Mitochondrial ADCK3 Employs an Atypical Protein Kinase-like Fold to Enable Coenzyme Q Biosynthesis

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SUMMARY

The ancient UbiB protein kinase-like family is involved in isoprenoid lipid biosynthesis and is implicated in human diseases, but demonstration of UbiB kinase activity has remained elusive for unknown reasons. Here, we quantitatively define UbiB-specific sequence motifs and reveal their positions within the crystal structure of a UbiB protein, ADCK3. We find that multiple UbiB-specific features are poised to inhibit protein kinase activity, including an N-terminal domain that occupies the typical substrate binding pocket and a unique A-rich loop that limits ATP binding by establishing an unusual selectivity for ADP. A single alanine-to-glycine mutation of this loop flips this coenzyme selectivity and enables autophosphorylation but inhibits coenzyme Q biosynthesis in vivo, demonstrating functional relevance for this unique feature. Our work provides mechanistic insight into UbiB enzyme activity and establishes a molecular foundation for further investigation of how UbiB family proteins affect diseases and diverse biological pathways.

INTRODUCTION

Protein kinase-like (PKL) superfamily members share an active site that catalyzes adenosine triphosphate (ATP)-dependent phosphorylation (Kannan et al., 2007), but evolution of divergent sequence features and unique accessory domains has enabled adoption of a broad range of biological functions. Crystallographic studies have revealed the unique features of 10 of the 20 known PKL families (Hon et al., 1997; Kang et al., 2008; Knighton et al., 1991; Ku et al., 2007; LaRonde-LeBlanc and Wlodawer, 2004; Mao et al., 2008; Walker et al., 1999; Yamaguchi et al., 2001; Young et al., 2003; Zheng and Jia, 2010); however, members of the UbiB family have remained refractory to purification and biochemical analyses.

UbiB kinases are present in archaea, bacteria, and eukaryotes (Leonard et al., 1996) and comprise an estimated one quarter of all microbial PKLs (Kannan et al., 2007). The founding member of the UbiB family, UbiB from Escherichia coli, is required for the aerobic biosynthesis of the redox-active lipid ubiquinone, also known as coenzyme Q (CoQ) (Poon et al., 2000). In eukaryotes, UbiB homologs are found exclusively in mitochondria (Pagliarini et al., 2008) and plastids (Lundquist et al., 2012, 2013; Martinis et al., 2013), where they have been implicated in stress responses (Jasinski et al., 2008), copper homeostasis (Schlecht et al., 2014), pigmentation (Lundquist et al., 2013), phospholipid metabolism (Tan et al., 2013), and isoprenoid lipid biosynthesis (Do et al., 2001; Lundquist et al., 2013; Martinis et al., 2013).

The human genome contains five UbiB family genes, ADCK1–5 (Figure 1A). Mutations in ADCK3 and ADCK4 cause a cerebellar ataxia (Horvath et al., 2012; Lagier-Tourenne et al., 2008; Mollet et al., 2008; Pineda et al., 2010) and a steroid-resistant nephrotic syndrome (Ashraf et al., 2013), respectively, that are each associated with CoQ deficiency. Silencing of ADCK1 expression alters epithelial cell migration (Simpson et al., 2008), and silencing of ADCK2 significantly decreases the viability of cells derived from glioblastoma multiforme (Wiedemeyer et al., 2010) and estrogen receptor-positive breast tumors (Brough et al., 2011). As such, ADCK proteins are promising therapeutic targets that await rigorous biochemical characterization.

ADCK3 (CABC1, COQ8), the focus of this work, has been only minimally characterized at the biochemical level. Phylogenetic analyses defined ADCK3 as an “atypical kinase” (Manning et al., 2002) and suggested that ADCK3 and ADCK4 are co-orthologs of the yeast protein Coq8p (Lagier-Tourenne et al., 2008). Coq8p is known to stabilize a complex of CoQ biosynthesis proteins in yeast (He et al., 2014), but the underlying mechanism is undefined. ADCK3 can weakly rescue the respiratory
growth defect of Coq8p knockout (coq8Δ) yeast, and this rescue partially restores the phosphorylation state of Coq3p, Coq5p, and Coq7p in vivo, but whether this effect is direct or indirect is unknown (Xie et al., 2011). ADCK3 is known to reside in the mitochondrial matrix (Pagliarini et al., 2008; Rhee et al., 2013), and we recently demonstrated that ADCK3 contains a transmembrane (TM) domain that can drive homodimerization (Khadria et al., 2014).

The primary gaps in knowledge about the UbiB family center around its undefined enzymatic activity. Although UbiB proteins share some sequence features with protein kinases, whether UbiB proteins actually adopt a PKL fold was unknown. Moreover, previous attempts by our laboratory and others to demonstrate kinase activity for UbiB proteins in vitro were unsuccessful for unknown reasons.

Here, to provide molecular insight into the activity of UbiB family proteins, we integrate bioinformatics, crystallography, in vitro activity assays, and investigation of in vivo CoQ production. By solving a crystal structure of a UbiB family protein, human mitochondrial ADCK3, we show that UbiB proteins adopt an atypical PKL fold with multiple UbiB-specific features positioned to inhibit protein kinase activity. We demonstrate that mutating one of these unique features relieves enzyme inhibition and enables autophosphorylation in vitro, while showing that this same mutation inhibits CoQ production in vivo. These results suggest a model wherein inhibition of protein kinase activity is important for the mechanism by which ADCK3 enables CoQ biosynthesis. Our work also suggests a mechanism by which human ADCK3 and ADCK4 mutations disrupt protein stability to cause disease and provides a foundation for therapeutic targeting of UbiB family proteins.

RESULTS

The UbiB Family Has Numerous Unique Sequence Motifs

The PKL domain of the UbiB family possesses an N-lobe insert and an N-terminal extension with uncharacterized sequence features (Figure 1A), including a unique and invariant KxGQ motif (Figure 1B). We conducted a statistical analysis of the features that distinguish UbiB proteins from other PKLs, which revealed...
that the UbiB family also has an atypical AAAS motif in an alaninerich (A-rich) loop that replaces the canonical glycine-rich (G-rich) nucleotide-binding loop, an ExD motif in the N-lobe insert, a modifed catalytic loop, and a conserved arginine or lysine residue in the F helix (\(xF\)) (Figure 1C and Figure S1A available online). However, the insolubility of full-length UbiB proteins (Figure S1C) has thus far hampered efforts to determine how these unique sequence elements enable UbiB-specific functions.

**Mature Mitochondrial ADCK3 Is Truncated**

To generate a tractable system for biochemical analyses, we tested the solubility of various ADCK3 truncation constructs. Mature forms of mitochondrial UbiB proteins are likely processed to remove their mitochondrial targeting sequences (MTSs), and they contain predicted single-pass TM domains that could limit solubility (Figure 1A). However, the MTS of ADCK3 could not be predicted in silico, so we determined it experimentally. Immunoblot and Edman degradation analyses of mitochondrial ADCK3 (Figure 1D) immunoprecipitated from human cells revealed that the mature form of ADCK3 is truncated by 162 residues to yield a 55 kDa protein (Figures 1E and 1F)—a size consistent with the observed yeast Coq8p truncation (Vögtle et al., 2009) and the predicted ADCK4 truncation (Figure 1A). Most of the sequence removed upon import into mitochondria lacks conservation among metazoan orthologs (Figure S1B). While the endogenous form of ADCK3 remained refractory to large-scale purification and crystallization, we were able to purify constructs with N-terminal truncations of 250 residues (ADCK3(NA250)) or 254 residues (ADCK3(NA254)). These constructs retain the PKL domain and the unique N-terminal extension, the combination of which represents the structural core of the UbiB family (Figure 1A). These results, combined with previous localization of ADCK3 to the mitochondrial matrix (Rhee et al., 2013), allowed us to create models for the ADCK3 precursor, the mature mitochondrial form, and the crystallized construct (Figures 1G and 1H).

**ADCK3 Adopts an Atypical PKL Fold**

We crystallized and solved the structure of ADCK3(NA254) at a resolution of 1.6 Å (Figure 2A; Table 1). ADCK3 adopts a core fold similar to that of well-characterized PKLs (Figure S2A), such as protein kinase A (PKA) (Knighton et al., 1991; Zheng et al., 1993) (Figure 2D), whereby the N-lobe folds into a \(\beta\) sheet and a single \(\alpha\) helix (\(\alphaC\)), and the C-lobe folds into a series of \(\alpha\) helices and \(\beta\) strands. The overall topologies of ADCK3 and PKA are also similar; but ADCK3 contains a long N-terminal extension (GQ\(_{1-25}\)), an N-lobe insert between \(\beta3\) and \(\alphaC\) (GQ\(_{25-53}\)), and a C-lobe insert between \(\beta9\) and \(\alphaF\) (GQ\(_{41-52}\)) (Figures 2C and 2F)—unique features that likely afford UbiB family-specific functions.

Unexpectedly, the N-terminal extension and the N-lobe insert fold into \(\alpha\) helices that pack together and rest directly over the A-rich loop and the PKL cleft, where they place the signature KxGQ motif into the substrate-binding pocket (Figure 2A). We have named this region of ADCK3, composed of the insert and extension, the “KxGQ domain” and its component \(\alpha\) helices “GQ\(_{1-25}\)” (Figure 2C). The KxGQ domain completely occludes the cleft that binds peptide substrates in typical protein kinases (Figures 2B and 2E). Moreover, in stark contrast to the architecture of ADCK3, the N-terminal extensions of all other structurally characterized PKL families are positioned far from the active site (Figures S2B–S2K). Even the N-terminal extension and the N-lobe insert of Rio family proteins, which exhibit the highest structural similarity to ADCK3 (Figure S2A), fold away from the nucleotide pocket, and leave the PKL cleft open to accept a peptide substrate (Figure S2B).

Within the KxGQ domain of ADCK3, we observed a salt bridge between K276 of the KxGQ motif and E405 of the ExD motif (Figures 3A and 3D). The polar and ionic interactions between Q279, K276, and E405 suggest that they act as a functional triad, which we have named the QKE triad. The QKE triad folds directly into the active site near the A-rich loop and highly conserved PKL residues such as D507 and N493 (Figure 3A). This position of the QKE triad overlaps with that of the substrate phosphoryl acceptor in structures of typical protein kinases. Collectively, this structural analysis of the KxGQ domain and the QKE triad suggests that they could function to inhibit protein kinase activity.

**ADCK3 Contains an Atypical Active Site**

Given that demonstration of kinase activity for a UbiB protein has remained elusive for well over a decade (Leonard et al., 1998), we examined the ADCK3 structure for additional features that could inhibit kinase activity. Comparing the structures of PKA and ADCK3 reveals significant differences within the catalytic loop. In PKA, D166(D166\(^{325}\)) is oriented toward the terminal phosphate of the ATP substrate, where it is thought to act as a catalytic base for the phosphoryl acceptor. In contrast, the homologous ADCK3 residue, D488\(^{ADCK3}\), is oriented in the opposite direction, where it forms a bidentate salt bridge with \(\alphaF\) through R611\(^{ADCK3}\) (Figures 3C and 3E).

While features such as the QKE triad and the D488/611 salt bridge could inhibit protein kinase activity, our PKA-ADCK3 superposition also shows that ADCK3 has hallmarks of an active kinase, including a salt bridge between K358 and E411 (Figure 3C), cation-binding residues (N493 and D507) poised for catalysis (Figure 3C), and intact kinase “spines” (Kornev et al., 2006) (Figures S3A and S3B). These hallmark features allow active kinases to bind MgATP and catalyze phosphoryl transfer. ADCK3’s retention of these features suggests that it would be capable of performing catalysis following removal of inhibitory constraints, but neither nucleotide binding nor catalytic properties had yet been demonstrated for a UbiB protein.

**ADCK3 Exhibits an Unusual Selectivity for Binding ADP over ATP**

To test whether ADCK3 can bind ATP, we quantified ligand-induced changes in ADCK3 melting temperature (\(\Delta T_m\)). In the presence of divalent cations, adenosine nucleotides significantly stabilized ADCK3(NA250), whereas other nucleotides did not (Figure 3F). Surprisingly, ADCK3 exhibited an unusual selectivity for binding ADP over ATP. PKA, an example of a more typical kinase, binds MgATP and MgADP with similar affinity, with dissociation constants of \(~10\) \(\mu\)M (Bhatnagar et al., 1983). ADP binding could inhibit ATP-dependent kinase activity, so we aimed to define the mechanism by which ADCK3 selectively binds ADP over ATP.
The location of the UbiB-specific KxGQ motif near the active site raises the question of whether it mediates nucleotide binding and determines selectivity for ADP. To measure the effects of ADCK3 point mutations on nucleotide binding, we quantified $D_{Tm}$ values over a range of ligand concentrations to generate ligand-binding curves, which allowed us to determine apparent dissociation constants ($K_{d, app}$) and maximum $D_{Tm}$ values ($D_{Tm, max}$). As expected, mutation of canonical PKL ATP-binding residues dramatically decreased ATP and ADP binding; however, mutation of the KxGQ motif had minimal effects, suggesting that it is not part of the core nucleotide-binding pocket.

To further examine the structural basis for nucleotide binding, we used deuterium exchange mass spectrometry (DXMS). DXMS revealed that ADCK3 has a stable hydrophobic core anchored by the F helix, while the KxGQ domain and portions of the unique C lobe are more mobile. Dramatic decreases in deuterium exchange rates in the presence of nucleotides were observed for the conserved DFG motif, which coordinates a cation that mediates nucleotide binding (see D507 in Figure 3C), and for the unique A-rich loop. This direct involvement of the UbiB-specific A-rich loop in nucleotide binding nominated it as a possible structural determinant of selectivity for ADP.

The A-Rich Loop of ADCK3 Is a Structural Determinant of Coenzyme Selectivity

Comparing the conformation of the A-rich loop of ADCK3 to the G-rich loop of PKA reveals significant differences. (Figure 4A). The steric and conformational flexibility of G52$_{PKA}$ allow...
a backbone amide of the G-rich loop to hydrogen bond with the γ phosphate of ATP. Thus, the A-rich loop of ADCK3 adopts a different conformation, and the homologous backbone amide is not ideally positioned to bind ATP. Furthermore, in superimposed structures of ADCK3 and PKA, A339GADCK3 clashes with the nucleotide bound to PKA.

To test the hypothesis that the A-rich loop of ADCK3 is a determinant of coenzyme selectivity, we mutated its alanine residues to glycine residues, thereby converting it into a more typical G-rich loop. An A339G mutation of ADCK3 significantly enhanced affinity for MgATP without affecting MgADP binding. As such, the A339G mutation affords a 6-fold increase in selectivity for ATP over ADP (\( K_d^{MgADP}/K_d^{MgATP} \)) (Figures 4B and S4A). Like the A339G mutation, an A337G,A339G double mutation also enhanced selectively for ATP (Figures 4C and S4A). However, in contrast, an A337G mutation alone did not flip coenzyme selectivity. These results demonstrate that A339ADCK3 is a major structural determinant of coenzyme selectivity and suggest that it could also be an important determinant of ADCK3 enzyme activity.

A Single A-to-G Mutation of the A-Rich Loop Enables Autophosphorylation

The G-rich loop of typical protein kinases is important for MgATP binding, peptide substrate binding, and catalysis (Sosse-I 1994; Hemmer et al., 1997). To test the idea that the ADCK3 A339G mutation might similarly enable protein kinase activity, we examined the ability of ADCK3 A-rich loop mutants to autophosphorylate. The A339G and A337G,A339G double mutations both enabled autophosphorylation (Figure 4D), which we found to be dependent on time and ATP concentration (Figures 4E–4G). MgATP-dependent phosphorylation of the N-terminal serine residue of ADCK3 A339G was also observed by liquid chromatography high-resolution tandem mass spectrometry (LC-MS/MS) (Figures S4B–S4D). Autophosphorylation was inhibited by an A339G,D507N double mutation (Figure 4H), which also inhibited MgATP binding (Figure 4I), demonstrating that the phosphorylation is ADCK3 dependent and not due to a contaminating kinase. Furthermore, the autophosphorylation was Mg\(^{2+}\) dependent (Figure 4H), a typical feature of kinase activity. Under these reaction conditions, we also observed nonspecific phosphorylation of all three ADCK3 variants with MnATP, a result that has been observed previously in reactions with MnATP (Schieven and Martin, 1988). Collectively, these results demonstrate that ADCK3 A339 inhibits protein kinase activity in vitro.

Mutation of the Conserved KxGQ Motif Enhances Autophosphorylation

The activity of the ADCK3 A339G mutant enabled us to test our structure-based hypothesis that the KxGQ domain inhibits protein kinase activity. In the A339G background, KxGQ motif mutations markedly enhanced autophosphorylation activity (Figure 4J). Interestingly, the enhanced autophosphorylation activity was not observed with KxGQ motif single mutants. Under these conditions, the A339G mutation appears to be necessary for autophosphorylation activity in vitro.

In vivo, accessory factors could induce conformational changes of the A-rich loop and the KxGQ domain to enhance activity. Based on our structure, we generated two hypothetical models for how the KxGQ domain could move (Figures S4A). However, the enhanced autophosphorylation activity was not observed with KxGQ motif single mutants. Under these conditions, the A339G mutation appears to be necessary for autophosphorylation activity in vitro.

UbIb-Specific Features Are Required for CoQ Biosynthesis

To test the direct functional relevance of novel UbIb family sequence elements, we performed in vivo structure-function analyses with Coq8p. Using our ADCK3 structure, we generated
Figure 3. ADCK3 Adopts an Atypical PKL Active Site that Binds Nucleotides

(A) Structure of the A-rich loop and the QKE triad of ADCK3^N^D^254^ colored as in Figure 2.

(B) Fold changes in apparent $K_d$ for ADCK3^N^D^250^ mutants compared with WT, as assessed by differential scanning fluorimetry (DSF) (mean ± SD, n = 3).

(C) Superposition of the nucleotide binding pockets of ADCK3^N^D^254^ (darker colors and black text) and PKA (PDB: 2QCS) (Kim et al., 2007) (lighter colors and gray text) colored as in Figure 2. The nucleotide (AMPPNP) (green) and cations (black spheres) are from the PKA structure. Red arrows highlight the unusual conformation of D488^ADCK3^.

(D) Simulated annealing composite omit maps ($2mF_o-DF_c$) contoured at 1.4σ (gray mesh) of the QKE triad and the serine of the AAAS motif.

(E) Simulated annealing composite omit map ($2mF_o-DF_c$) of D488^ADCK3^ and R611 contoured at 1.8σ (gray mesh).

(F) $T_m$ of ADCK3^N^D^250^ due to addition of various ligands and cations (mean ± SD, n = 3 independent $T_m$ determinations).

(G) Average differences (over five time points) in deuterium exchange of ADCK3^N^D^250^ due to the presence of ATP^γS or ADP mapped onto the structure of ADCK3^N^D^254^.

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a homology model of Coq8p (Figures S5A and S5B), which allowed us to design Coq8p mutations and examine their structural effects. We then expressed Coq8p variants in coq8Δ yeast, which exhibit a respiratory growth defect on nonfermentable carbon sources caused by CoQ deficiency. Mutation of conserved nucleotide pocket residues eliminated respiratory growth, both on solid media with nonfermentable carbon sources (Figures 5A and 5C) and in liquid media with depleted glucose (Figure S5C).

Using high-resolution LC-MS, we demonstrated that these growth phenotypes closely mirrored decreases in CoQ abundance (Figure 5B). Together, this panel of assays enables rapid and precise measurement of the phenotypic effects of mutations to conserved Coq8p and ADCK3 residues.

We next used these yeast assays to define essential residues in the A-rich loop and the KxGQ domain. Importantly, the mutation homologous to ADCK3 A339G, Coq8p A197G, caused a
significant decrease in CoQ abundance (Figure 5B), despite its activation of ADCK3 protein kinase activity in vitro, demonstrating the functional relevance of this conserved alanine residue. Mutation of the serine residue of the AAAS motif also markedly decreased respiratory growth (Figure 5A) and CoQ abundance (Figure 5B), further emphasizing the importance of the A-rich loop. Finally, mutations of the QKE triad eliminated respiratory growth and CoQ production (Figures 5A and 5B), demonstrating an essential role for the QKE triad in the core function of UbiB proteins.

Pathogenic ADCK3 and ADCK4 Mutations Disrupt Protein Stability

Our ADCK3 structure provides an opportunity to investigate the molecular basis of human diseases associated with UbiB family members. ADCK3 mutations that cause a cerebellar ataxia map to multiple regions of the protein, including the KxGQ domain (Figure 6A), further validating the central importance of this unique region of the protein (Horvath et al., 2012; Lagier-Tourenne et al., 2008; Mollet et al., 2008; Pineda et al., 2010). We used our structure to predict the deleterious effects of ADCK3 mutations (Figure 6B), most of which show decreased protein thermal stability or affinity for nucleotide (Figures 6C and 6D). By generating a homology model of ADCK4, we were likewise able to infer the deleterious effects of mutations in this kinase that cause a kidney disease (Ashraf et al., 2013) (Figure S6).

DISCUSSION

Insights into the Enzyme Activity of the UbiB Family

Since the classification of UbiB family protein sequences as PKL (Leonard et al., 1998), it has been assumed that UbiB family members are protein kinases. However, the structure, ligand-binding properties, and activity of a UbiB protein had not been described. Our results demonstrate that a UbiB family protein, ADCK3, does in fact adopt a PKL fold and bind adenine nucleotides in a divalent cation-dependent manner, strengthening the hypothesis that UbiB proteins are bona fide kinases.

This work also provides rationale for why demonstration of UbiB protein kinase activity has been so elusive. Our structural and biochemical investigations show that multiple UbiB-specific features inhibit ADCK3 protein kinase activity. With a single A-to-G mutation of the A-rich loop, we were able to release one of
Molecular Insight into ADCK3 Activity

Figure 6. Pathogenic ADCK3 Mutations Disrupt Protein Stability
(A) Residues mutated in patients with cerebellar ataxia mapped onto the ADCK3\textsuperscript{N254S} structure as spheres. Domains are colored as in Figure 2.
(B) Predicted structural effects of pathogenic ADCK3 mutations.
(C) Fold changes in $\Delta T_m$ of ADCK3\textsuperscript{N254S} mutants compared with WT, as assessed by DSF (mean ± SD, n = 3 independent $\Delta T_m$ determinations).
(D) Fold changes in apparent $K_d$ (ADP) for ADCK3\textsuperscript{N254S} mutants compared with WT as assessed by DSF (mean ± SD, n = 3 independent $K_d$ app determinations). *p < 0.05, **p < 0.01.

These inhibitory mechanisms and demonstrate kinase activity for a UbiB family protein.

Our work also shows that the QKE triad is essential for function in vivo and provides insight into the biochemical function of the conserved KxGQ domain. If UbiB proteins catalyze phosphorylation of proteins, as previously hypothesized (Martinis et al., 2013; Xie et al., 2011), then the KxGQ domain is likely to be an autoinhibitory domain because it fills the space normally occupied by peptide or protein substrates in typical protein kinases. This idea is supported by the enhanced autophosphorylation activity of KxGQ mutants in the A339G background. However, our analyses raise a competing hypothesis: ADCK3 may phosphorylate a small molecule, with the KxGQ domain functioning as a substrate-binding domain. This idea is supported by the observation of a potential small molecule binding pocket that could be accessed with minimal conformational changes and would be even more accessible with subtle movements of the KxGQ domain, such as those predicted by our NMA. The observed phosphorylation of an N-terminal serine is concordant with the protein kinase hypothesis, but this moderately hydrophobic N-terminal sequence could also mimic an elongated small molecule, such as a lipid.

A final hypothesis related to our observation of UbiB-specific inhibitory mechanisms is that ADCK3 is a pseudokinase. However, the retention of all residues necessary for catalysis, and the requirement of these residues for in vivo function suggest that this is unlikely, as all characterized pseudokinases are missing one or more essential catalytic residues (Zeqiraj and van Aalten, 2010).

Implications for Our Understanding of the PKL Superfamily
Our investigation of UbiB family proteins has widespread implications for our understanding of the ubiquitous PKL superfamily. One UbiB-specific feature, the A-rich loop, is a striking deviation from common G-rich nucleotide binding motifs. G-rich motifs are not only widespread in the PKL superfamily; they are also found in the Rossmann fold (Rossmann et al., 1974) and the p loop (Walker A motif) (Saraste et al., 1990). Generally, these G-rich motifs enable coordination of nucleotide phosphates by backbone amides. The second glycine in the G-rich loop (GxGxxG) was known to be critical for ATP binding and protein kinase catalysis by PKA (Bossemeyer, 1994; Hemmer et al., 1997), but it was not known what effects this residue had on nucleotide selectivity. Our work demonstrates that the analogous A-rich loop of ADCK3 confers an unusual selectivity for ADP over ATP. Moving forward, it may be important to test the hypothesis that UbiB proteins can use ADP as a phosphoryl donor. An ADP-dependent glucokinase has been described (Ito et al., 2001), but no PKL superfamily members are known to be ADP-dependent kinases.

Insight into the Biosynthesis of Isoprenoid Lipids
Many UbiB family proteins are required for the biosynthesis of isoprenoid lipids, such as CoQ, plastoquinone, phylloquinone (vitamin K), plastochromanol, and $\alpha$-tocopherol (vitamin E) (Cardazzo et al., 1998; Do et al., 2001; Lundquist et al., 2013; Martinis et al., 2013; Poon et al., 2000). Our work provides a molecular foundation for investigating this requirement. We show that UbiB-specific features, such as the A-rich loop and KxGQ motif, are required for the ability of Coq8p to enable mitochondrial CoQ biosynthesis. Furthermore, our structure-activity investigations suggest that inhibition of protein kinase activity is important for the mechanism by which UbiB proteins enhance CoQ biosynthesis.

Ongoing work in our laboratory seeks to understand why this inhibition of UbiB protein kinase activity is important for isoprenoid lipid metabolism. The yeast ortholog of ADCK3, Coq8p, has an established role in stabilizing a membrane-associated complex of proteins that comprises the CoQ biosynthetic machinery (He et al., 2014), and we currently favor two competing hypotheses for how Coq8p could mechanistically fulfill this role. One hypothesis is that Coq8p is a protein kinase that phosphorylates other proteins in the CoQ complex to stabilize their interactions. If this is true, then our work suggests that this protein kinase activity must be carefully regulated to enable proper phosphorylation of the CoQ complex. However, as detailed above, our work raises the alternative possibility that UbiB proteins are small molecule kinases. If this is true, then Coq8p might phosphorylate a prenyl lipid, which has been suggested to be a part of the mature CoQ biosynthetic complex (He et al., 2014). We recently demonstrated that another
Expression and Purification of ADCK3

Selenomethionine-labeled 8His-MBP-[TEV]-ADCK3 was expressed in E. coli using IPTG induction essentially as described (Gromek et al., 2013). Protein was purified using a nickel ion metal affinity chromatography (IMAC) column, MBP cleavage by TEV protease (Blommel and Fox, 2007), a second IMAC purification to remove 8His-MBP, and gel filtration (see Supplemental Experimental Procedures for further details).

Cocrystalization

Crystals of selenomethionine-labeled ADCK3 were obtained using the high throughput screening and optimization platform developed at the Center for Eukaryotic Structural Genomics (Markley et al., 2009). Crystallization screens were set with a TTP Labtech Mosquito robot, and a Tecan Genesis was used for optimization solutions. The best crystals were obtained by microseeding into 5 μl of 5 mg/ml selenomethionyl ADCK3, 12.5% PEG 3350, 150 mM (NH4)2SO4, 5 mM MgCl2, and 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) sodium salt (pH 7.5).

Expression and Purification for In Vitro Assays

8His-MBP-[TEV]-ADCK3 was expressed in E. coli by autoinduction (Fox and Blommel, 2009). Proteins were purified using cobalt IMAC resin, tobacco etch virus (TEV) cleavage, and a second subtractive IMAC purification to remove 8His-MBP (see Supplemental Experimental Procedures for further details).

Differential Scanning Fluorimetry

ADCK3 (1 μM) was mixed with nucleotide and MgCl2 (20 mM) in an aqueous buffer (100 mM HEPES, 150 mM NaCl [pH 7.5]) with SYPRO Orange Dye (5×; Life Technologies) (final concentrations), and the fluorescence of the mixture was measured as the temperature was increased from 15°C to 95°C using a Real Time PCR System (Applied Biosystems, ViiA7). The fluorescence data were analyzed with Protein Thermal Shift software (Applied Biosystems) to determine Tm values (see Supplemental Experimental Procedures for further details).

DXMS

ADCK3 (50 μM) was mixed with MnCl2 (2.5 mM), with or without ATP/S (2 mM) or ADP (2 mM), incubated (20°C, 20 min), and then cooled to 0°C. Deuterium exchange was initiated by diluting 2 μl of the ADCK3 solution with 10 μl of D2O buffer (8.3 mM Tris, 150 mM NaCl, in D2O, pH 7.2) at 0°C. At 10, 100, 1,000, 10,000, and 100,000 s, the exchange reactions were quenched with 18 μl of 3.2 M GuHCl, 0.8% formic acid, and 16.6% glycerol. The samples were digested with pepsin and analyzed by LC-MS/MS. The centroids of the isotopic envelopes of nondeuterated, partially deuterated and equilibrium-deuterated peptides were measured using DXMS Explorer (Sierra Analytics) and then converted to corresponding deuterium level (see Supplemental Experimental Procedures for further details).

In Vitro Kinase Auto phosphorylation Assays

Unless otherwise indicated, ADCK3 (50 μM) was mixed with [γ-32P]ATP (0.2 μCi/μl, 100 μM [ATP]pooled) and MgCl2 (20 mM) in an aqueous buffer (100 mM HEPES, 150 mM NaCl [pH 7.5]) and incubated (37°C, 10 min) (final concentrations). For the divalent cation screen, MgCl2, MnCl2, or CaCl2 was used at 20 mM. Reactions were quenched with 4xLDS buffer, [γ-32P]ATP was separated from ADCK3 by SDS-PAGE. The gel was stained (Coomassie), dried, and imaged (see Supplemental Experimental Procedures for further details).

Yeast Growth Assays

Saccharomyces cerevisiae (W303 background strain) coq8Δ yeast were transformed with p426 GPD plasmids encoding for Coq8o variants. To assay yeast growth on agar plates, serial dilutions of yeast were dropped onto Ura- media plates containing either glucose (2%, w/v) or glycerol (3%, v/v) and incubated (30°C, 2 days). To assay yeast growth in liquid media, yeast from a starter culture were swapped into Ura- media with glucose (0.1%, w/v) and glycerol (3%, v/v) at an initial density of 5 x 10⁶ cells/ml, and growth was monitored by optical density at 600 nm (OD600) (see Supplemental Experimental Procedures for further details).

S. cerevisiae CoQ quantitation by LC-MS

Yeast were grown past the diauxic shift in Ura- media (10 g/l glucose), isolated by centrifugation, and lysed with glass beads. CoQ10 was added as an internal standard. Lipids were extracted and dried under N2(g) by normalization peak areas to the CoQ10 internal standard. Student’s t test was used to determine statistical significance (see Supplemental Experimental Procedures for further details).

Implications for Human Diseases Involving UbiB Family Proteins

Mutations of ADCK3 are responsible for a neurodegenerative disease (Horvath et al., 2012; Lagier-Tourenne et al., 2008; Mollet et al., 2008; Pineda et al., 2010), and mutations of a closely related homolog, ADCK4, are responsible for a steroid-resistant nephrotic syndrome (Ashraf et al., 2013). The results presented here provide a structural framework for understanding how these ADCK mutations cause human disease.

Other ADCK proteins, such as ADCK2, have been linked to cancer cell viability (Brough et al., 2011; Wiedemeyer et al., 2010), suggesting inhibition of ADCK proteins as a potential therapeutic strategy. Kinases are proven chemotherapeutic targets for small molecule inhibitors, but off-target kinase inhibition can be problematic. Encouragingly, our analyses reveal that the architecture of the UbiB family nucleotide-binding pocket, especially the placement of the KxQQ motif, is likely sufficiently distinct to enable the development of specific inhibitors. Furthermore, ADCK2 has unique sequence features that distinguish it from the other human ADCKs, which should assist development of ADCK2-specific inhibitors. Our structure of ADCK3 provides a foundation for building homology models of ADCK2 for structure-based inhibitor design.

Collectively, our investigation of how ADCK3 uses a significant variation on the PKL fold to enable ubiquinone biosynthesis has broad implications on our understanding of kinase enzyme regulation, catalysis, and substrate recognition. These results also provide a framework for further biochemical analysis and therapeutic targeting of the widespread UbiB family.

EXPERIMENTAL PROCEDURES

Statistical Analysis of Unique Sequence Motifs

MAPGAPS (Neuwald, 2009) was used to align over 150,000 sequences belonging to the PKL superfamily, as done previously (Kannan and Neuwald, 2005). The curated alignment was used to delineate uniquely conserved residue patterns in the UbiB family using the program CHAIN (Neuwald et al., 2003, Neuwald, 2007; see Supplemental Experimental Procedures for further details).

Expression and Purification of ADCK3 for Crystallization

Selenomethionine-labeled 8His-MBP-[TEV]-ADCK3 was expressed in E. coli using IPTG induction essentially as described (Gromek et al., 2013). Protein was purified using a nickel ion metal affinity chromatography (IMAC) column, MBP cleavage by TEV protease (Blommel and Fox, 2007), a second IMAC purification to remove 8His-MBP, and gel filtration (see Supplemental Experimental Procedures for further details).

Cocrystalization

Crystals of selenomethionine-labeled ADCK3 were obtained using the high throughput screening and optimization platform developed at the Center for Eukaryotic Structural Genomics (Markley et al., 2009). Crystallization screens were set with a TTP Labtech Mosquito robot, and a Tecan Genesis was used for optimization solutions. The best crystals were obtained by microseeding into 5 μl of 5 mg/ml selenomethionyl ADCK3, 12.5% PEG 3350, 150 mM (NH4)2SO4, 5 mM MgCl2, and 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) sodium salt (pH 7.5).

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Molecular Insight into ADC3 Activity

ACCESSION NUMBERS

The coordinates and structure factors have been deposited in the Protein Data Bank with accession code 4PED.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2014.11.002.

AUTHOR CONTRIBUTIONS


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