Chapter 1

Structure and Function of the APP Intracellular Domain in Health and Disease

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Additional information is available at the end of the chapter

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1. Introduction

Talking about Alzheimer's disease (AD) on a biochemical level needs to highlight the molecular „*corpus delicti*": the amyloid or senile plaques [1]. These plaques are extracellular fibrillar deposits in the cortex and hippocampus mainly composed of a single proteinaceous compound, the Aβ peptide comprising predominantly 40 or 42 amino acid residues (Aβ40, Aβ42) [2]. The Aβ peptides originate by sequential ectodomain shedding and regulated intramembrane proteolysis (RIP) of the amyloid precursor protein (APP), a type I integral membrane protein highly expressed in neurons including synaptic compartments. The responsible proteases, the famous β- and γ-secretase respectively, have been reviewed in detail and will not be part of this paper [3, 4]. Since the cloning of APP 25 years ago, more than 9,000 publications (about one per day!) are listed for this protein in the PubMed database indicating its pivotal position in the amyloid cascade hypothesis [5], which constitutes the widely accepted pathogenic cascade ultimately leading to AD. While some years ago the plaques themselves were thought to be the primary cause of disease, it is nowadays well recognized that soluble Aβ oligomers are responsible for many of the neurotoxic properties causing memory dysfunction and finally dementia.

Despite intense research efforts AD can so far only be insufficiently treated in a purely symptomatic way and disease-modifying drugs are most wanted but are still not available [6]. In order to get a glimpse of understanding AD pathology at a biochemical level, we therefore have to understand the molecular structure of the key-player APP and its connected protein network. The structure, however, needs to be correlated with the physiological functions and the deregulating mechanisms causing toxicity, cell death, and disease [7, 8]. Bearing this in mind, the simultaneously generated sister peptides of Aβ deserve a major focus, namely the amino-terminal fragment (N-APP286) derived from sAPPβ as a ligand for the death receptor

6 (DR6) [9], and the APP intracellular domain as created by the ε-cut of γ-secretase during the RIP process [3], which is the topic of this paper. We will start by getting the architecture of APP into place.

2. Architecture of the APP protein

APP can be divided into three domains (Figure 1). As a single pass type I membrane protein, the N-terminal ectodomain of APP (residues 18 to 624 neglecting the signal peptide, numbers refer to the neuronal splice form APP695, UniPROT entry: P05067-4) locates to the extracellular space. The single hydrophobic transmembrane domain (TMD, residues 625 to 648) is followed by the rather short APP intracellular domain (AICD, residues 649 to 695). More important than this topological classification is the distinction according to the fragments produced by secretase cleavage events [10]. The products produced by ectodomain shedding are sAPPα (residues 18 to 612; cleaved by α-secretases, members of the ADAM family of zinc metalloproteases) and sAPPβ (residues 18 to 596; cleaved by β-secretase, an aspartic protease also known as BACE1 in the nervous system and BACE in peripheral tissue). The C-terminal fragments (CTFs) generated by ectodomain shedding are the still membrane embedded αCTF (CTF83) and βCTF (CTF99), respectively. The CTFs are subsequently cut in the RIP process by the intramembrane aspartate protease presenilin (1 or 2) as part of the γ-secretase complex, with αCTF being split into the p3 peptide and the AICD (residues 646 to 695) and βCTF into the Aβ peptide (Aβ40: residues 597 to 636; Aβ42: residues 597 to 638) and again the AICD.

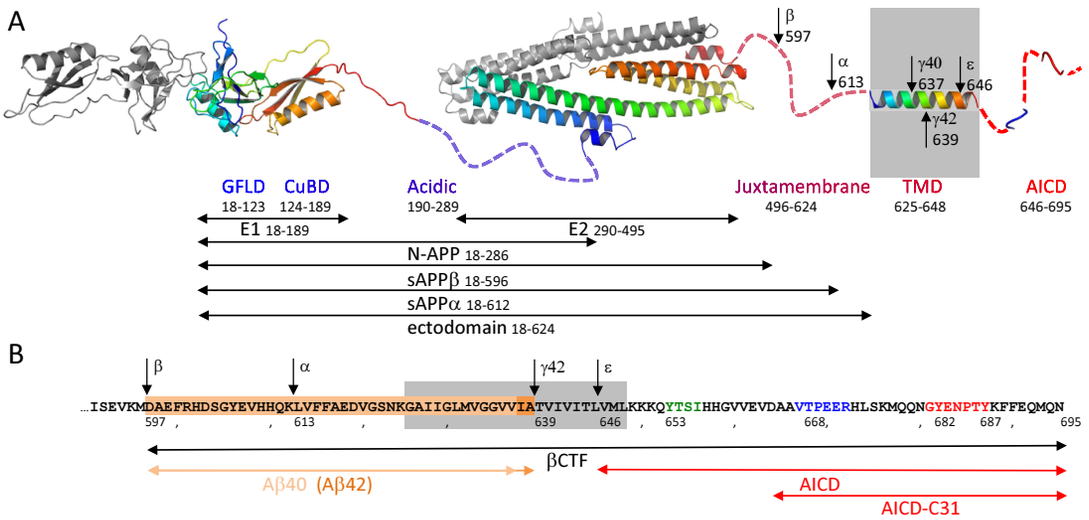

Figure 1. Architecture of APP and of its proteolytic fragments. A. Domain architecture of the neuronal splice variant APP695. Domains with known atomic structures (E1 and E2) and the TMD are shown as ribbon diagrams in a colour code from blue (N-terminus) to red (C-terminus). Dashed lines give structurally unknown regions. Proposed homodimeric interactions within E1 and E2 are shown in gray. Positions of secretase cleavage events and the respective breakdown products are labeled. B. Sequence and proteolytic fragments within βCTF. Aβ peptides, the TMD (gray), and sequence fingerprints within the AICD are colour coded.

In terms of three-dimensional structure, only substructures within the large APP ectodomain have been solved as independently folded subdomains. The N-terminal E1 domain is a two-lobe structure consisting of the growth factor like domain (GFLD, residues 18-123) and a copper-binding domain (CuBD, residues 124 to 189), both comprising mixed $\alpha\beta$ topologies rigidified by disulfide bridges [11-13]. The E1 domain is followed by a highly acidic, and probably unfolded, stretch of about 100 residues that passes on to the E2 domain (residues 290 to 495), consisting of two coiled-coils connected through a continuous central helix and resembling a spectrin family fold [14]. E1 and E2 domains have been implicated in APP dimerization [14-16], which is reported to be modified by the extracellular matrix [17], and to have significant impact on localization and cleavage events. In addition, dimerization might also involve the TMD region [16]. Besides dimerization, APP architecture (and likely function) is also influenced by a series of post-translational modifications, mainly by N- and O-glycosylation and phosphorylation [18], which will be discussed in detail below. The reminder of the ectodomain between E2 and the TMD, the so-called juxtamembrane region (residues 496 to 624), is again intrinsically disordered based on secondary structure prediction and contains the cleavage sites for the α- and β-secretases. The single TMD is clearly α helical, although with partial propensity in forming β structures. This propensity extends also to the juxtamembrane region with the fatal consequence, that after secretase cleavage the amyloid peptide folds into a β hairpin structure and aggregates to form the toxic oligomers and finally the amyloid fibrils. Finally, the AICD itself is again intrinsically disordered as shown by NMR and CD experiments [19, 20]. Importantly however, this small C-terminal stub has recently been shown to adopt different conformations reflecting its versatile functions. The structure-function relationship of the AICD shall be described in the following.

3. Biology of the AICD: Tales of a tail

When talking about the AICD, a clear distinction has to be made: the function (and probably also the structure) is different for AICD as part of APP at the membrane and for AICD as peptide generated by ε-cleavage of γ-secretase and first described by Passer *et al.* [21]. Within the AICD three sequence motifs have been identified to be of functional relevance. The first one is the ^{653}YTSI sequence, which has been implicated in the basolateral sorting of APP in polarized MDCK cells [22] and which is reminiscent to the YXXΦ (X: any residue; Φ: aromatic or large hydrophobic residue) consensus motif as tyrosine-based and clathrin-mediated endocytic sorting signal [23]. Indeed, when Tyr653 is mutated to alanine, APP is equally distributed on apical and basolateral membranes in MDCK cells [24]. Somewhat surprisingly, in neurons polarized sorting occurs independently of this signal [25]. Subcellular trafficking and neuronal APP sorting is still poorly understood [26] and remains a topic of intense investigation. This first motif contains three phosphorylatable residues (YTS), and it has been reported that at least Thr654 and Ser655 are phosphorylated in the adult rat brain under physiological conditions [27].

Much more attention has been drawn to the second fingerprint ^{667}VTPEER, as this site seems to be also critically involved in pathophysiological processes. While the function of the residues has remained elusive prior to the availability of structural data, Thr668 has since been established as the major phosphorylation site of APP and its physiological function has been investigated in the adult rat brain, post mitotic differentiating neurons and dividing cells [18]. Whereas pT668 in neurons is dominant in the fully-glycosylated mature APP, in differentiating cells the purely N-glycosylated immature protein as present in the endoplasmic reticulum and the early Golgi is of relevance. Accordingly, different kinases are responsible for Thr668 phosphorylation. In neurons, it is glycogen synthase kinase-3β (GSK3β) and cyclin-dependent kinase 5 (Cdk5), while Cdk1 and cdc2 kinase phosphorylate this residue in dividing cells. Moreover, when cells are exposed to stress, phosphorylation is taken over by c-Jun N-terminal kinase (JNK) [28].

Phosphorylation on Thr668 of APP depends on the presence of Pro669 and strongly affects Aβ production [29]. This is reminiscent of the Tau protein, where the phosphorylation of certain serine and threonine residues depends on adjacent proline residues and leads to tangle formation [29]. A first molecular explanation for the proline-dependency was revealed by studies showing that the prolyl isomerase Pin1, catalyzing the *cis-trans* isomerization of the Thr-Pro peptide bond, increases amyloidogenic APP processing and selectively elevates Aβ42 levels. Intriguingly, Pin1 is down regulated and/or inhibited by oxidation in neurons of Alzheimer's disease patients and *Pin1* knockout causes neurodegeneration (and tauopathy). Pin1 binds to Thr668-phosphorylated APP and accelerates Pro669 isomerization (by a factor of 10^3). Thus, the AICD swaps between two conformations, as visualized by NMR [29]. This conformational switch may in turn have crucial consequences with regard to the AICD protein interacting network, as shown for the neuronal adaptor protein Fe65 (Figure 2 and see below) [20, 30]. To evaluate in as much the phosphorylation state of Thr688 controls APP processing *in vivo*, knockin mice were generated in which Thr668 was changed to alanine ($APP_{TA/TA}$) [31, 32]. The $APP_{TA/TA}$ mutation, and thus absence of phosphorylation, did not significantly alter APP localization, processing, and Aβ generation, thus questioning the i*n vivo* role of Thr668 phosphorylation. However, these studies cannot rule out the possibility that a pathological increase in Thr668 phosphorylation, as found in AD patients [33], will also modulate its function. In line with this notion, Thr668 phosphorylation has also been reported to influence APP cleavage by caspases between residues Asp664 and Ala665, producing the cytotoxic AICD-C31 fragment, a process that has been strongly implicated in AD pathogenesis [34].

The third and most intensely studied fingerprint within the AICD is the ^{681}GYENPTY sequence containing an NPXY motif, a well-established internalization signal for membrane proteins [35]. NPXY is a classical tyrosine-based sorting signal for transmembrane proteins to endosomes and lysosomes [23]. However, the signal has been shown to only mediate rapid internalization of a subset of type I membrane proteins, including APP as well as members of the low-density lipoprotein (LDL) receptor family and integrin β. These proteins are internalized via clathrin-coated pits. Nevertheless evidence for a direct interaction of NPXY motifs with the coat or the AP-2 adaptor is weak.

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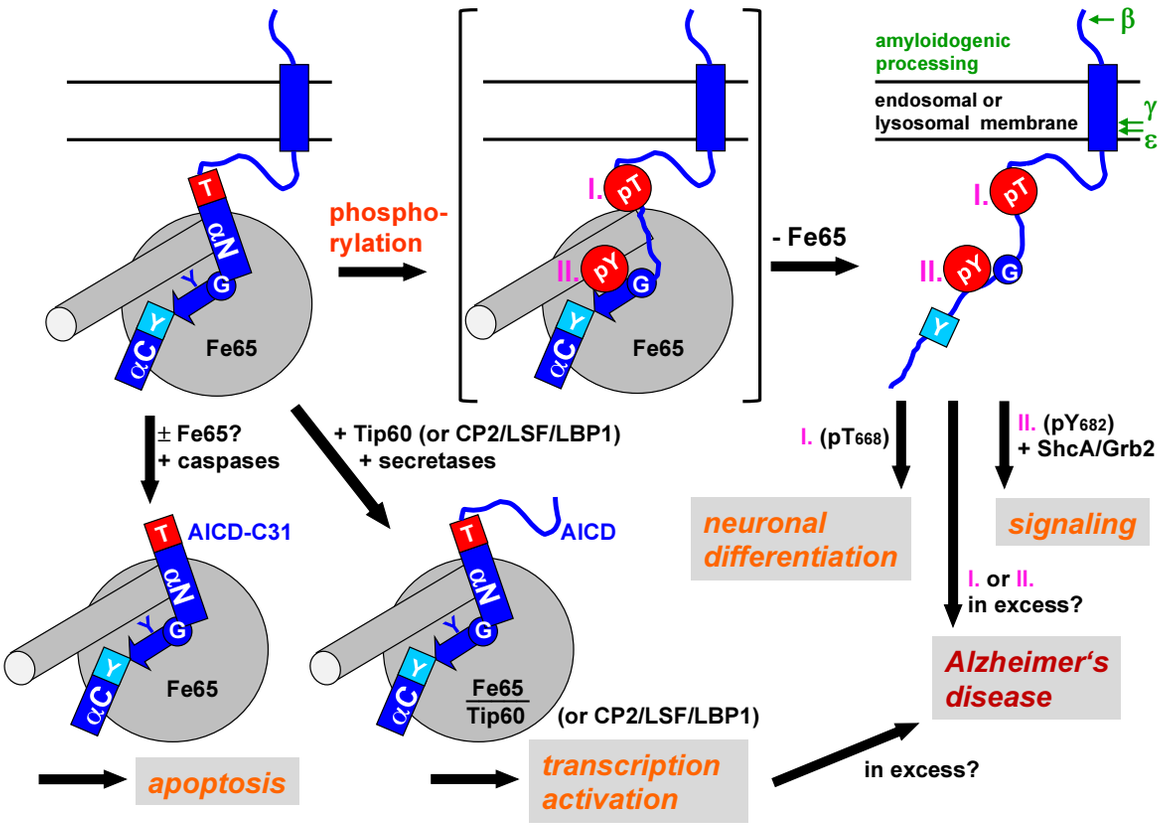

Figure 2. AICD in health and disease. Different fates of the AICD are exemplified for the main AICD interaction with Fe65-PTB2 (red T-box: TPEE, cyan Y-box: NPTY, G: glycine hinge, gray cylinder: C-terminal helix of Fe65-PTB2). In the non-phosphorylated state, AICD forms a stable complex with Fe65-PTB2 that assembles in ternary complexes with i.e. Tip60 or CP2/LSF/LBP1 via Fe65-PTB1. Upon cleavage by the secretases, the liberated complexes are involved in transcription activation. Alternatively, caspase cleavage within the AICD results in cytotoxic AICD-C31, which might compete with AICD for Fe65-PTB2 binding and induce apoptosis. Phosphorylation of either Thr668 (I.) or Tyr682 (II.) results in a destabilization of the Fe65-PTB2/AICD interaction (shown in brackets) and results in complex dissociation. Phosphorylation stimulates (I.) neuronal differentiation or (II.) initiates signaling cascades. Deregulation of the Fe65-PTB2/AICD interactions is strongly implicated in Alzheimer's disease progression.

Instead, the NPXY motif is well known to interact with adaptor proteins containing a domain known as phosphotyrosine-binding (PTB) or phosphotyrosine-interacting domain (PID) [36]. PTB domains reveal a fine tuned plasticity in ligand recognition, and besides recognizing phosphorylated NPXpY motifs, most PTB adaptor proteins can also bind to their ligand in a pY-independent manner. Accordingly, *in vitro* phosphorylation of Tyr687, which does not seem to occur in the brain [18], does i.e. not alter the binding affinity of AICD to its major PTB-containing adaptor protein Fe65.

In APP, the NPXY signal is extended by three residues at the N-terminal side (GYE), with especially Tyr682 being most critical for function [31, 37, 38]. The motif is present in many lysosomal glycoproteins that are endocytosed and targeted to the lysosomes [39]. In cell-culture studies, Tyr682 can be readily phosphorylated by the nerve growth factor receptor TrkA and the tyrosine kinases Abl and Src [40]. In brains of AD patients, it is known that at least βCTF is phosphorylated, whereas this is not the case for αCTF [41]. In addition, phos-

phorylation regulates both AICD peptide formation and AICD-dependent cellular responses (Figure 2). These data point to a sorting function regulated by Tyr682 phosphorylation, with non-phosphorylated APP kept at the plasma membrane and therefore processed by α-secretase, and a phosphorylation-dependent re-localization resulting in β-cleavage. Sorting implies docking to respective intracellular trafficking machineries and their adaptors, including PTB domain containing proteins. Consistently, an $APP_{YG/YG}$ mutation introduced into the endogenous APP locus by knock-in led to a marked shift toward the non-amyloidogenic pathway in brain with increased levels of full length APP, sAPPα, αCTF, unaltered βCTF and reduced sAPPβ and Aβ40 levels [31].

Sorting due to differentially phosphorylated residues is one side of the medal, signaling is the other [40]. Two signaling proteins are well known to require Tyr682 phosphorylation for binding to APP-CTFs, namely ShcA and Grb2. ShcA is a member of a family of cytoplasmic adaptor proteins (ShcA, ShcB, ShcC) that interacts with its PTB and Src homology2 (SH2) domains with receptor tyrosine kinases (RTKs) and activated growth factor receptors, which is the case also for SH2/SH3 domains containing Grb2 [42]. The initiated cascades are involved both in cell proliferation and gene transcription events, like i.e. the MAP kinase pathway. Again, binding occurs only to pTyr682 of βCTFs but not of αCTFs [41] (Figure 2). Whereas the reasons for the different binding preferences remain elusive, the underlying structural transitions within the AICD itself modulating sorting and signaling have been studied in some detail.

4. Structural transitions within the AICD

First structural insights on the AICD peptide came from NMR experiments, revealing most of the AICD to be unstructured. The transient structure (also termed intrinsic disorder: ID) of cytoplasmic domains of membrane proteins is well suited for the molecular recognition in intracellular signaling events for a number of reasons [43]: (i) modulation of the structural propensity provides ID proteins with the capability to combine high specificity with low affinity; (ii) binding diversity in which one region specifically recognizes differently shaped partners by structural accommodation at the binding interface, a phenomenon known as one-to-many signaling; (iii) binding commonality in which distinct sequences recognize a common binding site (with eventually different folds); (iv) the formation of large interaction surfaces as the ID region wraps up or surrounds its binding partner, making it possible to overcome steric restrictions; (v) faster rates of association by reducing dependence on orientation factors and by enlarging target sizes; (vi) faster rates of dissociation by unzipping mechanisms; (vii) the precise control and simple regulation of the binding thermodynamics; and (viii) the reduced life-time of ID proteins in the cell, possibly representing a mechanism of rapid turnover of important regulatory molecules. A prominent example of intrinsically disordered proteins is α-synuclein, a protein critically involved in Parkinson's disease, which binds to a multitude of partners differentially by alternative folding [44], a feature that equally applies to the intracellular domain of APP.

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Although NMR experiments revealed the AICD to be intrinsically disordered, the TPEE and NPTY motifs where found to form type I β-turns and TPEE forms part of a helix-capping box [19] (Figure 3). Type I turns are the most frequent reverse turns in protein structures, which in total involve about 1/3rd of all residues. Turns usually occur on the exposed protein surfaces and represent molecular recognition sites. In a capping box, the side chain of the first helical residue forms a hydrogen bond with the backbone of the fourth helical residue and, reciprocally, the side chain of the fourth residue forms a hydrogen bond with the backbone of the first residue [45]. These boxes are known to stabilize the N-termini of α-helices, and preordering of the elements is thought to guide recognition of the intracellular protein network and to reduce the entropic costs for complex formation, a feature that applies as well for APP. In addition, the conformation of the TPEE motif and the propensity of forming the N-terminally capped α helix critically depend on the phosphorylation status of Thr668 [20, 46]. This structure-function relationship can be explored by the study of the AICD with its cytoplasmic interaction partners.

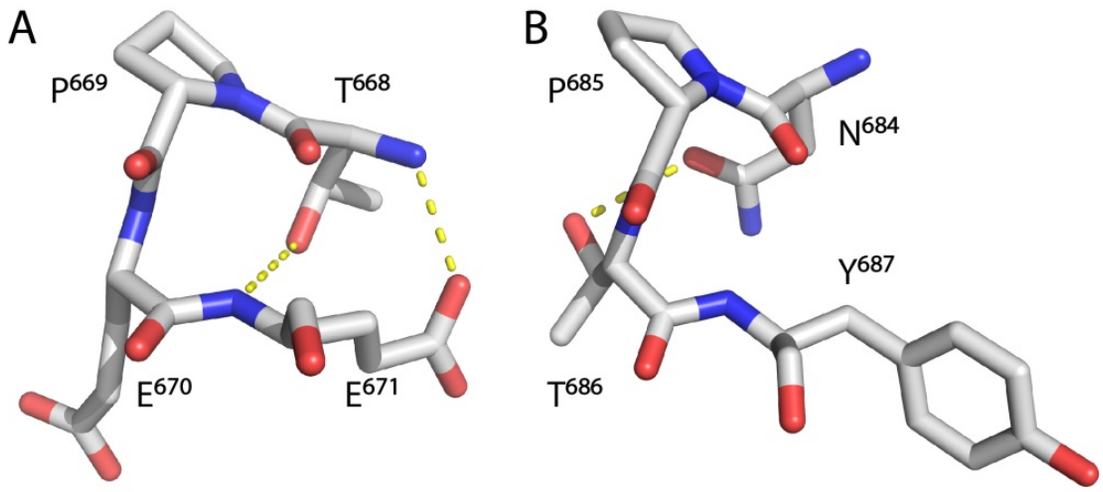

Figure 3. The TPEE and NPTY motifs. A. The TPEE motif forms a type I β-turn and a helix capping box with two characteristic hydrogen bonds (dashed yellow lines). B. The NPTY motif forms a similar type I β-turn.

5. Interaction partners of the AICD

More than 20 proteins have been reported to interact with the AICD [47] (Table 1). However, little is known whether these complexes occur also *in vivo* and what relevance they may have for cell physiology or AD pathogenesis. Basically, they can be classified in modifying, sorting, or signaling interactions. The modifying enzymes have been already mentioned and account for phosphorylation and prolyl *cis*/*trans* isomerization events. Basolateral sorting is guided by the protein PAT1, which is the only protein that has been shown to directly interact with the ^{653}YTSI motif and is associated with microtubules [48].

Knowledge about the interaction partners for the ^{667}VTPEER motif is similar scarce. Major binder for the motif, and as well for the complete AICD, are the multi-domain adaptor/scaffolding proteins of the Fe65 family (Fe65, Fe65L1, and Fe65L2) [49]. The only additional binding partner to the ^{667}VTPEER motif is the dimeric adaptor protein 14-3-3γ, which seems to stabilize the AICD/Fe65 interaction [50]. Fe65 is enriched in brain, whereas Fe65L1 and Fe65L2 are more widely expressed. All three members contain a WW domain and two PTB domains (PTB1 and PTB2). Through the PTB2 domain, they interact with the AICD and can alter APP processing. After proteolytic processing of APP and release of the AICD to the cytoplasm, Fe65 can translocate to the nucleus to participate in gene transcription events (Figure 2), which is modulated by 14-3-3γ. This role is further mediated by interactions of Fe65-PTB1 with the transcription factors CP2/LSF/LBP1 [51] and Tip60 [52] and the WW domain with the nucleosome assembly factor SET [53]. Possible target genes identified by reporter assays include GSK3β, Neprilysin, KAI1, and the low-density lipoprotein receptor-related protein 1 (LRP1), but the physiological relevance for endogenous transcriptional regulation has been discussed controversially [54]. Fe65-PTB1 also interacts with two cell surface lipoproteins receptors, namely LRP1 [55] and ApoEr2 [56], forming trimeric complexes with APP. The Fe55 WW domain further binds to mammalian Ena (mEna) [57], through which it functions in regulation of the actin cytoskeleton, cell motility, and neuronal growth cone formation [49]. The interaction has been implicated in a role for AICD signaling, in synaptic plasticity and memory [58]. Moreover, Fe65 family proteins have attracted attention, as Fe65 or Fe65L1 double knockout mice revealed defects in cortical development with neuronal mispositioning and ectopia, resembling human lissencephaly type 2 [59]. Interestingly, very similar cortical defects were also found in APP-/-APLP1-/-APLP2-/- triple knockout mice lacking all APP family members, suggesting a lack of APP/Fe65 dependent signaling as the underlying cause of defects in both mouse mutants [60].

Fe65 binding to the AICD is unique, as its extended binding interface ranges from the ^{667}VTPEER up to the ^{681}GYENPTY motif and thus includes almost the entire AICD-C31 fragment (Figures 2 and 4A). Most other AICD interacting proteins recognize the ^{671}GYENPTY motif and neighbouring residues, with the interaction site spanning only about 10 residues. As ^{681}GYENPTY is essential for APP trafficking, the respective complexes can also alter APP processing. Like Fe65, the binders for this motif are PTB-containing proteins including members of the X11/Mint, JIP, Dab, and Shc families, as well as the Numb protein.

Mints consist of a divergent N-terminal region and conserved C-terminal sequences composed of one PTB domain and two tandem PDZ domains. Although their regulatory role for APP metabolism and transport is unresolved, it seems that they slow cellular APP processing and reduce Aβ40 and Aβ42 secretion [61] by suppressing translocation of APP into BACE- and γ-secretase-rich detergent-resistant membrane (DRM) domains, the so-called rafts [62, 66]. In addition, there is evidence for a functional role of the AICD interaction with X11/Mints for synapse formation [62, 67] and synaptic neurotransmitter release [68]. c-Jun N-terminal kinase (JNK) interacting protein-1 (JIP1), a scaffolding protein for the JNK kinase cascade, has been suggested to mediate anterograde transport of APP by the molecular motor kinesin-1. However, this initial view has been challenged recently, as in contrast to this model, APP

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constructs lacking the AICD are still transported to the nerve terminal by the fast axonal transport mechanism [63].

Protein	Interacting domain	Interacting region within AICD	Function	Processing*	Selected citations
PAT1	n.a.	YTSI	Basolateral sorting	α↑, β↓**	[48]
Fe65,	PTB2	AICD-C31:	Endocytosis, signaling and	β↓	[49]
Fe65L1, -L2	n.a.	VTPEER + GYENPTY	transcription activation, ...	n.a.	[50]
14-3-3-γ	PTB	VTPEER	AICD/Fe65 stabilization	β↓	[61] [62]
X11/Mint		GYENPTY	Exocytosis, synapse formation, ...		
JIP1	PTB	GYENPTY	Transport	β↓	[63]
Dab1	PTB	GYENPTY	Transport, signaling	α↑, β↓	[64]
ShcA/Grb2	PTB/SH2	G(pY)ENPTY	Signaling	-	[42]
Numb	PTB	GYENPTY	Notch crosstalk	***	[65]

Table 1. Selected interaction partners of the AICD. *Data depend on cell line studied and are sometimes conflicting. **Due to basolateral sorting and independent of PAT1 binding. Pat1 binding as such increases Aβ levels [48]. ***Numb isoform dependent. ↓ denotes changes of non-amyloidogenic (α) or amyloidogenic (β) APP processing.

The Dab family member Dab1 regulates neuronal migration in mammals as an essential component of the Reelin signaling pathway. Dab1 binds not only to APP family proteins [64] but is well known to also bind to ApoE receptors (ApoEr2, VLDLR, and LRP) [69]. Dab1 increases cell surface expression of APP and ApoEr2, increases α-cleavage of APP and ApoEr2, and decreases APP βCTF formation and Aβ production in transfected cells and in primary neurons. The Dab family represents a prototype of PTB domains that bind their ligands in a pY-independent manner [36]. In addition Dab proteins bind specifically to the phosphoinositide (PI) PI-4,5-P_2, which is predominantly located at the cellular membrane [70]. Binding of PTB domains to PIs is a common principle to locate and orientate the adaptors at the target membrane and to facilitate downstream events that accompany NPXY peptide binding. Since PTB domains structurally belong to the pleckstrin homology (PH) superfold family and PH domains are the prototypical PI binding domains, this function seems to be evolutionarily conserved within PTB domains [36]. The crystal structures of ternary complexes of Dabs bound to ApoEr2 [71] or APP [72] peptides and lipid revealed the lipid head group (IP_3) to be recognized by a large basic patch opposite the peptide-binding groove (Figure 4A). This patch, also termed as "phospholipid binding-crown", is conserved in many PTB domains [36].

Finally, binding of the AICD to the Numb PTB domain has been found to inhibit Notch signaling [65], thereby establishing a crosstalk between the APP family and Notch in the development of the peripheral nervous system (PNS) [73]. Like APP, the Notch receptor undergoes a series of proteolytic cleavages that release the Notch intracellular domain (NICD) that functions in transcriptional activation and subsequent signal transduction events, including proliferation, differentiation, or apoptotic cues [74]. Similar to the NICD, the AICD has been also found to regulate PI-mediated calcium signaling through a γ-secretase depend-

ent pathway [75, 76]. Cells lacking APP were shown to exhibit deficits in calcium storage that could be reversed by transfection with APP constructs containing an intact AICD. Constructs lacking the AICD were not able to rescue the phenotype, strongly indicating that this domain is critically involved in endoplasmic reticulum (ER) calcium filling [76]. The multitude of interactions with the AICD raises the question of the spatial and temporal regulation of all these complexes, which needs a detailed structural analysis and a thorough biochemical characterization.

6. Structure-function relationship of AICD complexes

The structure-function relationship of AICD complexes is governed by the one-to-many principle with the intrinsically disordered AICD folding onto its manifold adaptor proteins, in particular the PTB domain containing proteins. The recurrent interaction pattern includes the recognition of the ^{681}GYENPTY sequence, which shall be described in the following. High resolution structures for this interaction are known for Dab1 and 2 [72], X11α [77], and the Fe65-PTB2 domains [30] (Figure 4A). All PTB domains comprise a pleckstrin homology (PH) fold consisting of a central β sandwich structure and a C-terminal α helix. Overall, complex formation can be described as an induced-fit docking of the AICD to a rigid PTB domain scaffold. Common to all the complexes is the binding of the ^{681}GYEN sequence to the β5 strand of the respective PTB domain by a mechanism called β completion, where a (antiparallel) β sheet is created between two polypeptide chains (*in trans*) (Figure 4D). This interaction occurs between the protein backbones and therefore strong sequence conservation is not present on the PTB domain side. The conservation of AICD Gly681 is explained as longer side chains would cause steric clashes with the PTB domains, as shown for the Fe65-PTB2/AICD interaction, where a G681A mutation abolishes the binding and Gal4-Tip60-dependent transactivation [78]. The importance of the flexible glycine becomes evident when comparing the solved PTB/AICD complexes (Figure 4B), revealing that Gly681 forms a hinge that allows for different AICD conformations in the N-terminal direction. The hinge function correlates with a peptide-flip of the glycine [30].

The side chain of Tyr682 is accommodated in the center of the interface and faces the C-terminal helix of PTB domains (Figures 3A and 3D). In all complexes it lays in a hydrophobic pocket, however, the conformations between the Fe65-PTB2 and Dab1 in respect to X11α and Dab2 complexes are different. The hydrophobic nature of the pocket explains the general conservation of a tyrosine or phenylalanine in this position in the context of NPXY sequences. All crystallized complexes are specific for non-phosphorylated Tyr682, which can be readily explained, as there is no space available to accommodate the extra phosphate moiety. This is in contrast to ShcA, where the binding site is more open [79], which apparently allows for binding of a phosphorylated Tyr682 (although no structure of this complex is available). The readout of the conserved glutamate is again different in the PTB complexes, although its function as selectivity filter seems to be minor. Whereas it forms i.e. a salt bridge with an arginine of X11α, in the Fe65-PTB2 complex it is fixed *in cis* to Lys688 following the NPTY motif.

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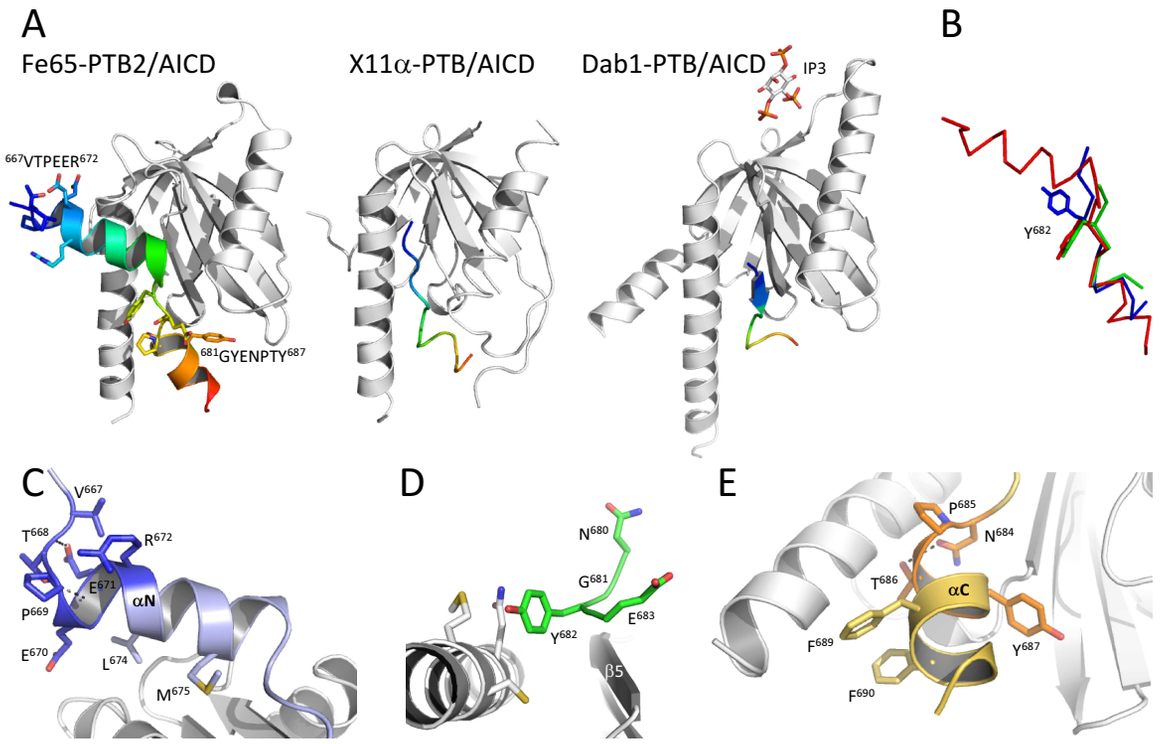

Figure 4. Structure of the AICD in PTB domain complexes. A. Crystal structures of AICD peptides in complex with PTB domains: Fe65-PTB2/AICD (PDB code 3DXC), X11α-PTB/AICD (1X11), and Dab1-PTB/AICD (1OQN). AICD peptides are colour coded from blue (N-terminus) to red (C-terminus) and PTB domains are given in gray. In Fe65-PTB2/AICD, the visible AICD structure corresponds to AICD-C31 and includes both the ^{667}VTPEER and the ^{681}GYENPTY sequences. Dab1 is also bound to the polar head group of the lipid PI-4,5-P_2 (IP3: inositol-1,4,5-triphosphate). B. Superposition of the three AICD fragments as shown in Figure 3A (complex with Fe65-PTB2: red; X11α: blue; Dab1: green). The alternative side chain conformations of Tyr682 are highlighted. C. Close-up view on the AICD helix αN in complex with Fe65-PTB2. The ^{667}VTPEER motif is highlighted in blue and hydrogen bonds within the capping box are given as dashed lines. D. Interaction of the ^{680}NGYE motif with Fe65-PTB2. The AICD stretch forms a β sheet *in trans* with strand β5 from the PTB domain. The side chain of Tyr682 is accommodated in a hydrophobic pocket created by the C-terminal helix of the PTB domain. E. Interaction of the ^{684}NPTY motif and helix αC of the AICD with Fe65-PTB2. Tyr687 is rather solvent exposed and helix αC is fixed to the PTB domain by hydrophobic interactions of two subsequent phenylalanines.

As already described, the ^{684}NPTY sequence is forming a type I β-turn structure, which is retained within the complexes and forms the N-terminal cap of an induced α-helix at the very C-terminus of AICD (helix αC) (Figure 4E). Asn684 has a conserved structural role, with the carboxamide of the side chain hydrogen bonding to the main chain of Thr686. As the carboxamide is also tightly bonded to the PTB domains, the preformed NPTY conformation is a major determinant and probably also a starting point for AICD folding and complex formation. The conserved proline initiates and stabilizes the subsequent helix as found in many α helices. The most prominent residue, however, is Tyr687, as the tyrosine at this position is the discriminator for the classification in pY-dependent and pY-independent PTB domains [36]. In all structurally solved AICD/PTB domain complexes the peptide is non-phosphorylated, which reflects the *in vivo* situation within neurons. The pY-independence is readily explained, as the binding pocket is rather solvent exposed, and besides some van-der-Waals interactions of the benzene ring the tyrosine is not coordinated further. The binding mode is quite different in pY-

dependent Shc or IRS1 peptide complexes, where the phosphate moiety is read out by a set of conserved arginine residues and the binding pocket is much more pronounced [36].

The NPTY sequence is followed by the ^{688}KFFEQMQN^{695} sequence, which forms the C-terminus of the AICD (Figures 4A and 4E). The conformation of this region is slightly different and not always present in the structures, as the complexes have mostly been formed with truncated synthetic peptides. In the Fe65-PTB2 (which contains the entire C-terminus) and X11α complexes, the region is part of the C-terminal helix αC. The helix is fixed to the PTB domains by hydrophobic interactions of the two phenylalanines (Phe689 and Phe690) with the C-terminal helices of the respective PTB domains. These helices are three turns longer than those of Shc [79] and IRS1 [80] PTBs, and therefore the total interaction surfaces are significantly larger.

In most PTB domain complexes bound to an NPXY motif the described surfaces comprise the entire interaction, however, there is a single exception to the rule: the Fe65-PTB2/AICD complex, where the interface is about three times as large and includes an additional α helix (helix αN, ^{669}PEERHLSKMQQ^{679}) N-terminal to the ^{681}GYENPTY sequence (Figure 4C) [30]. This helix is N-terminally capped by the ^{667}VTPEER motif comprising the phosphorylatable Thr668 as already described. Like helix αC, helix αN is of amphipathic character and binds on a hydrophobic patch on the Fe65-PTB2 surface located in between strand β5 and the N-terminus of the C-terminal helix, which is almost perpendicularly crossed by helix αN. Whereas Leu674 and Met677 cover the hydrophobic patch, Glu670, His673, and Gln678 are involved in polar interactions with the PTB domain. With the exception of Glu670, the ^{667}VTPEER capping box is not touching the PTB domain, which is somewhat astonishing, as it was afore known that phosphorylation of Thr668 is detrimental to complex formation [20]. As described for free AICD, the side chain of Thr668 is hydrogen-bonded to the main chain of Glu671, and Pro668 is *in trans* configuration. Furthermore, the side chain of Glu671 is tied back to the main chain nitrogen of Thr668, and thus completing the rigid helix cap.

The most important question arising from structural data is how phosphorylation is able to regulate Fe65-PTB2/AICD complex formation in a process that is critically involved in Aβ generation and AD pathogenesis? Phosphorylation induces a *cis* configuration of Pro669 [46], which is incompatible with the formation of helix αN. As found by mutational studies [30], the destruction of the helix cap increases the entropy of the system and reduces the binding affinity, and once the helix is dissolved, the remaining interfaces are not sufficient for maintaining the complex. This molecular switch model is only valid for the Fe65-PTB2/AICD interaction, as all other PTB domains do not contact Thr668 and phosphorylation does therefore not alter their binding affinity [20]. Intriguingly, the Fe65-PTB2/AICD interface spans almost the entire AICD-C31 fragment, which has been implicated in apoptotic events. This raises the next question: what determines stability, lifetime, and eventually toxicity of the AICD?

7. AICD turnover

The turnover of APP is very fast (with a half life of cell surface APP of about 30-40 minutes only [81] and only about 10% of APP are estimated to reach the cellular membrane, whereas

the majority of APP locates to the Golgi apparatus and trans-Golgi network [10]. APP not shed at the surface is internalized within minutes [82], delivered to endosomes, and if not degraded in lysosomes recycled to the cell surface [83]. AICD is even more difficult to study, as due to its small size it is rapidly degraded once it is released from the membrane by the insulin degrading enzyme (IDE) [84], that also degrades the Aβ peptide, by the proteasome [85], or by the endosomal/lysosomal system [86]. However, AICD found in the nucleus appears to be more stable, suggesting that AICD involved in signal transduction escapes rapid degradation [87]. Nuclear AICD is stabilized via interaction with Fe65 [88, 89], which accordingly has a dominant function in AICD mediated physiological and pathophysiological processes.

From a structural viewpoint it is evident that the enlarged and unique protein-protein interface coupled with high affinity binding prevents the AICD from degradation. Interestingly, AICD-C31 (starting at Ala665), which is believed to induce apoptosis and is enriched in AD brains [34], fits exactly in length with the AICD part interacting with Fe65-PTB2. Hence, two scenarios comprising a modulating role for Fe65 in AICD-C31 mediated neurotoxicity might be envisaged: (i), under physiological conditions Fe65 protects the AICD from caspase cleavage occurring at Asp664 and might therefore inhibit apoptosis as shown previously [90] and (ii), increased levels of AICD-C31 compete with AICD binding as part of full-length APP and therefore interfere with physiological Fe65 functions including nuclear signaling and trafficking of APP. In any case, modifying the protein-interacting network around the AICD seems to be a valid target for decreasing neurotoxicity and the treatment of AD.

8. Conclusion

Despite enormous efforts to develop an efficient treatment for AD, only symptomatic treatments with modest impact on the progress of the disease are available [6]. Drugs currently approved for the treatment of AD are either acetylcholine esterase inhibitors to increase the level of the neurotransmitter, which is depleted in AD brains, or antagonize the NMDA receptor to prevent abnormal neuronal stimulation [91]. None of them directly targets the amyloid cascade and would thereby allow for a disease-modifying treatment. Many current therapeutic approaches for AD focus on the reduction of the Aβ load either by inhibiting the involved secretases BACE and γ-secretase, or by augmenting the elimination of amyloid peptides, e.g. by active or passive immunotherapy [6]. Finally, a smaller number of trials have targeted ApoE4 levels or either tau phosphorylation or tau aggregation. None of the approaches was successful so far, which means that either there were not enough clinical trials or the ideas were too simplistic to be potent for a complex disease. Like for other complex diseases (i.e. hypertension or AIDS), a combination of drugs that have different modes of action could be the key to success.

In this sense, the AICD might be re-evaluated as a potential drug target. In contrast to Aβ, the AICD is a physiological highly relevant protein domain modulating a diverse set of important APP functions including trafficking and signal transduction. As both processes are also directly affecting Aβ production, upstream targeting of AICD might be

beneficial as the Aβ pathology is prevented *a priori*. Moreover, the pathophysiology of the AICD and its breakdown product AICD-C31 has come into the focus of AD research and would be tackled directly. As the AICD by its nature is created intracellular, efficient compounds need to be able to pass the plasma membrane and to accumulate within neurons, as is i.e. the case for the NMDA receptor antagonist memantine [92]. However, the AICD is intrinsically disordered, and therefore the protein interaction network around the AICD might be the crucial target rather than the AICD itself. Major binding partners are the PTB domains, with their known ability to modulate Aβ production (like Fe65, ShcA, and X11α) and to specifically recognize and fold the AICD. Although protein-protein interactions are notoriously difficult to be targeted, the urgent need for a disease-modifying and efficient treatment for this devastating disease seems worth the trial.

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