Crystal Structures of GCN2 Protein Kinase C-terminal Domains Suggest Regulatory Differences in Yeast and Mammals*

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In response to amino acid starvation, GCN2 phosphorylation of eIF2 leads to repression of general translation and initiation of gene reprogramming that facilitates adaptation to nutrient stress. GCN2 is a multidomain protein with key regulatory domains that directly monitor uncharged tRNAs which accumulate during nutrient limitation, leading to activation of this eIF2 kinase and translational control. A critical feature of regulation of this stress response kinase is its C-terminal domain (CTD). Here, we present high resolution crystal structures of murine and yeast CTDs, which guide a functional analysis of the mammalian GCN2. Despite low sequence identity, both yeast and mammalian CTDs share a core subunit structure and an unusual interdigitated dimeric form, albeit with significant differences. Disruption of the dimeric form of murine CTD led to loss of translational control by GCN2, suggesting that dimerization is critical for function as is true for yeast GCN2. However, although both CTDs bind single- and double-stranded RNA, murine GCN2 does not appear to stably associate with the ribosome, whereas yeast GCN2 does. This finding suggests that there are key regulatory differences between yeast and mammalian CTDs, which is consistent with structural differences.

The atomic coordinates and structure factors (codes 4OTN and 4OTM) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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In response to environmental stresses, eukaryotic cells rapidly reduce protein synthesis, which lowers expenditure of energy and resources and facilitates a reprogramming of gene expression designed to restore cell homeostasis. A central mechanism directing this translational control involves phosphorylation of eukaryotic initiation factor 2 (eIF2) (1–3). This is illustrated during starvation for amino acids, where phosphorylation of eIF2 by the protein kinase GCN2 (EIF2AK4) reduces the exchange of eIF2-GDP to eIF2-GTP that is required for delivery of initiator Met-tRNA\textsuperscript{Met} to the translation machinery. As a consequence, there is repressed initiation of global protein synthesis, thus lowering the utilization of limiting amino acids. Accompanying this global translational control, eIF2 phosphorylation leads to preferential translation of select mRNAs such as that encoding the transcription factor ATF4 (4, 5). In turn, ATF4 activates the transcriptional expression of genes involved in metabolism and nutrient uptake, anti-oxidation, and protein folding and assembly, which collectively can serve to ameliorate stress damage (6).

GCN2 is a member of the family of eIF2 kinases that together serve as direct sensors of cellular stress. GCN2 can be activated by amino acid depletion, infection with single-stranded (ss) RNA viruses (7–9), UV irradiation (10, 11), and disruption of proteasomal degradation of proteins (12). Additional eIF2 kinases include PERK (PEK(EIF2AK3), induced by endoplasmic reticulum stress, PKR (EIF2AK2), activated by double-stranded RNA produced during viral infection, and HRI (EIF2AK1), which functions to couple protein synthesis in erythroid tissues to iron availability (1–3). Together these eIF2 kinases are essential for health, with loss of GCN2 in mice leading to increased morbidity during nutrient depletion (13), and in humans, EIF2AK4 (GCN2) mutations cause pulmonary veno-occlusive disease, a form of pulmonary hypertension (14). Disruptions of PERK trigger neonatal diabetes, and disruptions in bone, liver, and digestive systems in humans (Wolcott-Rallison syndrome) and in mouse models (15–17).

The abbreviations used are: eIF2, eukaryotic initiation factor 2; HisRS, histidyl-tRNA synthetase-like domain; CTD, C-terminal domain; mCTD, murine GCN2 CTD; yCTD, yeast GCN2 CTD; EMP, ethyl mercuric phosphate; MEF, mouse embryonic fibroblast; r.m.s.d., root mean square deviation; ds-doubled stranded; ss-, single stranded; Bis-Tris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)propane-1,3-diol.
Crystal Structures of Yeast and Murine GCN2 CTDs

The mechanisms by which eIF2 kinases recognize stress signals in cells involve regulatory regions that are juxtaposed to the protein kinase domain. Mechanistic features largely derived from genetic and molecular studies in yeast *Saccharomyces cerevisiae* indicate that GCN2 contains two central regulatory regions, a histidyl-tRNA synthetase-like domain (HisRS) and a C-terminal domain (CTD), which function together to sense nutrient depletion through a mechanism that is not yet fully understood (18–24). Proposed models include a role for both domains in binding uncharged tRNA, which accumulate during amino acid deprivation, followed by a conformational change that activates the kinase domain of GCN2, facilitating autophosphorylation and induction of eIF2 phosphorylation (25). The binding of uncharged tRNA by the HisRS is supported by its sequence conservation with the histidyl tRNA synthetase. The mechanism by which the CTD would bind tRNA has yet to be established. Other proposed mechanistic features mediated by the CTD are dimerization and ribosome binding. The CTD of yeast GCN2 was shown to be sufficient to mediate binding of this eIF2 kinase to ribosomes as judged by co-migration with ribosomes separated by sucrose gradient centrifugation (19, 21, 24). In yeast, GCN2 association with the translational machinery has been proposed to be important for facilitating binding to accumulating uncharged tRNAs in the context of the A site of ribosomes (25). Furthermore, the CTD of GCN2 can bind double-stranded (ds) RNA through a cluster of lysine residues, which had led to the proposal that GCN2 associates with ribosomes through direct interactions with rRNA (21). The sequences of the CTD of GCN2 in vertebrates share little sequence similarity with their yeast counterpart; therefore, it is not currently known whether the functional features attributed to the yeast CTD GCN2 are functionally germane to mechanisms regulating GCN2 phosphorylation of eIF2 in mammals.

In this study we explore the structural and functional properties of the CTD of GCN2 from both yeast and mammals with the goal of answering the following key mechanistic questions. How does the CTD facilitate dimerization of yeast GCN2? Given the sequence divergence of the CTD between yeast and mammals, are there conserved structural features? Is dimerization important for GCN2 function in mammalian cells subject to amino acid deprivation? Addressing these questions will provide insight into the mechanisms controlling the activation of GCN2 eIF2 kinase and the ensuing translational control triggered by deprivation of nutrients.

**EXPERIMENTAL PROCEDURES**

**Preparation of Murine and Yeast CTD Proteins**—Several expression vectors encoding N-terminal His₆-tagged or N-terminal His₆-SUMO-tagged CTD proteins of yeast and murine GCN2 with different N termini were used in this study. N-terminal His₆ constructs include cDNA encoding the murine GCN2 C-terminal domain (mCTD) from residues 1493–1648, inserted between the Ndel and BamHI sites of plasmid pET28a (Novagen). Previously reported yeast GCN2 CTD (yCTD) residues 1498–1659 or 1536–1659 were also introduced between the Ndel and BamHI sites into plasmid pET15b (Novagen) (21). N-terminal His₆-SUMO constructs included cDNA encoding mCTD residues 1514–1648 or yCTD encoding residues 1519–1659 inserted into pSMT3 vector (gift from Dr. Christopher Lima, Sloan-Kettering Institute) between restriction sites BamHI and XhoI. Crystallographic studies were carried out using mCTD 1514–1648 and yCTD 1519–1659 proteins.

All plasmids were transformed into Rosetta (DE3) *Escherichia coli* (Novagen, Inc.); cultures were grown at 37 °C in Luria broth containing 20 μg/ml kanamycin (pET28, pSMT3) or 100 μg/ml ampicillin (pET15) and 34 μg/ml chloramphenicol until the optical density at 600 nm reached 0.6 and then induced overnight at 18 °C by adding 1 mM isopropyl-β-D-thio-galactoside. Selenomethionine-derivatized yeast N-terminal His₆-SUMO-CTD (1519–1659) was expressed in M9 medium as described (26). In this method, six amino acids, leucine, isoleucine, lysine, phenylalanine, threonine, and valine, were added to the medium to inhibit methionine synthesis, thereby forcing the bacterial cells to use the supplied selenomethionine.

Both N-terminal His₆-SUMO fusion and N-terminal His₆ proteins were purified similarly with the exception of the cleavage step as described here. *E. coli* cell pellets were resuspended in 50 mM sodium phosphate, pH 7.8, 0.3 M NaCl, 10 mM imidazole, and lysed two times by using a French press (SLM-AMINCO, Spectronic Instruments, Rochester, MN) at 1000 p.s.i. and then subjected to ultracentrifugation at 35,000 rpm for 30 min. The supernatant was then incubated with nickel-nitrilotriacetic acid (Qiagen) beads for 1 h at 4 °C. Protein bound beads were applied to a column and washed with 50 mM sodium phosphate buffer, pH 7.8, 0.3 M NaCl, and 20 mM imidazole until the absorbance reading at 280 nm reached background level. The His₆-SUMO affinity tag was removed by on-column cleavage with the addition of Ulp1 protease, which was prepared using an expression plasmid kindly provided by Dr. Chris Lima (Sloan-Kettering Institute). Ulp1 protease was added at an estimated mass ratio of 1:1000 (Ulp1: His₆-SUMO-protein) and incubated overnight at 4 °C. The His₆-SUMO-tagged samples were subjected to thrombin cleavage (2 units/mg of protein) at 4 °C overnight to remove the His₆ tag. The mCTD protein was then loaded on a heparin column buffered in 50 mM Tris-HCl (pH 8.5) and eluted with a linear 50 to 1000 mM NaCl gradient.

Finally, mCTD-containing fractions were concentrated and then subjected to Superdex 75 gel filtration column chromatography buffered in 50 mM Tris (pH 8.0), 300 mM NaCl, and 1 mM DTT. After purification, the mCTD protein used for crystallization migrated as a single band as judged by SDS-PAGE and Coomassie staining. The native and selenomethionine yCTD samples were purified as described above for the mCTD. Purified CTD samples were concentrated, filtered to remove any particulate matter, and then stored at −80 °C.

**Crystallization and Data Collection**—mCTD (resides 1514-1648) or yCTD (resides 1519-1659) was diluted to a final concentration of 24 and 13 mg/ml, respectively, in a solution of 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, and 1 mM DTT for vapor diffusion hanging drop crystallization trials. Diffraction quality crystals of mCTD were obtained from the precipitant solution including 0.1 M Bis-Tris (pH 6.5) and 2.0 M (NH₄)₂SO₄ in drops containing 1 μl of protein and 1 μl of precipitant solution. Crystals of yCTD were obtained from 0.1 M Bis-Tris (pH 5.5) and 21–28% (w/v) polyethylene glycol 2000 (PEG2K).
As mCTD lacks Met residues, efforts to solve the phase problem focused on obtaining a heavy atom derivative. Ethyl mercuric phosphate (EMP) was added directly into crystallization drops to a final concentration of 1 mM overnight. Crystals of mCTD were removed from the mother liquor with a fiber loop, soaked in the Cryo solution (0.1 M Bis-Tris (pH 6.5), 2.1 M (NH₄)₂SO₄, 10% ethylene glycol, and 1 mM EMP) for about 1 min, and then immediately frozen in liquid nitrogen. Selenomethionine derivative crystals of yCTD were obtained from identical conditions as used for the native crystals. A heavy atom derivative of yCTD was prepared by soaking crystals overnight in a solution of 0.1 M Bis-Tris (pH 5.5), 25% PEG2K, and 1 mM EMP. Crystals were cryocooled in 0.1 M Bis-Tris (pH 5.5), 21–28% (w/v) PEG2K, 10% ethylene glycol, and 1 mM EMP. All data were collected at beam line GM/CA CAT 23-ID at the Advanced Photon Source, Argonne National laboratory, and processed with HKL-3000 (27). mCTD crystals belong to P₃,2₁ space group; yCTD crystals belong to space group P2₁2₁2₁ (see Table 1). There remains some disorder associated with this structure that is not easily modeled and contributes to higher R values than obtained for the mCTD structure. Consistent with this view is the small number of water molecules associated with this structure, 109 as compared with 169 for the murine structure, and the disordered loop between β1 and α1 in each subunit of the structure. Coordinates and structure factors have been deposited with the Research Collaboratory for Structural Bioinformatics.

**Preparation of Functional Mutants**—By using site-directed mutagenesis, alanine substitutions were introduced in the mCTD and full-length murine GCN2 for residues predicted to play important roles in either stabilizing the dimeric structure or binding RNA. The QuikChange® site-directed mutagenesis kit (Stratagene Inc.) was used for creating mCTD mutants, whereas the QuikChange® XL site-directed mutagenesis kit (Stratagene Inc.) was used for GCN2 full-length mutants. The mutations were confirmed by DNA sequencing. Substituted mCTD proteins were purified as His₅-SUMO fusion proteins using the same protocol as described for mCTD, excluding the proteolytic removal of the N-terminal His₅ SUMO tag. Use of the His₅-SUMO fusion proteins facilitated characterization as several of the substituted proteins (Y1614A, L1564A, L1561A, Y1639A, I1646A, L1587A) were found to degrade readily after removal of the N-terminal affinity tag, and dimerization of the wild-type protein was not significantly affected by inclusion of the tag. Size exclusion chromatography was used to characterize the estimated molecular weight of the wild-type and substituted mCTD samples by using a Superdex 200 10/300 GL (GE Healthcare) column bufferred in 50 mM Tris (pH 8.0) and 0.30 M NaCl. Although several different size exclusion chromatography experiments were performed on tagged and untagged versions of mCTD mutants, ultimately the mCTD mutants were analyzed for dimerization as fusion proteins with results provided in Table 2 for a single representative experiment. For characterization of RNA binding activity, substituted mCTD proteins were prepared as described for mCTD that was used in the crystallographic studies in which the N-terminal affinity tag was removed.

**Cell Culture and Luciferase and Immunoblot Assays**—Wild-type and GCN2⁻⁻ mouse embryonic fibroblast (MEF) cells were derived previously (31). MEF cells were cultured in Dulbecco’s modified eagle media (DMEM) supplemented with 1 mM nonessential amino acids, 10% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin at 37 °C. The P₉K-ATF4-Luc activity measurements were similar to those previously described (5). Briefly, GCN2⁻⁻ MEF cells were plated at a density of 10⁵ cells per well in 6-well plates and grown to ~40% confluency. An N-terminal FLAG-tagged cDNA encoding the wild-type mouse GCN2 (p767) or the indicated CTD mutation was inserted into a derivative of the pcDNA3 expression plasmid that was transfected along with the pP₉K-ATF4-Luc reporter using the FuGENE 6 transfection reagent (Roche Applied Science). The Renilla luciferase plasmid was co-transfected and served as an internal control for transfection efficiencies (Promega). 24 h after transfection cells were treated with 1 μM thapsigargin, 5 mM histidinol, or no stress and cultured for 8 h. Lysates were prepared by 1X passive lysis buffer (Promega), and dual-luciferase assays were per-
formed as described by the Promega instruction manual. Values reported are a measure of the ratio of firefly versus Renilla luciferase units (relative light units) and represent the mean values determined for three independent transfections for each plasmid. Renilla luciferase values did not change significantly in the dual reporter assays. The results represent the means, and the S.D. is shown as an error bar. Statistical significance was calculated by using the two-tailed Student’s t test.

In parallel GCN2−/− MEF cells were transfected with the wild-type or mutant GCN2 expression plasmids or parent pcDNA3 vector alone using FuGENE transfection reagents (Promega). Transfected cells were cultured in DMEM as described above. Lysates were prepared, and equal amounts of protein were separated by SDS-PAGE followed by transfer to nitrocellulose filters as described (32). GCN2 levels were measured using polyclonal antibody-specific to this eIF2 kinase (Cell Signaling catalog #3302) followed by incubation with horseradish peroxidase-tagged secondary antibody. FLAG-tagged GCN2 was measured by immunoblot analysis using a monoclonal antibody that specifically recognizes the FLAG epitope (Sigma catalog #F8104). As a control for equal protein loading, actin levels were also measured by immunoblot using a polyclonal antibody specific to this eIF2 kinase protein. Both nitrocellulose and DEAE membranes were dried and quantitated by FLA-5100 (Fuji Image Plate BAS E2 2325 for 1 h. Thus, the total poly(C) in each binding assay was the sum of quantitated radiolabeled poly(C) from nitrocellulose and DEAE membranes. A ligand binding curve was plotted as the fraction of RNA molecules bound ([RNA]nitrocellulose/[RNA]nitrocellulose + [RNA]DEAE), representing bound divided by total as a function of mCTD concentration. The background obtained on the filter with RNA in the absence of protein was subtracted from all values of bound RNA. The data with the log of protein concentration versus the fraction of RNA bound was fit with a sigmoidal dose-response curve (variable slope) using Sigmaplot Version 11.0 (Systat Software Inc.). Use of standard one-site or two-site ligand binding models produced unsatisfactory fits to the data. We report the concentration for which half-maximal binding (the inflection point of the sigmoidal curve) was obtained from this analysis as an approximation of the relative binding affinity of WT mGCN2 to nucleic acid in this assay.

For fluorescent nucleic acid binding assays, the 35-mer oligonucleotides 5′-rhodamine-labeled ssRNA (5′-AUAGCUUAGCCUCAGUAGUGGCGUAUUUCUCAGGC-3′) and 5′-rhodamine-labeled ssDNA (5′-ATAGCCTAGCTAGTGCGTCGTAATTCCTAGCG-3′) were synthesized and desalted using gel filtration chromatography (Midland Certified Reagent Co., Midland, TX). The oligonucleotides were resuspended in water to give a final concentration of 100 μM. Double-stranded-labeled RNA and DNA oligomers were obtained by incubating Midland synthesized unlabeled complementary strands of ssRNA (5′-CGCUAGGAUUACGCGCAUCUAGCGCUAGCUAU-3′) and ssDNA (5′-GCCTAGAAATTACGGCAGTACTGAGGTGAAGCTAT-3′), respectively, at 70 °C for 10 min and cooling gradually at room temperature to allow annealing of complementary strands.

Filter binding assays with WT mCTD were carried out using 30 nM 5′-rhodamine-labeled ssRNA, dsRNA, or dsDNA in a similar manner as that described above for the binding assay using radiolabeled poly(C). The mCTD concentrations used to address binding of these oligonucleotides varied from 0.05 to 7.5 μM. Binding of γCTD to ssRNA and dsRNA was also tested by using the filter binding assay with γGCN2 concentrations ranging from 0.05 to 7.5 μM. Both nitrocellulose and DEAE membranes were dried and quantitated by using radiolabeled ssDNA from nitrocellulose (FLA-5100 β-emission imaging system (Fujifilm) using a 532-nm laser. CTD binding curves for all nucleic acids tested were plotted as described above. Moreover, mCTD mutants, R1547A, K1540A, and K1603A, were also analyzed for their association with ssRNA using the filter binding assay. 30 nM 5′-rhodamine-labeled ssRNA was used with the same protein concentrations ranging from 0.05 to 7.5 μM. The data analysis utilized a sigmoidal dose-response curve with variable slope as described above.

Ribosome Association Assays—GCN2−/− MEF cells were transfected with plasmids expressing wild-type versions of full-length GCN2 or mCTD, each tagged with a FLAG epitope at the N terminus. Cells were cultured as described above and treated with 50 μg/ml cycloheximide 10 min before harvesting. Collected cells were washed with a cold solution of phosphate-buffered saline (pH 7.4) containing 50 μg/ml cycloheximide, and cell lysates were prepared in a solution of 20 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 100 mM NaCl, and 0.4% Nonidet P-40 supplemented with 50 μg/ml cycloheximide. Cell lysates were
processed by passage through a 23-gauge needle and preclerified by microcentrifugation (10,000 rpm for 10 min at 4°C). The supernatant was then layered onto a 10–50% sucrose gradient solution containing 20 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 100 mM NaCl, and 50 μg/ml cycloheximide and then subjected to centrifugation in a Beckman SW-41Ti rotor for 2 h at 40,000 rpm at 4°C. Gradients were fractionated using a Biocomp Gradient Station, and absorbance of RNA at 254 nm was recorded using an in-line UV monitor. Proteins from equivalent volumes of the gradient fractions were precipitated with TCA and separated by SDS-PAGE, and FLAG-tagged GCN2 was measured by immunoblot analysis using antibody that recognizes the FLAG epitope.

To measure ribosome association of murine GCN2 expressed in yeast S. cerevisiae, full-length murine GCN2, which was tagged with a FLAG epitope at the N terminus, was expressed in yeast strain J82 (MATa ura3-52 leu2-3 leu2-112 gcn2Δ trp1Δ-63 p1098[SU2-S51A LEU2]). The murine GCN2 cDNA was encoded in a URA3-marked high copy number plasmid that was transformed into the yeast strain and expressed using the galactose-inducible-CYC1 hybrid promoter, as described (34). Strain J82 expresses elf2α-S51A, rendering the strain insensitive to growth inhibition by expressed murine GCN2 phosphorylation of the yeast elf2α. This would allow for sufficient levels of the GCN2 proteins to be detected by immunoblot analyses. Lysates were prepared from the yeast strain expressing murine GCN2, and lysates were subjected to sucrose gradient centrifugation and fractionation using Biocomp Gradient Station as described (21). The FLAG-tagged murine GCN2 was visualized in the fractions by immunoblot analysis using monoclonal antibody specific for the FLAG epitope. As a control we also expressed full-length yeast GCN2 tagged at the N terminus with FLAG expressed using a galactose-inducible promoter in yeast (23). Lysates were prepared, and yeast GCN2 was similarly analyzed for association with ribosomes.

RESULTS

Crystal Structures of Yeast and Murine GCN2 CTDs

Crystallization of C-terminal Domains of GCN2—Crystallization of the CTD from both murine and yeast GCN2 proteins, designated mCTD and yCTD, respectively, required identification of an optimal N terminus for each. We initially purified an N-terminally His8-tagged mCTD (1493–1648) and then proteolyzed the sample with thrombin to remove the N-terminal tag. However, after cleavage, we observed two distinct electrospray ionization masses consistent with two different N termini, the expected N terminus for thrombin cleavage and a second N terminus beginning at residue 1514. We then expressed and purified mCTD (1514–1648) and obtained diffraction-quality crystals of this protein. Similarly, initial crystals of yCTD (1498–1659 or 1536–1659) were very small, but diffraction-quality crystals were subsequently obtained for yCTD including residues 1519–1659, analogous to the mCTD (1514–1648) (see “Experimental Procedures”). Crystals of both murine and yeast CTD contain two polypeptides in the asymmetric unit.

The CTDs from Yeast and Murine GCN2 Form Interdigitated Dimeric Structures—The crystal structures of murine and yeast CTDs reveal novel interdigitated dimeric structures with no obvious matches to known three-dimensional structures (Fig. 1). Both CTD structures were determined by using experimental phasing methods (see “Experimental Procedures” and Table 1). The mCTD structural model includes residues 1530–1648 for the A chain and 1526–1648 for the B chain; the yCTD includes residues 1537–1549 and 1559–1659 for the A chain and residues 1538–1555 and 1560–1659 for the B chain. Missing residues in yCTD are disordered and correspond to the connecting loop region between the N-terminal β-strand and α-helix. Formation of the mCTD and yCTD dimers buries an unusually large accessible surface area for each molecule, 4200 and 4300 Å2, respectively, potentially contributing to the stability of each molecule.

In the mCTD, each subunit comprises an N-terminal β-strand (β1) and a long bent α-helical region (α1, α2, n1) from one polypeptide chain along with three β-strands (β2, β3, β4) and three α-helices (α3, α4, α5) from the second polypeptide, that together constitute a four-stranded β-sheet and a three-helical bundle (Fig. 1). The yCTD structure has a similar dimeric arrangement with an N-terminal β-strand (β1), but in this case a single long straight α-helix (α1) from one polypeptide. The same arrangement of three β-strands (β2, β3, β4) and three α-helices (α2, α3, n1, α4) from the second polypeptide make up each subunit. The dimeric structures in the murine and yeast CTDs are organized similarly (Figs. 1 and 2) but have distinctly different three-dimensional structures due to the relative positions of the two subunits in each structure.

In comparing a single subunit of the murine and yeast CTD structures, the r.m.s.d. is 2.1 Å for superpositioning of 74 Ca atoms, consistent with the same overall fold (Fig. 2A). However, the N-terminal regions have different relative dispositions despite sharing a conserved core structure. As shown in the structure-based sequence alignment in Fig. 2, the lengths of the comparable secondary structural elements and intervening loop regions differ significantly in the two structures with only 12 residues that are identical in the two sequences. There are also important structural differences in the overall shape of the dimeric CTD structures as well as the relative positioning of secondary structural elements within these dimeric structures despite similarities in overall organization.

The dimeric structures of the murine and yeast CTDs have three interfaces that are each situated between the two polypeptide chains. We refer to these interfaces as I, II, and III, with III being the central interface between the two subunits (Fig. 3). Interface I in both the yeast and murine CTDs arises principally from hydrogen-bonding interactions between the N-terminal β-strand 1 and β-strand 2 from the second polypeptide chain. Interface II results from the interaction of the N-terminal α-helical region in the mCTD structure with the second polypeptide chain within each subunit. This interface features the following residues, His1549, Gln1552, Val1553, Leu1557, Thr1560, Leu1561, and Leu1564 from the A chain and residues Ile1571, Ile1573, Phe1635, Tyr1637, Tyr1639, and Tyr1644 from the B chain, with A and B chains referring to the two different polypeptides that make up the dimer (Fig. 3A). By comparison in interface II of yCTD, the N-terminal α-helix from one polypeptide interacts with the other N-terminal α-helix involving residues Leu1574 and Leu1577 from each poly-
Crystal Structures of Yeast and Murine GCN2 CTDs

FIGURE 1. Crystal structures of murine and yeast CTDs reveal novel dimers. Ribbon renderings are shown for the dimeric CTD structures murine, one polypeptide chain in red, the second in green (A) and yeast, one polypeptide in magenta and the second in cyan (B). The murine and yeast CTDs are oriented such that the β-sheets in the murine red chain and yeast cyan chain are in approximately the same view. C, a portion of the experimental electron density map for the mCTD structure is shown contoured at 1.5σ with the ball-and-stick final model (yellow, C; yellow: red, O; blue, N) for two β-strands, left strand including residues 1643–1648, the C-terminal strand, and the right strand residues 1632–1637. Experimental phasing was obtained for a mercury derivative as described under “Experimental Procedures.” The final model was superimposed on the initial model built for the mercury phased map.

peptide. This interaction is unique to yCTD; the long α-helices in mCTD do not interact directly with one another. Additional interactions involve the long α-helix include residues Trp-1641, Tyr-1562, Ile-1573, Ser-1569, and Ala-1566 from the A chain and Tyr-1645, His-1647, and Ser-1652 from the B chain (Fig. 3B). Interface II in mCTD and yCTD is only approximately equivalent due to structural differences. Residues involved in interface II that are located in structurally similar locations include the following (murine/yeast): His-1549/Tyr-1562, Gln-1552/Ala-1565, Leu-1557/Ser-1569, and Thr-1560/Ile-1573 in interface II that are located in structurally similar locations.

Table 1

Crystallographic data

M refers to murine CTD, Y refers to yeast CTD. Rmerge = (Σ(|Fcalc − Fobs|)/Σ|Fcalc|), where |Fcalc| refers to the average intensity of multiple measurements of the same reflection. Rmerge and Rfree = (Σ|Fcalc| − Fobs|/Σ|Fcalc|) where Rfree was calculated over 5% of the amplitudes not used in refinement. Values in parentheses for Rmerge, completeness, and I/σ of native mCTD and yCTD datasets represent values for the highest resolution shells (1.93–1.90 and 1.98–1.95 Å, respectively).

| Cell parameters and data processing | M native | M Hg | Y native | Y Hg | Y SeMet |
|------------------------------------|----------|------|----------|------|----------|
| Wavelength, Å                      | 0.97952  | 1.007 | 1.00587  | 1.00392 | 1.00392  |
| Resolution limit, Å                | 1.9      | 2.2   | 1.95     | 2.4   | 3.0      |
| Space group                        | P3,2,1   | P3,2,1 | P2,1,2   | P2,1,2 | P2,1,2   |
| a, Å                               | 85.494   | 85.688 | 85.719   | 85.687 | 85.468   |
| b, Å                               | 85.494   | 85.688 | 118.803  | 119.985 | 120.766  |
| c, Å                               | 73.04    | 74.009 | 46.609   | 46.698 | 46.567   |
| β, °                               | 120      | 120   | 120      | 120   | 120      |
| Rmerge, %                          | 0.045 (0.64) | 0.079 | 0.040 (0.26) | 0.115 | 0.054    |
| Completeness, %                    | 99.6 (99.3) | 99.8 | 94.4 (81.9) | 99.6 | 97.0     |

| Refinement statistics               |          |      |          |      |          |
|------------------------------------|----------|------|----------|------|----------|
| Rmerge, %                          | 19.5     | 22.2 | 19.5     | 22.2 | 19.5     |
| Rfree, %                           | 23.6     | 27.5 | 23.6     | 27.5 | 23.6     |
| c.r.m.s.d.                          | 0.008    | 0.008 | 1.104    | 1.071 | 1.071    |
| No. atoms                          | 2212     | 1857 | 2212     | 1857 | 2212     |
| Protein                            | 169      | 109  | 169      | 109  | 169      |
| B-factor, Å (Å²)                   | 41.5     | 35.4a| 41.5     | 35.4a| 41.5     |
| Water                              | 49.3     | 48.1 | 49.3     | 48.1 | 49.3     |
| Ramachandran plot (%)              | 93.5     | 96.7 | 93.5     | 96.7 | 93.5     |
| Favored regions                    | 6.5      | 3.3  | 6.5      | 3.3  | 6.5      |
| Allowed regions                    |          |      |          |      |          |

* Average isotropic temperature factor after TLS refinement in PHENIX.
vide a basis for evaluating the role of specific residues in dimeric interactions that may regulate function.

**GCN2 Dimerization Occurs in Vitro and Is Required for Translational Control**—To assess the role of residues within interfaces II and III of the mCTD in dimerization, we introduced alanine substitutions for the following residues: Leu-1561, Leu-1564, Leu-1587, Tyr-1614, Tyr-1639, and Ile-1646 (Fig. 3E). Residues Leu-1561, Leu-1564, and Tyr-1639 are involved in forming hydrophobic dimer interface II; Leu-1587 and Ile-1646 form hydrophobic interface III; Tyr-1614 stacks with Pro-1538 and hydrogen bonds to Leu-1536 of another polypeptide chain. As interface I is formed primarily by backbone hydrogen-bonding interactions, no residues were selected from this interface for further analysis. We also selected residues that may have a role in ribosome/RNA binding activities, functions previously attributed to the yeast CTD (21), and characterized their dimerization properties through alanine substitution of Lys-1540, Arg-1547, and Lys-1603 (Fig. 3E). These mCTD residues are located on the surface of mGCN2 and roughly define possible RNA binding surfaces. Lys-1540 is located in a loop between H10 and H11; Lys-1552 and Lys-1553 are located in this same region of the structure in yCTD. Arg-1547 is located within H11, and the structurally equivalent residue in yCTD is the solvent-exposed residue Trp-W1560. Lys-1603, located in a4, is structurally equivalent to Arg-1609 in yCTD. The substituted mCTDs were expressed and purified as N-terminal His6-SUMO fusion proteins from E. coli and then characterized by size exclusion chromatography. The percentage of dimeric protein observed for each of the substituted fusion proteins is shown in Table 2. Substitution of residues within the interfaces II or III, with the exception of Leu-1587, significantly reduced the amount of dimeric protein observed by size exclusion chromatography. The CTD featuring L1587A exhibited intermediate behavior, with approximately half of the protein observed in a dimeric state, whereas all of the other CTDs were ~25% or less dimer. Given the nature of the dimeric mCTD structure, substitutions of buried residues within these interfaces may also affect the overall stability or folding of the protein. In contrast, substitution of residues predicted to play a role in the RNA binding activity of murine CTD had no effect on the percentage of dimer, with each displaying wild-type levels of dimerization.

To determine the contribution of the CTD dimerization to GCN2 function, WT and mutant versions of full-length murine GCN2, which were tagged with the FLAG epitope at the N terminus, were expressed in GCN2/−/− MEF cells. Although there were some variations in the levels of the mutant versions of GCN2 compared with WT, each was expressed at substantially higher levels than that measured for endogenous GCN2 protein in GCN2/−/− MEF cells (Fig. 4A). Next, we measured the translational expression of ATF4 using a well-characterized reporter containing sequences encoding the 5′ leader of the ATF4 mRNA inserted between the constitutive TK promoter and the firefly luciferase reporter. There was a 2-fold increase in ATF4 expression in the GCN2/−/− MEF cells upon treatment with histidinol, a drug that induces a starvation response and thereby elicits GCN2 phosphorylation of eIF2 (Fig. 4B). There was no increase in luciferase activity in GCN2-deficient cells upon histidinol treatment. Further illustrating the selectivity for activation of GCN2 in response to nutrient depletion, ATF4
translational expression was induced independent of this eIF2 kinase in response to treatment with thapsigargin, a potent inducer of endoplasmic reticulum stress and an alternative eIF2 kinase PERK. Transfection of a cDNA expressing the WT GCN2 restored ATF4 translational expression in the GCN2−/− cells, with more than a 4-fold increase in luciferase activity upon nutrient stress (Fig. 4B). By comparison, for each of the GCN2 proteins with substitutions of important residues in interfaces II or III, significantly reduced ATF4 expression was observed, consistent with the idea that CTD dimerization is required for GCN2 activity. GCN2 containing the L1587A expressed at levels higher than wild-type GCN2 but exhibited intermediate levels of CTD dimerization in the in vitro assay and also displayed partial induction of ATF4 expression during nutrient deprivation, suggesting that its luciferase activity is correlated with dimerization. Finally, the MEF cells expressing GCN2 with Ala substitutions of Lys-1540, Arg-1547, and Lys-1603 displayed sharply lowered levels of ATF4 translational expression, suggesting that these surface-accessible basic residues in the CTD are required for induced GCN2 activity (Fig. 4B).

**Murine CTD Binds to Both Single-stranded and Double-stranded Nucleic Acid**—To determine whether the WT mCTD binds RNA, we investigated the interactions between RNA and mCTD by using a filter binding assay. The binding of WT mCTD to 32P-poly(C) followed a sigmoidal dose-response curve with a half-maximal binding value of 5.6 μM (Fig. 5A). To
further verify if ssRNA binding was length- or sequence-specific, we carried out filter binding assays with a 5′ rhodamine-labeled 35-mer ssRNA as the substrate for WT mCTD. Quantification of the results indicated that binding of rhodamine-labeled ssRNA and wt mCTD followed a sigmoidal dose-response curve that was similar to its binding curve with 32P-labeled poly(C). However, mCTD bound the shorter ssRNA with a half-maximal binding value of 0.52 μM (Fig. 5B) as compared with 5.6 μM for poly(C). This difference in apparent binding affinity may be due to the length of the ssRNA molecules used for each experiment. For each ssRNA substrate, the sigmoidal dose-response curve is consistent with non-sequence specific binding of mCTD. The steeper slope associated with the binding to poly(C) likely reflects binding of more mCTD molecules per poly(C) due to its length. To determine whether WT mCTD also binds other nucleic acids, we carried out filter binding experiments for dsRNA and dsDNA. WT mCTD bound dsRNA and dsDNA with relative binding affinities similar to that exhibited for ssRNA.

To assess the contribution of conserved basic residues in the mCTD binding of ssRNA, filter binding experiments were performed with mutants, K1540A, R1547A, and K1603A, identified as defining potential nucleic acid binding surfaces in mCTD using the rhodamine-labeled 35-mer ssRNA. K1540A, R1547A, and K1603A mCTD mutants exhibited a maximal binding of 15.5, 57.6, and 71.3%, respectively, as compared with
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WT mCTD. This suggests that substitution of charged lysine and arginine residues with alanine negatively impacts the ability of the CTD of mGCN2 to bind ssRNA.

Murine CTD Does Not Stably Associate with Ribosomes—We next addressed whether the CTD mediates interaction of mGCN2 with ribosomes through fractionation of lysates by sucrose gradient centrifugation as previously described for yGCN2 (19, 21, 32). We followed this procedure for ribosome association using lysates prepared from Gcn2−/− MEF expressing full-length or the CTD portion of murine GCN2, each tagged with the FLAG epitope at the N terminus. Cell lysates were applied to sucrose gradients and subjected to centrifugation, and after fractionation, ribosomes were visualized (Fig. 6). Tagged mGCN2 proteins in the fractions were visualized by immunoblot. Yeast GCN2 was reported to stably associate with free 60 S ribosomes and translating ribosomes independent of stress (19, 21). We did not observe significant association of the full-length mGCN2 or the mCTD proteins with ribosomes in the MEF cells (Figs. 6A). In each case the GCN2 proteins were localized to the top portion of the gradient fractions with no GCN2 observable in the ribosomal fraction. As expected ribosomal S6 protein was found in the ribosome fractions. Tagged mGCN2 was also found largely free of ribosomes when the MEF cells were subjected to histidinol treatment before harvesting (Fig. 6A).

The N-FLAG murine GCN2 was previously expressed in yeast gcn2Δ mutant cells and shown to complement for the function of this eIF2 kinase (34). We wished to determine whether expression of the full-length murine GCN2 tagged at the N terminus with FLAG in yeast displayed association with ribosomes that were separated by sucrose gradient centrifugation. Although the majority of mGCN2 protein was localized to the top portion of the sucrose gradient, free of ribosomes, we could reproducibly detect a minor portion of the expressed murine GCN2 that co-migrated with free ribosomes and polysome upon overexposure (Fig. 6B). By contrast, yGCN2 that was also N-FLAG-tagged co-migrated with the 60 S ribosomal subunit and monosomes. These results suggest that mGCN2 does not stably bind to ribosomes under the same experimental conditions that were successfully used to demonstrate ribosomal association with yeast GCN2.

DISCUSSION

GCN2 protein kinase is expressed among virtually all eukaryotes, suggesting that phosphorylation of eIF2 and translational control is central to eukaryotic stress responses to starvation for nutrients. Most of our understanding of the regulation of GCN2 activity comes from studies in yeast S. cerevisiae in which the activity of the eIF2 kinase domain of GCN2 is controlled by the flanking HisRS-related domain and CTD in response to nutrient availability. Whereas the GCN2 protein kinase and HisRS-related domains show sequence similarities that are broadly shared among eukaryotes, the CTDs show considerably more variation, as illustrated by the sequence alignments from yeast and mouse GCN2 (Fig. 2B).

The dimeric structures of the murine and yeast CTD are unusual in lacking similarity to any other known structures and in their organization including secondary structural elements from two different polypeptide chains within each subunit. In contrast to three-dimensional domain-swapped dimers, the CTD dimer is more accurately described as interdigitated with the core B-sheet formed through interactions of strands from two different polypeptide chains. A potential benefit of this dimeric arrangement is that it may provide additional stability to the dimeric structure. This idea is supported by the unusually large surface area of ~4000 Å² that is buried upon dimer formation in each CTD. Perhaps the most surprising result of our...
structural analysis was that both yeast and murine GCN2s share a similar dimeric organization and a common core structure. At the same time, there are significant structural differences including the overall shape and size of the dimers dictated largely by interface III in each of the structures. There are also significant functional differences between the yeast and murine CTDs.

Functional activities associated with yCTD include dimerization, nucleic acid binding, and ribosome association. The dimeric mCTD structure provides a basis for assessing the role of specific subunit interactions in dimerization and the functional consequences of disrupting these interactions in the mammalian system. Collectively, size exclusion chromatographic characterization of substituted mCTD proteins and analysis of the ability of these same substitutions to disrupt induced ATF4 translational expression in cells supports the hypothesis that dimerization of the CTD is important for function in mGCN2. In this structure-based approach, we found that substitution of any of the residues involved in interactions within dimer interfaces negatively impacted the ability of GCN2 to activate ATF4 expression after treatment with histidinol. Thus, as was reported for yGCN2 (22, 24, 25), dimerization appears to be important for the function of mGCN2, and in this regard the proteins are similar.

One of the reported regulatory features associated with yCTD is ribosomal association. Yeast GCN2 was found to stably bind to free and translating ribosomes that were separated by sucrose gradient centrifugation (19, 21), and it has been proposed that association with the translational machinery provides GCN2 with access to bind uncharged tRNA that enters the A site of ribosomes during periods of amino acid starvation (25). We carried out a sucrose gradient analysis using lysates from MEF cells expressing FLAG-tagged full-length GCN2 or mCTD and found minimal binding to ribosomes (Fig. 6A). Furthermore, when we expressed the full-length mGCN2 in yeast, we observed minimal ribosome association, with the majority of the protein situated at the top of the gradients, free of ribosomes (Fig. 6B). Thus, in contrast to its yeast counterpart, mGCN2 does not appear to exhibit stable ribosomal association, suggesting a regulatory difference between the mGCN2 and its yeast counterpart.

Binding to dsRNA by the CTD has been proposed as the basis for the association of yGCN2 with ribosomes. Because mGCN2 does not appear to stably associate with ribosomes, one possibility was that mGCN2 does not bind dsRNA. However, this proved not to be the case, as both yCTD and mCTD bind ssRNA and dsRNA as analyzed by filter binding assay. These results are consistent with a general affinity for nucleic acid, and in accord with this idea, mCTD also binds dsDNA. Binding of mCTD to ssRNA is consistent with its role in sensing infection by ssRNA viruses as previously reported (7, 8). Interestingly, GCN2 was also shown to provide resistance to DNA viruses mouse cytomegalovirus (MCMV) and human adenovirus, and loss of GCN2 (EIF2AK4) was shown to block eIF2 phosphorylation upon MCMV infection (35). This would suggest that GCN2 can be activated in response to a broader range of nucleic acids than previously suggested. Given the lack of selectivity between ss- and dsRNA and dsDNA, it is likely that the interaction of the CTD with nucleic acid is governed in part by electrostatic binding. This idea is supported by the moderate binding affinities that we infer from the filter binding experiments.

Both mCTD and yCTD have similar nucleic acid binding properties, and yet mGCN2 does not appear to be stably associated with ribosomes. This suggests that the nucleic acid binding properties of mCTD may be central for mGCN2 activation in response to diverse stress signaling involving nucleic acids, including those involving virus infection. Furthermore, the GCN2 CTD may serve in conjunction with the adjacent HisRS-related domain to bind different uncharged tRNAs that accumulate during nutrient deprivation, facilitating activation of the eIF2 kinase (23). The functional relevance of nucleic acid binding by mCTD was further investigated by identifying structurally conserved basic residues found in both yCTD and mCTD. Three conserved basic residues, Lys-1540, Arg-1547, and Lys-1603, were substituted with Ala in mGCN2 and then tested for dimerization, ATF4 translational expression, and nucleic acid binding. None of these conserved basic residues was found to impact dimerization, but all were found to decrease ATF4 expression after treatment with histidinol, suggesting that these residues are important for GCN2 function. All three substituted proteins showed reduced binding to ssRNA as compared with the WT mCTD. Of these, K1540A resulted in the most significant reduction in binding activity, retaining only 15% of the activity observed for WT mCTD.

Taken together, our results suggest that although some aspects of GCN2 regulation involving the CTD are conserved between yeast and mammals, others such as ribosomal association are not. Furthermore, our studies are consistent with a broader functional role for the CTD through its recognition of viral nucleic acid through a bipartite binding site including the HisRS-related domain.

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