S92 phosphorylation induces structural changes in the N-terminus domain of human mitochondrial calcium uniporter

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The mitochondrial calcium uniporter (MCU) plays essential roles in mitochondrial calcium homeostasis and regulates cellular functions, such as energy synthesis, cell growth, and development. Thus, MCU activity is tightly controlled by its regulators as well as post-translational modification, including phosphorylation by protein kinases such as proline-rich tyrosine kinase 2 (Pyk2) and AMP-activated protein kinase (AMPK). In our in vitro kinase assay, the MCU N-terminal domain (NTD) was phosphorylated by protein kinase C isoforms (PKCβII, PKCδ, and PKCε) localized in the mitochondrial matrix. In addition, we found the conserved S92 was phosphorylated by the PKC isoforms. To reveal the structural effect of MCU S92 phosphorylation (S92p), we determined crystal structures of the MCU NTD of S92E and D119A mutants and analysed the molecular dynamics simulation of WT and S92p. We observed conformational changes of the conserved loop2-loop4 (L2-L4 loops) in MCU NTD of S92E, NTD of D119A, and NTD of S92p due to the breakage of the S92-D119 hydrogen bond. The results suggest that the phosphorylation of S92 induces conformational changes as well as enhancements of the negative charges at the L2-L4 loops, which may affect the dimerization of two MCU-EMRE tetramers.

Under physiological conditions, mitochondria, which uptake and sequester Ca2+ into the matrix, play essential roles in the regulation of ATP synthesis through the tricarboxylic acid cycle (TCA), buffering of cytosolic Ca2+, and cell growth and development1. However, prolonged overload of mitochondrial Ca2+ uptake can trigger the production of large amounts of reactive oxygen species (ROS), induce opening of the mitochondrial permeability transition pore, cause disruption of mitochondrial membrane potential, and eventually lead to apoptotic and necrotic cell death1. The malfunction of mitochondrial Ca2+ homeostasis causes pathological diseases, including ischemia reperfusion, myocardial infarction, and epilepsy2–4.

A key pathway for mitochondrial Ca2+ uptake across the inner mitochondrial membrane (IMM) is through the mitochondrial calcium uniporter (MCU) complex, which facilitates Ca2+ entry into the IMM through the electrochemical potential gradient driven by the mitochondrial membrane potential ($\Delta$ψ = approximately −180 mV)5,6. The MCU is the pore-forming subunit of the MCU complex and acts as a selective Ca2+ channel. The MCU complex consists of MCU, along with its regulatory proteins, MCU paralog (MCUb), mitochondrial calcium uptake 1, 2 & 3 (MICU1, MICU2, and MICU3), essential MCU regulator (EMRE), and mitochondrial

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we determined two crystal structures of MCU NTD S92E, an S92p mimic, and NTDD119A mutants at a resolution of 2.50 Å and 2.85 Å, respectively, and analysed the molecular dynamics simulation for NTDWT and NTDS92p. We proposed that phosphorylation at S92 induces conformational and electrostatic changes in the L2-L4 loops of the mitochondrial Ca2+ channel for mitochondrial Ca2+ uptake at a “fight-or-flight” response13–15.

The role of the MCU as an essential Ca2+ channel for mitochondrial Ca2+ uptake is supported by functional studies of the MCU in human cells and mouse models24–16. Inhibition of Ca2+ uptake in the matrix has been previously demonstrated by blocking the MCU pore with ruthenium red (Ru360)16, and by genetic ablation of MCU7,11. Previous research has also shown that the microRNA miR-25 can reduce the mRNA level of MCU and directly down-regulate MCU expression, thus inhibiting the mitochondrial Ca2+ uptake12. In the MCU pore-forming region, mutations of negatively charged acidic residues (E257, D261, E264) in human MCU, have also been shown to inhibit the MCU activity16. Although the MCU knock-out mouse was reported as the mild phenotype, unexpected compensatory changes that affect cytosolic Ca2+ homeostasis or modulate mitochondrial Ca2+-dependent metabolism impair the short-term mitochondrial Ca2+ uptake at a “fight-or-flight” response13–15.

The N-terminal domain (NTD) of the MCU (MCU NTD) plays essential roles in the dimerization of two MCU-EMRE tetramers, MCUR1 interaction, MCUb NTD interaction, Mg2+ binding selectivity, redox sensor, and regulation of MCU Ca2+ uptake activity17–19. In addition, the MCU NTD can be altered by post-translational modifications in the mitochondrial matrix space20,21. Under inflammatory and hypoxic conditions, MCU undergoes S-glutathionylation in a highly conserved C97 residue and functions as a mitochondrial ROS sensor in the mitochondrial matrix7. Phosphorylation of the MCU (predicted Y158 in the NTD and Y289, Y317 in the C-terminal domain) by proline-rich tyrosine kinase 2 (Pyk2) induces an increase in the mitochondrial Ca2+ uptake by facilitating formation of the MCU channel via MCU oligomerization20, S57 phosphorylation in the MCU by AMP-activated protein kinase (AMPK) facilitates mitochondrial Ca2+ entry during mitosis and boosts mitochondrial respiration to maintain energy homeostasis24. Regulatory functions of MCU Ca2+ uptake by Ca2+/calmodulin-dependent protein kinase II (CaMKII) still remain controversial22–26, although Nguyen et al. suggest S92 phosphorylation (S92p) of MCU by CaMKII in vivo25. Since our study focuses on the S92p of MCU by protein kinase C (PKC), we exclude an argument about the functional role of MCU driven by CaMKII.

PKC isoforms, a heterogeneous family of serine/threonine (Ser/Thr) kinases, are encoded by nine genes (α, β, γ, δ, ε, η, θ, ϵ, ζ) in human27, and PKCζI (one of the splice variants of PKCζ), PKCδ, and PKCε, are localized in the mitochondrial matrix and regulate in response to ROS27–29. PKC directly phosphorylates a wide range of cellular substrates and regulates various cellular functions, such as cell migration, differentiation, proliferation, senescence, and apoptosis27–29.

In this study, we observed that the MCU S92 was phosphorylated by mitochondrial PKC isoforms, including PKCζI, PKCδ, and PKCε, via in vitro kinase assay. To uncover the structural effects of phosphorylated S92 (S92p), we determined two crystal structures of MCU NTD S92E, an S92p mimic, and NTDD119N at a resolution of 2.50 Å and 2.85 Å, respectively, and analysed the molecular dynamics simulation for NTDWT and NTDS92p. We propose that phosphorylation at S92 induces conformational and electrostatic changes in the L2-L4 loops of the MCU NTDWT due to the breakage of S92-D119 hydrogen bonds. As a result, it may affect the dimerization of the two MCU-EMRE tetramers.

Results

The MCU NTD S92 is phosphorylated by PKCζ, PKCζI, and PKCδ. The MCU NTD sequence, which is encoded by exon 3 and 4 (residues 75–165) of the MCU gene, was highly conserved based on 230 MCU NTD homologous protein sequences in the ConSurf server (Fig. 1A–C, Supplementary Fig. S1)28. The MCU NTD has six serines (S87, S92, S105, S107, S129, and S138) and four threonines (T76, T100, T139, and T157). Among these, the highly conserved S92 in the 89-RLPS-92 sequences (the RxxS motif, where x is any residue) was determined to be a putative recognition site for phosphorylation by Ser/Thr kinases containing CaMKII, cAMP-dependent protein kinases (PKA), and PKC, using the KinasePhos 2.0 server and Group-based Prediction System (GPS) 2.0 softwares (Fig. 1C, Supplementary Fig. S1)22,31–33. Previously, Nguyen et al. isolated mitochondria from vascular smooth muscle cells (VSMC) and detected the S92p of MCU by CaMKII using specific MCU S92p antibodies in an immunoblotting25. To further investigate whether S92 is phosphorylated by other Ser/Thr kinases such as PKA and PKC localized in the mitochondrial matrix26,35, we performed in vitro kinase assays with myelin basic protein (MBP; positive control), MCU NTDWT, MCU NTD S92E (all alanine mutations of the nine Ser/Thr residues in the NTD except S92) and [γ32P]ATP. In control experiments, MBP, a multiple phosphorylation target by Ser/Thr kinases36,37, was phosphorylated by PKA and PKC isoforms (α, β, γ, δ, ε, η, θ, ϵ, ζ) (Supplementary Fig. S2). Under the same conditions, MCU NTDWT was phosphorylated by PKC, but not by PKA (Fig. 1D) and MCU NTD S92E was also phosphorylated by PKC (Fig. 1F). In all nine PKC isoforms, three PKC isoforms, including PKCζ, PKCδ, and PKCε, are localized in the mitochondrial matrix and regulate the reactive oxygen species (ROS) formation in the matrix27–29. In addition in vitro kinase assays, we observed that PKCζ, PKCδ, and PKCε, phosphorylated S92, and that S92 phosphorylation activities by PKCζ, PKCδ, and PKCε, were stronger than that of PKCζ (Fig. 1E,F). Thus, we suggest that PKCζ, PKCδ, and PKCε, localized in mitochondrial matrix can phosphorylate the S92 in the MCU NTD.

In details of conformational and electrostatic changes of MCU NTD by S92 phosphorylation. To reveal the structural effect of S92 phosphorylation in the MCU NTD, we generated the S92E mutant, an S92p mimic, of MCU NTD fused with the bacteriophage T4 lysozyme at the N-terminal end of MCU NTD.
(T4-MCU NTDS92E) to improve protein solubility for crystallographic studies. We determined the structure of T4-MCU NTDS92E at a resolution of 2.50 Å by molecular replacement using the MCU NTD WT (PDB ID: 4XSJ) and T4 lysozyme (PDB ID: 2LZM) structures as templates (Fig. 2A; Table 1).

The overall structure of MCU NTD S92E was similar to the structure of MCU NTD WT (PDB ID: 4XSJ) with root-mean-square deviation (RMSD) of 0.57 Å for 87 Cα atoms, and consisted of two helices (α1 and α2) and six β-strands (β1−β6). The S92-D119 in the L2-L4 loops of MCU NTD WT formed a hydrogen bond at a distance of 2.5 Å; the R93 interacted with the E118 to form a salt bridge and stabilized the closed form of L2 loop (Fig. 2B,C). The mutation of S92 to E92 results in atomic clashes of the side chains between E92 and D119, broke the S92-D119 hydrogen bond, and induced conformational changes from the closed form of the L2 loop in MCU NTDS92E to the open form (Fig. 2B,C). The peptide backbone of the L2 loop in the MCU NTD WT moved away from L2 loop of MCU NTDS92E (Cα atom distance of 4.6 Å), and the side chain of R93 moved up to the position of S92 and formed a new hydrogen bond with E92 and D119. The MCU NTD S92E L90 in the hydrophobic interior, which also contained V88, L115, I122, V125, and I153 in MCU NTDS92E, moved away from that of MCU NTDS92E at a distance of 2.2 Å (Fig. 2B,C). Based on the MCU NTD S92E structure, we can suggest that the additional phosphate group by the S92 phosphorylation breaks the S92-D119 hydrogen bond due to atomic clashes between the phosphate group of S92p and the carboxyl group of D119, and induces a conformational change similar to that of the MCU NTDS92E.

In our previous studies, we unintentionally observed that the S92A mutation abolish the S92-D119 hydrogen bond in the structure of the MCU NTD S92A (Supplementary Fig. S3A). Intriguingly, the conformational changes of the L2-L4 loops in MCU NTDS92E were similar to that of MCU NTDS92A (Supplementary Fig. S3B) and were maintained in the open form of L2 loop in comparison with the closed form of MCU NTDS92E (Fig. 2C,

![Figure 1](image-url)
Supplementary Fig. S3A), hypothesizing that S92 phosphorylation might modulate the open or closed conformation of the NTD L2-L4 loops.

To further investigate whether the S92-D119 hydrogen bond is important for maintaining the closed conformation of the L2-L4 loops, we prepared the D119A mutant to break the S92-D119 hydrogen bond. We determined the structure of the MCU NTDD119A mutant fused with N-terminus T4-lysozyme fusion (T4-MCU NTDD119A) at 2.85 Å resolution. Overall, the structures of MCU NTDWT and the MCU NTDD119A mutant were similar, with an RMSD of 0.61 Å for 86 Cα atoms (Fig. 2B). As expected, the MCU NTDD119A also broke the S92-D119 hydrogen bond from the L2-L4 loops of MCU NTD WT and caused structural changes in the L2-L4 loops, similar to that observed in the structure of MCU NTDS92E (Fig. 2B,D). The L2 loop conformation of the MCU NTDD119A moved away at a Cα atom distance of 5.1 Å from that of MCU NTDWT, while the side chain of R93 residue, which moved up to the position of S92, did not form a new hydrogen bond because of lack of a hydrogen bonding counterpart by D119A mutation (Fig. 2B,D).

In addition, to understand whether the S92-D119 hydrogen bond disruption by S92p might contribute to flexibility of L2-L4 loops in the MCU NTD, we performed the ensemble refinement using PHENIX and calculated the root-mean-square fluctuation (RMSF) (Å) from the ensemble refinement results of the MCU NTDWT and the mutants (S92E and D119A)41,42. Overall structures of two mutants showed similar RMSF scores in dynamics to that of the MCU NTDS92E (Fig. 2B,D). The L2 loop conformation of the MCU NTDS92E moved away at a Cα atom distance of 5.1 Å from that of MCU NTDWT, while the side chain of R93 residue, which moved up to the position of S92, did not form a new hydrogen bond because of lack of a hydrogen bonding counterpart by D119A mutation (Fig. 2B,D).

To investigate whether phosphorylation of S92 in the MCU NTD affected electrostatic charges, we calculated and compared the side chain charges of the residues S92, S92p, S92E, and D119A, at the mitochondrial matrix pH of approximately 7.8 using the Henderson–Hasselbalch equation43,44. Negative charges in the mutant S92E (pKa

Figure 2. Structural comparison between the MCU NTDWT and the NTDS92E or NTDD119A mutants. (A) Overall structure of MCU NTDS92E mutant (green) fused with bacteriophage T4 lysozyme (gray) at the N-terminus end of the MCU NTD. (B) Ribbon diagrams of superimposed MCU NTDWT (PDB ID: 4XSJ) and mutant structures of MCU NTDS92E and MCU NTDD119A. The ribbon diagrams are represented in different colours: magenta (WT), green (S92E), and blue (D119A). The side chains of residues are shown in stick, the red dashed lines denote hydrogen bonds, and the L2 loop of conformational changes are represented by black arrows. (C,D) Detailed view of superimposed L2-L4 loops of MCU NTDWT (magenta) and MCU NTDS92E (green) (C) or MCU NTDWT (magenta) and MCU NTDD119A (blue) (D). The backbone and side chains of residues are represented in ribbon and stick, respectively. The arrows represent movement of the residues participating in hydrophobic interaction (black) or hydrogen bonds (red). Dashed-lines (green in S92E and magenta in WT) denote hydrogen bonds.
~4.3) and S92p (pKa1 ~1.5, pKa2 ~6.3) by deprotonation of the hydroxyl group were increased by −1.0 and −2.0, respectively, whereas the negative charge of −1.0 in the D119A mutant (pKa ~3.9) was reduced in comparison with the MCU NTDWT. In agreement with the changes of the negative charge, electrostatic surface charge was enhanced in the L2-L4 loops of the MCU NTDS92E and the S92p model structures, whereas the positive surface charge of MCU NTDD119A was increased compared to the MCU NTDWT (Fig. 3C).

Collectively, these findings suggest that the S92-D119 hydrogen bond formation or disruption, which depends on S92 phosphorylation, regulates the conformation of L2-L4 loops and additional negative charges in the phosphate group of S92p in the MCU complex.

Molecular dynamics simulation analysis of NTDWT and NTDs92p monomers. Molecular dynamics (MD) simulations were performed on the NTDWT and NTDS92p monomer structures to identify the intra structural changes caused by phosphorylation of S92 in the NTD. The MD simulations clearly showed the flexibility change of the L2-L4 loop region (Fig. 4A,E,F). The fluctuations of all amino acid residues in NTDWT and NTDS92p monomer structures were measured by plotting of the RMSF. The RMSF values of the L2 and L4 loops of the NTDs92p structure were significantly higher than the values of the NTDWT (Fig. 4B). The average RMSF values of the L2 loop for the NTDWT and NTDS92p were 0.76 Å and 1.41 Å, respectively; the values of the L4 loop were 0.63 Å and 0.93 Å, respectively.

To investigate details of the atomic interaction between the residues near the L2 and L4 loops, the final MD trajectory structure was extracted. In NTDWT, the S92 and R93 in the L2 loop were hydrogen-bonded with D119 in the L4 loop (Fig. 4C). Conversely, in NTDS92p, only R93 participated in the hydrogen bond interaction, as the interaction of S92 with D119 was broken (Fig. 4D). Therefore, it can be inferred that phosphorylation on S92 can break the interaction between the S92 and D119.

| Proteins          | T4 lysozyme-MCU NTD S92E | T4 lysozyme-MCU NTD D119A |
|-------------------|--------------------------|--------------------------|
| PDB ID:           | 6JG0                     | 6KVX                     |

**Table 1.** Data collection and refinement statistics. aBeamline 5C at Pohang Acceleratory Laboratory (PAL) in South Korea. bValues in parentheses are for highest-resolution shell. c<sup>R</sup>merge = ΣₙΣᵢ |I(h)ᵢ − ⟨I(h)⟩|/ΣₙΣᵢ I(h)ᵢ, where I(h) is the intensity of reflection of h, Σₙ is the sum over n reflections and Σᵢ is the sum over i measurements of reflection h. dCC₁/₂ in outer shell were calculated from HKL2000. e<sup>R</sup>work = Σ₀[|Fₒ| − |Fₑ|]/Σ₀|Fₒ|; 5% of the reflections were excluded for the <sup>R</sup>free calculation.

~4.3) and S92p (pKa1 ~1.5, pKa2 ~6.3) by deprotonation of the hydroxyl group were increased by −1.0 and −2.0, respectively, whereas the negative charge of −1.0 in the D119A mutant (pKa ~3.9) was reduced in comparison with the MCU NTDWT. In agreement with the changes of the negative charge, electrostatic surface charge was enhanced in the L2-L4 loops of the MCU NTDs92E and the S92p model structures, whereas the positive surface charge of MCU NTDs119A was increased compared to the MCU NTDWT (Fig. 3C).

Collectively, these findings suggest that the S92-D119 hydrogen bond formation or disruption, which depends on S92 phosphorylation, regulates the conformation of L2-L4 loops and additional negative charges in the phosphate group of S92p in the MCU complex.
Effects of S92 phosphorylation on the dimerization of the MCU-EMRE tetramer. Upon examination of the structures of the MCU NTD<sub>WT</sub>, MCU NTD<sub>S92E</sub>, and MD simulated S92p models, we expected that S92 phosphorylation in the MCU induces conformational changes as well as enhancements in the negative charges in the local L2-L4 loops (Figs. 2 and 4). Wang <i>et al</i>. recently reported that the tetrameric MCU-EMRE channels underwent extensive interactions with each other resulting in the formation of dimers at the MCU NTDs, including the L2-L4 loops (Fig. 5A, B, D). Additionally, the MCU-EMRE channel interactions formed a V-shaped tetrameric MCU-EMRE dimer. Moreover, a single mutation, D123R, in the L4 loop of the MCU NTD abolished the dimerization of the two MCU-EMRE channels, possibly by disrupting the electrostatic interactions with the neighboring arginine residues, R93 and R124<sup>19</sup>. To elucidate the effect of S92p on the dimerization of tetrameric MCU-EMRE, we compared the binding energy difference for the dimerization of the tetramer between WT and S92p NTDs using the PRODIGY web server<sup>45</sup>. The binding free energy of NTD<sub>S92p</sub> (−7.4 kcal/mol) was higher than that of WT (−10.5 kcal/mol), suggesting that the conformational changes and enhancement of negative charges by S92 phosphorylation may affect the dimerization of two MCU-EMRE channels (Fig. 5C–F).

To confirm this hypothesis, we performed MD simulations for the NTD<sub>WT</sub> and NTD<sub>S92p</sub> octamer structures. To compare the distance between the two tetramers (NTD-A and NTD-B), the three key monomer pair distances (M2-M8, M3-M7, and M4-M6) were monitored during 10 ns simulations times (Fig. 6). The snapshot structures at 10 ns (Fig. 6A, B) and the distance trajectory during the MD simulation (Fig. 6C–E) show that the distances between the paired monomers in NTD<sub>S92p</sub> were significantly increased compared to that of the NTD<sub>WT</sub> by approximately 1.5 to 5 Å. It suggests that the additional negative charges from the phosphate group might contribute to push each tetramers (NTD-A and NTD-B) away. Overall, our MD simulation studies suggest that the S92 phosphorylation can weaken dimerization of the MCU-EMRE tetramer.
Discussion

MCU activity is modulated by its regulatory proteins, including MICU1, MICU2, MCUb, EMRE, and MCUR1, as well as post-translational modifications such as phosphorylation. In addition, the MCU NTD plays pivotal roles in MCU interaction, MCUb NTD interaction, Mg$^{2+}$ binding selectivity, phosphorylation, redox sensor, oligomerization of MCU-EMRE channel complexes, and regulation of MCU Ca$^{2+}$ uptake activity. Thus, we believe functional roles of MCU NTD for its Ca$^{2+}$ uptake activity warrants further investigation, although recent studies of NTD deletion of MCU appears to be functionally dispensable in mitochondrial Ca$^{2+}$ uptake.

Protein kinases can be localized in the sarcoplasmic reticulum (SR) and mitochondria, and modulate function of Ca$^{2+}$ channels by phosphorylation. Phosphorylation of Ca$^{2+}$ channels containing ryanodine receptor 2 and inositol 1,4,5-trisphosphate receptors regulates Ca$^{2+}$ release in the SR through PKA and CaMKII. The MCU is directly phosphorylated by Pyk2 and AMPK and phosphorylated MCU facilitates Ca$^{2+}$ entry into the mitochondria. The conserved S92 in the MCU is a putative recognition site (89-RLPS-92; RxxS motif) for phosphorylation by Ser/Thr kinases such as CaMKII, PKA, and PKC on the basis of prediction of KinasePhos 2.0.

Figure 4. L2-L4 structure comparison between NTD$_{\text{WT}}$ and NTD$_{\text{S92p}}$ monomers during 10 ns molecular dynamics (MD) simulation. (A-D) MD snapshot structures of NTD$_{\text{WT}}$ and NTD$_{\text{S92p}}$ structures at 10 ns. (A) NTD$_{\text{WT}}$ and NTD$_{\text{S92p}}$ structures are superimposed and colored in pink and cyan, respectively. The S92 and S92p residues are displayed in a stick model with the same color. (B) The root-means-square fluctuation (RMSF) plots for C$_{\alpha}$ atoms of the NTD$_{\text{WT}}$ and the NTD$_{\text{S92p}}$ are shown as pink and cyan lines, respectively. The RMSF values of the L2 and L4 loops are highlighted by the boxes in the same color. (C,D) Atomic interaction analysis between residues of L2 and L4 loops on NTD$_{\text{WT}}$ (pink) and NTD$_{\text{S92p}}$ (cyan). Hydrogen bond interactions are shown as dashed lines. (E,F) MD snapshot structure superimposition. The 20 trajectories are extracted every 0.5 ns during 10 ns simulation time and aligned for NTD$_{\text{WT}}$ (pink) (E) and NTD$_{\text{S92p}}$ (cyan) (F).
server and GPS 2.0 software. Nguyen et al. observed S92 phosphorylation of MCU by CaMKII using MCU S92 phospho-specific antibody in vivo, although the regulatory functions of MCU activity by CaMKII still remain controversial. Instead, our in vitro kinase assay results indicated MCU S92 was phosphorylated by PKC isoforms (PKC βII, PKC δ, and PKC ε) localized in the mitochondrial matrix, but was not phosphorylated by PKA (Fig. 1D,F). Further studies are needed to understand the functional roles of MCU NTD phosphorylation by PKC isoforms.

Free radicals, such as ROS and reactive nitrogen species (RNS), generate in a well-modulated manner to maintain cellular homeostasis as signalling second messengers, and play critical roles in the activation of enzymes and alteration of lipids, protein, and DNA. Under physiological conditions, the MCU uptakes Ca²⁺ ions into the

Figure 5. Structural comparison between the NTD<sup>WT</sup> and the NTD S92-phosphorylated (NTD<sup>S92p</sup>) model in the dimerization of two MCU-EMRE channels. (A) The overall structure of two MCU-EMRE channels (PDB ID: 6O58). MCU and EMRE are shown in grey and magenta colored ribbons, respectively. (B) Detailed view of the interacting surfaces of NTD-A and NTD-B in dimer of the two MCU-EMRE channels. The residues (R93, D123, and R124) forming salt bridges and hydrogen bonds are shown in sticks. (C) Detailed view of the superimposed MCU NTD<sup>S92p</sup> MD simulated model structures (10-nsec snapshot) onto NTD<sup>WT</sup>-A or -B of the two MCU-EMRE channel complexes. The MCU NTD<sup>S92p</sup> residues that are expected to disrupt salt bridges and hydrogen bonds (R93, D123, and R124) are depicted as orange sticks and cyan sticks, respectively. Atomic clashes between NTD-A and NTD-B of the MCU NTD<sup>S92p</sup> are denoted by red spheres. (D–F) Differences between the electrostatic surface charges of the interacting interfaces of the MCU NTD<sup>WT</sup>-A or -B (M1–M8) superimposed on the MCU NTD<sup>S92p</sup>-A or -B (M1–M8). Enhancement of negative charges in the MCU NTD<sup>S92p</sup> are highlighted with black arrows. Blue surfaces and red surfaces in the NTD<sup>WT</sup> and in the MD simulated NTD<sup>S92p</sup> indicate positive and negative charges, respectively.
matrix, Ca^{2+} ions play an essential role modulating ATP synthesis through TCA cycle and the electron transfer chain, and finally induce ROS production as by-products in the mitochondria. However, continuous overload of mitochondrial Ca^{2+} entry can produce large amounts of ROS and eventually lead to apoptotic or necrotic cell death. Upon production of ROS, PKCβII, PKCδ, and PKCε translocate to the mitochondria and modulate functions of enzymes and Ca^{2+} channels by Ser/Thr phosphorylation, as well as ROS production. In our studies, we observed that PKCβII, PKCδ, and PKCε phosphorylated the MCUS92 in vitro. We speculate that regulation of the S92 phosphorylation by the PKC isoforms under physiological conditions play important roles in ROS homeostasis or programmed cell death by excessive ROS, driven by the MCU Ca^{2+} uptake. Additional experimental evidence will be required to clarify the functional roles of PKC isoforms in the MCU.

In conclusion, we identified that the PKC isoforms, PKCβII, PKCδ, and PKCε, are capable of phosphorylating S92 in the MCU NTD. We also characterized local conformational changes in our structural determination of MCU NTD\textsubscript{s92} and NTD\textsubscript{d119a} as well as in MD simulation analysis of the WT and S92p. The conformational changes and enhancement of negative charge of the L2-L4 loops in the MCU NTD by S92 phosphorylation may be essential for regulating MCU activity, despite there lacks of functional data for the MCU activity modulation by S92 phosphorylation. Further studies are required to reveal the functional effects of MCU S92 phosphorylation by the PKC. The results provide a framework for further studies investigating the functional and structural roles of MCU phosphorylation by PKC.

Figure 6. Distance comparison between NTD\textsubscript{WT} and NTD\textsubscript{s92p} octamers using MD simulation structures. (A-B) The 10 ns-snapshot structures of NTD\textsubscript{WT} (A) and NTD\textsubscript{s92p} octamers (B) are shown in pink and cyan, respectively. Each of the eight monomers are designated as M1 – M8. The distances between the center of monomer were calculated. (C-E) The three key monomer pair distances in the central region of the octamer were measured during 10 ns simulations times: M2-M8 (C), M3-M7 (D), and M4-M6 (E). The Cα atom of F111 (red sphere) located at the nearest position of the center of monomer was selected for the distance measurement.
Materials and Methods

DNA constructs. For structural studies, human MCU NTD (residues 75–165), including N-terminal His6-tagged bacteriophage T4 lysozyme (residues 2–161; triple mutants of D20N/C54T/C97A)26,28 in the modified pET21a vector (Novagen), was constructed as previously described. Point mutagenesis using polymerase chain reaction (PCR) was performed to construct the S92E or D119A mutants. For the in vitro kinase assay of the MCU NTD S92, we designed all Ser/Thr to Ala mutants (T76, S87, S92, T100, S105, S107, S129, S138, T139, and T157; MCU NTDΔα). PCR was used to synthesize the MCU NTDΔα construct using 12 oligonucleotides; then, MCU NTDΔα_s92 was generated using the A92S mutation from the MCU NTDΔα.

Purification of MCU constructs. The T4 lysozyme–MCU NTD_s92 or T4 lysozyme–MCU NTD_D119A was purified using the same method for T4 lysozyme–MCU NTD_wt as previously described. The proteins were expressed in the Escherichia coli strain BL21-CodonPlus (DE3), followed by purification using Ni-NTA affinity and size exclusion chromatography (SEC) on a HiLoad 16/60 Superdex 75 column (GE Healthcare Life Science). The samples were concentrated by centrifugation using Amicon Ultra-15 10 K filter units (Millipore) to 5 mg mL−1 in final buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5% (v/v) glycerol, and 1 mM DTT.

For in vitro kinase assays, MCU NTD_wt and MCU NTDΔα_s92 were purified by a similar procedure using the following buffers: lysis buffer [50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 10 mM imidazole, 5% (v/v) glycerol, 1 mM PMSE, 1 mM β-mercaptoethanol], wash buffer [50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 40 mM imidazole, 5% (v/v) glycerol], and elution buffer [50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 500 mM imidazole, 5% (v/v) glycerol]. The samples were then purified using SEC on a HiLoad 16/60 Superdex 75 column (GE Healthcare Life Science) pre-equilibrated with a buffer [50 mM Tris-HCl (pH 7.5) and 150 mM NaCl]. Then, the fractions containing human MCU NTD_wt and MCU NTDΔα_s92 were collected. The protein was concentrated using an Amicon Ultra-15 10 K filter unit (Millipore) at a concentration of 0.5 mg mL−1. Final human MCU NTD proteins were stored at −80 °C.

In vitro kinase assays. Six micrograms (6 μg) of purified MCU NTD_wt, 20 μg of purified MCU NTDΔα_s92, and 32 μg of commercially obtained MBP (Enzo, ALX-202-075) were phosphorylated by PKA (Promega, V5161), PKC mixtures (α, β, and γ isosforms with lesser β and γ; Promega, V5261), PKCβII (Promega, V3741), PKCγ (Promega, V3401), and PKCα (Promega, V4036). In vitro phosphorylation of PKA was performed in 25 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 2 mM DTT, 5 mM β-glycerophosphate, 0.1 mM Na3VO4, 0.2 mM Mg-ATP, and 3 μmol of [γ-32P]ATP (3000 Ci/mmol) with 20 ng PKA for MBP and 100 ng PKA for MCU NTD_wt and MCU NTDΔα_s92. In vitro phosphorylation assays of PKC mixtures, PKCβII, PKCγ, and PKCα, were performed in 1 × reaction buffer A (SignalChem, K03-09) [20 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 0.02% (v/v) Tween-20], 2 mM DTT (SignalChem, DB6-09B), 1 × PKC lipid activator (SignalChem, L51-39), 0.2 mM Mg-ATP, and 3 μmol of [γ-32P]ATP (3000 Ci/mmol) with 100 ng PKC, 50 ng PKCβII, 200 ng PKCγ, and 200 ng PKCα for MCU NTD_wt, and MCU NTDΔα_s92. All in vitro kinase assays were performed at 30 °C for 60 min. The reaction was halted by the addition of SDS-PAGE sample buffer. Then, reaction samples were resolved by SDS-PAGE and visualized by autoradiography.

Crystallization. The T4 lysozyme–MCU NTD_s92 or T4 lysozyme–MCU NTD_D119A was crystalized using the same method for T4 lysozyme–MCU NTD_s92, as previously described. Crystals of T4 lysozyme–MCU NTD_s92 or T4 lysozyme–MCU NTD_D119A were produced using the hanging drop vapour diffusion and microseeding method, using T4 lysozyme–MCU NTD_wt crystals as seeds in the reservoir solution containing 20% (w/v) polyethylene glycol (PEG) 3350, 5% (v/v) glycerol, 0.3 M (NH4)2SO4, and 0.1 M Bis-Tris-HCl (pH 5.5). Once the microcrystals (<0.01–0.02 mm) of the T4 lysozyme–MCU NTD_wt grew at 20 °C, 2 μL of T4 lysozyme–MCU NTD_s92 or T4 lysozyme–MCU NTD_D119A proteins and 2 μL of the reservoir solution were added directly to the 1 μL drop containing T4 lysozyme–MCU NTD_wt seed crystals. The final T4 lysozyme–MCU NTD_s92 or NTD_D119A crystals were grown at 20 °C in 5 μL mixtures containing the WT and the S92E or D119A mutant at a 1:4 molar ratio. The crystals were directly flash-frozen in liquid nitrogen.

Data collection, structure determination, and refinement. Diffraction data of T4 lysozyme–MCU NTD_s92 or T4 lysozyme–MCU NTD_D119A crystals were collected at 100 K using synchrotron X-ray sources on beamlines 5 C at the Pohang Acceleratory Laboratory (PAL) (Pohang, South Korea). We finally collected diffraction data for T4 lysozyme–MCU NTD_s92 at a resolution of 2.50 Å and for T4 lysozyme–MCU NTD_D119A at 2.85 Å using a single wavelength, 0.9795 Å. The diffraction data were processed using the HKL2000 suite57. Molecular replacement was carried out using Phaser in the CCP4 suite58, using the structures of the bacteriophage T4 lysozyme (PDB ID: 2LZM) and MCU NTD (PDB ID: 4XTB) as templates. The obtained models were subjected to iterative rounds of model building and refinement using programs Coot29 and REFMAC5 in CCP4 suite34. The details of data collection and refinement statistics are provided in Table 1.

Structural analysis. All structural figures were generated using PyMOL version 1.5.0.4 (Schrödinger LLC). The amino acid sequence and protein surface conservation of the MCU NTD were calculated using the ConSurf suite58. The amino acid sequence and protein surface conservation of the MCU NTD were calculated using the ConSurf suite58. The electrostatic surface charges of MCU NTDs (WT, S92E, S92p, and D119A) were analysed using the PDB2PQR server60 and visualized using PyMOL version 1.5.0.4 (Schrödinger LLC).

Ensemble refinement. Ensemble refinement for T4 lysozyme–MCU NTD_wt (PDB ID: 4XSJ), T4 lysozyme–MCU NTD_s92, and T4 lysozyme–MCU NTD_D119A was performed using structures and structural
factors by phenix.ensemble_refinement. Default parameters were used in the phenix.ensemble_refinement, including rTLS = 0.8 and Tresh = 5 K, and solvent updated every 25 cycles. The simulations have an equilibration phase (10 ns) in which the temperature, X-ray weight and averaged structure factors stabilized, followed by an acquisition phase (10 ns). The output structures by ensemble refinement were visualized using PyMOL version 1.5.0.4 (Schrödinger LLC) with a script 'ens_tool.py'. The RMSF difference histogram for the MCU NTDS92 and mutants (S92E and D119A) was plotted using SigmaPlot 12.

**Molecular dynamic simulations.** Four molecular dynamics (MD) simulations of NTDS92 and NTDS92E monomer and octamer structures were run out using GROMACS (GROningen Machine for Chemical Simulations) 2018.4 package with amber99sb-starILDNP force field. Molecular topologies for phosphorylated S92 were generated by AnteChamber Python parser interface (AC PyType) with generalized AMBER force field 2 (GAFF2). All four systems were solvated with TIP3P water molecules in a dodecahedron box and Na\(^+\) counter ions were added to neutralize the net changes of the systems by replacing water molecules. In all cases, bond lengths were constrained with LINCS and long-range electrostatics were calculated using the smooth particle mesh Ewald (PME) method with a cut-off of 1.0 nm. A cut-off of short-range non-bonded interactions, van der Waals (vdW), were truncated at 1.0 nm. All MD simulations were conducted energy minimization using the steepest descent method. Equilibration was then performed in two phases, during which position restraints applied to all heavy atoms of the protein. First, the simulations were run under NVT conditions at 300 K, using Berendsen's coupling algorithm for 100 ps. The second phase of equilibration was carried out an NPT ensemble for 10 ns without any restraint and under the same conditions as the NPT ensemble. All analyses of MD simulation results were performed using the analysis tools in the GROMACS package.

**Accession numbers.** Atomic coordinates and structure factors of T4 lysozyme-MCU NTDS92 and T4 lysozyme-MCU NTDS92E have been deposited in the PDB with the accession numbers, 6JG0 and 6KVX, respectively.

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Author contributions

S.H.E. and Y.L. planned and organized the experiments. Y.L. performed purification, crystallization, collection of X-ray diffraction data, structure determination and analysis, in vitro kinase assays, ensemble refinement, and data analysis. P.J. performed purification, collection of X-ray diffraction data, and data analysis. G.L., S.Y., and K.W.L. performed the molecular dynamics simulation analyses. Y.L. and P.J. carried out gene cloning and expression. T.G.K., T.Y., C.K.M., and D.H.K. contributed to experimental design and data interpretation. S.H.E., Y.L., and K.W.L. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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