Cleavage and polyadenylation specificity factor 30: An RNA-binding zinc-finger protein with an unexpected 2Fe–2S cluster

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Edited by Jacqueline K. Barton, California Institute of Technology, Pasadena, CA, and approved March 1, 2016 (received for review September 8, 2015)

Cleavage and polyadenylation specificity factor 30 (CPSF30) is a key protein involved in pre-mRNA processing. CPSF30 contains five Cys3His domains (annotated as “zinc-finger” domains). Using inducibly coupled plasma mass spectrometry, X-ray absorption spectroscopy, and UV-visible spectroscopy, we report that CPSF30 is isolated with iron, in addition to zinc. Iron is present in CPSF30 as a 2Fe–2S cluster and uses one of the Cys3His domains; 2Fe–2S clusters with a Cys3His ligand set are rare and notably have also been identified in Mitoflavin, a protein that was also annotated as a zinc finger. These findings support a role for iron in some zinc-finger proteins. Using electrophoretic mobility shift assays and fluorescence anisotropy, we report that CPSF30 selectively recognizes the AU-rich hexamer (AUA AAAA) sequence present in pre-mRNA, providing the first molecular-based evidence to our knowledge for CPSF30/RNA binding. Removal of zinc, or both zinc and iron, abrogates binding, whereas removal of just iron significantly lessens binding. From these data we propose a model for RNA recognition that involves a metal-dependent cooperative binding mechanism.

Significance

Cleavage and polyadenylation specificity factor 30 (CPSF30) is part of a complex of proteins, collectively called CPSF, that direct pre-mRNA processing. CPSF30 is classified as a “zinc-finger” protein because it contains five repeats of three cysteine and one histidine residues that serve as ligands for zinc. When zinc binds to these ligands, the domain adopts the structure necessary for function (1–4).

Zinc-finger proteins (ZFs) are a large class of proteins that use zinc as structural cofactors (1–4). ZFs perform a variety of functions ranging from the modulation of gene expression through specific interactions with DNA or RNA to the control of signaling pathways via protein–protein interactions. The general feature that defines a ZF protein is the presence of one or more domains that contain a combination of four cysteine and/or histidine residues that serve as ligands for zinc. When zinc binds to these ligands, the domain adopts the structure necessary for function (1–4).

ZFs are typically identified by the presence of cysteine and histidine residues in regular repeats and are categorized into classes based upon the number of cysteine and histidine residues, and the spacing between the residues (1, 2). At least 14 distinct classes of ZFs have been identified to date. ZFs are highly abundant, with more than 3% of the proteins in the human genome annotated as ZFs, based upon their sequences (1, 2). In some cases, there are conserved in vitro and in vivo data that support the annotation of proteins as ZFs, whereas in other cases the only evidence that a protein is a ZF comes from its amino acid sequence. The best-studied class of ZFs comprises the “classical” ZFs. This class is composed of ZFs that contain a Cys5His2 domain (CysX2–CysX12–HisX3–His). Classical ZFs adopt an alpha-helical/antiparallel beta-sheet structure when zinc is coordinated and bind DNA in a sequence-specific manner (2, 4). The remaining classes of ZFs are collectively called “nonclassical” ZFs (1). One class of nonclassical ZFs is the Cys4His domain (CysX2–CysX4–CysX2–CysX2–His). The first protein of this class to be identified was tristetraprolin, which contains two Cys4His domains and regulates cytokine mRNAs via a specific ZF domain/RNA binding interaction (1). With the publication of genome sequences this domain has been found in a myriad of proteins. The National Center for Biotechnology Information (NCBI) conserved domain architecture tool identifies 404 distinct proteins (both hypothetical and experimentally validated) that contain this domain, and humans contain at least 60 (Fig. 1). As a class, these proteins are predicted to be involved in RNA regulation; however, the function(s) of most of these proteins have not yet been established (1, 2, 10, 11).

One important Cys3His ZF protein is cleavage and polyadenylation specificity factor 30 (CPSF30) (2, 12). CPSF30 contains five Cys3His domains. CPSF30 is part of a complex of proteins, collectively called CPSF, that are involved in the polyadenylation step of pre-mRNA processing (16). The other members of CPSF are CPSF160, CPSF73, CPSF100, Figl, and Wdr53 (16). Polyadenylation occurs at a specific region of the pre-mRNA called the polyadenylation cleavage site (PAS). The PAS consists of an upstream element with the conserved sequence AAUAAA (also called the AU-hexamer), a stretch of bases where cleavage occurs, after which a conserved GU-rich or U-rich sequence is present (usually between 40–60 nt after the cleavage site) (12, 13). CPSF73 is the endonuclease that cleaves the RNA; the roles of the other CPSF proteins are less clear (12, 13). Initially, CPSF160 was identified as the protein within the CPSF complex that recognizes the AU-hexamer (14–16); however, two recent studies using cell-based methods found that CPSF160 does not play this role (17, 18).

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Instead, CPSF30 and Wdr33 were identified as the proteins involved in AU-hexamer recognition (17, 18). These findings are intriguing in light of evidence that the H1N1 human influenza virus protein NS1A targets CPSF30 to obstruct cellular mRNA processing (19–21), suggesting that the link between NS1A and cellular mRNA processing is RNA recognition by CPSF30.

Given these cell-based results that CPSF30 is involved in recognition of the AU-hexamer of pre-mRNA along with our emerging understanding that CCCH-type ZFs are a general ZF motif involved in AU-rich RNA sequence recognition, we sought to determine whether CPSF30 directly recognizes the AU-hexamer of pre-mRNA. To our surprise, CPSF30 was a reddish-colored protein upon isolation and purification, which suggested the presence of an iron cofactor. Here, we report that CPSF30 contains iron, which was confirmed by measuring the metal content. In a given preparation, both zinc and iron were observed, on average 3.78 ± 0.02 nmol and 0.51 ± 0.05 mol per protein. A ferrozine assay independently confirmed the presence of iron (24). Together, these studies revealed that CPSF30 contains iron, which is generally invariant.

**Results and Discussion**

**CCCH ZF Proteins.** CPSF30 belongs to a class of proteins that are annotated as ZFs in genome databases. To determine how frequently this “CCCH ZF domain” occurs in eukaryotes, we searched UniProt, a search tool that includes reviewed and total protein sequences. Using UniProt was performed (22). This resulted in 516 reviewed proteins and 25,610 total proteins when all organisms were considered. To provide further context, the search was narrowed to include only “CCCH ZF domains” found in *Homo sapiens*. This led to the identification of 60 reviewed and 222 total proteins. The 60 reviewed proteins were then grouped based upon the number of CCCH domains present and a consensus sequence for each domain within the context of the number of domains was determined (Fig. 1). The proteins with CCCH ZF domains had between one and five domains, with the proteins having one domain being the most abundant (30 of the 66 proteins had only one domain). The organization of the domains (i.e., spacing between cysteine and histidine ligands) was generally invariant.

**CPSF30 Contains Iron.** A series of CPSF30 constructs that contained just the five CCCH ZF domains were prepared recombinantly and expressed under standard conditions. Unexpectedly, all of the constructs turned red upon protein overexpression, suggesting that iron was coordinating to the protein in addition to or in lieu of zinc (Fig. S1). The most soluble construct, which contained a maltose-binding fusion tag (MBP), was purified and used for subsequent studies. The UV-visible spectrum of the purified CPSF30 exhibited peaks at 420, 456, and 583 nm, which are indicative of iron (particularly iron–sulfur clusters), in addition to the expected peaks around 220–280 nm for protein backbone and aromatic amino acid peaks around 280 nm (Fig. 3). Inductively coupled plasma mass spectrometry (ICP-MS) of CPSF30 was performed to measure the metal content. In a given preparation, both zinc and iron were observed, on average 3.78 ± 0.02 nmol and 0.51 ± 0.05 mol per protein. A ferrozine assay independently confirmed the presence of iron (24). Together, these studies revealed that CPSF30 contains iron, which is generally invariant.

**Table 1. Survey of the CCCH domain containing proteins in *H. sapiens*.**

<table>
<thead>
<tr>
<th># of CCCH ZF Domains</th>
<th>Consensus Sequence</th>
<th>Frequency in Human Genome</th>
<th>Examples</th>
<th>Overall Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>2F1</td>
<td>AAUAAA</td>
<td>29 Putative ATP-dependent RNA helicase, Leschonygote receptor cluster member 9</td>
<td>RNA helicase</td>
<td></td>
</tr>
<tr>
<td>2F1</td>
<td>AAUAAA</td>
<td>9 SRA-hydrolases, erythroblastin</td>
<td>SRA synthesis, RNA splicing interactions, post-translational regulation</td>
<td></td>
</tr>
<tr>
<td>2F1</td>
<td>AAUAAA</td>
<td>7 E3 ubiquitin-protein ligase maskrin 3</td>
<td>mRNA transport, poly(A) RNA binding</td>
<td></td>
</tr>
<tr>
<td>2F1</td>
<td>AAUAAA</td>
<td>6 Cleavage and polyadenylation specificity factor subunit 4, Poly (ADP-ribosyl) polymerase 12</td>
<td>Pre-mRNA regulation</td>
<td></td>
</tr>
<tr>
<td>2F1</td>
<td>AAUAAA</td>
<td>5 WDR33, CPSF100, CPSF70, CPSF160</td>
<td>Multi-functional</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 1.** Survey of the CCCH domain containing proteins in *H. sapiens*.
CPSF30’s CCCH ZF domains have both zinc and iron cofactors, of which zinc is more readily chelated.

X-Ray Absorption Spectroscopy of the Iron Sites. X-ray absorption spectroscopy (XAS) was used to characterize the protein-bound Fe coordination geometry and ligand environment. The X-ray absorption near edge structure (XANES) portion of the XAS spectrum for Fe bound to CPSF30 is shown in Fig. 4A. A Fe pre-edge transition peak, observed at ~7,112.4 eV with a corresponding peak area of 23.7 × 10⁻² eV², is consistent with a four-coordinate tetrahedral ferric complex (25, 26). The Fe edge inflection energy, determined from the first inflection point of the rising edge, occurs at 7,120 eV and is again consistent with a Fe(III) species characteristic of an oxidized 2Fe(III)–2S cluster (27). The Fe extended X-ray absorption fine structure (EXAFS) (Fig. 4B) was best simulated in the nearest-neighbor environment with ca. three S ligands at an average bond length of 2.26 Å and ca. one O/N ligand at 2.03 Å (Table S1). Long-range scattering includes an Fe–Fe vector at a bond length of 2.67 Å. In addition, multiple Fe–C interactions were observed at 3.17, 3.44, and 3.95 Å. These bond lengths represent average values obtained from two independent samples and are consistent with reported values for oxidized 2Fe–2S clusters bound by three Cys and one His residues (28). Thus, we speculate that iron is bound to CPSF30 as a 2Fe–2S cluster with a CCCH ligand set.

XAS of the Zinc Sites. XAS was also used to characterize protein-bound Zn coordination geometry and metrical parameters. The Zn edge inflection energy at 9,662.5 eV is consistent with Zn(II) (Fig. 5A). The postedge region of the spectrum shows features at both 9,665.7 eV and 9,671 eV, consistent with independent sulfur and oxygen/nitrogen ligand environments published for ZnN₁S₃ peptides (29, 30), suggesting similar coordination environments. The Zn EXAFS region (Fig. 5B) was best fit in the nearest-neighbor ligand environment with ca. one O/N ligand at 2.01 Å and ca. 2.5 S ligands at 2.31 Å (Table S1). The Fourier transform of the Zn XAS for CPSF30 is consistent with a ZnN₁S₃ peptide system published previously (30). Long-range carbon scattering was also observed at 3.07, 3.26, 3.43, and 4.03 Å. These data are consistent with Zn coordinated to CPSF30 in a tetrahedral CCCH ligand environment.

RNA Binding Studies: CPSF30 Binds to the AU-Rich Hexamer of α-Synuclein Pre-mRNA in a Cooperative Manner. CPSF30 is part of a complex of proteins, collectively called CPSF, that regulate pre-mRNA processing. CPSF30 has been proposed to be involved in recognition of the PAS present in pre-mRNA, because the signal for α-Syn oligomers and the Rβ control RNA oligomer because it does not contain the polyadenylation hexamer and is not enriched in adenosine and uridine nucleotides (Table 1).

Electrophoretic mobility shift assays (EMSA) were performed with four α-Syn oligomers and the Rβ control. CPSF30 formed complexes with all of the α-Syn oligomers interrogated, as evidenced by shifting of the RNA oligomers (Fig. 6A and Fig. S3A), whereas no binding was observed for CPSF30 with Rβ or with the MBP tag with any of the RNA oligomers (Fig. S3B).

Fluorescence anisotropy (FA) was performed to determine the binding affinity of CPSF30 for the α-Syn36, α-Syn52, and α-Syn54 oligomers. Binding was observed for all three α-Syn RNA targets, but not for Rβ. Anisotropy values, corrected for the change in quantum yield, were plotted versus the concentration of CPSF30 protein titrated. The data were fit to two models: a 1:1 binding model and a cooperative binding model (32). The cooperative binding model gave the best fit, with [P]₁/₂ values of 93.5 ± 2.7 nM, 115.0 ± 3.6 nM, and 143.8 ± 3.8 nM, with Hill coefficients of 1.67 ± 0.07, 1.63 ± 0.08, and 1.58 ± 0.07 for α-Syn36, α-Syn52, and α-Syn54, respectively (Fig. 6B). Binding was not observed when these RNA targets were titrated with either apo-CPSF30 or iron-loaded (zinc-deplete) CPSF30. The zinc-loaded (iron-deplete) CPSF30 exhibited significantly weakened affinity compared with the iron-loaded zinc-loaded CPSF30 ([P]₁/₂ 570.5 ± 23.4) nM with α-Syn53. No binding was observed for MBP alone with any of the RNA targets. To determine whether the AU-rich sequence is the site of RNA binding, titrations of CPSF30 with three altered RNA sequences were performed (Table 1). The sequences all lacked the AU-hexamer. In two cases the AU-hexamer was replaced with polyC (CCCCCCC) or polyU (UUUUUU), and in the third the GU-rich sequence (UGUUUU) near the polyuridine site that has been proposed as an alternative target sequence for CPSF30 (33).
CPSF30 showed no binding to any of these sequences (in the Fe/Zn, Fe-only, Zn-only, or apo forms) (Fig. 6C).

Identification of Iron and Zinc Domains. The five CCCH domains of CPSF30 are highly homologous (Fig. S4A), and from sequence comparison it is not apparent which domain is loaded with the Fe–S cluster and which is loaded with zinc. A series of mutants in which each domain was modified at the coordinating cysteine and histidine ligands (CCCH to AAAA named ΔZF1, ΔZF2, ΔZF3, ΔZF4, and ΔZF5) were prepared to identify where iron and zinc bind. The mutant proteins were overexpressed and purified following the identical conditions used for WT-CPSF30. All of the mutants were reddish in color, and by analysis by ICP-MS (Fig. S4B) revealed that they all were loaded with iron, but lost between one and two equivalents of Zn, with ΔZF2 losing the most Zn (2.4 equivalents) and ΔZF1 the least (1.3 equivalents). The affinities of these mutants for RNA (αSyn24) was subsequently measured (Fig. S5) ΔZF4 retained comparable binding affinity to WT CPSF30, ΔZF1, ΔZF3, and ΔZF5 exhibited two- to threefold weaker affinities, and the ΔZF2 mutant did not bind to αSyn24 RNA with any appreciable affinity. Like WT-CPSF30, none of the mutants exhibited any affinity for polyC RNA. Taken together, these data support a model in which the sites of Fe and Zn binding are flexible, with iron loading occurring first. The data also suggest that ZF2 is critical for tight RNA binding, whereas the other ZFs seem to be less important.

Model of CPSF30/RNA Binding. The data for CPSF30/RNA binding were best fit to a cooperative binding model with a minimum stoichiometry of 2 CPSF30:1 RNA and an average Hill coefficient of 1.63 ± 0.07 (Fig. 6B and C). CPSF30 alone is a monomer, as evidenced by gel filtration chromatography data (Fig. S2), yet exhibits positive cooperativity upon RNA recognition, perhaps indicating RNA-induced protein dimerization. Dimerization of proteins that bind to RNA is not unprecedented; for example, the Cys5His ZF ZAP is a monomer in solution that then dimerizes upon binding to the ZAP-responsive RNA element (34). Other RNA-binding proteins that recognize their targets in a cooperative manner are the human zincode-binding protein IMP-1, HIV-1 Rev protein, hordeiviral yb protein, human La protein, and tomato bushy stunt virus p33 protein (35–38). There are also other AU-rich RNA-binding proteins such as AUf-1 (p42), Hsp70, and HuR, which bind to their respective RNA partners in a cooperative manner (39–41). Moreover, a two-domain construct of CPSF30 were first crystallized bound to the NS1 influenza A virus protein, where it is present as a dimer (21).

Conclusions

Several important conclusions can be drawn from the work presented here: (i) CPSF30 is the protein within the CPSF complex that directly recognizes PAS RNA; (ii) CPSF30 contains an unexpected 2Fe–2S cluster, in addition to zinc; (iii) CPSF30 is always loaded with iron first, then zinc; and (iv) the sites of iron and zinc binding are flexible but high-affinity RNA binding requires that one Fe site and at least two Zn sites be occupied. The CPSF complex directs mRNA 3′ maturation via a mechanism of RNA recognition, cleavage, and polyadenylation (Fig. 2); however, the roles of the proteins that make up the CPSF complex are not clearly defined. Studies of the complex in a cellular setting have provided tantalizing support for CPSF30 as the protein that binds to the PAS signal. The work described here provides direct evidence that CPSF30 selectively recognizes the AU-rich hexamer of pre-RNA via its CCCH domains. Unexpectedly, the work also shows that CPSF30 contains a 2Fe–2S site with a CCCH ligand set, in addition to its predicted zinc sites. These 2Fe–2S sites with CCCH ligand sets are rare; 2Fe–2S sites were first identified in the 1960s, with two principal types identified: ferredoxin (1962), which uses a CCCH ligand set, and rieske type (1964), which uses a CCHH ligand set. However, it was not until the late 2000s that a 2Fe–2S site with a CCCH ligand set was identified (23). This site was found in a protein called mitoNEET, a mitochondrial protein that is a target of the type-2 diabetes drug pioglitazone (23). Remarkably, like CPSF30, mitoNEET was annotated as a ZF protein based on the presence of a CCCH domain (with spacing of CXCX2CX3HX4 compared with CXCX2CX2H for CPSF30 and its homologs) and turned red upon protein expression and purification (42). MitoNEET contains a singular 2Fe–2S cluster with a CCCH ligand set and no zinc sites. The two homologs of MitoNEET, Miner1 (or NAF1) and Miner2, also contain between one and two 2Fe–2S clusters bound to CCCH sites (43–45). This suggests that the annotation of a protein as a ZF protein simply based upon its amino acid sequence (i.e., repeats of cysteine and histidine residues) may not always be correct and care must be taken in defining a ZF protein based just upon amino acid sequence. There is also evidence for a CCCH ligated 2Fe–2S cluster in proteins involved in iron–sulfur cluster assembly in Escherichia coli IscB and IscU (46, 47). In addition, in yeast, the GxxX4/Fra2 signaling proteins have been shown to interact via a 2Fe–2S cluster that has a CCCHX ligand set (48).

The biological significance of the 2Fe–2S cluster identified in CPSF30 is not yet known. Fe–S sites have been shown to play roles in electron transfer, oxygen sensing, iron sensing, substrate activation, and catalysis (49). Similarly, in mitoNEET there is not yet a definitive role for the 2Fe–2S site; however, there is evidence that it may be involved in trafficking iron via a redox sensing mechanism (50–54). Redox sensing may also be important for CPSF30: The CPSF30 yeast homolog, YH1, responds to hypoxic stress (loss of O2) by shuttling to the cytoplasm from the nucleus (38).

Table 1. RNA oligomers tested

<table>
<thead>
<tr>
<th>RNA oligomer</th>
<th>Length, nt</th>
<th>Sequence (5′–3′)</th>
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<tbody>
<tr>
<td>α-Syn28</td>
<td>38</td>
<td>CCCAUCUCACUUUAUAAAUAUAUAUAUAUAUGCUAAAGC</td>
</tr>
<tr>
<td>α-Syn30</td>
<td>30</td>
<td>UCCGCAUCUUUAUAAAUAUAUAUAUAUAUGCUAAU</td>
</tr>
<tr>
<td>α-Syn24</td>
<td>24</td>
<td>CACUUUAUAUAAAUAUAUAUAUAUAUGCU</td>
</tr>
<tr>
<td>GU-rich24</td>
<td>24</td>
<td>CCGAAGGUGUUGAUUGAUAGC</td>
</tr>
<tr>
<td>Poly U24</td>
<td>24</td>
<td>UUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUU</td>
</tr>
<tr>
<td>Poly C24</td>
<td>24</td>
<td>CACGUUUUAUAAAUAUAUAUAUAUAUGCU</td>
</tr>
<tr>
<td>Rfl31</td>
<td>31</td>
<td>UGGCAGAAGCGCUGGCUGACAAUAUAAUACUG</td>
</tr>
</tbody>
</table>

The AU hexamer region is shown in bold.
nucleus (55), and the Fe–S cluster may facilitate the protein localization (50–54). Additionally, the Arabidopsis homolog of CPSF30, which contains just three CCCH domains, has been shown to be involved in redox signaling, via its cysteine ligands (56). Another possible role for the 2Fe–2S cluster of CPSF30 is to regulate oligonucleotide binding a redox-dependent manner; Fe–S clusters are emerging as key cofactors in a range of regulatory proteins. In some proteins for which the Fe–S cluster plays a regulatory role, such as the base excision repair proteins, the Fe–S cluster’s DNA binding affinity is dependent on the oxidation state of the Fe–S cluster and modulated by DNA charge transfer (57–62). In others, such as Aft2 (which also contains a structural zinc site), it is the presence or absence of the Fe–S cluster that drives DNA binding (63).

Taken together, our experimental data for CPSF30 reveal that CPSF30 contains both a 2Fe–2S site and a zinc site. Both sites are important for sequence-specific RNA binding, with the Zn site playing a larger role. CPSF30 selectively recognizes and binds to the polyadenylation AU-rich hexamer of α-synuclein pre-mRNA in a cooperative manner with high affinity. The location of the zinc and iron sites within the SCCH domains of CPSF30 seem to be flexible; however, iron is loaded before zinc. CPSF30 is a target of the human homolog of CPSF30, which also contains a structural zinc site, it is the presence or absence of the Fe–S cluster that drives DNA binding (63).

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ACKNOWLEDGMENTS. This work was supported by the National Science Foundation Grant CHE1306208 (to S.L.J.), American Heart Association/Friedreich’s Ataxia Research Alliance Grant 12PRE11720005 (to A.V.R.), American Heart Association Grant 11PRE6900008 (to B.E.Z.), NIH Grants CA102428 (to G.M.W.) and DK068139 (to T.L.S.), and NIH training Grant T32GM066706-13 (to G.D.S.). Portions of this research were carried out at the Stanford Synchrotron Radiation Lightsource, a directorate of the Stanford Linear Accelerator Center, National Accelerator Laboratory, and an Office of Science User Facility operated for the US Department of Energy Office of Science by Stanford University. The SSLR Structural Molecular Biology Program is supported by the DOE Office of Biological and Environmental Research, and by NIH–National Institute of General Medical Sciences (including Grant P41GM103393). Use of the National Synchrotron Light Source, Brookhaven National Laboratory, was supported by the US Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract DE-AC02-98CH10886.