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Characterization of the Interactions of Mammalian RNA Polymerase I Associated Proteins PAF53 and PAF49

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Abstract

Masami Muramatsu's laboratory demonstrated the critical role of RNA polymerase I (Pol I)-associated factor PAF53 in mammalian rRNA transcription. They have also identified a second polymerase associated factor, PAF49. Both PAF49 and PAF53 copurify with that fraction of the RNA polymerase I molecules that can function in transcription initiation *in vitro*. PAF49 and PAF53 are the mammalian homologues of two subunits of yeast RNA polymerase I, A34.5 and A49, that form a TFIIF-related subcomplex in yeast RNA polymerase I. In light of those publications, we investigated the interactions between various deletion and substitution mutants of mammalian PAF49 and PAF53 with the purpose of identifying those domains of the mammalian proteins that interact. Comparison of our results with structural studies on yeast A34.5 and A49, demonstrate that the yeast and mammalian proteins may in fact share structural similarities. In fact, the deletion mutagenesis data confirmed and extended the structural studies. For example, amino acids 41–86 of PAF49 were sufficient to provide the basis for heterodimerization. *In silico* structural analysis predicted that this region could assume a structure similar to the homologous region of yeast A34.5. Those similarities are insufficient, by themselves, for the proteins to form inter-specific heterodimers. However, substitution of amino acids 52–98 of yeast A34.5 with amino acids 41–86 of mammalian PAF49 resulted in a protein that could heterodimerize with mouse PAF53.

Ribosomal gene activity is essential to cell growth, varying dramatically with growth rate, and in response to a wide variety of factors (reviewed [1–3]). During periods of rapid cell growth, rRNA synthesis can account for more than 70% of the total RNA synthesis of the cell. In rapidly growing tumor cells the cellular ribosomal RNA content must be replicated every 8–10 hours, whereas in normal hepatocytes, the half-life of rRNA is measured in weeks. Cells have evolved various mechanisms for regulating rDNA transcription. For example, the rDNA transcription factor UBF is regulated by expression [4, 5] phosphorylation [6], acetylation [7, 8] and through interactions with the protein product of the retinoblastoma susceptibility gene [9–11]. The regulation of UBF activity modulates the formation of the preinitiation complex as well as elongation [12]. Changes in the cellular levels of UBF can affect the levels of transcribable chromatin [13]. Similarly, the species-specific transcription factor, SL1 is also regulated at different levels [14–16].

The three nuclear RNA polymerases contain catalytic cores that consist of ten subunits that are shared or homologous [17, 18], and they also contain peripheral subcomplexes [19, 20]. In yeast RNA polymerase I one subcomplex consists of A14 and A43. The A43 subunit of RNA polymerase I mediates the recruitment of Rrn3 to the polymerase. Rrn3 bridges

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Supporting Information Available: Supplemental Figures 1–3 are available free of charge via the Internet at http://pubs.acs.org.

between the polymerase and the promoter complex, [21–24] and is required for the formation of the stable and competent preinitiation complex[24, 25] by the core polymerase. Only 5–10% of the molecules of RNA polymerase I within the cell contain Rrn3 (discussed in references [26, 27]). Rrn3 is the best characterized example of a group of proteins that distinguish the transcription competent form of RNA polymerase I. Prior to the discovery of Rrn3, Hanada *et al.* reported that the form of RNA polymerase I that supported specific transcription could be distinguished from that which couldn't by the association of two additional proteins, PAF53 and PAF49 [28]. Consistent with their report, we have also reported that only 60% of the RNA polymerase I molecules in rapidly growing, rat, hepatoma cells contain PAF53 [29], indicating that not all core polymerase complexes contain PAF53. PAF53 and PAF49 in mammalian cells are the homologues of yeast the A49 and A34.5 subunits [28, 30–32]. Although the report of Hanada *et al.* would suggest that PAF53 and PAF49 are essential for rDNA transcription, studies on the yeast homologues suggest the contrary. For example, both A34.5 and A49 are nonessential subunits of RNA polymerase I [33, 34]. However, A49−/− yeast grow at only 5–10% of the wild type rate. More recently, Geiger *et al.* reported that the A34.5/A49 subcomplex in yeast functioned as an elongation factor for RNA polymerase I [31]. Any consideration of the role/s of A49/A34.5 and PAF53/PAF49 in rDNA transcription requires that apparently contradictory observations concerning the biochemistry and biology of the proteins need to be reconciled.

In order to add to our understanding of the roles of PAF53/PAF49 in rDNA transcription, we have mapped the domains of mammalian PAF53 and PAF49 that mediate their interaction. Our results both confirm and extend those studies that have focused on the mammalian proteins. In addition, our studies agree with the structural studies on yeast A34.5/A49 that mapped those domains of the yeast homologues of PAF53 and PAF49 that are necessary for the heterodimerization. Interestingly, deletion mutagenesis of mouse PAF49 demonstrated that a region of only 46 amino acids (41–86) was sufficient to mediate the interaction with PAF53. In contrast the yeast structural studies suggested that a much larger region of A34.5 was participated in the dimerization with A49. However, despite apparent structural similarities, yeast A34.5 did not heterodimerize with mouse PAF53. When we substituted amino acids 41–86 of mouse PAF49 for amino acids 52–98 of yeast A34.5, the resulting chimera interacted with PAF53. Similar results were obtained when regions in PAF53 that had been identified by mutagenesis were substituted into yeast A49. These data demonstrated that despite apparently conserved structures and functions, the amino acid sequences of the A34.5/A49 and PAF53/PAF49 homologues have evolved independently to the point that they represent species-specific evolution.

Materials and Methods

Tissue Culture and Transfection

NIH 3T3, NIH 3T6 and HEK293 cells were grown as described previously [35, 36]. HEK293 and HEK293Tcells were plated in DMEM, 5% FBS at 2×10^6 cells per 100mm dish, and transfected the next day using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Transfections were carried out for 24 or 48 hr for 293 and 3T3 cells, respectively. Cells were scraped into lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, containing protease inhibitors (Complete, Roche Molecular Biochemicals) and either used immediately or frozen at −80°C until needed [37].

Transfection, Ligand and Immunoaffinity Purification

Transfections were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Forty-eight hours post transfection, whole cell lysates were prepared as described previously [29, 38, 39] and the lysates were tumbled with anti-FLAG-

agarose,, immobilized nickel (Ni-NTA agarose, Qiagen) or GSH-agarose for 2h at 4 °C. The bound proteins were eluted with FLAG peptide (500 μg/ml), imidazole (500 mM) or reduced GSH (50 mM), or the beads were boiled in SDS sample buffer, and the eluted proteins were analyzed by SDS-PAGE and Western blotting using anti-FLAG, anti-GST, anti-HA or antibodies specific to PAF53 or PAF49 as described previously [38].

Immunoblotting

Protein determinations were performed using the Bio-Rad D-C assay kit with bovine serum albumin as the protein standard. Western blots were carried out as described previously [29]. Proteins were detected by incubating the filters with a 1:2000 dilution of the appropriate antibody, followed by incubation with horseradish peroxidase-conjugated anti-rabbit antibodies (Bio-Rad). FLAG-tagged proteins were detected by incubating the filters with a 1:2000 dilution of the M2-anti-FLAG antibody (Sigma) followed by incubation with horseradish peroxidase-conjugated anti-mouse antibodies (Bio-Rad). Immunoreactive proteins were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo). The molecular sizes of the immunodetected proteins were verified by comparison to the migration of prestained protein markers (Bio-Rad) electrophoresed in parallel lanes [4]. Antibodies to PAF53 and mammalian A127 have been described previously [29]. The antibodies to GFP were obtained from Clontech, the antibodies to GST were obtained from Sigma.

Mutagenesis of PAF53 and PAF49

Full-length or mutants of PAF53 or PAF49 were cloned into pcDNA3.1 (Invitrogen), PKH3 BSENX, and/or pEBG as required. Various epitope tags were added to the coding regions by PCR, and the constructs were confirmed by DNA sequencing. Deletion mutants were constructed by PCR-directed mutagenesis using overlapping primers based on the sequences of the cDNA and the vector. The internal deletion mutant of PAF49, between amino acids 41–50, was generated by overlapping PCR. The chimeric mouse/yeast proteins were constructed similarly. All the amplification reactions were performed using iProof polymerase (Bio-Rad). All mutant constructs were confirmed by DNA sequencing. The initial clones for PAF53 and PAF49 were obtained from the laboratory of Dr. Masami Muramatsu. The clones for yeast A34.5 and yeast A49 were obtained from Dr. David Schneider.

Results

PAF53 and PAF49 both heterodimerize and interact with RNA polymerase I

We have previously reported [40] that transiently transfected PAF53 associates with RNA polymerase I, and we confirmed and extended this observation for PAF49. 293T cells were transfected with vectors expressing tagged-PAF53 or tagged-PAF49 or both proteins simultaneously. Whole cell extracts were harvested and protein-protein interactions detected by western blots after either immunoaffinity pull-down assays (anti-FLAG) or immobilized nickel affinity assays (IMAC) pull-down assays. The starting material and pull-down fractions were probed for A127 (the second largest subunit of RNA polymerase I), using antibodies raised to recombinant A127 protein [29], and ectopically expressed PAF49, and PAF53 as indicated. As shown in Figure 1, when either PAF49 or PAF53 were overexpressed in 293 cells, we found evidence that the two proteins interacted both with one another and with RNA polymerase I. Specifically, in lanes 4, 6 and 9 ectopically expressed PAF49 and PAF53 are shown to interact. As shown in lane 4 and 11, ectopically expressed PAF49 and PAF53 can interact with endogenous RNA polymerase I. When we used His-tagged PAF53 in pull-down experiments, there was significant background binding of Pol I (note the band marked A127 in Fig 1, lanes 7–9). Hence, we repeated the pull-down assay

using FLAG-tagged PAF53 (Fig 1, lanes 10 and 11). The background signal at 127 kDa was greatly reduced (Fig 1 lane 10) and the interaction between PAF53 and A127 was apparent (Fig 1, lanes 10 and 11). These experiments demonstrated that both PAF53 and PAF49 could interact with RNA polymerase I and confirmed that the two proteins interacted with one another either directly or indirectly [30, 41]. In the course of these experiments, we observed that when PAF53 and PAF49 were overexpressed simultaneously, we observed a greater accumulation of both proteins than that observed when the proteins were transfected individually (compare the levels of PAF53 and PAF49 in Fig 1, lanes 1 and 2 *vs.* 3). These results suggested that the formation of a PAF53/PAF49 heterodimer acted to stabilize the two proteins.

Identification of the Domain in PAF49 that Interacts with PAF53

In order to better understand the interactions between PAF53, PAF49 and RNA polymerase I, we next sought to define the domains in PAF53 and PAF49 that mediated this interaction. Figure 2 summarizes the PAF49 mutants used in this study and which mutants were able to interact with PAF53.

The first series of PAF49 deletions demonstrated that the interaction domain with PAF53 was between amino acids 1–100 of PAF49. Deletion mutants of PAF49 containing amino acids 1–300 and 1–200 interacted with PAF53 (data not shown). As shown in supplemental Figure 1, PAF49 constructs containing amino acids 1–100 (Figure 1, Panel A) were capable of interacting with PAF53. Thus, the first 100 amino acids of PAF49 are sufficient for binding to PAF53. This was supported by the observation that deletion of amino acids 1–100 abrogated the interaction with PAF53 (supplemental Figure 1, Panel B). Thus, amino acids 1–100 of PAF49 are necessary and sufficient to mediate the interaction between PAF49 and PAF53, and there does not appear to be any other, independently acting binding site. This result extends the result published by Yamamoto *et al.* [30] who reported that amino acids 1–270 of PAF49 were required for the interaction with PAF53.

In order to better delineate the amino acids required for the interaction with PAF53, a series of deletion mutants were constructed that focused on the N-terminal amino acids. Analysis of the interactions of these mutants with PAF53 (Figure 3) narrowed the binding site to between amino acids 41–100 (data not shown). Constructs containing amino acids 1–40, 50–110 and 60–120 of PAF49 did not interact with PAF53. Only one construct in this series of mutants, PAF49 41–100, was able to interact with PAF53 (data not shown). Subsequently, we dissected the region between amino acids 40 and 100 of PAF49 to determine the minimum region that would be sufficient to mediate an interaction between PAF49 and PAF53. The results of these experiments demonstrated that the C-terminal boundary lies between amino acids 80–86 of PAF49. Constructs expressing amino acids 30–76 (Figure 3A, lane 8) or amino acids 41–80 (Figure 3B, lane 7) of PAF49 didn't bind PAF53, but constructs expressing amino acids 30–86 (Figure 3A, lane 9) and amino acids 41–86 (Figurer 3B, lane 8) did bind PAF53. These experiments also demonstrate that the N-terminal boundary of the binding domain lies between amino acids 41–45 of PAF49 and would include a conserved block of amino acids (discussed below). A construct expressing amino acids 45–100 did not bind PAF53 (Figure 3B, lane 6), but one that expressed amino acids 30–96 did bind PAF53 (Figure 3A, lane 10). The consensus of these experiments was that amino acids 41–86 were sufficient to mediate the interaction (Figure 3B, lane 8).

Interestingly, multiple sequence alignment using Pfam [42] (Wellcome Sanger Institute) analysis of the region from 1–100 of mouse PAF49 with yeast and other A34.5 homologues, identified a conserved block of 9 amino acids (PAF49 amino acids 41–49) (Supplemental Figure 2A). This suggested that these 9 AA may be critical for function. Accordingly we deleted amino acids 41–50 of PAF49, and found that the protein could no longer interact

with PAF53 (Supplemental Figure 2B). These nine amino acids are necessary for the interaction between PAF49 and PAF53.

These data are consistent with the X-ray crystallographic data published by Geiger *et al.* [31] wherein it was reported that amino acids 22–144 of yeast A34.5 constituted the heterodimerization domain for the interaction with A49. However, our data would suggest that the region required for the interaction is actually smaller, 45 amino acids. Analysis of the predicted secondary structure of the region encompassed by amino acids 41–86 using I-Tasser [43, 44] demonstrated a similar arrangement of β-sheets and α-helical regions as that found in the region that includes the predicted β2 through β5 regions of A34.5 [31] (Supplemental Figure 3).

Identification of the Domain(s) in PAF53 that Interacts with PAF49

We next determined the region of PAF53 required for the interaction with PAF49. The constructs used in this series of experiments and their ability to interact with PAF49 are summarized in Figure 4. As shown in Figure 5, Panel A, amino acids 1–331 and 1–222 of PAF53 supported an interaction with PAF49, but amino acids 1–114 did not. Moreover, amino acids 216–435 of PAF53 did not support an interaction with PAF49, suggesting that the region between amino acids 1 and 222 was both necessary and sufficient for binding of PAF53 to PAF49 (Fig. 5, panel B). Subsequent experiments with PAF53 70–222,114–222 and 1–160 demonstrated that amino acids 70–160 were insufficient to mediate the interaction with PAF49 (Fig 5, panels C&D). Taken together, these data demonstrated that the N-terminal boundary of the region of PAF53 required for the interaction with PAF49 lies between amino acids 1 and 70. Moreover, the C-terminal boundary is defined as between amino acids 114 and 160 (aa1–160 was sufficient to mediate an interaction, but aa 1–114 was not). This region is in good agreement with the predicted dimerization domain defined by the three-dimensional studies of Geiger *et al.* [31]. In this same light, it is interesting to note that Hanada *et al.*[28] reported that PAF53 and A49 contained two regions with conserved amino acids between amino acids 34–59 (PAF53) and 135–160. Our PAF53 deletion analysis presented here, and the three dimensional analysis in Geiger *et al.*[31], suggests that the region in PAF53 (yeast A49) that interacts with PAF49 (yeast A34.5) is discontinuous and may only consist of the domains referred to as β1, β8 and β9 in A49 [31.]

The mammalian and yeast homologues of PAF53 and PAF49 do not heterodimerize

In silico analysis (I-TASSER) of the structure of the domain in PAF49 that interacts with PAF53 predicts that it generates a structure similar to that predicted for yeast A34.5 (Supplemental Figure 3) [43]. Further, I-TASSER analysis of amino acids 1–60 of PAF53 demonstrated that they might be structurally similar to amino acids 5–48 of *C. glabrata* A49 (Figure 6). This led us to investigate the possibility that the yeast and mammalian homologues might interact with one another, *i.e.* yeast A34.5 might interact with mammalian PAF53 and yeast A49 might interact with mammalian PAF49. As a first step to investigating this question, we determined if the two yeast proteins would interact when coexpressed in a mammalian cell line.

HEK 293T cells were transfected with yeast A34.5 and A49 and we determined if the two proteins would coimmunoprecipitate. As shown in Figure 7, panel A, the yeast proteins did interact with one another when coexpressed in 293 cells. We then sought to determine if the yeast proteins would interact with the mammalian proteins. As shown in Figure 7, panels B and C, neither combination heterodimerized, *e.g.* yeast A49 did not interact with mammalian PAF49. This suggested that despite the apparent conservation of structure between A34.5 and PAF49, there was a need for a specific amino acid sequence. Pfam analysis of the sequences of yeast A34.5 and mouse PAF49 indicated that amino acids 41–86 of the mouse

protein were the “equivalent” of amino acids 52–98 of the yeast. In order to test this model, we constructed a chimeric protein in which amino acids 41–86 of mammalian PAF49 were substituted for amino acids 52–98 of yeast A34.5 (Figure 8, panel A). We then determined if this chimeric protein would interact with mammalian PAF53. The chimeric yeast/mouse/yeast form of A34.5 was cotransfected with GST-tagged PAF53, and the ability of the two proteins to heterodimerize was assessed. As shown in Figure 8, panel A, lane 6, the chimeric form of yeast A34.5 interacted with mammalian PAF53.

Our deletion mutagenesis studies on the sites of mouse PAF53 required for the interaction with mouse PAF49 indicated that amino acids 1–70 and amino acids between 114–160 were required for that interaction. To confirm and extend those observations, we substituted amino acids 1–70 and 114–160 of mouse PAF53 for amino acids 71–113 and 109–135 of yeast A49. We then asked if this chimeric protein could interact with mouse PAF49. As shown in Figure 8, panel B, this construct could interact with mouse PAF49 (lane 6). Hence, amino acids amino acids 1–70 and 114–160 of mouse PAF53 contain information necessary for the interaction.

The results of the pull-down assays as well as *in silico* analysis of the mammalian and yeast proteins indicate that similar domains of PAF53/A49 and PAF49/A34.5 are involved in the formation of the heterodimer. These results are summarized in Figure 9. The top panel, adapted from Geiger et al.[31] indicates both the dimerization domains of A34.5 and A49 as reported [31] and the dimerization domains of PAF49 and PAF53 based on the results reported herein. The bottom panel shows a colorized version of the predicted 3D structure of a portion of the yeast A34.5/A49 heterodimer (2nffa; pdb) in which the corresponding mammalian heterodimerization domains are colored in green (PAF49) or orange (PAF53).

Discussion

Previous studies from our laboratory and others have demonstrated that either the levels of PAF53 and PAF49 or their association with RNA polymerase I in mammalian cells are regulated coordinately with transcription by RNA polymerase I[28, 35]. We have previously reported that PAF53 levels correlated with the rate of rDNA transcription in insulin-treated 3T6 and H4-E2 cells [35]. In addition, we have reported that the molar ratio of PAF53 to core RNA polymerase I subunits in affinity purified polymerase complexes was 0.6 to 1 [29]. These data were consistent with the original observations reported by Hanada *et al.* [28] and with those of Yamamoto *et al.* [30] who reported that only transcriptionally competent RNA polymerase I contained PAF53.

Hanada *et al.* [28] reported that when 3T6 cells were serum starved, PAF53 translocated from the nucleolus and that this corresponded with a decreased rate of rDNA transcription. Similarly, Yamamoto *et al.* [30] reported that when 3T3 cells were serum starved, PAF49 translocated from the nucleolus. Moreover, both manuscripts reported that they could biochemically separate two forms of RNA polymerase I. Yamamoto *et al.* [30] reported that both PAF49 and PAF53 were present in one subpopulation of RNA polymerase I (fraction IB) and absent from a second population (Fraction IA). They also demonstrated that fraction 1B, the polymerase population that contained PAF49, was the transcriptionally competent form of the enzyme. Interestingly, the yeast homologues of PAF53 and PAF49, A49 and A34.5, can be dissociated from otherwise intact RNA polymerase I [32, 45] resulting in Pol I lacking the A49/34.5 heterodimer and the intact A49/A34.5 heterodimer [31]. While it has been reported that neither A49 nor A34.5 are essential subunits, mutants in A49 demonstrate reduced growth rates at 30°C and reduced viability at nonpermissive conditions [33] and Rpa51 of fission yeast, the homolog of budding yeast A49, is required to maximize rDNA transcription [46]. In addition, Kuhn *et al.* have demonstrated that the A49/A34.5 heterodimer

is required for normal Pol I cleavage activity on synthetic templates and processivity [32]. Interestingly, while *S. pombe* Rpa51 can functionally replace *S. cerevisae* A49, mouse PAF53 cannot [46]. These results argue that the association of A34.5/PAF49 and A49/PAF53 with the polymerase, especially PAF53 and PAF49, and with one another might be subject to regulation. Moreover, they suggest that the interactions of A34.5/PAF49 and A49/PAF53 might also be species-specific, *i.e.* different elements are required for the formation of the heterodimer. Interestingly, the structure of the heterodimerization domains of the yeast A34.5/49 complex is similar to that of the TFIIF complex involved in transcription by RNA polymerase II [47] suggesting a conservation of function that survived the divergence of the polymerases themselves.

These observations raise the question as to whether the interactions between PAF53/PAF49 are similar to those between A34.5 and A49. The yeast complex plays a significant role in rDNA transcription, and the elaboration of the domains involved in the formation of the mammalian heterodimer would add to our understanding of the evolution of the polymerases and their function. Interestingly our studies indicate there is evidence for a conservation of the structure of the heterodimerization domains of the two proteins, at least with respect to the heterodimerization domains of A34.5 and PAF49. We demonstrated that yeast A34.5 cannot heterodimerize with PAF53. However, when amino acids 41–86 of PAF49 were substituted for amino acids 52–98 of yeast A34.5, the chimeric protein heterodimerized with PAF53. While this 41 amino acid long domain, is significantly shorter than that domain predicted by the crystallography studies [31], it is consistent with those studies. The predicted three dimensional model of this domain matches that of the yeast homologue. However, yeast A34.5 could not heterodimerize with mouse PAF53 indicating that there are sequence-specific requirements as well as a structural requirement.

Analysis of the domains of PAF53 required for the interaction with PAF49 suggested that both the N- and C-terminal segments of the amino acids from 1–160 were required for the interaction. Amino acids 1–70 and 70–160 alone did not heterodimerize with PAF49 (summarized in Figure 4). When we aligned this segment with the structure of the yeast heterodimer, it suggested that the interaction might be mediated by two separate elements. To test this model, we inserted amino acids 1–70 and 114–160 of mouse PAF53 into yeast A49. The chimeric protein interacted with mouse PAF49. This was both consistent with the results of the deletion analysis and the secondary structure predicted by the crystallography.

Our data demonstrate that the yeast A34.5/A49 and mammalian PAF49/PAF53 heterodimeric complexes interact through species-specific sequence elements that appear to assume similar structural motifs. This is consistent with the model that the components of the RNA polymerase I transcription have evolved away from one another over time, while maintaining a conservation of function (molecular coevolution). Confirmation of our predictions with respect to structure, will require the determination of the structure of amino acids 41–86 of PAF49 and amino acids 1–160 of PAF53. In addition, our results are consistent with the model that the mammalian heterodimer may function in elongation similar to the yeast complex. In this extent it is interesting to note that while there is evidence for the existence of endogenous mammalian RNA polymerase I complexes that lack the heterodimer [28–30], there is no published evidence for such a complex in yeast cells. Whether this indicates that the regulation of the assembly of the heterodimer with the core polymerase is different in yeast and mammalian cells remains to be seen. In preliminary experiments, we have found that while the assembly of the PAF49/PAF53 complex with Pol I is sensitive to growth conditions, the formation of the heterodimer is not.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Pol I	RNA polymerase I
rRNA	ribosomal RNA
rDNA	ribosomal DNA
Rpa34 or A34.5	the 34.5 kDa subunit of yeast RNA polymerase I, a member of the A34.5 protein family that includes PAF49

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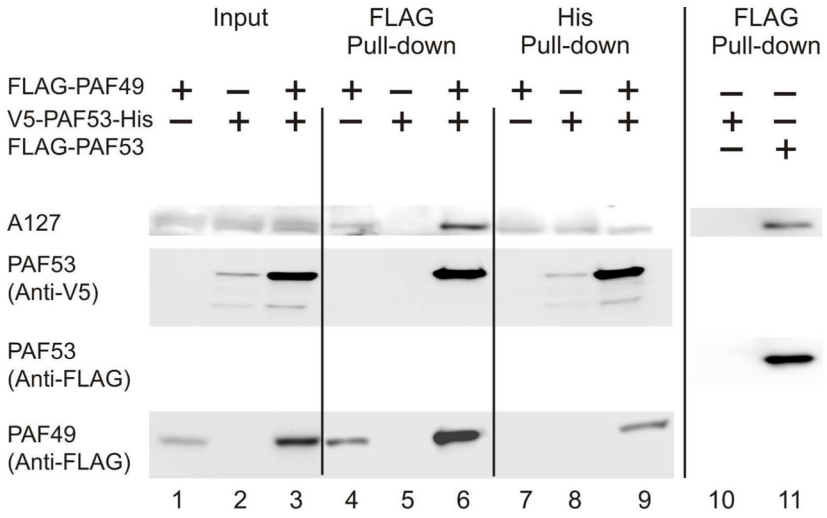


Figure 1.

Ectopically expressed PAF53 and PAF49 interact with one another and with RNA polymerase I. HEK 293 cells were transfected with vectors driving the expression of the indicated proteins. Forty-eight hours post-transfection, whole cell lysates were prepared and incubated with either immobilized anti-FLAG antibodies (FLAG Pull-Down) or immobilized nickel (His Pull-Down). The bound proteins were eluted either with FLAG peptide or EDTA and analyzed by SDS-PAGE and western blotting as described in Materials and Methods. Note the background signal for A127 in the His Pull-down lanes (7).

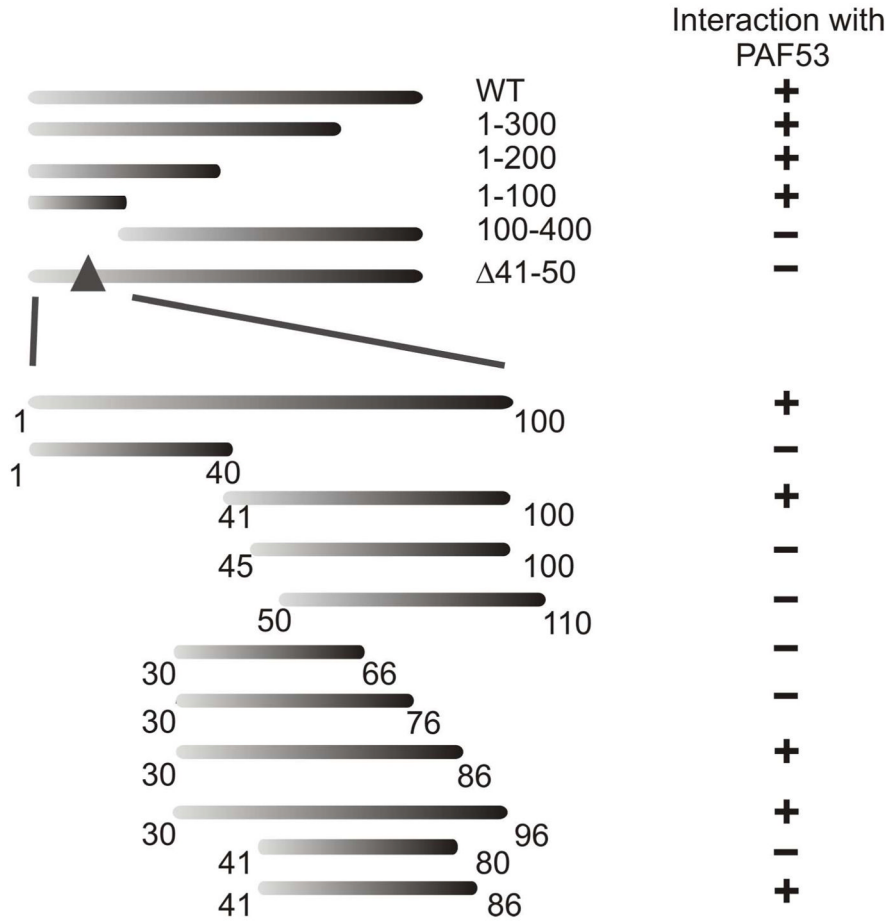


Figure 2.
Schematic summary of PAF49 mutants and their ability to interact with PAF53.

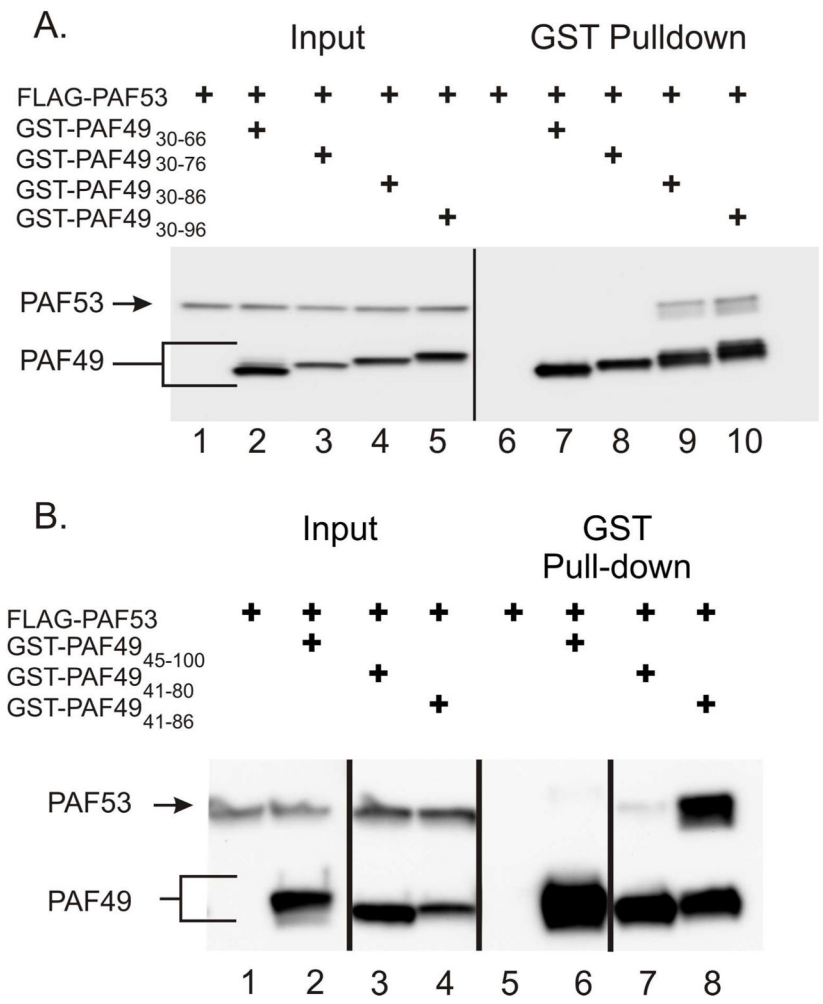


Figure 3.

Amino acids 41–86 of PAF49 are sufficient for the interaction with PAF53. A. Deletion analysis demonstrates a C-terminal boundary circa −86. B. Further analysis, demonstrates a N-terminus for heterodimerization at amino acid 41 and confirms the C-terminus boundary between amino acids −80 and −86. HEK 293T cells were cotransfected with the indicated deletion mutants of PAF49 and V5-tagged PAF53. The interacting proteins were analyzed as described in the legend to Figure 1.

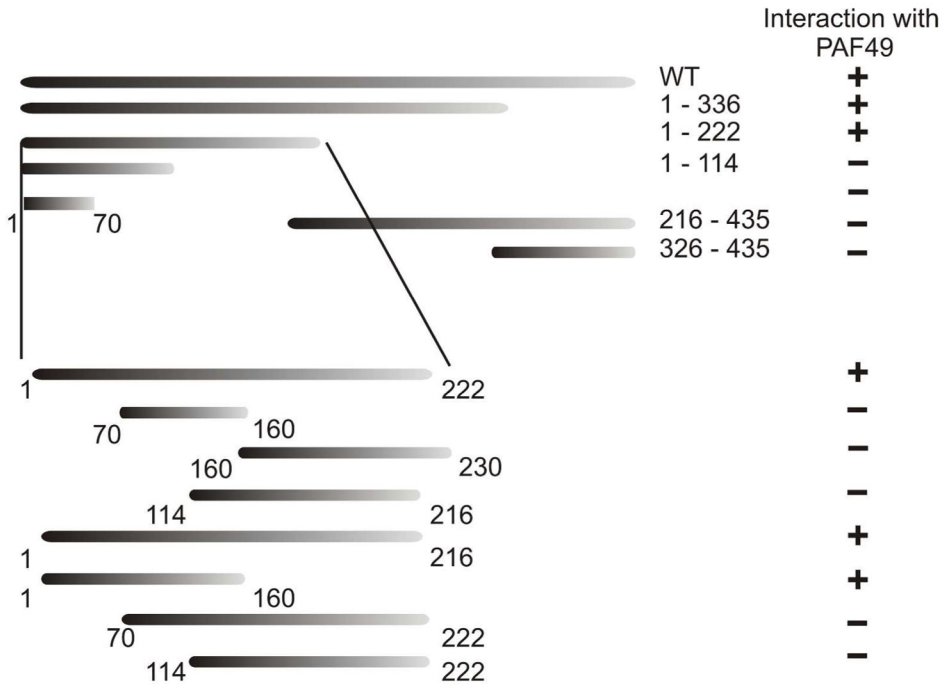
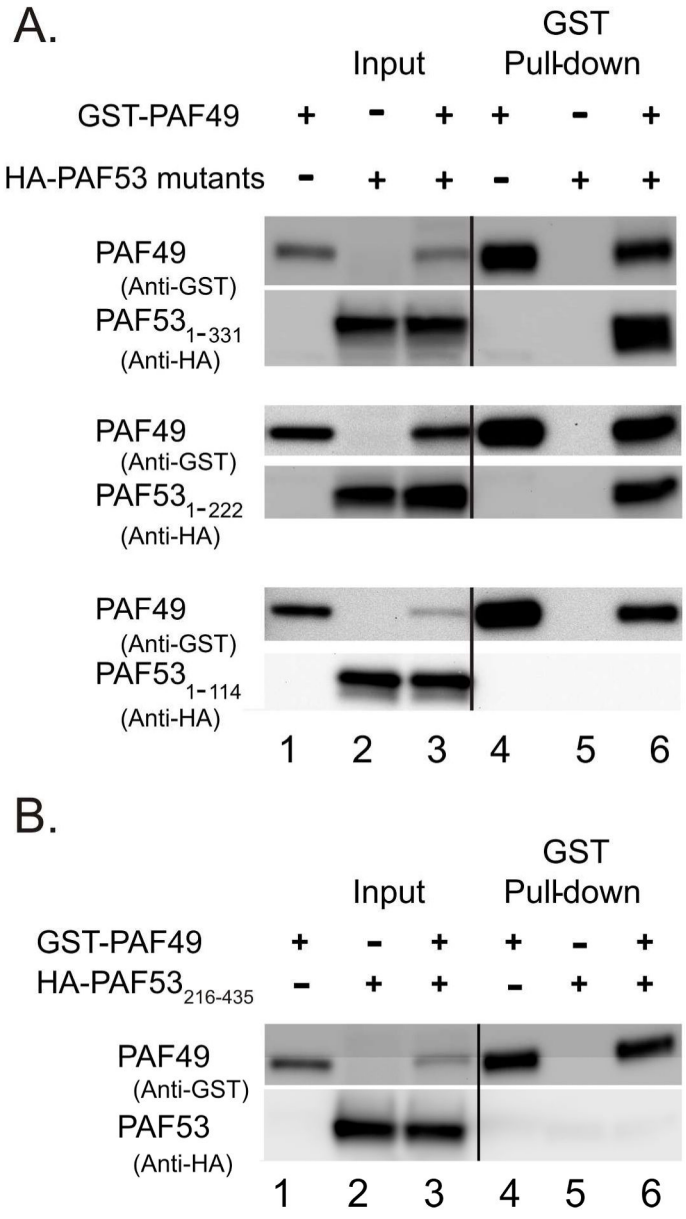


Figure 4.
Schematic summary of PAF53 mutants and their ability to interact with PAF49.



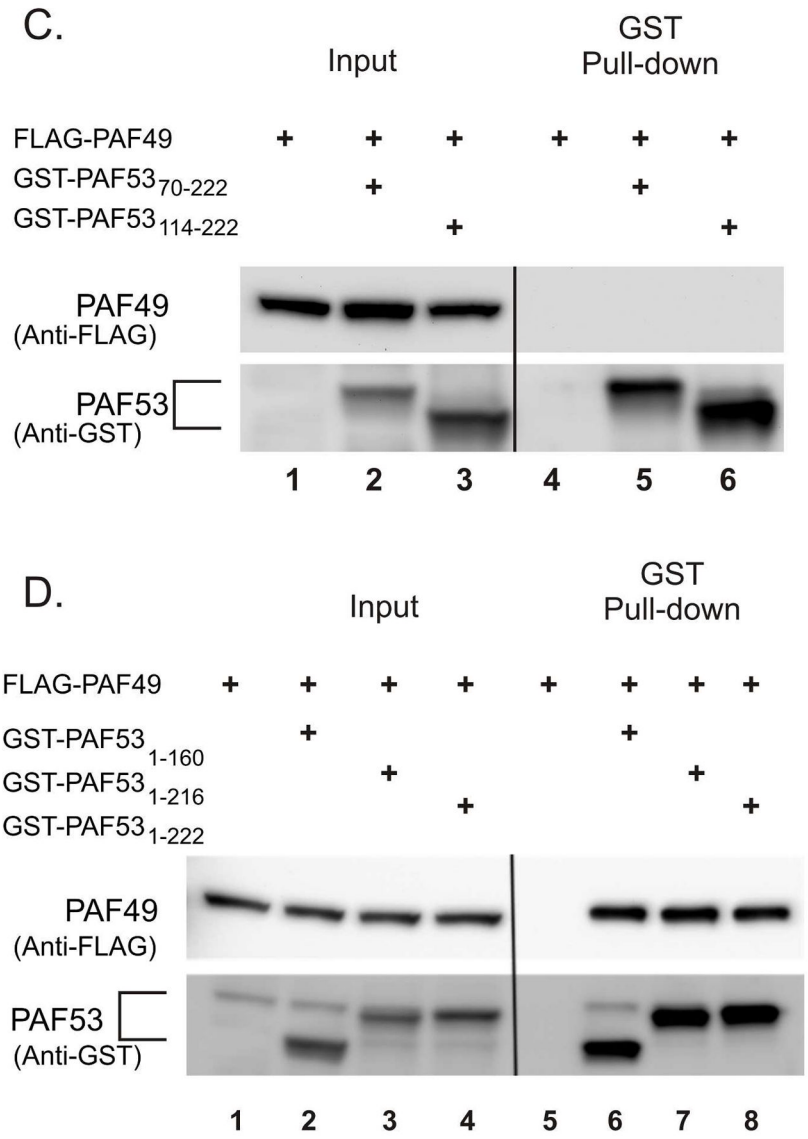


Figure 5.

The N-terminal 160 amino acids of PAF53 are necessary and sufficient for the interaction with PAF49. Deletion mutagenesis demonstrates that the C-terminal domain required for the interaction between PAF49 and PAF53 lies between amino acids 114–222 (A), and that the C-terminal domain (aa 216–435) of PAF53 does not interact with PAF49 (B). Within this 220 amino acid region, amino acids between 1–70 and 114 and 222 (C) of PAF53 are required for the interaction with PAF49, but amino acids 1–160 are sufficient to mediate heterodimerization. HEK 293 cells were cotransfected with vectors driving the expression of the indicated deletion mutants of HA-PAF53 along with a vector driving the expression of GST-PAF49. The interacting proteins were analyzed as described in the legend to Figure 1.

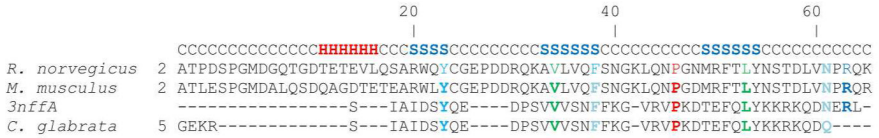


Figure 6.

I-Tasser alignment of the first 70 amino acids of rat or mouse PAF53. The top alignment obtained using either the mouse (or rat) PAF53 sequence was 3nffa, *C. glabrata* A49, using HHSEARCH threading template. The alignment had a normalized Z-score of 1.89. A Z-score >1means a good alignment. All the residues are colored in black; however, those residues in the template which are identical to the residue in the query sequence are highlighted in color. The coloring scheme is based on the property of amino acids, where polar are brightly colored while non-polar residues are colored in dark shade. H-helix, C-coil and S-sheet

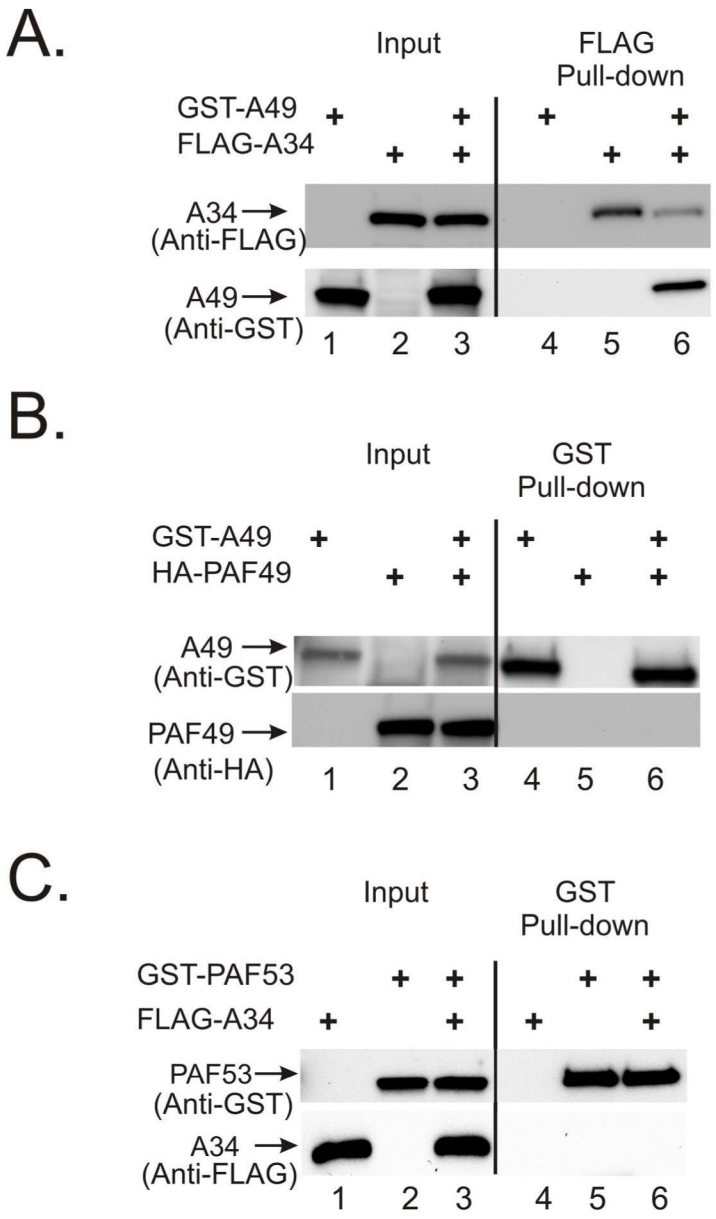


Figure 7.

Yeast A49 and A34.5 do not heterodimerize with mammalian PAF49 and PAF53, respectively. (A) Yeast A49 and A34.5 can be expressed in mammalian cells and the expressed proteins heterodimerize. The yeast and mammalian forms of A34.5/PAF49 (B) and A49/PAF53 do not heterodimerize (C). HEK 293T cells were cotransfected with plasmids encoding the indicated yeast proteins A34.5 and A49. Forty-eight hours post transfection, lysates were prepared and fractionated as indicated. The interacting proteins were analyzed as described in the legend to Figure 1.

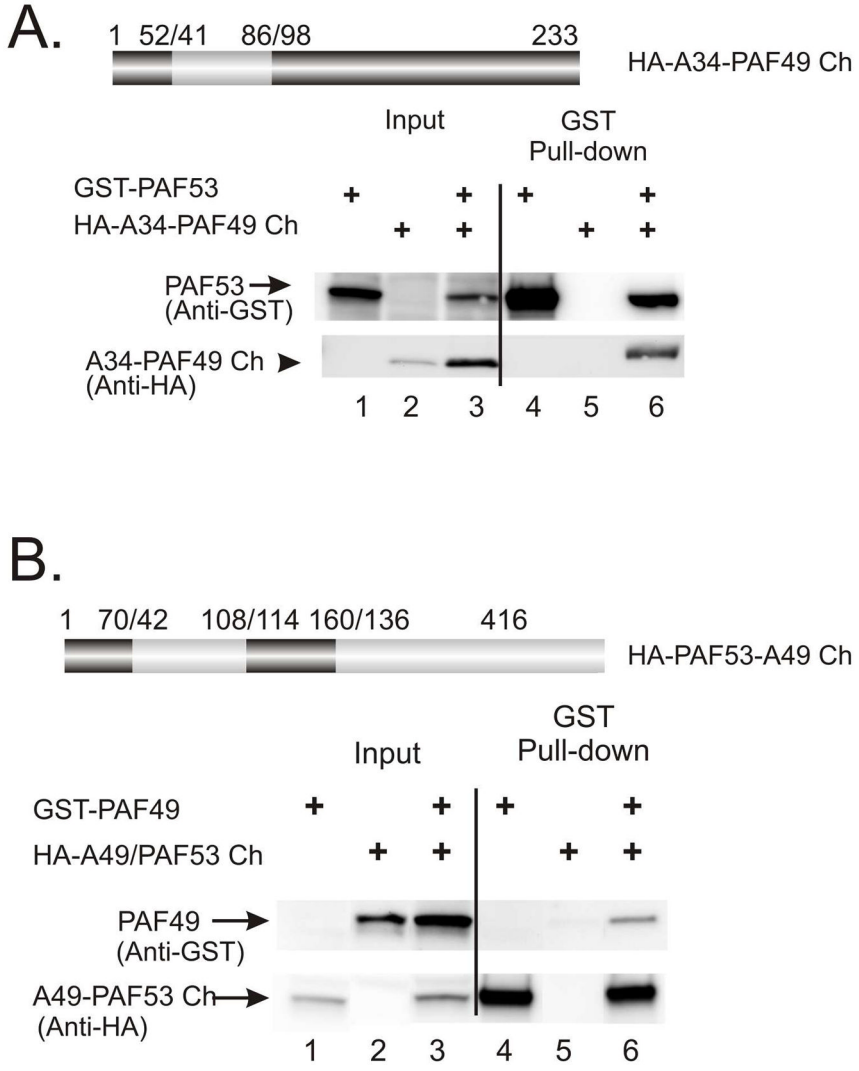


Figure 8.

Insertion of amino acids 41–86 of mouse PAF49 into yeast A34.5 directs it to interact with mammalian PAF53 (A) and insertion of amino acids 1–70 and 114–160 of PAF53 into A49 directs it to interact with mammalian PAF49 (B). HEK 293T cells were cotransfected with vectors driving the expression of the indicated chimeric proteins. Forty-eight hours post transfection, lysates were prepared and fractionated by the indicated pull-down method. For the experiments described in Panel A, amino acids 52–98 of yeast A34.5 were replaced with amino acids 41–86 of mouse PAF49. For the experiments described in Panel B, amino acids 1–42 and 109–136 of yeast A49 were replaced with amino acids 1–40 and 114–160 of mouse PAF53. The ability of the various proteins to heterodimerize was assayed as described in Materials and Methods and in the legend to Figure 1.

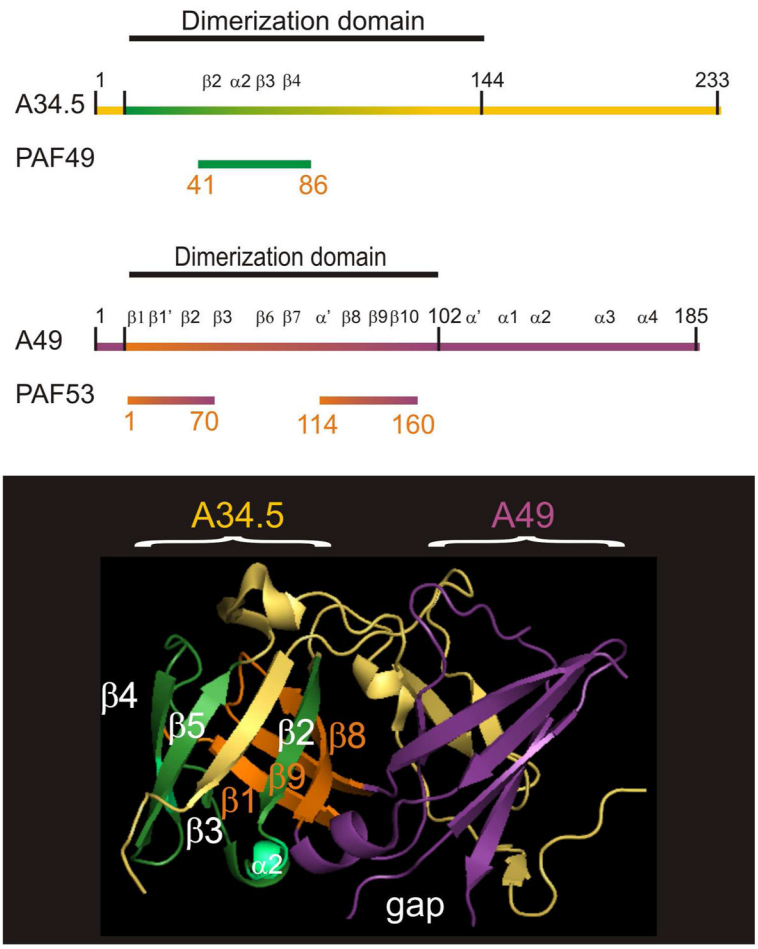


Figure 9.

Comparison of the heterodimerization domains of yeast A34.5 and A49 with those of mammalian PAF49 and PAF53. (Top panel). This panel, adapted from Geiger et al.[31], indicates both the dimerization domains of A34.5 and A49 as reported [31] and the dimerization domains of PAF49 and PAF53 based on the results reported herein. (Bottom Panel) This panel shows a colorized version of the predicted 3D structure of a portion of the yeast A34.5/A49 heterodimer (3NFF; pdb) in which the corresponding mammalian heterodimerization domains are colored in green (PAF49) or orange (PAF53) against the structure of the yeast heterodimer. The pdb file was downloaded and the corresponding amino acids were colorized using The PyMol.