IDENTIFICATION OF CHCHD3 AS A NOVEL SUBSTRATE OF PKA USING AN ANALOG-SENSITIVE CATALYTIC SUBUNIT
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Running Title: Novel PKA Substrate, ChChd3

Due to the numerous kinases in the cell, many with overlapping substrates, it is difficult to find novel substrates for a specific kinase. To identify novel substrates of cAMP-dependent protein kinase (PKA), the PKA catalytic subunit was engineered to accept bulky N6-substituted ATP analogs, using a chemical genetics approach initially pioneered with v-Src (1). Methionine 120 was mutated to glycine in the ATP binding pocket of the catalytic subunit. To express the stable mutant C-subunit in E. coli required co-expression with PDK1. This mutant protein was active and fully phosphorylated on Thr197 and Ser338. Based on its kinetic properties, the engineered C-subunit preferred N6-(benzyl)-ATP and N6-(phenethyl)-ATP over other ATP analogs, but still retained a 30 μM Km for ATP. This mutant recombinant C-subunit was used to identify three novel PKA substrates. One protein, a novel mitochondrial ChChd protein, ChChd3, was identified, suggesting that PKA may regulate mitochondria proteins.

PKA is expressed in all mammalian cells and plays a major role in processes such as metabolic control, memory, growth, development, and apoptosis. Thus, it has many substrates which usually are highly tissue specific. One such example is the bifunctional enzyme phosphofructokinase-2 (PFK2), which upon phosphorylation by PKA is converted to its phosphatase active mode in liver, but is stabilized in its kinase mode in heart, and is not a PKA substrate in skeletal muscle (1). Although PKA has been widely studied in most cells, the direct substrates of PKA are difficult to distinguish from downstream substrates that are phosphorylated as a result of the PKA activation. A possible way to determine whether identified substrates are modified by the kinase directly or indirectly via an intermediary kinase is to engineer the ATP binding pocket of the kinase in question to accept a bulky ATP analog that cannot be used by the wild type kinase. This strategy was developed initially for a tyrosine kinase, v-Src, (2) and has subsequently been used for other protein kinases (3). Shah et al., found that a single mutation conferred specificity for ATP analogs that have a large substituent on the nitrogen at position 6 (N6) on the purine ring of ATP (4). Two residues that were found were 5 Å of the N6 position of ATP were based initially on the structures of PKA and CDK2 (cyclin-dependent kinase 2) since the crystal structure of v-Src was not available at the time (2). In v-Src, these residues are Val323 and Ile338, while the corresponding residues in PKA are Val104 and Met120. Although the Val323 mutation was found to be not important for conferring specificity in v-Src, mutation of Ile338 to Ala did alter the specificity of v-Src and allowed the enzyme to use an orthogonal bulky ATP analog. Once the crystal structure of hematopoietic cell kinase (Hck), a Src family tyrosine kinase was available, molecular modeling was employed to explore more fully the binding pocket of Hck to find the best analog for
the single mutation (5). They found that this single mutation opened up a new pocket that was otherwise inaccessible and thus termed this residue the “gatekeeper.” The best analog, based on the modeling, was found to be N°(phenethyl)-ATP and experimentally, N°(phenethyl)-ATP was also preferred by the mutant but was a poor substrate for wild type v-Src.

Substitution of the gatekeeper residue has now been applied to find direct substrates of over 30 kinases to date including v-Src (6) and most recently Yersinia PKA (YpkA) (7). In general, this approach can be used for cell extracts but not for intact cells due to the inability of cells to take up the labeled ATP analogs. Ubersax et al employed this method to identify the direct substrates of CDK1 on a proteome-wide scale in budding yeast using extracts (8). Approximately 200 CDK1 substrates were identified, revealing that CDK1 is involved in global regulatory functions, in addition to its accepted role in phosphorylating cell-cycle regulatory proteins, thus giving insight into the complexity of the network controlled by this kinase.

We have engineered a mutant form of the C-subunit to identify direct PKA (PKA-Cα) substrates in mitochondria. Since tyrosine phosphorylation of complex I in response to cAMP has been reported (9) and at least one AKAP, DAKAP1/AKAP120, is known to recruit both Src and PKA to mitochondria, it is important to unravel the specific PKA signaling pathways that contribute to mitochondrial structure and function. We thus engineered a mutant form of the PKA catalytic subunit (PKA-Cα-analog sensitive-1, PKA-Cα-as1) that accepts an orthogonal ATP analog. Co-expression of the mutant protein with PDK1 in E. coli yields active enzyme that was readily purified. Based on its kinetic properties, we demonstrate a clear preference for N°(benzyl)-ATP for PKA-as1, but not for wild type C-subunit. Using [32P]-labeled N°(benzyl)-ATP and the purified engineered catalytic-subunit, we identified three novel PKA substrates in purified mouse mitochondria including ChChd3 and protein disulfide isomerase A5. ChChd3 is endogenously expressed in mitochondria and may play a roll in metal binding similar to Cox17 and 19 (10).

MATERIALS AND METHODS

Computer Modeling - InsightII (Molecular Simulations, Inc; San Diego, CA) was used to examine and model the ATP binding site of PKA. The adenosine complex (IBKX=Protein Data Bank code) was modified to simulate mutations in the ATP binding pocket of ATP.

Engineering of C-Subunit Mutants – The oligo used for the mutation for Met 120 to Gly is: 5’-TACATGTCgggGAGTATGTAGCTG-3’ (Genbase). The oligo used for the mutation for Met 120 to Ala is: 5’-TACATGTCgcgGAGTATGTAGCTG-3’. Lower case letters represent the amino acid mutated. Mutations in the mouse C-subunit in the pRSETb vector were made using the Kunkel template method (11). His6-tagged C subunit in pET15b was made by cloning out of pRSETb (Invitrogen) at the NdeI and HindIII sites. Mutations introduced in the His-tagged C subunit pET15b construct were done using Quikchange by Stratagene. Constructs were then transformed into E. coli BL21 (DE3) bacteria. For co-expression of the M120G mutant and PDK1 we used the pET15b vector for the C-subunit and pGEX vector for PDK1.

Expression of the M120G Mutant - Colonies tested for expression of C-subunit were grown to an optical density of 0.6 at 600 nm and then induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside for 6 hours. Cells were collected by centrifugation, resuspended in Laemmli sample buffer, and then run on SDS-PAGE (10%). To test for solubility, the harvested cell pellets were resuspended in lysis buffer (50 mM NaHPO4, 20 mM Tris-HCl, 100 mM NaCl, pH 8.0) and subjected to 3 freeze/thaw cycles. The samples were then centrifuged for 30 min. at 4°C (16,000 rpm) in a microfuge to separate the soluble (supernatant) and insoluble (pellet) fractions. The proteins were then separated on an SDS-PAGE (10%) gel.

Preparation of [γ-32P] N°(Benzyl) ATP ([γ-32P] A*TP) – N6(Benzyl) ATP was synthesized as previously described (6).
Purification of the M120G PKA Catalytic Subunit— After induction, cultures from E. coli BL21 (DE3) bacteria were harvested, spun down, resuspended in lysis buffer (50 mM NaHPO₄, 20 mM Tris-HCl, 100 mM NaCl, pH 8.0) and lysed by one pass in a French pressure cell at 1,000 p.s.i. The resulting lysate was then centrifuged for 40 minutes at 15,000 rpm in a Beckman JA20 rotor at 4°C. The His₆-tagged C-subunit was then purified using the procedure for Talon Nickel resin (Clontech). Supernatant was batch-bound to 1.0 ml resin/L culture for 1 hr at 4°C and then washed twice with lysis buffer and once with wash buffer (same as lysis buffer but at pH 7.0). The His-tagged protein was then eluted with 4 imidazole elutions ranging from 50 mM to 500 mM.

Western Blot Analysis – Lysates from transformed E. coli were run on a SDS-PAGE gel and then transferred to PVDF (polyvinylidene fluoride) membrane at 30 V for 60 min. The blots were probed with an anti-phospho-threonine 197 antibody (kindly provided by A. Newton, UCSD) and anti-phospho-Serine 338 antibody (SynPep Corp.), a custom antibody described previously (12). The secondary antibody was an anti-rabbit horseradish-peroxidase (HRP) conjugate (Pharmacia Biotech). The membranes were blocked in 5% milk in TBST (Tris-Buffered Saline Tween-20) overnight. The blocking solution was removed and the membranes were rinsed twice with TBST. The primary antibody was incubated with the blot at room temperature for 1 hour with gentle shaking. After the primary antibody was removed, the membranes were rinsed 4 times with TBST. The second antibody, anti-rabbit-HRP, was added at a dilution of 1:2000 and the blots were incubated for 30 min. at room temperature with gentle shaking. The blots were rinsed with TBST twice and then incubated with SuperSignal West Pico chemiluminescent substrate detection kit (Pierce) for one minute. Bands were detected with ECL Hyperfilm (Pharmacia Biotech).

Inhibition Assays – The assay mixture consisted of MOPS, MgCl₂, 100 μM cold ATP or ATP analog, [γ-³²P]-ATP (1 pM), 200 μM Kemptide and 100 nM WT or mutant enzyme in a 30μL volume. The samples were incubated at 37°C and quenched with 20μL of 50% acetic acid. Fifty μL of the mixture was spotted on phosphocellulose disks. The disks were washed for 10 min. in 0.5% phosphoric acid four times, followed by one brief rinse with acetone. They were counted in a scintillation counter. Assays were done in duplicate.

Kinetic Assays – Two assays were used to evaluate the catalytic efficiency of the mutant enzymes. Radioactive assays were performed in triplicate using [γ-³²P]-ATP. The assay mixture was comprised of 50 mM MOPS, 5 mM MgCl₂, 0.5 mM DTT, 100 μM Kemptide, cold ATP or cold N⁶(benzyl)ATP or N⁶(phenethyl)ATP analogs, and purified recombinant WT and mutant enzyme (0.25-0.5nM). The samples were incubated at 30°C for 5 min. Thirty microliters of each sample were spotted on phosphocellulose disks, washed, and radioactivity was counted in a scintillation counter as described above. The computer program Prism (GraphPad Software, Inc.) was used to calculate Km and kcat values.

Activity Assay – Activity was also monitored using a coupled assay as described by Cook et al. (13). In this assay, 200 μM Kemptide, 100 mM MOPS, 10 mM MgCl₂, 1 mM PEP (phosphoenolpyruvate-dependent enzyme), 1 mM ATP, 15U/ml LDH (lactate dehydrogenase), 7U/ml PK (pyruvate kinase), and 0.2 mM NADH, were incubated with 0.3 mg/ml C-subunit. All components except Kemptide and PKA were mixed. The reaction was initiated by adding 990 μL of assay mix to 10 μL of PKA C-subunit. The mixture was blanked in a spectrophotometer and 10 μL of Kemptide was added to start the reaction.

Two-dimensional electrophoresis/MALDI-TOF - Proteins were diluted in 2D electrophoresis buffer (20 mM DTT, 8 M urea) containing 0.5% ampholytes (pI range 3-10), and passively absorbed onto immobilized pH gradient strips (pI range 3-10) for 16 h at 20°C, followed by isoelectric focusing on the IPG runner (Invitrogen) for 1,200 V-h and standard SDS-PAGE. The samples were stained with colloidal coomassie or silver and imaged on a VersaDoc 4000 using the PDQuest software (Bio-Rad;
Excised proteins were washed three times in 50% acetonitrile/10 mM NH$_4$HCO$_3$ followed by a brief dehydration in 100% acetonitrile. Proteins were incubated overnight in 50% acetonitrile/10 mM NH$_4$HCO$_3$ with 0.05 µg trypsin (Roche). Isolated peptides were washed and concentrated in C$_{18}$ ZipTips (Millipore) according to the manufacturer’s protocol. Samples were directly eluted onto a 100 spot platform using a buffer containing 4-alpha hydroxy cinnamic acid (Agilent), 50% acetonitrile, 10 mM diammonium citrate, and 0.1% trifluoroacetic acid. MALDI-TOF analysis was performed on an Applied Biosystems Q-Star XL hybrid mass spectrometer. MALDI-TOF fingerprint data was analyzed with the online database at the University of California, San Francisco (http://prospector.ucsf.edu).

**RESULTS**

**Mutagenesis of the Catalytic Subunit** - Computer modeling of the PKA catalytic subunit and the v-Src studies suggested that mutating only Met120 was sufficient to allow the kinase to accept an orthogonal ATP analog (Figure 1). The Kunkel template method (11) was then used to introduce mutations into the catalytic subunit sequence in the pRSETb vector. Constructs were engineered to convert Met120 to both Gly and Ala. In addition to these single mutants a double mutant was engineered where Val 104 was replaced with Ala. Expression was carried out as described previously (15).

**Expression** - When the M120G mutant was expressed initially in *E. coli*, the protein was highly insoluble. Efforts to purify the protein via the established protocol for wild type C-subunit using P-11 resin and MonoS chromatography also failed. Consequently, the mutated PKA was cloned into a vector for 6HIS expression (pET156b). This allowed us to purify the protein using a nickel resin. Expression improved under these conditions; however, the protein remained mostly insoluble. It is not uncommon for mutant PKA C-subunits to be insoluble due to defective phosphorylation of the protein. Typically when the wild type C-subunit is expressed in *E. coli* it is fully active and contains 2-4 phosphates due to autophosphorylation (16). Using an antibody generated against the activation loop of PKC that works well for PKA (17), we demonstrated that the His-tagged M120G mutant was not phosphorylated on either Thr197 in the activation loop or on Ser338 in the C-terminal tail.
(Supplemental Figure 1A and C) under typical expression conditions.

To generate soluble and active enzyme, His₆-tagged M120G was co-expressed with PDK1 which efficiently phosphorylates threonine 197 and resulted in soluble protein (Supplemental Figure 1B and C).

Western blots (Supplemental Figure 1C), confirmed that both Thr197 and Ser338 were phosphorylated only when the mutant C-subunit was co-expressed with PDK1. Under these conditions, there is still some insoluble protein, but a clear change in the phosphorylation state of the protein and in the yield of the pure protein was seen. Clearly, the mutation disrupts the ability of the protein to autophosphorylate. Either the mutation leads to a conformational change that causes the protein to aggregate or the mutant has an impaired ability to accept ATP so that it is no longer capable of autophosphorylation in E. coli.

Biochemical Characterization of the M120G mutant C-subunit – Computer modeling suggested that N⁶(phenethyl)-ATP or N⁶(benzyl)-ATP would fit best into the newly available hydrophobic pocket (Figure 1). To determine qualitatively which orthogonal analogs would be preferred by the mutant C-subunit (PKA-Cα-as1) an inhibition assay was performed.

As seen in Figure 2, the WT C-subunit was not inhibited by the N⁶-methylbenzyl and N⁶-phenethyl ATP despite a 100+ fold excess of the analog to radiolabeled ATP, while only a slight inhibition of the wild type kinase was seen for the analog having the smallest N⁶-substitutions such as N⁶(benzyl) ATP and N⁶(cyclopentyl) ATP suggesting that the WT C-subunit can bind to this analog to some degree. The mutant catalytic subunit, but not wild type C-subunit, showed a significant amount of inhibition by all of the cold analogs, with the N⁶(phenethyl) ATP showing complete inhibition, and the N⁶(benzyl) ATP nearly complete. The smallest N⁶-substituted analog also competed least well with the radiolabeled ATP.

Kinetics assays were then performed using [³²P]-ATP, [³²P]-N⁶(phenethyl)-, and [³²P]-N⁶(benzyl)-ATP analogs to determine the Km and kcat. As summarized in Table 1, N⁶(phenethyl)-ATP and N⁶(benzyl)-ATP had the highest affinity for the mutant C-subunit (at least 15-fold better than for ATP), but neither was turned over as quickly as the wild type C-subunit with ATP. This may be due to a slower off-rate for ATP. The N⁶(benzyl)-ATP showed a two-fold faster kcat/Km (0.53) than N⁶(phenethyl)-ATP (0.24). Other analogs, such as N⁶(cyclopentyl)-ATP, did not have a significant advantage over ATP (data not shown). The modified C-subunit still accepts ATP with a slightly lower affinity than wild type catalytic subunit. The pure mutant protein has a high specific activity with ATP and Kemptide of approximately 28 U/mg compared to around 20-24 U/mg for wild type catalytic subunit.

To characterize other properties of the mutant enzyme, we also tested its ability to bind to the RIα subunit. Formation of holoenzyme with RIα has an absolute requirement for ATP (18). As seen in Figure 3, the mutant is also inhibited by RIα in a concentration dependant curve similar to the wild type catalytic subunit, demonstrating that the mutation of M120 to Gly did not disrupt regulation of the kinase. The mutant catalytic subunit was also inhibited by PKI similar to the wild type catalytic subunit. In the presence of PKI, the rate of M120G decreased from 4.74 x 10⁻⁴ U/mg to 4.14 x 10⁻⁵ U/mg. This was similar to the inhibition of WT, which dropped from 3.10 x 10⁻⁴ U/mg to 1.63 x 10⁻⁵ U/mg.

Having expressed a stable mutant of the C subunit that accepts bulky analogs of ATP, we went on to specifically identify mitochondrial PKA substrates. The M120G mutant was incubated for 10 minutes with mitochondria purified from mouse brain and [³²P]-N⁶(benzyl)-ATP. The mitochondria were then pelleted, redissolved in lysis buffer, and run on a 2D gel where the first dimension was separated on the basis of isoelectric point and the second on the basis of molecular weight (Figure 4A). Initially, we focused on the low molecular weight proteins because they were best resolved. Based on our mass spectrometry analysis, three proteins were identified that had putative PKA phosphorylation sites (Table 2). These included a cytoplasmic protein, smoothelin large isoform , an ER protein, a homolog to the protein disulfide isomerase precursor A5, and the mitochondrial protein ChChd3. Of these we chose to focus on ChChd3 because it is of unknown function. ChChd3 is a previously uncharacterized protein that was
shown to be a putative mitochondrial protein of unknown function that contains a coiled coil-helix, coiled coil helix motif. The sequence of ChChd3 is shown in Figure 4B. The peptides that were identified by mass spectrometry analysis are underlined while the putative PKA phosphorylation sites are highlighted in red. The domain organization and localization motifs of ChChd3 will be described in a separate manuscript.

In initial studies, we wanted to determine the subcellular localization of endogenous ChChd3. A polyclonal antibody was generated and confocal microscopy was used to visualize endogenous protein in 10T½ cells. The protein colocalized with Mitotracker (Molecular Probes) confirming that endogenous ChChd3 is a mitochondrial protein (Figure 5). Next, we used the recombinant GST-ChChd3 for an in vitro kinase assays to determine whether in vitro phosphorylation by PKA was direct. Figure 6 demonstrates that PKA directly phosphorylates recombinant ChChd3 with a stoichiometry of 0.3 moles of phosphate incorporated per mol of ChChd3. Although three potential PKA phosphorylation sites exist in ChChd3, threonine 10 represents the most likely site to be phosphorylated. A GST fusion protein of ChChd3 T10A was generated and found to be a poor PKA substrate (data not shown).

Once we determined that ChChd3 could be phosphorylated by PKA in vitro, we next wanted to determine whether PKA could phosphorylate ChChd3 in cells. Because of the low endogenous levels of ChChd3 in HEK 293 cells, we were unable to detect phosphorylation of ChChd3 without using transient transfection. (Figure 7B, panel 1). Therefore, a Flag tagged ChChd3 construct was transfected into HEK 293 cells, and immunofluorescence was used to demonstrate that Flag-tagged ChChd3 was localized to the mitochondria (Figure 7A). Subsequently, HEK 293 cells were transfected with Flag-ChChd3. After a 24 hour transfection, the cells were stimulated with 100 uM CPT-cAMP for 30 min. The cells were lysed, separated by SDS-PAGE, and the phosphorylation of ChChd3 was determined using a polyclonal antibody we generated to a peptide containing a phosphothreonine at position 10 in ChChd3 (Figure 7B). A clear increase in ChChd3 phosphorylation was observed in the presence of cAMP (panel 2). This effect could be blocked by pre-incubation with a phosphopeptide to ChChd3 containing phosphothreonine 10 (panel 3), but not the unphosphorylated peptide (panel 4). Antibodies to the Flag epitope and tubulin were used as loading controls (panels 5 and 6). Taken together, these data demonstrate that the mitochondrial protein ChChd3 is a substrate for PKA in cells when cAMP levels are elevated.

**DISCUSSION**

Based on modeling of the ATP binding pocket, mutating Met120 to Gly appeared to be the best candidate for accepting a bulky analog of ATP and for discriminating between the wild type and mutant C-subunits. Modeling also predicted that either N\(^6\)(benzyl)- or N\(^6\)(phenethyl)-ATP would be good candidates to fit into the engineered ATP binding pocket while also blocking poor substrates for the wild-type kinase. The additional methylene group between the nitrogen and the benzene ring confers additional flexibility. A space filling CPK model in Figure 1 demonstrates best the occupation of the pocket by the benzene ring of the N\(^6\)(phenethyl)-adenosine analog in the M120G mutant. The methionine residue acts as a “molecular gate,” as described by Shokat of the isoleucine 338 in v-Src (4).

To understand why the mutant kinase would actually prefer the analog over ATP, as found in v-Src, the electrostatic surfaces in the mutant PKA were visualized using GRASP in Figure 1. The opened pocket accommodating the N\(^6\)(phenethyl)-adenosine in the M120G mutant revealed a hydrophobic surface upon which the hydrophobic phenethyl group could easily dock, possibly resulting in a faster “on” rate of the analog into the site. However, the rate limiting step for ATP turnover is its “off” rate (19).

Based on the modeling, the M120G mutation was engineered into the C\(_\alpha\) subunit of PKA. Expressing the M120G mutant in E. coli proved to be challenging. The protein initially was completely insoluble and unphosphorylated. This was surprising because the wild type C subunit is completely phosphorylated when expressed in E. coli, and this mutant uses ATP fairly well compared to wild type C subunit. The
mutation may disrupt folding or may disrupt the stability between the two lobes. In any case, the mutation disrupts the initial autophosphorylation event on Thr197 that leads to secondary phosphorylations in the C-subunit when the enzyme is expressed in E. coli. Phosphorylation of Serine 338 is thought to be intramolecular (20).

To overcome this problem, we co-expressed M120G PKA with 3-phosphoinositide-dependent kinase (PDK1), an upstream kinase for many AGC kinases including PKA (21). PDK-1 is constitutively active but appears to be regulated by its proximity to substrates through its pleckstrin homology (PH) domain which binds to PI-(3,4,5)-P3 (22). PDK1 binds to an F-X-X-F motif on the C-terminal tail of PKA (23) and its other substrates and phosphorylates PKA at Thr197. When PDK-1 was co-expressed with the M120G mutant, the mutant became phosphorylated on both Thr197 and Ser338 and was consequently soluble. This technique has proved to be highly useful for purification of other insoluble C-subunit mutants.

\[ \text{N}^6(\text{Benzyl})\text{-ATP} \] was the best choice for specificity based on the kinetic analysis. The Km was similar to \[ \text{N}^6(\text{phenethyl})\text{-ATP} \] but the turnover was faster. This is advantageous because the kinase will still be active. In other work done with this mutant, published after this work was completed, it was found that this mutation of the catalytic subunit (M120G or M120A) also conferred specificity to a class of pyrazolo[3,4-d]pyrimidine-based inhibitors (24). The inhibitors are similar to the PP1 inhibitor found to be specific for Src family kinases (25).

This engineered protein was then used to find direct PKA substrates by adding it to purified mitochondria with radiolabeled \[ \text{N}^6(\text{benzyl})\text{-ATP}. \] The \[ ^{32}\text{P} \]-labeled proteins were detected by first separating on two-dimensional SDS-PAGE, and then exposing the resulting gels to autoradiography film or a phosphoimager. Ideally, this mutant protein also could be expressed in tissue culture cells to probe for direct substrates; however, currently there is not a good way of getting the ATP analogs into the cells.

Crude preparations of mitochondria were used to identify PKA substrates. Partial contamination of endoplasmic reticulum and cytoplasm would account for the detection of smoothelin and protein disulfide isomerase precursor A5, respectively. An important question arising from these studies is whether PKA and radiolabeled \[ \text{N}^6(\text{benzyl})\text{-ATP} \] were able to enter the purified mitochondria or whether ChChd3 was released into the cytoplasm during the preparation process. Two lines of evidence suggest that PKA is able to phosphorylate ChChd3 within the mitochondria. First, endogenous PKA was detected in the outer membrane and inner membrane space of highly purified, fractionated mitochondria (Ma and Taylor, unpublished results). Second, upon stimulation of HeLa cells with CPT-cAMP, an increase in phospho-ChChd3 Thr10 is detected in intact mitochondria by confocal microscopy (Darshi and Taylor, unpublished results). Clearly, the precise location of ChChd3 and PKA in the different mitochondrial sub-compartments needs to be rigorously characterized and this work is now in progress.

There are many different protein kinases in the cell. Nearly 2% of the human genome codes for protein kinases (26) and each typically has multiple splice variants. This makes finding actual substrates, not just “biochemically feasible” substrates, of a specific kinase difficult. The molecular modeling results demonstrate how similar the ATP binding pockets are between Src family kinases and PKA. This demonstrates why efforts to create specific ATP-like inhibitors for kinases are challenging. Using ATP analogs that are labeled at the \( \gamma \)-phosphate with \[ ^{32}\text{P} \], one can identify substrates specific to that kinase that is labeled. The direct substrates can then become specific targets for drug design, in addition to the kinase.

ACKNOWLEDGEMENTS: This work was supported in part by NIH grant DK 5441 to SST and DK071228 to CCK. S. Schauble was supported by a Ruth L. Kirschstein National Research Service Award - NIH/NCI T32 CA009523.
FIGURE LEGENDS

Figure 1: **GRASP rendering of the ATP-binding pocket in the M120G C-subunit.** On the left is the M120G mutant C-subunit ATP binding pocket with adenosine. On the right is M120G with N\(^6\)(benzyl)-adenosine. This an estimation of how the ATP analog would fit into the mutated pocket. The mutation of Met120 to Gly opens up a hydrophobic space that accommodates the benzyl ring. These figures were generated using GRASP (27). White = hydrophobic, red = basic, blue = acidic.

Figure 2: **Competition of ATP analogs for [\(\gamma\)-\(32\)P]-ATP.** Each assay contained recombinant C-subunit (mutant or wild type), Kemptide, [\(\gamma\)-\(32\)P]-ATP (<1 pM), and one of the cold analogs (100 \(\mu\)M). After incubating at 37\(^\circ\)C for 30 minutes, samples were spotted on phosphocellulose disks, washed, and subsequently counted. Unlabeled phenethyl-ATP and benzyl-ATP competed best with [\(\gamma\)-\(32\)P]-ATP when the mutant catalytic subunit was used. The wild type catalytic subunit did use the analogs well and therefore there is little or no inhibition.

Figure 3: **R1\(\alpha\) inhibits PKA WT and M120G activity similarly.** Graph of inhibition of M120G PKA by R209K R1\(\alpha\) compared to wild type PKA C-subunit. Using the standard PKA activity assay, activity of the mutant and wild type was measured with increasing amounts of R209K R1\(\alpha\).

Figure 4: **Autoradiograph of phosphorylated proteins in 2D gel of a mouse brain mitochondrial sample.** A) The pI range for this autoradiograph is 4-7 as indicated. The region containing the most phosphorylated spots, corresponding to proteins in the lower molecular range is enlarged; the spots are circled and numbered. The numbers correspond to the spot numbers in Table 2. B) Amino acid sequence of the unknown protein identified as ChChd3. The putative phosphorylation sites are indicated in red. The peptide that was used subsequently for generating antibodies is in green. The peptides that were identified by mass spectrometry are underlined.

Figure 5: **Confocal images of endogenous ChChd3 in fixed 10 T \(\frac{1}{2}\) cells.** Antibodies to ChChd3 for immunocytochemistry. On the left in red is the fluorescence from MitoTracker red, a mitochondrial dye (Molecular Probes). In green (center) is the stain for ChChd3. On the right are the merges of the red and green. These show that the endogenous ChChd3 colocalizes primarily with mitochondria. Cells were incubated with MitoTracker one hour before fixing and imaged on a Bio-Rad MRC1024 confocal microscope.

Figure 6: **Purified, recombinant GST-ChChd3 is a PKA substrate.** The left panel is a coomassie stained gel (CB) showing recombinant ChChd3 and PKA. The middle panel is a western blot of the ChChd3 protein (W). Upon phosphorylation, a change in mobility of the ChChd3 protein is observed. On the right is the resulting autoradiograph of the gel when exposed to film (P\(^{32}\)). Assays consisted of glutathione-resin purified GST-ChChd3, \(32\)P-ATP, in the absence or presence of wild type PKA C-subunit.

Figure 7: **Elevated cAMP levels result in ChChd3 phosphorylation in HEK 293 cells.** A) Transiently transfected Flag-ChChd3 protein is localized to mitochondria. HEK 293 cells were incubated with MitoTracker (red) one hour to stain mitochondria. Cells were fixed, permeabilized, and incubated with anti-Flag antibodies and imaged on a Bio-Rad MRC1024 confocal microscope. B) HEK293 cells were incubated alone, or transiently transfected with wild type Flag-ChChd3, then starved (lane 1), or stimulated for 30 min with 100 \(\mu\)M CPT-cAMP (lane 2). Cells were lysed in detergent containing buffer and soluble proteins were separated on 10% SDS-polyacrylamide gels and Western blotted using an anti-
phosphoChChd3 (Thr 10) antibody. Untransfected cells (top panel), or cells transfected with Flag-ChChd3 alone (panel 2) were compared with HEK 293 cells transfected with Flag-ChChd3 in the presence of a blocking phosphopeptide (panel 3) or the non-phosphorylated peptide (panel 4). Soluble ChChd3 is shown in panel 5 and was normalized to endogenous levels of tubulin (panel 6).

Supplementary Figure 1: Expression, purification, and phosphorylation of the M120G Mutant C-subunit. SDS-PAGE gels were run as described previously and stained with Coomassie blue. A) Expression and purification of his-tagged M120G C-subunit alone. B) Expression and purification of his-tagged M120G C-subunit with PDK1. C) The upper two panels are Western blots using antibodies that are specific for the phosphorylated sites Ser338(top) and Thr197(middle) (12). The left two lanes represent His-M120G expressed in the presence (+) PDK1. The right two lanes are His-M120G expressed in the absence of PDK1 (-). The bottom panel was stained with Ponceau S to show the total amount of protein in each sample. MW = molecular weight markers; St = WT C-subunit standard; T= total cell lysate; P = Pellet or insoluble fraction; S = Supernatant or soluble fraction; eA, eB, and eC = elutions A, B, and C from the nickel resin.
Table 1: Kinetics properties of the native C-subunit compared to the M120G C-subunit. Assays were performed using [γ-32P]-ATP, [γ-32P]-phenethyl-ATP, or [γ-32P]-benzyl-ATP and spotted on phosphocellulose disks. Km and kcat values were determined by plotting counts per minute vs. concentration in GraphPad Prism. *The number for the wild type catalytic subunit are from previously published results (28).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Nucleotide</th>
<th>Km (μM)</th>
<th>kcat (s⁻¹)</th>
<th>kcat/Km (s*μM⁻¹)</th>
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<td>ATP</td>
<td>30.5</td>
<td>10.6</td>
<td>0.34</td>
</tr>
<tr>
<td>Mutant</td>
<td>Phenethyl-ATP</td>
<td>1.5</td>
<td>0.12</td>
<td>0.24</td>
</tr>
<tr>
<td>Mutant</td>
<td>Benzyl-ATP</td>
<td>1.1</td>
<td>0.5</td>
<td>0.53</td>
</tr>
<tr>
<td>WT</td>
<td>Benzyl-ATP</td>
<td>&gt;100mM</td>
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</tr>
</tbody>
</table>
Table 2: Best match proteins identified for spots 16, 18, and 19. Also indicated are the peptides sequences and the mass over charge numbers for the three peptides that were used to identify ChChd3. The peptides identified by MALDI-TOF that matched ChChd3 were as follows: AAANEQLTR (mass=973.5067); VTTEEYQK (mass=997.4842); and VTFEADENITVVK (mass=1707.8441).

<table>
<thead>
<tr>
<th>Spot</th>
<th>Match</th>
<th>Score</th>
<th>PKA phos. site</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>16</td>
<td>AK003989</td>
<td>88.4</td>
<td>No</td>
<td>Similar to smoothelin large isoform L2</td>
</tr>
<tr>
<td>18</td>
<td>12849144</td>
<td>88.3</td>
<td>Yes</td>
<td>Homolog to protein disulfide isomerase A5 precursor</td>
</tr>
<tr>
<td>19</td>
<td>ChChd3</td>
<td>163</td>
<td>Yes</td>
<td>Coiled-helix coiled-helix domain containing protein; unknown</td>
</tr>
</tbody>
</table>
Figure 1

+ Adenosine  + Benzyl-Adenosine
Figure 2

Relative Inhibition of Kemptide P32-phosphorylation by ATP and ATP-analogs

Wild Type
Mutant

methylation
phenethyl
cyclohexyl
biphenyl-ATP
Figure 3

% Inhibition

PKA Mutant
PKA WT

R1a (nM)
Figure 4

A.

- PKI

+ PKI

B

MGGTASTRRVTFEADENENITVVKIGRLSE
30

NVIDRMKESPSGSKSQRSSVYGASVSD
40

EDLKRRVAELALEQAKESEHQRRLQQA
50

RDLERERAANEQLTRALRERISSEEERM
60

KAKHLRQLEKDRVMQDKQDFAFYKEQLAR
70

LEERSSEFYKVTTEEEYQKAAEVEAKFKRY
80

EYHPVCADLQTKILQCYRQNTQTLSCSAL
90

ASQYMHCNVNAKQQSMLEKGG
100

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Figure 5

10T-1/2 Cells

MitoTracker  Endog. Ch Chad3  Merge
Figure 6

![Image of a gel with bands labeled CB, W, and P^32, indicating GST-ChChd3 and PKA proteins under different conditions with PKA activation.]
Figure 7

A.

MitoTracker  Flag-Chchd3  Merge

B.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>α-Phospho-Chchd3 Thr10 (non-transfected)</td>
</tr>
<tr>
<td>2</td>
<td>α-Phospho-Chchd3 Thr10</td>
</tr>
<tr>
<td></td>
<td>α-Phospho-Chchd3 Thr10 + phosphopeptide</td>
</tr>
<tr>
<td></td>
<td>α-Phospho-Chchd3 Thr10 + peptide</td>
</tr>
<tr>
<td></td>
<td>α-Flag</td>
</tr>
<tr>
<td></td>
<td>α-Tubulin</td>
</tr>
<tr>
<td></td>
<td>CPT-cAMP</td>
</tr>
</tbody>
</table>

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Supplementary Figure 1

A.  - PDK1
B.  + PDK1

C.  Std  Pell  Sup  Pell  Sup
    WT  +PDK  +PDK  -  -

Phospho Ser 338
Phospho Thr 197
Ponceau
REFERENCES