation constant, if possible. The ligands in the syringe were again at least 10 fold more concentrated. Titration curves were fitted to the data using ORIGIN (supplied by the manufacturer) yielding the stoichiometry N, the binary equilibrium constant K_a (= K_d^{-1}) and the enthalpy of binding ΔH. The entropy of binding ΔS was calculated from the relationship $\Delta G=-RT\cdot\ln K_a$ and the Gibbs-Helmholtz equation. The values were averaged from to two three titrations. Protein concentrations were determined by measuring the OD_{280}. Peptides were purchased at >95% purity from the Institute of Biomolecular Sciences, University of Southampton, UK and weighed on an analytical balance. Where possible, peptide concentrations were verified by measuring OD_{280} or OD_{257}. The resulting errors on the concentrations are estimated to be <10% for the proteins and the peptides. Unless otherwise stated, the values for the stoichiometry N were within this error region around N=1.

Crystallography and structure determination. Co-crystals of α-adaptin appendage and the synaptojanin WVxF peptide were grown by hanging-drop vapor diffusion against a reservoir containing conditions centered around 1.2M ammonium sulphate, 3% isopropanol and 0.05M sodium citrate. Hanging drops were 2µl and contained 222µM α-adaptin appendage and 277.5µM synaptojanin WVxF peptide (sequence: NPKGWVTFEEEE). Crystals were obtained after incubation for approximately 1 week at 18ºC. To obtain crystals containing peptides bound to the top side, α-adaptin appendage-WVxF co-crystals were soaked in a solution of mother liquor containing amphyphysin1 FEDNFVP peptide. Crystals were cryo-protected by transfer to Paretone-N (Hampton Research) and were flash-cooled in liquid nitrogen. Data to 1.6Å were collected at 100K at Station 9.6 SRS Daresbury, UK. Crystals were monoclinic and belonged to spacegroup C2 (a=146.6Å, b=67.3Å, c=39.7Å, β=94.53º). Data collection statistics are shown in Supp. Table 1. Reflections were integrated using MOSFLM (31) and were scaled using the CCP4 suite of crystallographic software (32). A difference Fourier, calculated using our model of the α appendage bound to the WVxF peptide (unpublished), revealed beautiful density for the amphiphysin peptide. The model was completed using COOT (33) and O (34) and was refined using REFMAC5 (35). The validated coordinates and structure factors for the crystal structure containing the synaptojanin WVxF and the amphyphysin1 FEDNFVP have been deposited in the protein data bank (36) (PDB id: XXXX). Figures were generated using Aesop (Martin Noble, personal communication) and the peptide interaction map was generated using the output from LIGPLOT (37) as a starting point.

Results

The region in amphiphysin1 necessary for binding to the AP2 complex has been mapped to residues 322-340 and binding is enhanced if the region is extended to 322-363 (38). This comprises the FEDNF[328] and the DPF[359] motifs (Fig. 1A). A further extension to include the PWxxW had no additional effect on binding. Using isothermal titration calorimetry, we found that a construct from residues 1-378 binds to the α-adaptin appendage with an affinity of 1.6μM but not to the β2-adaptin appendage (Fig. 1B) This domain forms a dimer due to the N-terminal BAR domain (26). The affinity is very similar to the one measured for the 12mer DNF-peptide INF**F**E**D**N**F**VPEI (7). The stoichiometry for interaction between the α-adaptin appendage and the amphiphysin protein as well as the DNF 12mer is 1:1. A longer construct, residues 1-390, comprising the FxDxF, DPF and the PWxxW motifs, had a similar affinity, though a contribution of a second very weak binding site becomes visible (data not shown). Thus, the FEDNF in amphiphysin1 is the major determinant for the binding to the α-adaptin appendage.

Adaptor binding by amphiphysin via FxDNF and DPF motifs. We extended these observations by mutagenesis of both motifs sequentially and simultaneously and show that AP2 binding to the DNF motif at residue 326 is stronger than binding to the DPF at residue 357 (Fig. 2A). Mutations of both DNF and DPF motifs to SGA are even more effective than the single mutants in disrupting binding. In pull-down experiments from brain and COS-7 cell extracts, α- and β-adaptins follow the same pattern, because they are part of the same complex (AP2). We already know from Fig. 1 that amphiphysin is specific for α-adaptin. There is also no interaction of γ and δ adaptins with amphiphysin, showing that AP1 and AP3 complexes in COS-7 extracts do not bind to amphiphysin (Fig. 2A). From this it is clear that both motifs contribute to AP2 binding but there is a clear difference in affinity for the α-adaptin appendage. In amph2 the FEDAF motif is the major binding sequence for AP2 adaptors (Fig. 2C).

Given the strong effect of mutagenesis of the FxDxF motifs in amphiphysin1 and 2 we tested how well other residues might substitute (Fig. 2B and 2C). The initial surprise was that FxDPF does not substitute for FxDNF but that FxDPW does. The structural basis for this is not clear but different peptides can bind in different conformations. The FEDNF peptide from amphiphysin and the FKDSF from synaptojanin bind in an extended conformation where the first F in this binds into the pocket formed by F836, F837 and W840 (7,18). This cannot be the case for most DxF/W peptides which do not have an equivalent Phe residue and the proline residue in the DPF/W motifs forces the peptide into a loop structure. Binding is also possible when the Asn residue in the FEDNF motif of amphiphysin1 is replaced by Ser, Ala, Asp or Ile, while Gly and Leu abolish the interaction. For amphiphysin2 we looked at a more limited set of substitutions (Fig. 2C), but again the change of FxDAF to FxDPF weakens the interaction. The FxDxF is the major binding motif of amphiphysins, however we should be very cautious in extending this observation to other proteins which have other non-conserved residues (see below). An interesting observation was that the presence of the hinge domain of the α-adaptin appendage increased the binding of amphiphysin1 to the top binding site (Fig. 2D). This is reminiscent of the interaction of the hinge region of β-adaptin with the clathrin terminal domain. Addition of a peptide derived from the proline rich region of dynamin did not abolish this effect so that an interaction between a proline rich stretch in the α-hinge with the SH3 domain of amphiphysin can be ruled out (data not shown).

Use of DxF in vivo. We and others have shown that overexpression of DxF domains can inhibit endocytosis (3,39-41). In the case of amphiphysin, overexpression of the full-length protein in COS-7 cells inhibits endocytosis of transferrin (27,38) and this can be rescued by co-expression of dynamin. Thus, part of the inhibitory phenotype is due to sequestration of dynamin. This effect can be mimicked by overexpression of the SH3 domain (42). Given that amphiphysin has a very low affinity for

dynamin in solution (100-200μM) but binds to dynamin very tightly when bound to beads implies that the clustering of amphiphysins results in a strong avidity for dynamin. Thus, if we can prevent the clustering of amphiphysin by blocking the recruitment to sites of endocytosis by mutagenesis of the DxF motifs we should prevent the inhibitory potential of the overexpressed protein. Fig. 3A shows that overexpression of WT amphiphysin1 inhibits transferrin uptake and results in a redistribution of AP2 adaptors, when compared to non-transfected control cells at the same plane of focus. Point mutants of the DNF and the DPF motifs partially rescue the transferrin uptake inhibition phenotype as well as the adaptor redistribution. The rescue is most efficient with the FxDNF+DPF double mutant (Fig. 3B). For the single mutants the FEDNF → FESGA protein shows a weaker inhibition of endocytosis than the DPF → SGA version. We conclude that both motifs of amphiphysin are indeed involved in binding AP2 adaptors in vivo. Also, as argued above, this experiment underlines that dynamin is recruited to sites of endocytosis by amphiphysin if it is clustered at active zones.

Affinities of FxDxF motifs. We have shown that DPF peptides have an affinity for the α-adaptin appendage in the range of 100μM (12). Recently, we found that the FxDxF peptides from amphiphysin1 and the 170 Kd isoform of synaptojanin have a higher affinity; in the range of 2-20μM (7). The mutagenesis presented here confirmed the stronger binding of the FxDxF motif of amphiphysin to the AP2 adaptor compared to the DPF motif. Therefore we tested peptides from these regions to measure their affinities directly with purified α-adaptin appendage domain in order to explain the contribution of individual residues (Fig. 4A, 4B and Table I). From our previous work we had found that a 7mer peptide (**F**E**D**N**F**VP) is sufficient for binding but residues distant to the motif contribute to binding leading to a tenfold stronger interaction of the Amph1-DNF 12mer (INF**F**E**D**N**F**VPEI). This peptide does not bind to the β2-adaptin appendage domain, again confirming our results from pull-down experiments, but is able to block the slow phase of endocytosis in isolated nerve terminals of bipolar cells (43). By mutagenesis we showed that the DPF motif of amphiphysin1 contributes less to binding of AP2 adaptors, and likewise a 12mer peptide containing this motif (LDLDF**DPF**KPDV) has a lower affinity (~200μM) which explains why we only saw a very small contribution from this motif in the titration of amph1 1-378 with the α-adaptin appendage or in the bipolar cells. Substitution of DNF for DPF in this peptide does not increase the affinity. However, substitution of DPF for DNF in the Amph1-DNF 12mer reduces the affinity by over 40 fold to about 100μM although the peptide still follows the proposed FxDxF consensus sequence. The reduction is smaller if FxDPW is used instead of FxDNF with a K_d of about 60μM. Obviously, the larger tryptophan can make stronger hydrophobic interactions with α-adaptin but we do not know whether it interacts with the pocket around W840 in the mode of a DPW motif or a with the more shallow pocket around Ile853 like the second phenylalanine in an FxDxF motif. The importance of the interaction of the first Phe from FxDxF motifs was tested by swapping the residues F and E before the FxDNF and this led to a drop of the affinity to about 180μM. Exchanging the FxDNF to FxDDF or FxDSF (corresponding to the residues of human synaptojanin1 p170) keeps the affinity below 10μM, and exchange to FxDAF or FxDIF (corresponding to the residues of amphiphysin2 and HIP1, respectively) results in a medium affinity of about 20-30μM. The FxDAF peptide from amphiphysin2 has a similar affinity as the FxDNF to DAF exchange in amphiphysin1.

Taken together, the changes of the affinities perfectly match the order we obtained from the pull-down experiments with amphiphysin mutants (Fig. 2B and 2C). All the peptides tested have a stoichiometry of 1:1 except Amph1-DPF 12mer where two peptides bind to each appendage domain. This peptide contains an overlapping clathrin box (LLDLD) and also contains a version of an FxxF motif which can also interact weakly with α-adaptin (7,11).

Given the different affinities of amphiphysin peptides that all follow the FxDxF consensus motif, we looked at possible high affinity

interaction motifs in other endocytic proteins by sequence comparisons. A 12mer peptide of the FxDLF sequence in Dab2 had a very low affinity. Previously, we found weak affinities for an FxDPW peptide from epsin1, and the same applies to an FxDPF peptide from Eps15, an FxDAF peptide from AP180 and an FxDPF peptide from the phospholipase A2 activating proteins (PLAA). A medium affinity was found for an FxDGF peptide from mouse Eps15. However, we know that this is not dependent on the FxDxF but the overlapping presence of an FxxFxxL motif (7). Another medium affinity interaction was found for an FxDIF from Huntingtin Interacting Protein 1 (HIP1). From this we conclude that FxDxF is not a general high affinity binding motif for α-adaptin but that certain residues at the fourth position are favorable for binding (N, S, A, I and D) and that additional determinants in the surrounding contribute as well. Recently, a variation of the FxDxF α-adaptin binding motif has been described in the AP180 homolog clathrin assembly lymphoid myeloid leukemia protein (CALM), where the aspartic acid is replaced by a serine (44). We could not detect binding of a corresponding 12mer peptide to the α-adaptin appendage but changing the FEDNF in the amphiphysin1 12mer to FESNF had only a very mild effect leading to a threefold decrease in affinity.

In summary, the affinity of the DNF 12mer from amphiphysin1 explains how this protein can be a major ligand for the AP2 complex although it contains only two binding motifs, especially when amphiphysin dimers bind to clustered AP2 in assembly zones. Therefore, by predicting endocytic proteins only on the basis of having multiple DxF/W motifs we would have missed such proteins. If DxF/W motifs are simply a means of ensuring recruitment to an adaptor complex, then one high affinity motif will substitute for multiple low affinity motifs which will work rather like Velcro.

Affinities of DxF/W motifs. Many accessory proteins contain multiple copies of DxF/W and not necessarily a high affinity FxDxF of WxxΦ motif and some of them are still able to interact significantly with the α-adaptin appendage. In order to check whether the affinity of the DPF motif of amphiphysin1 is typical also for other DxF/W motifs, we measured the binding of several peptides derived from Eps15, epsin1, PLAA and Dab2 (Fig. 4A and 4B). None of these peptides containing a single DxF/W motif bound tighter than 200μM and the affinities were only slightly greater in the case of double DPF peptides from Eps15 and Dab2. It is worth noting that these peptides bound with a 1:1 stoichiometry to the α-adaptin appendage implying the need for a certain distance between DxF/W motifs to bind simultaneously to two α-adaptin appendages. This had also been observed with complete motif domains, where the maximum number of the α-adaptin appendage domains bound was lower than the number of DxF/W motifs (7).

High resolution structure. In order to better understand the interaction between the FxDxF motif from amphiphysin and the α-adaptin appendage domain we solved the structure of the core Amph1 FxDNF 7mer peptide bound to the α-adaptin appendage at 1.6Å resolution. The structure was obtained by soaking the FEDNFVP peptide into a crystal of the α-adaptin appendage in complex with a WVxF peptide from synaptojanin (sequence: NPKGWVTFEEEE) bound to the side of the sandwich domain. An amphiphysin peptide has previously been crystallized with the α-appendage(7,8,12,18) but our higher resolution allows us to analyze the interaction of the α-adaptin appendage with the peptides in greater detail (Fig. 5). The core of the sandwich subdomain-binding WVxF peptide interacts as in the previously published structure (PDB ID 1W80 (7)). All residues of the amphiphysin peptide, with the exception of the side-chain of Glu, are well resolved in the structure and all except the Glu and the Pro are involved in the interaction with the α-adaptin appendage. The two bulky side-chains of the Phe residues interact with the hydrophobic sites around W840 and Y915 as previously described. The side chain of the Glu corresponding to the first x of the FxDxF is flexible and not visible. This Glu may bond, via a water, to R920 of the α-adaptin appendage, though clearly the side chain is

highly flexible. This interaction is also possible for the FEDNF from rat synaptojanin and maybe the FDDxF motifs from amphyphysin2, HIP1 and CALM but not for the FKDSF from human synaptojanin which was used in our previous structure. A crucial interaction for the motif is the salt bridge between the conserved Asp and R916 from the α-adaptin appendage. However, recent data by Meyerholz et al. (44), as well as our Amph1 FxSNF peptide, suggests that a hydrogen bonding partner like S is sufficient for binding in certain contexts. The interactions with R905 of the α-adaptin appendage determine the specificity at the x2 position of the FxDxF sequence. In this and the previous structures, the x2 residue work as acceptors for a hydrogen bond. The interaction with an FxDDF could even increase the contribution from this position by ionic attraction. It is not clear, however, why Ile is tolerated at this position but Val and Leu are not. Taken together, the core consensus sequence for a medium to high affinity binding motif for the α-adaptin appendage is F[E/D][D/S][N/S/D/A/I]F.

Predictions of trafficking proteins that bind appendage domains. Many of the endocytic proteins that bind to adaptors contain multiple copies of DxF/W motifs clustered in so-called motif domains. Using a specially written computer program (http://www.mrc-lmb.cam.ac.uk/genomes/madanm/harvey/) we find that DxF/W motifs occur more often than expected in human, worm, fly, slime mold and yeast protein non-redundant databases. We found that the easiest way to filter the results with confidence was to search for the co-occurrence with other endocytic motifs. A number of other motifs appear in endocytic proteins like the α-adaptin appendage binding motifs WVxF or FxxFxx[F/L], the clathrin terminal domain-binding motifs LLDLD or PWxxW, or the Eps15-homology (EH) domain binding sequence NPF. The program allows the user to search any subset (or even all) of the results for the coincidence of any motifs. For example, a search for F[E/D][D/S][N/S/D/A/I]F, to look only for high affinity α-adaptin appendage binding proteins, and the co-ocurrence of a DPF motif, leads to about ten hits in the human database. As well as the expected appearance of amphiphysin and HIP1, a MAPKKK, a tyrosine kinase and a Kelch-BTB homologue were also identified. This program will facilitate the search for any novel motif-containing proteins across a wide range of sequenced genomes. This may be of interest to any investigators working in fields where short motifs are important binding determinants.

Discussion

Amphiphysin fulfils a critical function in clathrin-mediated endocytosis by inducing curvature at the neck of a coated-pit while at the same time recruiting the scission molecule dynamin to this position (45). Amphiphysin itself is recruited to sites of endocytosis via its interactions with phospholipids in the plasma membrane (26,28,46), with the α-adaptin appendage domain of the AP2 complex (12,47) and the N-terminal domain of clathrin (5,48). It contains only two binding motifs for the α-adaptin appendage domain and interacts only weakly with the β2-adaptin appendage domain. This is in contrast to many other endocytic proteins like human Eps15 with 15 DPF, epsin1 with 9 DPW or AP180 with 4 DPF motifs. Nevertheless, amphiphysin is a major AP2 binding protein (7,27). Therefore we set out to investigate the molecular basis for the high affinity that the motifs in amphiphysin must have in order to achieve efficient binding.

In recent crystallization experiments, the platform subdomain of the α-adaptin appendage has been proposed to bind ligands in two different modes. The DPF/W motif binds with the Phe or Trp in a large hydrophobic pocket centered around W840. In the second binding mode, FxDxF motifs bind with the first Phe in the W840 pocket (7,18). In the first mode the proline will allow the necessary kink in the backbone to allow the Asp to form a hydrogen bond with the backbone of the peptide and also to interact with R905 via a water. A first proposal of this mode of binding would be that the surrounding residues will have very little influence on the binding affinity. We found that in amphiphysin1 the FxDxF motif is the major determinant for the interaction with the α-

adaptin appendage domain *in vitro* and *in vivo*. This is underscored by the similar affinities of the DNF 12mer peptide and the construct comprising the N-terminal BAR domain and the motif domain. The DPF motif is also able to interact with the α-adaptin appendage domain although with an affinity that is at least an order of magnitude weaker than the FxDxF motif.

The FxDxF motif was proposed to be responsible for the interactions of other endocytic proteins containing this sequence (7,13,18). We showed that this is a strong binding motif for the α-adaptin appendage in amphiphysin1 and the 170 kDa isoform of synaptojanin (7), however, the generalization does not hold true for many other proteins such as Dab2, Eps15, AP180 and epsin1. In fact, by mutating this motif in the motif domain of amphiphysin1 and by titrations of peptides with different variants of this motif we found that the fourth residue in the FxDxF motif is critical for a high affinity interaction. The interaction of this residue with the α-adaptin appendage is strong with residues like Asn, Ser and Asp. These residues are found in motifs from amphiphysin1 and the 170 kDa isoform of synaptojanin which have a high affinity because they can interact with the side chain of R905 of the α-adaptin appendage. Small residues like Ala, Ile are also tolerated and give rise to a medium affinity, such as that observed in amphiphysin2 and HIP1. Other residues like Val or Leu weaken the interaction such that the affinity is similar to a standard DPF/W motif. The results were confirmed in titration experiments using FxDxF peptides from different proteins (Table I). The weak FxDxF motifs have affinities in the same range as isolated DPF/W motifs and may well contribute to binding when they are clustered together like in AP180, CALM or Dab2 (12,44,49) or combined with a β-sandwich binding motif like in connecdenn (21).

The first x in the FxDxF is very flexible in our structure, indicating looser requirements here. In the case of the FxDPW motifs of epsin1 and PLAA the precise binding mode has not been conclusively resolved by our study and further structural data are needed to clarify whether they adopt the extended FxDxF or the DPF/W loop conformation. Certainly, we conclude from this that there are different modes of binding centered around the large hydrophobic pocket on the α-adaptin appendage involving F836, F837 and W840 with the surrounding charged and hydrophobic residues influencing the strength of the interactions. Together with the WVxF motif binding to the side of the β-sandwich sub-domain and the still unidentified binding site for the FxxFxxL motif, the α-adaptin appendage domain is able to interact with up to four different types of motifs.

Recently, it has been reported that the N-terminal region of NECAP also interacts with FxDxF motifs with a similar affinity to the α-adaptin appendage (50). The structure of this domain revealed that it belongs to the PH domain superfamily but the lipid binding residues are not conserved. The relative affinities for FxDxF motifs from several endocytic proteins showed that the NECAP PHear binding consensus sequence differs from that of α-adaptin appendage domain. Since NECAP also interacts strongly with the side site of the α-adaptin appendage domain via its C-terminal WVxF motif they may indeed serve as a third appendage domain of the AP2 complex.

Short sequence motifs occurring in repeats also play a role in other trafficking pathways. Well studied examples are the FG motifs of nuclear pore proteins, often placed in FSFG or GLFG motifs, which interact with cargo carriers (51-53). Similarly, FxxΦ motifs have been shown to interact with γ-appendage domains in the AP1 complex and GGA proteins (11,14,54). Many endocytic proteins including epsin, CALM, stonin2 and Dab2 have NPF motifs which mediate the interaction with Eps15 homology (EH) domains of proteins like Eps15 and intersectin (55,56). In contrast to single high-affinity motifs such as phospho-YxxΦ motifs binding to SH2 domains, NPxY motifs binding to PTB domains or the FxDxF or WVxF for the α-adaptin appendage discussed here, a chain of repeated low affinity sequences offers additional mechanisms for regulation. They can sample density of binding partners using avidity effects, and by placing other types of binding motifs (e.g. for clathrin) into the repeat they can interact with different binding partners in a cooperative or exclusive manner depending on

the number, the sequential order and the spacing of the motifs (5,7,8,18,22,24). We and others have shown that the same mechanism is also used in AP1 and GGA dependent trafficking from the Golgi, where FxxF/W motifs interact with the γ- and GGA-appendage domains (11,14,16,57-59).

A further level of complexity is added by the fact that several α- or γ- appendage binding proteins also interact with the β1- and β2-adaptin appendage domains which are also present in the AP1 and AP2 complexes (7,8,11,17,24,60,61). Since the binding sites on the appendage domains and the binding motifs are at least partially overlapping, the affinity, copy number and the relative position of the motifs determines the specific recruitment of an accessory protein to a clathrin-coated pit. Amphiphysin is the major recruiter of dynamin to the neck of a clathrin-coated pit at the plasma membrane and is specific for the α-adaptin appendage only. There should be other proteins taking over this function at the Golgi. Good candidates are sorting nexin 9 and the pacsins, which, like amphiphysin, contain BAR domains for membrane interactions and SH3 domains for dynamin recruitment.

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Footnotes

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Abbreviations

AAK: Adaptor associated kinase
Amph: Amphiphysin
AP180: Adaptor protein of 180 kDa
AP2: Adaptor protein complex 2
BAR: Bin2/amphiphysin/Rvs
CALM: Clathrin assembly lymphoid myeloid leukemia protein
CCV: Clathrin-coated vesicle
Dab2: Disabled 2
Eps15: Epidermal growth factor receptor pathway substrate 15
Epsin: Eps15 interacting protein
GAK: Cyclin G-associated kinase
GST: Glutathione-S-transferase
HIP1: Huntingtin interacting protein 1
ITC: Isothermal titration calorimetry
PLAA: Phospholipase A2 activator

Figure Legends

Fig. 1. Interaction of amphiphysin with appendage domains. *A*, schematic representation of the domain architecture of mammalian amphiphysin. The motif domain is located between the N-terminal BAR and the C-terminal SH3 domain. The blow-up shows the sequence conservation between rat amphyphysin1 and 2. Red blocks represent the DxF motifs which were used as peptides to determine the specificity of the interaction and yellow blocks represent clathrin binding motifs. *B*, profiles of typical calorimetric titrations of the α-adaptin and β2-adaptin appendage domain into a solution of GST-amphiphysin1 (1-378), (upper panel) at 10 °C and the integrated data normalized to the concentration of the appendage domain (lower panel). The fit yields a dissociation constant of 1.6μM, ΔH of -16kcal/mol and ΔS of -30 cal/mol*K.

Fig. 2. Contributions of the two DxF motifs in amphiphysins 1 and 2. *A*, GST-fusions of the N-terminal 378 residues of rat amphiphysin1 (Amph1AB) and mutations of the DxF motifs to SGA were used to probe interactions with AP1, AP2 and AP3-complexes in rat brain and COS7 extracts. *B*, mutational analysis of the FEDNF motif in Amph1AB binding to the AP2 complex. Mutations are made on the background of a functional DPF motif. Longer exposure of the blots confirmed that the limited contribution made by this motif is still functional (data not shown). *C*, mutational analysis of the FDDAF in motif in Amph2AB binding to the AP2 complex. *D*, enhanced binding of amphiphysin1 to the α-adaptin appendage+hinge domain.

Fig. 3. Function of DxF motifs *in vivo*. *A*, full-length amphiphysin1 overexpressed in COS7 cells (green) causes a redistribution of the AP2 puncta (red) from the membrane and inhibits uptake of transferrin (blue). *B*, the double mutant FxSGA / FxSGA has a small effect on AP2 distribution. *C* and *D*, the single SGA mutant of the DPF motif shows a stronger inhibition of the AP2 distribution than the mutant of the DNF motif, consistent with their affinities for AP2 adaptor complexes. Scale bar – 10 μm. *E*, quantification of the data for which representative cells are shown in *A*-*D*. Shown for each transfection are the mean ± standard deviation σn-1 for the percentage of transfected cells showing a wild-type AP2 distribution (black bars) and wild-type transferrin uptake (grey bars). The number of cells quantified for each data point are as follows: WT – 87; FxSGA / FxSGA – 76; FxSGA / FxDPF – 148; FxDNF / FxSGA – 85.

Fig. 4. Specificity of FxDxF/W and DxF/W motifs for the α-adaptin appendage domain. *A*, profiles of typical calorimetric titrations of Dab2-FxDLF, HIP1-FxDIF and Amph1-DNF 12mer peptides into a solution of the α-adaptin appendage (upper panel, top to bottom) and the integrated data, normalized to the concentration of the DNF-peptides (lower panel). *B*, profiles of typical calorimetric titrations of PLAA-DPF1, Amph1-DPF 12mer and a triple DPF peptide into a solution of the α-adaptin appendage (upper panel, top to bottom) and the integrated data, normalized to the concentration of the DNF-peptides (lower panel). The results of the fits are listed in Table 1

Fig. 5. High resolution structure of FxDxF peptide with the α-adaptin appendage domain. *A*, ribbon diagram of the 1.6 Å structure of the α-adaptin appendage with the WxxF peptide from synaptojanin bound to the side of the sandwich subdomain (green) and the FEDNFVP 7mer peptide from amphiphysin1 bound to the platform sub-domain (gold). *B*, The density for the amphiphysin peptide. All residues can be clearly seen, with the exception of the E, as discussed in the text. *C*, details of the FxDxF peptide binding sites showing interacting α-adaptin appendage residues and coordinated waters (blue) involved in binding. *D*, the FEDNFVP peptide displayed as a linear chain showing hydrogen bonding and ionic interactions (dashed green lines) and hydrophobic interactions (grey lines). The most important peptide residues are in italics. *E*, A comparison of the modes of

binding of the synaptojanin FKDSF motif (cyan) (pdb id 1W80), the amphiphysin FEDNF (purple, this work) and a DPW from epsin (blue) (pdb id 1KYD).

Table I Binding constants of peptides measured by ITC. Dissociation constants for the interactions of peptides and the α-adaptin appendage as determined by ITC at 10°C. Note that the dissociation constant Kd is the inverse of the association constant Ka obtained from the binding experiments. The values for ΔH and TΔS are given for the association reaction. The experiments marked with an asterisk have been determined previously (7).

Table 1

Peptide name	sequence	K_d (μM)	ΔH (kcal mol^{-1})	TΔS (kcal mol^{-1})
Amph1-DNF 7mer*	**F**E**DNF**VP	21	-6.9	-0.8
Amph1-DNF 8mer	**F**E**DNF**VPE	28	-7.5	-1.6
Amph1-DNF 12mer*	INF**F**E**DNF**VPEI	2.5	-9.8	-2.5
Amph1-DNF to DPF 12mer	INF**F**E**DPF**VPEI	100	-6.7	-1.5
Amph1-DNF to DPW 12mer	INF**F**E**DPW**VPEI	52	-11.1	-5.6
Amph1-DNF 12mer swap	INFE**FDNF**VPEI	180	-7.7	-2.8
Amph1-DNF to DDF 12 mer	INF**F**E**DDF**VPEI	3.7	-10.2	-3.1
Amph1-DNF to DSF 12 mer	INF**F**E**DSF**VPEI	5.4	-10.6	-3.7
Amph1-DNF to DAF 12mer	INF**F**E**DAF**VPEI	21	-6.5	-0.4
Amph1-DNF to DIF 12mer	INF**F**E**DIF**VPEI	29	-8.7	-2.8
Amph1-DNF to SNF 12mer	INF**F**E**SNF**VPEI	7.4	-10.1	-3.5
Amph1-FKDNF 12mer	INF**F**K**DNF**VPEI	4.4	-11.8	-4.8
Amph2-DAF 12mer	LSL**F**D**DAF**VPEI	12	-9.4	-3.0
Amph1-DPF 12mer	LDLDF**DPF**KPDV	190	-4.4	0.4
Amph1-DPF to DNF	LDLDF**DNF**KPDV	> 300		
Syjn-FKDSF*	LDG**F**K**DSF**DLQG	27	-14.3	-8.4
Syjn-FEDNF*	LDG**FE****DNF**DLQS	4.5	-11.9	-5.0
Dab2-FxDLF	QSN**F**L**DLF**KGNA	> 300		
Eps15-FxDPF	**DMF**C**DPF**TSST	> 300		
Eps15-FxDGF*	S**F**G**DGF**ADFSTL	140	-12.7	-5.5
Eps15-FxGGF*	S**F**G**GGF**ADFSTL	120	-14.0	-4.6
AP180-FxDAF	I**DLF**G**DAF**GSSA	> 300		
Epsin1-FxDPW*	APA**F**S**DPW**GGSP	200	-10.0	-5.2
PLAA-FxDPF	NPS**F**S**DPF**TGGG	250	-3.2	1.4
Hip1-FxDIF	DNK**F**D**DIF**GSSF	94	-14.7	-9.5
CALM-FxSVF	NVD**FE****SVF**GNKS	> 300		
TripleDPF	**DPF**KD**DPF**VGS**DPF**	96	-8.2	-2.9
Dab2-DPF	PNP**DPF**RD**DPF**AQP	250	-14.4	-9.7
Eps15-double	TST**DPF**TTSST**DPF**SAS	140	-8.2	-3.2
Eps15-DCF	PFAS**DCF**FKQT	> 300		
Epsin-DPW	GPPSS**DPW**APAP	> 300		
PLAA-DPW	YNTSD**DPW**LTAY	> 300		
PLAA-DPF1	TLPTA**DPF**TGAG	290	-2.4	2.2
PLAA-DPF2	TMAGV**DPF**TGNS	230	-2.4	2.3
GAK-DPFDQF	TV**DPFDQF**LLPS	240	-4.7	0.0
GAK-DLF	KP**DLF**GEFLNSD	> 300		

Figure 1

A


BAR
SH3
rAmphiphysin1 321 INFFEDNFVPEINVTTPSQNEVLEVKKEE.TLLDLDFDPFKPDVTPAGSAAATHSPMSQTLPWDLW 385
rAmphiphysin2 360 LSLFDDAFVPEISVTTPSQFEAPGPFSEQASLLDLDFEPLPPVASPVKAPTPSG....QSIPWDLW 421

B

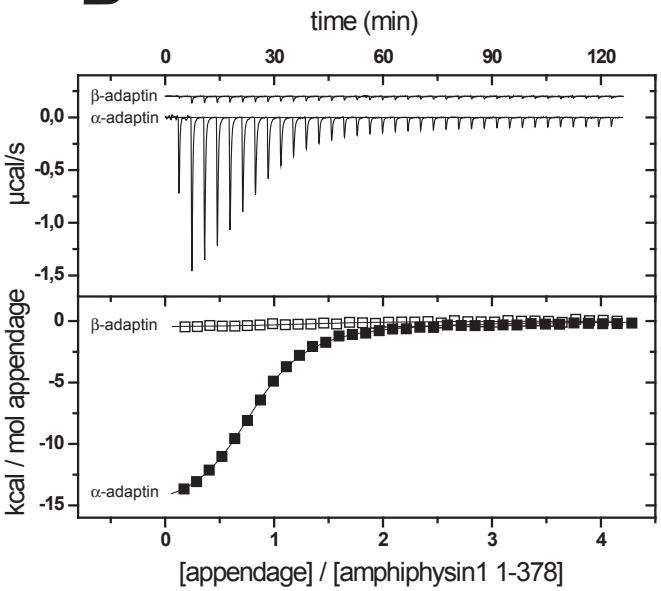

time (min)
0
30
60
90
120
β-adaptin
α-adaptin
0,0
-0,5
-1,0
-1,5
µcal/s
β-adaptin
α-adaptin
0
-5
-10
-15
kcal / mol appendage
0
1
2
3
4
[appendage] / [amphiphysin1 1-378]

Figure 2

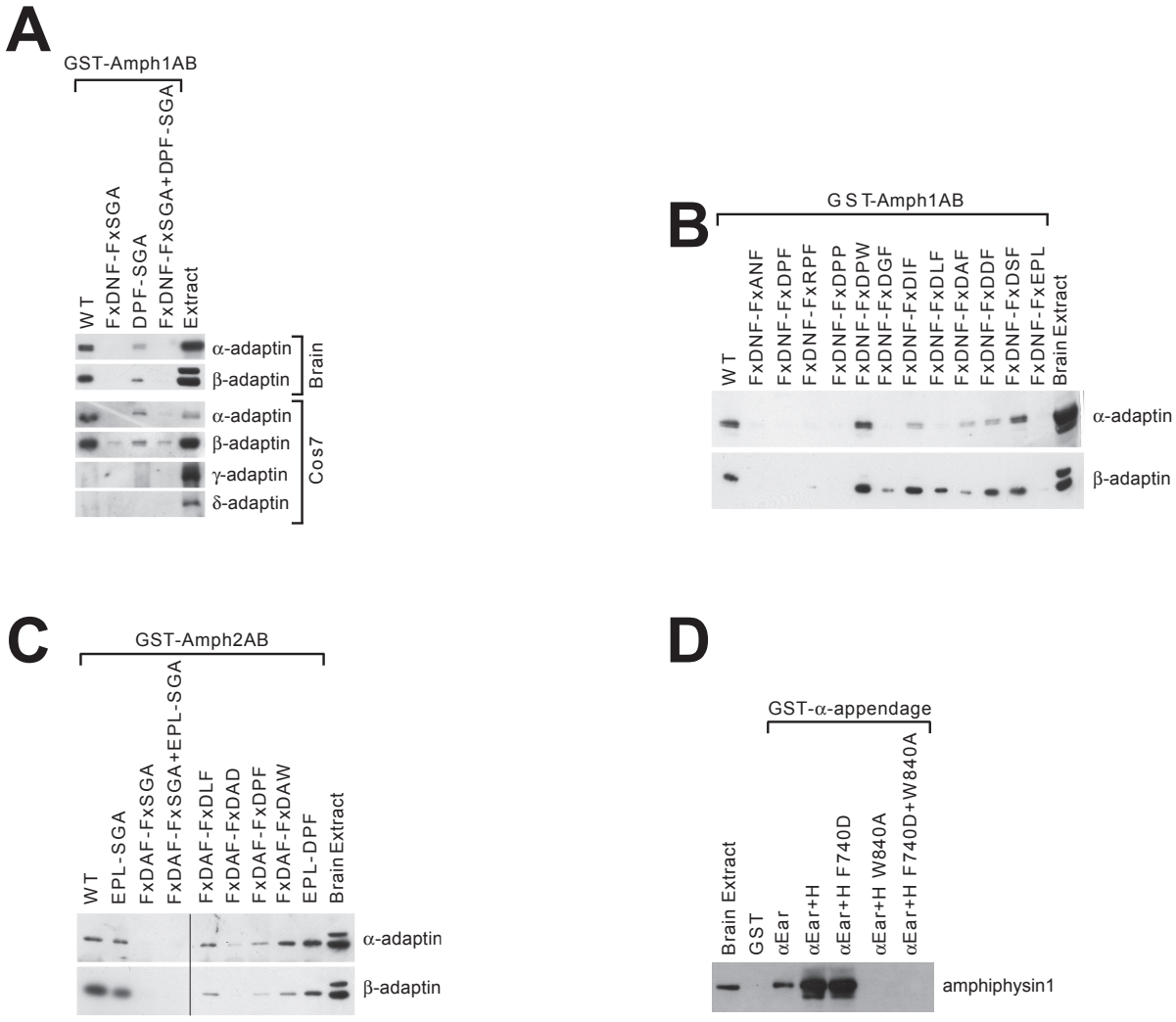
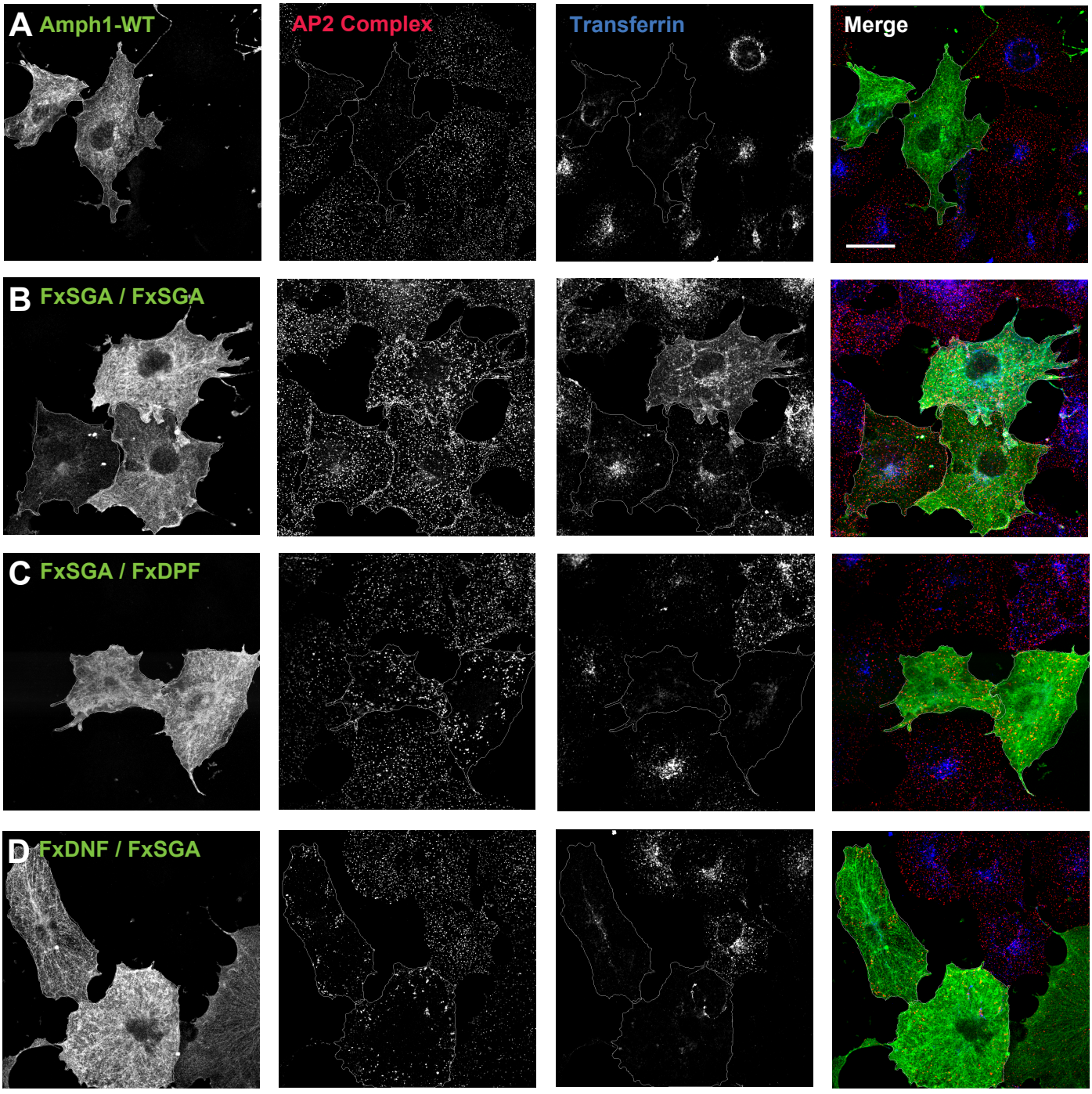

A
GST-Amph1AB
WT
FxDNF-FxSGA
DPF-SGA
FxDNF-FxSGA+DPF-SGA
Extract
α-adaptin
β-adaptin
Brain
α-adaptin
β-adaptin
γ-adaptin
δ-adaptin
Cos7
B
GST-Amph1AB
WT
FxDNF-FxANF
FxDNF-FxDPF
FxDNF-FxRPF
FxDNF-FxDPP
FxDNF-FxDPW
FxDNF-FxDGF
FxDNF-FxDIF
FxDNF-FxDLF
FxDNF-FxDAF
FxDNF-FxDDF
FxDNF-FxDSF
FxDNF-FxEPL
Brain Extract
α-adaptin
β-adaptin
C
GST-Amph2AB
WT
EPL-SGA
FxDAF-FxSGA
FxDAF-FxSGA+EPL-SGA
FxDAF-FxDLF
FxDAF-FxDAD
FxDAF-FxDPF
FxDAF-FxDAW
EPL-DPF
Brain Extract
α-adaptin
β-adaptin
D
GST-α-appendage
Brain Extract
GST
αEar
αEar+H
αEar+H F740D
αEar+H W840A
αEar+H F740D+W840A
amphiphysin1

Figure 3


A Amph1-WT
AP2 Complex
Transferrin
Merge
B FxSGA / FxSGA
C FxSGA / FxDPF
D FxDNF / FxSGA

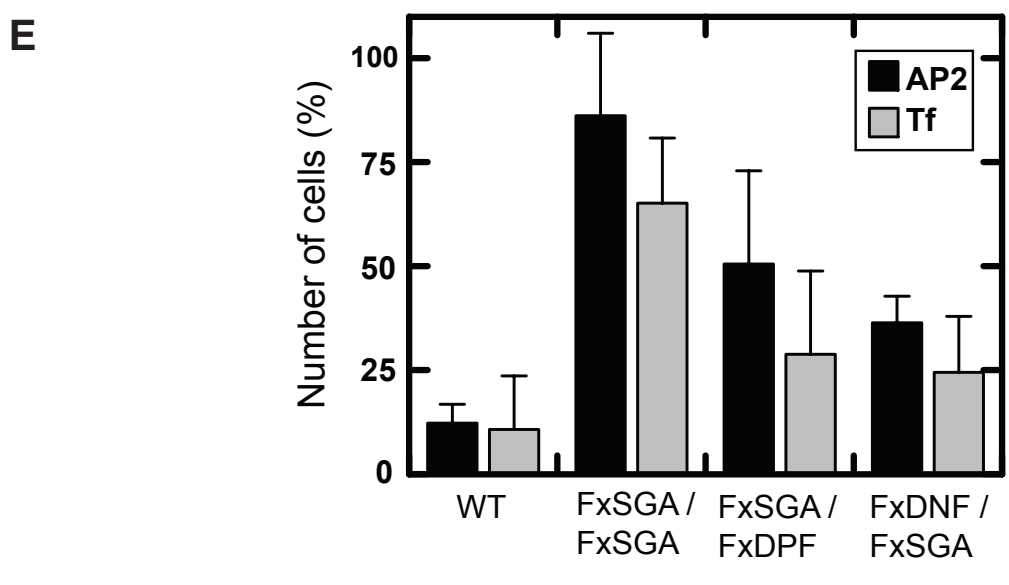

E
Number of cells (%)
100
75
50
25
0
AP2
Tf
WT
FxSGA / FxSGA
FxSGA / FxDPF
FxDNF / FxSGA

Figure 4

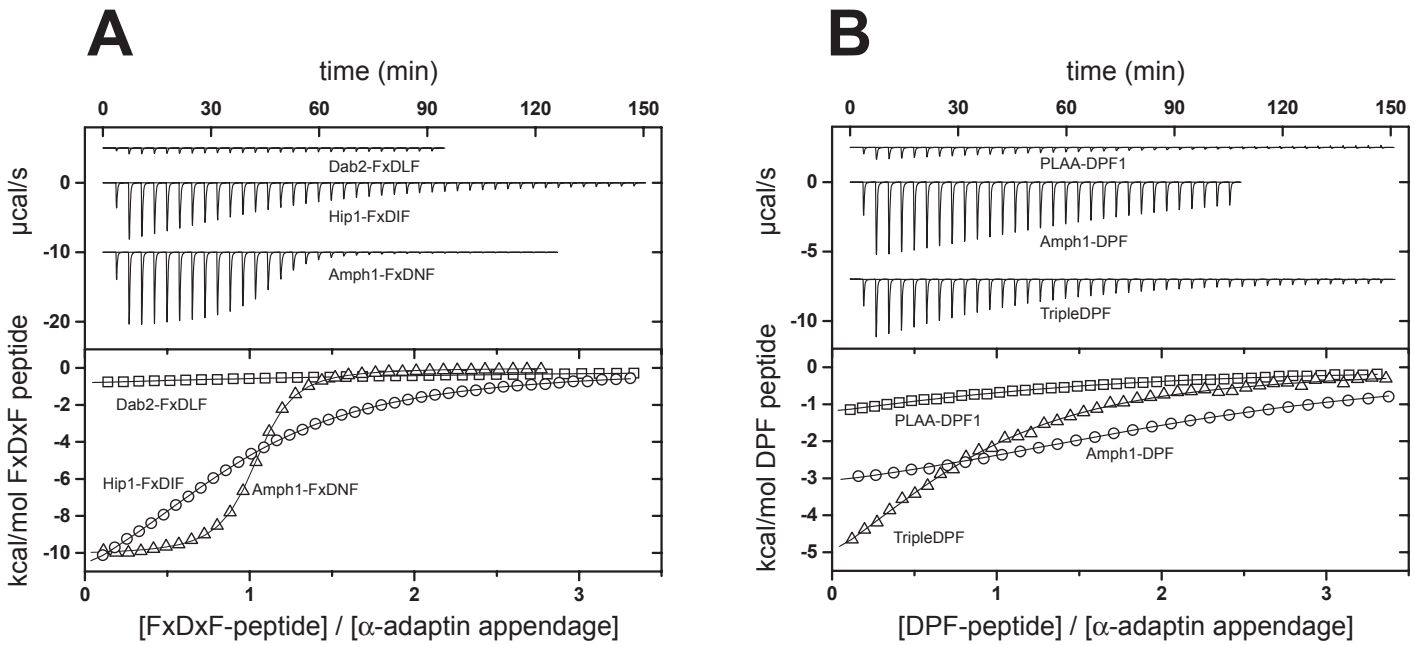
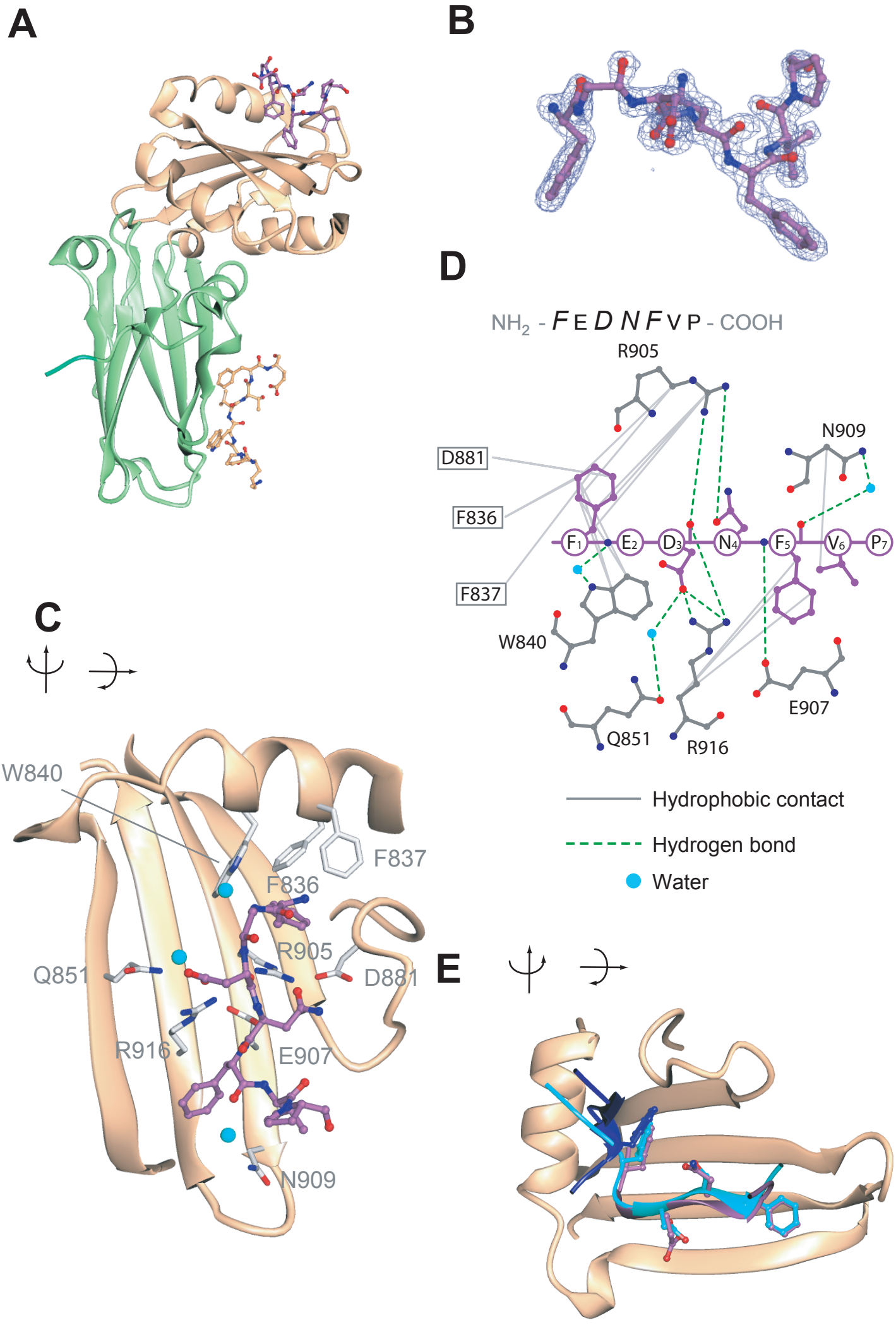

A
time (min)
0
30
60
90
120
150
µcal/s
0
-10
-20
Dab2-FxDLF
Hip1-FxDIF
Amph1-FxDNF
kcal/mol FxDxF peptide
0
-2
-4
-6
-8
-10
Dab2-FxDLF
Hip1-FxDIF
Amph1-FxDNF
0
1
2
3
[FxDxF-peptide] / [α-adaptin appendage]
B
time (min)
0
30
60
90
120
150
µcal/s
0
-5
-10
PLAA-DPF1
Amph1-DPF
TripleDPF
kcal/mol DPF peptide
0
-1
-2
-3
-4
-5
PLAA-DPF1
Amph1-DPF
TripleDPF
0
1
2
3
[DPF-peptide] / [α-adaptin appendage]

Figure 5


A
B
D
NH2 - F E D N F V P - COOH
R905
N909
D881
F836
F837
W840
F1
E2
D3
N4
F5
V6
P7
Q851
R916
E907
Hydrophobic contact
Hydrogen bond
Water
C
W840
F837
F836
R905
Q851
D881
R916
E907
N909
E