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Minireview

Functions of WW domains in the nucleus

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Abstract The WW domain is a protein module found in a wide range of signaling proteins. It is one of the smallest protein modules that folds as a monomer without disulfide bridges or cofactors. WW domains bind proteins containing short linear peptide motifs that are proline-rich or contain at least one proline. Although the WW domain was initially considered a 'cytoplasmic module', the proteins containing WW domains have also been localized in the cell nucleus. Moreover, these proteins have been documented to participate in co-activation of transcription and modulation of RNA polymerase II activity. The carboxy-terminal domain (CTD) of RNA polymerase II acts as an assembly platform for distinct WW domain-containing proteins that affect the function of the RNA polymerase II. The formation of complexes between CTD and WW domain-containing proteins is regulated by phosphorylation of the CTD. Since the CTD sequence is highly repetitive and a target of several post-translational modifications and conformational changes, it presents a unique structure capable of enormous molecular diversity. The WW domain has been implicated in several human diseases including Alzheimer's disease. The WW domain-containing iso-prolyl isomerase named Pin1, a protein known to be essential for cell cycle progression, was shown to be active in restoration of the microtubule-binding activity of Tau, a protein of neurofibrillar tangles found in the brains of Alzheimer's patients. It is the WW domain of Pin1 that interacts directly with Tau protein. In addition, the WW domain-containing adapter protein FE65 was shown to regulate processing of Alzheimer's amyloid precursor protein. It is expected that by understanding the details of the WW domain-mediated protein–protein interactions, we will be able to illuminate numerous signaling pathways which control certain aspects of transcription and cell cycle.

Key words: Protein–protein interaction; WW domain; Carboxy-terminal domain of RNA polymerase II; Phosphorylation; *O*-Glycosylation; *cis*/*trans* Conformation; Alzheimer's disease; Tau protein; Fe65 adapter protein

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Abbreviations: cdk, cyclin-dependent kinase; CTD, carboxy-terminal domain; K, lysine; L, leucine; MPM-2, mitotic phosphoprotein monoclonal-2; NLS, nuclear localization signal; P, proline; R, arginine; RNA Pol II, RNA polymerase II large subunit; S, serine; SH3, Src homology domain 3, a protein module originally identified by homology to the amino-terminal region of Src protein; T, threonine; W, tryptophan; WW, a protein module with two conserved Ws; Y, tyrosine; YAP, Yes kinase-associated protein; x, any amino acid

1. WW domain as a protein module

WW domains are small modules composed of 40 amino acids. The name refers to two signature tryptophan (W) residues that are spaced 20–22 amino acids apart and play an important role in its structure and function [1]. The delineation of the WW domain was prompted by the identification of repeated sequences in the murine isoform of Yes kinase-associated protein (YAP) [1]. Functionally, the WW domain resembles the Src homology domain 3, a protein module originally identified by homology to the amino-terminal region of Src protein (SH3 domain) by displaying affinity toward proline-rich or proline-containing ligands [2]. Recent studies have shown that certain molecular details of the domain–ligand complex are similar for SH3 and WW domains [3]. The structure of the WW domain reveals a compact antiparallel three-stranded-β-sheet that forms a shallow interface for binding linear peptide motifs of the ligands [4,5]. Based on the ligand predilection, WW domains fall into two major and two minor groups [6]. One major group (Group I) binds polypeptides with the minimal core consensus PPxY, and the other binds ligands with PPLP motif (Group II). Group III WW domains select poly-P motifs flanked by R or K, whereas Group IV WW domains bind to short sequences with phospho-S or phospho-T followed by P, in a phosphorylation-dependent manner [6].

The K_d of interaction for WW–ligand complex formation is in the high nM to low μM values for proline-rich ligands, and in the low μM values for phospho-SP- or phospho-TP-containing ligands. Phosphorylation of the terminal tyrosine in the ligand PPxY for Group I WW domains abolishes the binding in vitro, and possibly in vivo, suggesting that this modification could represent a negative regulation mechanism for a large subset of WW domains [6].

Shortly after its characterization, WW domain attracted attention because the signaling complexes it mediates have been implicated directly or indirectly in several human diseases including Liddle's syndrome of hypertension, muscular dystrophy, Alzheimer's and Huntington's diseases, and, more recently, cancer [6,7]. In the case of Alzheimer's disease, discussed herein, the implicated WW domains interface with cell cycle and transcription; and the binding specificity of one of the WW domains, Pin1, was illuminated with the help of antibody specific for phospho-epitopes found on mitotic proteins.

This brief contribution focuses on one aspect of WW domain, specifically its role in the nuclear processes. Many facets of the WW domain function in the nucleus are just emerging

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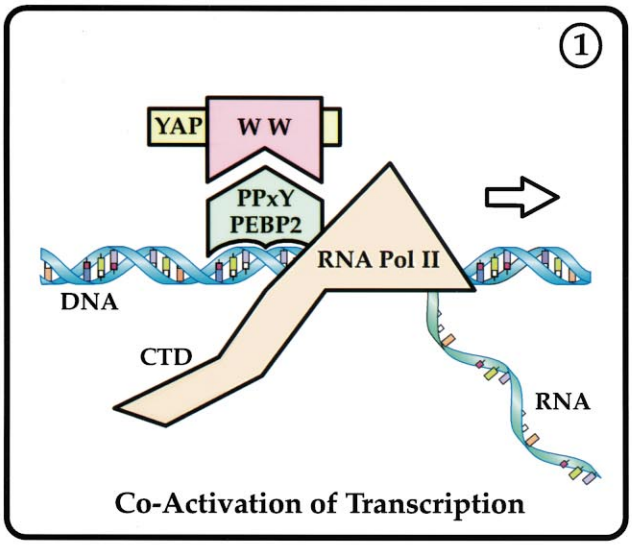
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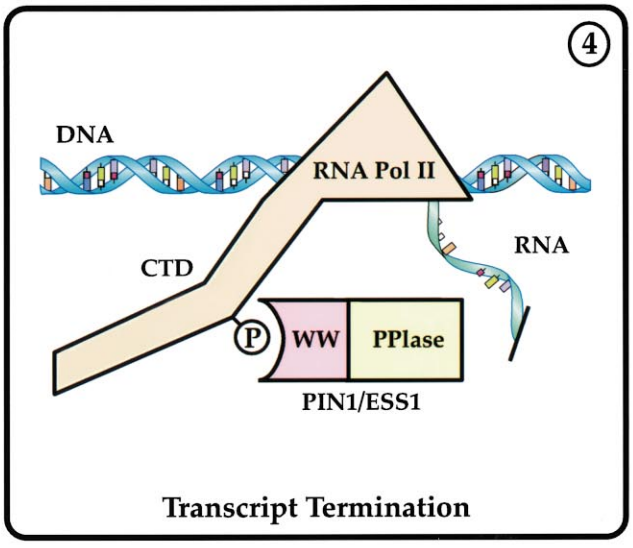
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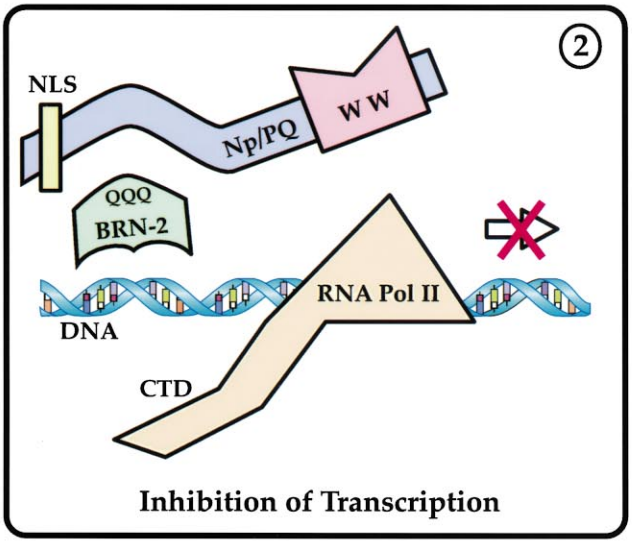
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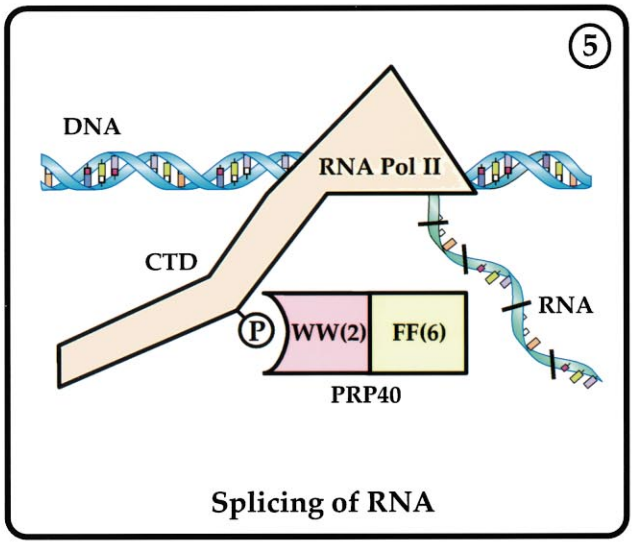
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CTD
RNA
P
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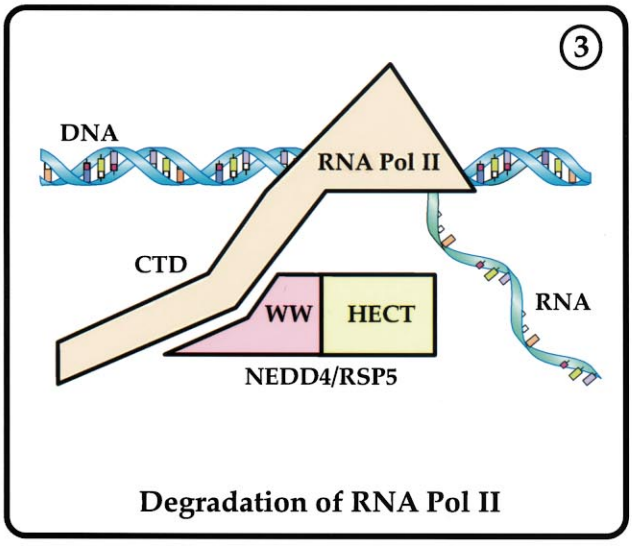
FF(6)
PRP40
Splicing of RNA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WW
HECT
NEDD4/RSP5
Degradation of RNA Pol II

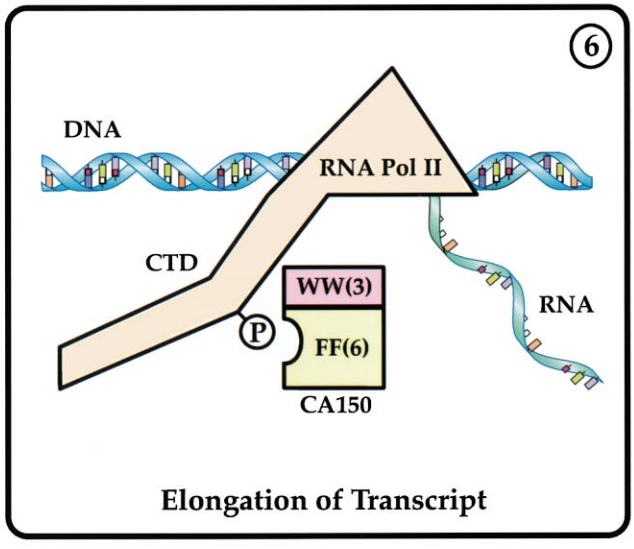
6
DNA
RNA Pol II
CTD
RNA
WW(3)
P
FF(6)
CA150
Elongation of Transcript

Fig. 1. Examples of WW domain-mediated complexes in the nucleus. The WW domain of Yes kinase-Associated Protein (YAP) binds Polyoma Enhancer Binding Protein 2 (PEBP2) acting as a co-activator of transcription (panel 1 and [8]). The WW domain-containing protein Np/PQ (named after two identical but independently characterized proteins Npw38 and PQBP1) binds to the poly Q region of transcription factor BRN-2 inhibiting transcription (panel 2, [10,39,40]). Carboxy-Terminal Domain (CTD) of RNA Pol II forms a complex with WW domains of mammalian Nedd4 (Rsp5 is the yeast homolog of Nedd4) protein that contains ubiquitin ligase activity (Hect domain). Formation of this complex leads to the degradation of RNA Pol II (panel 3, [6]). Phosphorylated CTD binds to the WW domain of Pin1 (Ess1 is the yeast homolog of Pin1) protein that contains iso-prolyl isomerase activity. Formation of this complex is correlated with transcriptional termination (panel 4, [6]). Phospho-CTD interacts with WW domains of a splicing factor Prp40 resulting in regulation of RNA processing (panel 5, [41]). Elongation of transcript is co-regulated by complex between phospho-CTD and CA150 adapter protein. Here the complex between phospho-CTD and CA150 is mediated by FF domains (panel 6, [42]).
←

and prevent us from presenting a uniform picture of signaling steps mediated by WW domains acting in nucleus. Nevertheless, this study should serve as a useful summary of what is currently known about the role of WW domain-based interactions in regulating transcription and the cell cycle. Additionally, we hope this review will stimulate interest in the field and serve as a harbinger of new observations.

2. WW domain and transcription

Among several examples of WW domain complexes functioning in the cell nucleus, the best documented one is the physical and functional complex formed between the WW domain of YAP and the PEBP2 (polyoma enhancer binding protein 2) transcription factor, in which the YAP WW domain acts as a transcriptional co-activator through interaction with a PPPY motif in PEBP2 [8] (Fig. 1). The PPxY motif has been observed in a number of transcription factors including NF-E2, AP2, and c-Jun, where it may play a role in transcriptional activation. For example, the hematopoietic transcription factor, NF-E2, contains two PPxY motifs, which can be recognized by the WW domains of E3 ubiquitin ligases [9]. Interaction of WW domain-containing proteins with this motif seems to be important for transcriptional activation, as mutant constructs expressing one of the two PPxY motifs in NF-E2 or the single mutated copy in PEBP2 are defective in their co-transcriptional activity [9]. It is speculated that the presence of the PPxY motif within transcription factors may recruit WW domain-containing proteins such as Npw38 or YAP, which have been known to act as transcriptional co-activators. Perhaps negative regulation of transcription by certain WW domains or by WW domain-containing proteins is also possible. PQBP-1, a novel polyglutamine tract binding protein with an WW domain, was shown to inhibit activation of transcription by Brn-2 [10] (Fig. 1).

3. WW domains, RNA polymerase II and cell cycle

The cell cycle and transcription by RNA polymerase II are closely related because they employ shared components [11]. The transcriptional activity of RNA polymerase II large subunit (RNA Pol II) is modulated during cell cycle. The phosphorylation status of the carboxy-terminal domain (CTD) of the largest subunit of RNA Pol II is cell cycle dependent and correlates with changes in transcription [11]. Several CTD kinases are also members of the cyclin-dependent kinase (cdk) family including cdk1, cdk7, cdk8, and cdk9. Each of these cdks, have been directly linked to the cell cycle regulatory events [11]. Other CTD kinases such as casein kinase II, and protein-tyrosine kinases such as c-Abl and Arg, have also been implicated in cell cycle modifications of the CTD. Therefore the same enzymes target and coordinate functionally linked events of cell cycle and RNA Pol II-regulated transcription.

Within the RNA Pol II largest subunit, the CTD is the primary target of phosphorylation. In the M-phase where transcription is globally inhibited, CTD is hyperphosphorylated in part by cdk1. In G_1 phase, CTD phosphorylation on S and T is somewhat lower. In the G_1/S phase transition, protein-tyrosine kinase activity of c-Abl toward CTD increases [11].

The CTD of mammalian RNA polymerase II largest subunit contains 52 tandem repeats of a heptapeptide with the consensus sequence, YSPTSPS. These heptapeptides may be either perfect or imperfect iterations of the consensus heptapeptide [11]. Gavva and colleagues were the first to show that WW domain-containing proteins such us Nedd4 and YAP could interact with RNA Pol II CTD even though CTD contained only partial P**P**x**Y** motif (x**P**x**Y**) [12]. Being a part of the repeated heptamers (YSPTS**PSY**SPTSPS) the **PSY** motif of CTD is present at a relatively high local concentration [13]. Subsequently, work from several laboratories, including ours, has demonstrated that CTD of RNA Pol II could bind various WW domain-containing proteins and that the binding was regulated by phosphorylation [6]. Ess1/Pin1 PPIase was shown to interact with the phosphorylated form of CTD, whereas Rsp5/Nedd4 ubiquitin ligase was documented to interact preferentially with unphosphorylated CTD [6,9,13]. Phosphorylation of CTD seems to play a role of a switch that selects which WW domain-containing proteins could assemble on the CTD tail. These exclusive complexes seem to correlate with distinct functions of RNA Pol II. For example, binding of Ess1/Pin1 to CTD of RNA Pol II may regulate the process of RNA termination. Complex of the CTD with Rsp5/Nedd4 is proposed to initiate degradation of RNA polymerase II by the ubiquitin-proteasome pathway [6] (Fig. 1). A number of other proteins with modular domains are known to nucleate on the CTD tail giving this essential part of RNA Pol II an important regulatory function. Indeed, considering the growing importance of the CTD of RNA Pol II as a sensor of numerous signaling pathways, the CTD is 'the tail that wags the dog' [14].

4. Potential diversity at the CTD of RNA Pol II

We know that CTD is a target of S, T, and Y phosphorylation, that Ess1/Pin1 peptidyl-prolyl *cis*/*trans* isomerase catalyzes the *cis*/*trans* conversion of the peptide bonds between phospho-S/T and P, and that *O*-glycosylation of S and T residues of CTD was documented and shown to be reciprocal with phosphorylation [11,15,16]. We therefore decided to calculate the number of potential variants that the highly re-

peated heptapeptide sequence of CTD could form. For our calculations we took only perfect heptapeptide repeats and ignored imperfect iterations. In the human CTD, there are 21 perfect repeats and in the yeast CTD there are 19 perfect repeats.

We considered a single YSPTSPS heptamer. Within the YSPTSPS heptamer, Y, Ss and T may be modified by phosphorylation. Each of Y, S and T can independently acquire one of two forms: phosphorylated and/or not phosphorylated. The number of possible variants of the YSPTSPS heptamer due to phosphorylation is 32 (each of five Y, S and T can appear in two variants: $2^5 = 32$).

Since we assume that each of the YSPTSPS heptamers is independent of any other in the entire CTD, the number of possible variants of CTD due to phosphorylation is 32^N, where N is the number of repeats of YSPTSPS heptamers in the entire CTD.

Using the same tools we calculated the number of possible variants due to *O*-glycosylation of S and T, as well as the number of possible variants of CTD due to alternative *cis*/*trans* conformations of S–P bonds (Table 1).

A surprisingly large number of variants was revealed when we took into account all three possible modifications to the YSPTSPS heptamer described above. The Y can appear in two forms (phosphorylated and/or not phosphorylated); each S and T may appear in three forms (phosphorylated, glycosylated or unmodified) and each of S–P peptide bond may appear in two forms (*cis* or *trans*). The number of possible variants of YSPTSPS due to all three modifications is 648; $(2\times3\times3\times3\times3\times4 = 648)$.

Since each of the YSPTSPS heptamers is considered independent of any other in the entire CTD, the number of possible variants of CTD due to all three modifications is 648^N, where N is the number of repeats of YSPTSPS heptamers in the entire CTD (Table 1).

Indeed, the numbers are astronomical, and they would be significantly higher if the imperfect repeats (e.g. additional 31 for the human RNA Pol II CTD) were included. The purpose of this academic exercise is to illuminate a tremendous potential of CTD sequences for forming unique structural and biochemical micro-environments. Probably the number of conformations, shapes or variants that are 'meaningful' or 'read' by the CTD interacting proteins is smaller. For example, phosphorylation or glycosylation may favor one particular conformation of the S–P peptide bonds within the CTD. The biological response could be initiated only if the stoichiometry of interacting proteins is reached; e.g. half of the CTD tail has to be occupied by binding partners or it has to assume a certain conformation in order for the RNA Pol II to change its activity. Without any doubt, the CTD of RNA Pol II remains an exciting challenge for experimental biology.

5. WW domains of Pin1 and Fe65 in Alzheimer's disease

A monoclonal antibody called mitotic phosphoprotein monoclonal-2 (MPM-2) has been an important tool for dissecting the role of phosphorylation in mitotic regulation [17]. It is through the use of the MPM-2 antibody that the connection between RNA Pol II CTD, cell cycle and one aspect of protein regulation in Alzheimer's disease has surfaced [11]. The MPM-2 antibody recognizes a phosphorylated epitope: phospho-S- or phospho-T-P epitope on ca. 50 proteins, which are localized to various mitotic structures [17]. MPM-2 antibody helped to uncover the novel binding function of Pin1 WW domain [11]. Pin1 and MPM-2 were shown to recognize phosphorylated CTD as well as phosphorylated Tau protein [11,18]. Tau, a microtubule-associated protein is an important component of neurofibrillary tangles present in the Alzheimer's brain. Tau as a component of neurofibrillary tangle is hyperphosphorylated and its phosphorylation causes a loss of Tau binding to microtubules. It was also shown that Pin1 has the ability to bind to phospho-T-P motif in Tau and restore Tau's ability to promote microtubule assembly in vitro [18].

Since changes in the phosphorylation status of RNA Pol II in Alzheimer's brain have been detected and Tau is hyperphosphorylated in the neurofibrillary tangles of Alzheimer's brain, it should be of interest to study the Pin1 WW domain in detail. The isolation of low molecular weight, non-peptide compounds that interact with the WW domain of Pin1 or the Pin1 protein and modulate its activity in neurons, would provide valuable reagents for basic and applied study.

Fe65 is one of three adapter proteins interacting with the cytodomain of Alzheimer's β-amyloid precursor protein (APP) [19]. APP is an integral membrane protein from which the Alzheimer's β-amyloid peptide (Aβ) derives as a consequence of its proteolytic processing [20]. This amyloidogenic processing is affected by the interaction of APP with Fe65 and with the other two PTB-containing ligands, X11 [21] and mDab1 [22]. The overexpression of Fe65 causes an increased generation of Aβ in cultured cells, [23,24] while overexpression of X11 inhibits the proteolytic processing of APP [25–27]. The WW domain of Fe65 binds several proteins, one of which is Mena, the mammalian orthologue of the product of the *enabled* gene of *Drosophila*, which is a genetic modulator of the phenotype induced by the Abl gene mutation in *Drosophila* [28]. The *Drosophila* Abl gene has a neuronal function and it is required for axonal outgrowth and fasciculation. The detection of Fe65–Mena/(enabled) protein complex is of particular interest, considering that mDab1 is the orthologue of the *disabled* gene of *Drosophila*, another modulator of the Abl mutation induced phenotype, whose action is counteracted by *enabled* [29]. The possible competition between mDab1 and

Table 1
Molecular diversity of the yeast and human CTD of RNA polymerase II

	Yeast CTD	Human CTD
Number of repeats of YSPTSPS in the CTD	19	21
Number of possible variants of CTD due to phosphorylation of Y, S and T positions	3.961×10^{28}	4.056×10^{31}
Number of possible variants of CTD due to glycosylation of S and T positions	7.556×10^{22}	1.934×10^{25}
Number of possible variants of CTD due to alternative conformations of S–P peptide bonds (*cis* and *trans*)	2.749×10^{11}	4.398×10^{12}
Number of possible variants of CTD due to phosphorylation, glycosylation and alternative conformations of S–P peptide bonds	2.630×10^{53}	1.104×10^{59}

the Fe65–Mena complex for the binding to APP could explain the opposite effects of *enabled* and *disabled* gene products in vivo.

Besides its involvement in the protein–protein interaction network centered at the cytosolic domain of APP, Fe65 has also been studied for its possible role in the regulation of transcription. Early results on this protein showed that it is able to strongly activate the transcription of a reporter gene when fused to an heterologous DNA binding domain [30]. More recently it was found that Fe65, through its PTB1 domain, forms complexes, in vitro and in vivo, with the transcription factor CP2/LSF/LBP1 and that these complexes are present both in the cytoplasm and in the nucleus [31]. This observation has acquired new significance following the discovery that CP2/LSF/LBP1 gene is a genetic determinant of Alzheimer's disease [32]. In fact, by examining 1139 Alzheimer's disease cases and 1317 controls, it was demonstrated that a G↔A polymorphism in the 3′-UTR of the CP2/LSF/LBP1 gene on chromosome 12 is associated with a modified risk of Alzheimer's disease. Individuals bearing one or two adenine alleles have a significantly lower risk of Alzheimer's disease than the subjects with the guanine–guanine genotype [32]. This observation suggests the existence of a functional link between Aβ generation from APP and the CP2/LSF/LBP1 factor – the tool of this link being Fe65. In support of this hypothesis, there is a recent evidence that Fe65 is targeted to the nucleus and that this phenomenon is regulated by APP, which functions as a cytoplasmic anchor preventing the Fe65 nuclear translocation [33]. Experiments based on the use of deletion mutants of Fe65 allowed us to demonstrate that a region of about 100 amino acids, containing the WW domain, is responsible for Fe65 nuclear targeting and that the same region is also sufficient to target to the nucleus a reporter protein, otherwise restricted to the cytoplasm. Considering that no known nuclear localization signal (NLS) is present in these 100 amino acids, it can be speculated that the translocation of Fe65 into the nucleus takes place by means of a cargo system and that the WW domain is involved in the docking of Fe65 to the nuclear-targeted cargo. There are several examples of proteins imported to the nucleus in the absence of any canonical NLS [34]. The question is how these proteins interact with the translocation machinery. As mentioned above, the WW domain of Fe65 interacts with several proteins, only one of which has been identified. In the light of the possible involvement of this WW domain in the nuclear targeting of Fe65, the identification of its ligands could lead to the discovery of new mechanisms of cytosol-nuclear trafficking not based on the classical NLS motif.

6. WW domain as an emerging therapeutic target

Since the WW domain is small and its ligand is rigid and well-structured, the WW domain–ligand complex is amenable for gene and drug therapy [35,36]. Small non-peptide compounds, perhaps mimicking *N*-substituted amino acids [3], may act as stimulators or inhibitors of signaling steps mediated by WW domain. Moreover, we are now in a position to use molecular repertoires to tailor individual WW domains toward new binding and signaling activities [37].

With the imminent advent of the WW domain proteomic chip based on a unique feature of the WW domain, namely its ability to form an active domain when chemically synthesized on solid support as in the case of the SPOT membrane assay ([38] and Sudol, M., submitted) we should be able to identify specific signaling targets for development of useful therapeutics.

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