Phosphorylation of ULK1 by AMPK regulates translocation of ULK1 to mitochondria and mitophagy

Weili Tian a,1, Wen Li a,1, Yinqin Chen b, Zeming Yan c, Xia Huang d, Haixia Zhuang d, Wangtao Zhong a, Yusen Chen a, Wenxian Wu a, Chunxia Lin a, Hao Chen a, Xiaoyan Hou e, Liangqing Zhang d, Senfang Sui e, Bin Zhao a, Zhe Hu d,⇑, Longxuan Li f,⇑, Du Feng b,⇑

a Guangdong Key Laboratory of Age-related Cardiac-cerebral Vascular Disease, Institute of Neurology, Affiliated Hospital of Guangdong Medical College, Guangdong Medical College, Zhanjiang 524001, Guangdong, China
b Department of Vascular Surgery, Thyroid and Mammary Gland Surgery, Guangdong Medical College, Zhanjiang 524001, China
c Department of Interventional Radiology, Guangdong Medical College, Zhanjiang 524001, China
d Department of Anesthesiology, Guangdong Medical College, Zhanjiang 524001, China
e State Key Laboratory of Biomembrane and Membrane Biotechnology, School of Life Sciences, Tsinghua University, Beijing 100084, China
f Department of Neurology, Gongli Hospital, Pudong New Area, Shanghai 200135, China

1 These authors contributed equally to this work.

Aberrations: ULK1, UNC-51 like kinase; AMPK, Adenosine 5'-monophosphate (AMP)-activated protein kinase; FUNDCT, FUN-14 domain containing protein; mTOR, Mammalian target of rapamycin; LC3, Light chain 3

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Corresponding authors at: Institute of Neurology, Affiliated Hospital of Guangdong Medical College, Zhanjiang 524001, China (D. Feng); Department of Anesthesiology, Guangdong Medical College, Zhanjiang 524001, China (Z. Hu); Department of Neurology, Gongli Hospital, Pudong New Area, Shanghai 200135, China (L. Li).

E-mail addresses: bzhuzhe@hotmail.com (Z. Hu), longxuanlee2006@yahoo.com (L. Li), feng_du@foxmail.com (D. Feng).

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Abstract

ULK1 like kinase (ULK1) translocates to dysfunctional mitochondria and is involved in mitophagy, but the mechanisms responsible for ULK1 activation and translocation remain unclear. Here, we found that hypoxia induces phosphorylation of ULK1 at Serine-55 by Adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK). Unlike wild-type ULK1, an ULK1 (S555A) mutant cannot translocate to mitochondria in response to hypoxia. Inhibition or knockdown of AMPK prevents ULK1 translocation and inhibits mitophagy. Finally, the phospho-mimic ULK1 (S555D) mutant, but not ULK1 (S555A), rescues mitophagy in AMPK-knockdown cells. Thus, we conclude that AMPK-dependent phosphorylation of ULK1 is critical for translocation of ULK1 to mitochondria and for mitophagy in response to hypoxic stress.

1. Introduction

Mitophagy is a process in which autolysosomes eliminate damaged mitochondria to sustain the energy balance or maintain cell survival [1–5]. At present, mitochondrial autophagy mediated by PARKIN/PINK1 is a relatively clear signal transduction pathway: In healthy mitochondria, PINK1 is constitutively expressed and imported, probably via the TIM/TOM complex, to the inner membrane where it is cleaved by presenilin-associated rhomboid-like protein (PARL) and ultimately proteolytically degraded. When the mitochondrial membrane potential is lost, PINK1 is stabilized on the outer mitochondrial membrane to recruit Parkin to depolarize the mitochondria, where Parkin ubiquitinates a subset of protein substrates to trigger mitophagy [1,4]. The autophagy initiating factor, ULK1, belonging to the Serine/Threonine kinase family, is required for autophagy induction [6–9]. ULK1 forms a complex with Atg13 and FIP200 to regulate the initial step of autophagy induction in mammalian cells. Formation of the ULK1–Atg13–FIP200 protein complex is not altered by nutrient conditions unlike its yeast counterpart, the Atg1–Atg13–Atg17 complex [10–12]. However, deletion of any of these factors does not lead to severe impairment of autophagy under starvation conditions [13]. In survival [1–5]. At present, mitochondrial autophagy mediated by PARKIN/PINK1 is a relatively clear signal transduction pathway: In healthy mitochondria, PINK1 is constitutively expressed and imported, probably via the TIM/TOM complex, to the inner membrane where it is cleaved by presenilin-associated rhomboid-like protein (PARL) and ultimately proteolytically degraded. When the mitochondrial membrane potential is lost, PINK1 is stabilized on the outer mitochondrial membrane to recruit Parkin to depolarize the mitochondria, where Parkin ubiquitinates a subset of protein substrates to trigger mitophagy [1,4]. The autophagy initiating factor, ULK1, belonging to the Serine/Threonine kinase family, is required for autophagy induction [6–9]. ULK1 forms a complex with Atg13 and FIP200 to regulate the initial step of autophagy induction in mammalian cells. Formation of the ULK1–Atg13–FIP200 protein complex is not altered by nutrient conditions unlike its yeast counterpart, the Atg1–Atg13–Atg17 complex [10–12]. However, deletion of any of these factors does not lead to severe impairment of autophagy under starvation conditions [13]. In
addition, even in ULK1−/−–ULK2−/− MEFs, long-term glucose withdrawal is able to induce autophagy [14,15], suggesting that in different cell types, the autophagy pathway responds in different ways to nutrient depletion [16].

Despite the confusion surrounding its function in starvation-induced autophagy, ULK1 plays a significant role during terminal erythroid maturation, because ULK1 null reticulocytes display delayed mitochondrial clearance during red blood cell development [17,18], indicating that ULK1 has a more specific role in mitophagy. Several proteins are involved in hypoxia-induced mitophagy in mammalian cells, including the mitophagy receptors NIX and FUNDC1 [19], or the regulatory kinases ULK1 and SRC [20–24]. In hypoxic conditions, mitochondrial outer membrane proteins like NIX and FUNDC1 are required for mitophagy by interacting with LC3 to recruit autophagic membranes. ULK1 or SRC regulates the phosphorylation status of FUNDC1 by enhancing or reducing FUNDC1-LC3 binding [17,21,22].

ULK1 with AMPK is influenced by phosphorylation of ULK1 by AMPK and directly regulates its kinase activity. The interaction of ULK1 at Serine-555 by AMPK is involved in translocation and the mechanism that triggers mitochondrial translocation of ULK1. Nevertheless, the phosphorylation status of ULK1 during hypoxia nes its function in autophagy. Loss of AMPK mTORC1 [30–32]. Therefore, the phosphorylation of ULK1 determines the decision to possibly induce autophagy. Additionally, the phosphorylation of ULK1 in turn acts in a feedback loop to phosphorylate and thereby regulate AMPK or mTORC1 [30–32]. Therefore, the phosphorylation of ULK1 determines its function in autophagy, mitophagy, and cell survival, and connects cellular energy sensing to mitophagy [25].

We recently identified FUNDC1 as a new substrate of ULK1. It recruits ULK1 to damaged mitochondria, regulating autophagic clearance of damaged mitochondria in hypoxic conditions [21]. Nevertheless, the phosphorylation status of ULK1 during hypoxia and the mechanism that triggers mitochondrial translocation of ULK1 have yet to be revealed. Here, we found that phosphorylation of ULK1 at Serine-555 by AMPK is involved in translocation of ULK1 to mitochondria and in mitophagy.

2. Materials and Methods

2.1. Antibodies and reagents

The following primary antibodies were used in this study: anti-AMPKα1 antibody (Cell Signaling Technology, #2532), anti-PRKAA1 (AMPKα) antibody (Thermo, MA5-15815), anti-phospho-AMPK α1 antibody (Cell Signaling Technology, #2535), anti-ULK1 antibody (H-240) (Santa Cruz, sc-33182), anti-Atg1/ULK1 antibody (Sigma, A7481), anti-ULK1 (D8H5) rabbit mAb (Cell Signaling Technology, #8054), anti-phospho-ULK1 (Ser757) rabbit mAb (Cell Signaling Technology, #6888), anti-phospho-ULK1 (Ser555) rabbit mAb (Cell Signaling Technology, #5869), anti-phospho-ULK1 (Ser317) rabbit mAb (Cell Signaling Technology, #12753), anti-p62/SQSTM1 antibody (Sigma, L7543), anti-LC3 polyclonal antibody (MBL, PM036), anti-LC3B polyclonal antibody (Sigma, L7543), anti-LC3 polyclonal antibody (MBL, PM036), anti-TIM23 (BD Biosciences, 611222), anti-TOM20 (FL-145) (Santa Cruz, sc-11415), anti-VDAC1 monoclonal antibody (Abcam, ab14734), anti-FUNDC1 polyclonal antibody (Aviva, ARPS280_P050), anti-HA Clone 16B12 monoclonal antibody (Covance, MMS-101R). Secondary antibodies used for western blotting were: HRP affinity pure goat-mouse IgG (Earthbox, E030110), HRP affinity pure goat anti-rabbit IgG (Earthbox, E030120) and HRP-conjugated goat anti-rabbit IgG Fc (SouthernBiotech, 4041-05). The following fluorescent secondary antibodies were obtained from Life Technologies: Alexa Fluor 488-labeled donkey anti-mouse IgG (A21202), Alexa Fluor 488-labeled donkey anti-rabbit IgG (A21206), Alexa Fluor 555-labeled donkey anti-mouse IgG (A31572), Bafilomycin A1 (50 nM, B9739) and Metformin hydrochloride (2 mM, PHR1084-500MG) were purchased from Sigma. Dorsomorphin 2HCi (1 μM, S7306) was purchased from Selleck. Protein A/G plus-agarose immunoprecipitation reagent (Santa Cruz, sc-2003), and Lipofectamine 2000 (Invitrogen, 11668027) were used according to the manufacturer’s protocol.

2.2. Plasmids and siRNA

HA-AMPK and HA-ULK1 S317A were given as gifts by Prof. Kun-Liang Guan. HA-ULK1 (S555A) was gifted by Dr. Georg Ramin. HA-hULK1 (deposited by Do-Hyung Kim) was obtained from Addgene. HA-AMPK KD (kinase dead), HA-ULK1 (S555D) and HA-ULK1 (S757A) were created by site-directed mutagenesis using HA-AMPK and HA-ULK1 as the templates. Mutations were confirmed by sequencing. siRNA sequences for AMPK α1 subunit, oligo1: 5’-CCCAUAUUUAUUGCCGUGUAdTdT-3’; oligo2: 5’-GAATCTGTGAACACGACAdTdT-3’.

2.3. Cell culture and transfection

HeLa, ULK1 (+/+) MEFS and ULK1 (−/−) MEFS (deposited by Dr. Sharon Tooze) were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin/streptomycin. Tapetum lucidum MEFS, long-term glucose with-
Equivalent protein quantities (30 μg) were subjected to SDS–PAGE, and transferred to PVDF membranes (Millipore). Membranes were probed with the indicated primary antibodies, followed by the appropriate HRP-conjugated secondary antibodies. Immunoreactive bands were visualized with Pro-light HRP (Tiangen) or Immobilon Western Chemiluminescent HRP Substrate (Millipore). For quantitation of the bands on the immunoblot, we scanned the films and analyzed the scans using ImageJ software (NIH). Beta-actin levels were used to correct for gel loading differences.

2.6. Immunofluorescence microscopy

When the treated cells had grown to 70% confluence on a coverslip, they were washed twice with PBS (pre-warmed), and fixed with freshly prepared 4% paraformaldehyde (pre-warmed) at 37 °C for 20 min. Antigen accessibility was increased by treatment with 0.1% Triton X-100 on ice. After blocking with 2% BSA (bovine serum albumin), fixed cells were incubated with primary antibodies for 1 h at room temperature, washed with PBS, then stained with a secondary antibody for a further 1 h at room temperature. Cell images were captured with a TCS SPS II Leica confocal microscope. Colocalization analysis was done by ImageJ software using the “RG2B colocalization” ImageJ plugin (using default parameters). LC3 puncta were counted automatically by ImageJ.

2.7. Immunoprecipitation

After culturing under hypoxic condition for 12 h, cells were lysed in 1 ml of lysis buffer (50 mM Tris–Cl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 30% glycerol) containing 1 mM PMSF and protease inhibitor cocktail (Roche Applied Science) for 1 h at 4 °C. The lysates were immunoprecipitated with specific antibody and protein A/G plus-agarose immunoprecipitation reagent (Santa Cruz) overnight at 4 °C. The precipitants were then washed 5 times with lysis buffer, and the immune complexes were eluted with sample buffer containing 1% SDS for 10 min at 100 °C and analyzed by 12% SDS–PAGE.

2.8. Electron microscopy

Electron microscopy was performed as described previously [33]. Briefly, cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, at 37 °C for 2 h, and then dehydrated in a graded ethanol series and embedded. Approximately 70 nm ultrathin sections were mounted on copper grids. The samples were then stained and visualized using a 120 kV Jeol electron microscope (JEM-1400) at 80 kV. Images were captured using a 120 kV Jeol electron microscope (JEM-1400).

2.9. Statistical analysis

Assays for characterizing cell phenotypes were analyzed by Student’s t-test, and correlations between groups were calculated using Pearson’s test. P values <0.01 were deemed statistically significant.

3. Results

3.1. Hypoxia induces changes in ULK1 phosphorylation and promotes translocation of ULK1 to mitochondria

How is ULK1 phosphorylated in response to hypoxic conditions? We first examined the expression of ULK1 and its phosphorylation at three candidate phosphorylation sites (Ser-317, Ser-555, or Ser-757) [34]. The protein level of ULK1 is markedly elevated in response to hypoxia; however, the three phosphorylation sites are modified differently (Fig. 1A). The phosphorylation of Serine-317 and Serine-555 increases, although the latter site shows much stronger phosphorylation than the former. In contrast, phosphorylation of Serine-757 decreases sharply (Fig. 1A). In the meantime, the phosphorylation of threonine-172 in AMPK α1 also increases in response to hypoxia, but the protein level of AMPK α1 is not significantly changed (Fig. 1A). Subcellular fractionation assays demonstrate that in hypoxic cells, a proportion of ULK1 and phosphorylated ULK1 (Ser-555) can be detected in the fraction that is enriched in mitochondrial components (Fig. 1B). Further, immunoprecipitation experiments reveal that the binding of ULK1 with AMPK α1 is much stronger in hypoxic than in normoxic conditions, while the interaction of ULK1 and mTORC1 is stronger in normoxic conditions (Fig. 1C). Immunofluorescence results show that mutating Ser-555 to alanine (S555A) abolishes the translocation of ULK1 to mitochondria under hypoxia. As a control, mutating Ser-757 to alanine (S757A), which removes the site phosphorylated by mTORC1, markedly affects the translocation of ULK1 (Fig. 1D and E). To be noted, the total number of ULK1 punctate has not significantly changed in cells transfected ULK1 (WT), ULK1 (S555A), or ULK1 (S757A) (Fig. 1F).

3.2. AMPK α1-dependent phosphorylation of ULK1 is critical for translocation of ULK1 to mitochondria in response to hypoxia

In order to investigate whether AMPK α1 is the critical factor for ULK1 phosphorylation and mitochondrial translocation under hypoxia, we determined the phosphorylation status of ULK1 following different treatments, including inhibition of AMPK α1 activity by the agonist Dorsomorphin 2HCl or siRNA knock-down of AMPK α1. As shown in Fig. 2A, Dorsomorphin 2HCl effectively blocks the phosphorylation of AMPK α1 at Thr-172 under hypoxic conditions. Meanwhile, the hypoxia-induced phosphorylation of ULK1 at Ser-555 and Ser-317 also vanishes completely, whereas phosphorylation of Ser-757 is not obviously changed. Similar changes in phosphorylation status are observed when the endogenous AMPK α1 is knocked down in MEF cells by siRNA. Phosphorylation of AMPK α1 at Thr-172 and ULK1 at Ser-555 and Ser-317 are abolished under hypoxia, whereas phosphorylation of ULK1 at Ser-757 decreases slightly (Fig. 2B). The protein level of ULK1 does not change after blocking AMPK α1 activity or knocking down endogenous AMPK α1 (Fig. 2B).

Immunofluorescence shows that ULK1 translocates to the mitochondria in hypoxic cells. However, after blocking the activity of AMPK α1 or knocking down the endogenous AMPK α1, ULK1 no longer translocates to the mitochondria when cells are exposed to hypoxia (Fig. 2C and D).

3.3. Ser-555 of ULK1 plays an important role in hypoxia-induced mitophagy

Next, we examined the role of ULK1 Ser-555 phosphorylation in hypoxia-induced mitophagy. Under hypoxic conditions, depletion of endogenous ULK1 inhibits degradation of mitochondrial proteins including VDAC, TOM20 and TIM23, as well as the autophagy substrate p62. Similar results were observed in ULK1 (WT) MEFs treated with Bafilomycin A1 (Baf1) under hypoxia (Fig. 3A and B). Reconstitution of exogenous ULK1 in ULK1 KO cells restores mitophagy under hypoxic conditions. Adding the ULK1 (S555A) mutant cannot restore mitophagy in hypoxic ULK1 KO cells. However, introducing the phospho-mimic mutant ULK1 (S555D) into ULK1 KO cells markedly stimulates mitophagy (Fig. 3A and B). Ultrastructure analysis by electron microscopy
 magnesium was used to compare the ability of ULK1 (S555D) and ULK1 (S555A) to mediate mitophagy in ULK1 null cells. ULK1 (S555D) restores the formation of mitochondrion-containing autophagosomes (mitophagosomes) while ULK1 (S555A) does not (Fig. 3C).

It was reported previously that during hypoxia-induced mitophagy, ULK1 phosphorylates FUNDC1 and thereby enhances the binding of FUNDC1 to LC3. Here, we found that when ULK1 was immunoprecipitated with an anti-FUNDC1 antibody, a considerable proportion was phosphorylated at Serine-555 (Fig. 3D).

3.4. AMPK α1 is required for ULK1-dependent mitophagy induced by hypoxia

To further elucidate the function of AMPK α1 and its substrate ULK1 in hypoxia-induced mitophagy, we examined normal or
kinase-dead AMPK α1 and ULK1 phosphorylation mutants in induction of mitophagy under hypoxia in AMPK α1-knockdown cells. In wild-type (WT) cells, AMPK is activated by hypoxia, as indicated by phosphorylation of Thr-172. The phosphorylation of ULK1 Ser-555 is significantly enhanced, while ULK1 Ser-757 is dephosphorylated compared to normoxia (Fig. 4A and B). In AMPK α1-knockdown cells, which do not undergo mitophagy under normoxia, phosphorylation of ULK1 Ser-757 is slightly elevated. When exogenous AMPK α1 or its constitutively phosphorylated substrate ULK1 (S555D) was reconstituted in AMPK α1-knockdown cells, the hypoxia-induced autophagic degradation of mitochondrial proteins TIM23, TOM20 and VDAC and the autophagy substrate p62 were restored. Both LC3-I and LC3-II also decreased under these conditions (Fig. 4A and B). Meanwhile, AMPK α1 kinase dead (AMPK α1 KD), wild-type ULK1 and ULK1 (S555A) all failed to rescue the mitophagy process in AMPK α1-knockdown cells exposed to hypoxia (Fig. 4A and B).

Next, we used immunofluorescence and EM to monitor the colocalization of LC3 and mitochondria and the formation of mitophagosomes. In hypoxic cells, extensive colocalization of LC3 and mitochondria was observed (Fig. 4C and D) and mitophagosomes were detected by EM (Fig. 4E). Metformin, an AMPK α1 kinase activator, is able to promote the colocalization of LC3 and mitochondria and induce mitophagosome formation even without hypoxic treatment (Fig. 4E). However, knocking down endogenous AMPK α1 or adding the AMPK inhibitor Dorsomorphin 2HCl...
inhibits the appearance of mitophagic puncta in hypoxic cells without obviously affecting the formation of single-membraned autolysosomes (Fig. 4E).

4. Discussion

AMPK is regarded as a potential candidate in mitophagy regulation because it maintains cellular energy homeostasis by sensing and manipulating energy status. It influences the erythrocyte life span by accumulating dysfunctional mitochondria and responding to reactive oxygen species (ROS) [35,36]. Defective autophagy-dependent mitochondrial clearance and accumulation of damaged mitochondria have been observed in the blood cells of AMPKα1/−/− mice; these abnormalities were corrected by transplantation of WT bone marrow [25]. Hypoxia can trigger AMPKα1 activation through ROS-dependent CRAC (calcium release-activated calcium) channel activation, leading to an increase in cytosolic calcium that activates the AMPKα1 upstream kinase CaMKKβ [35]. Regulation of ULK1, an autophagy initiating kinase, plays a central role in general autophagy, in particular in autophagosome formation [11]. However, recent studies indicate that it has a selective function in mitophagy upon stimulation by different environmental cues. ULK1 has been identified as the substrate of AMPKα1 which connects cellular energy sensing to mitophagy in glucose or nutrient starvation-induced autophagy [16,17,22]. Several potential ULK1 phosphorylation sites have been identified in these studies, but the dynamic changes in the phosphorylation status of ULK1 during hypoxia are not clear. In our study, hypoxia significantly induced AMPKα1 activation as indicated by phosphorylation at Thr-172 (Fig. 1A), which is consistent with AMPK activation in response to other stimuli including glucose withdrawal or energy depletion [25,26,32,35]. Phosphorylation of ULK1 at Ser-555 and Ser-317 also increased dramatically under hypoxia, while phosphorylation at Ser-757 decreased (Fig. 1A). Interestingly, the phosphorylation pattern of ULK1 under hypoxic conditions is similar to that in glucose or nutrient starvation-induced autophagy, suggesting that a common mechanism of ULK1 activation is shared between general autophagy and mitophagy [25,26,30,32,35].

The changes in phosphorylation status are possibly due to the actions of the different binding partners of ULK1 during hypoxia, since the ULK1/AMPKα1 interaction is increased in hypoxic cells while the ULK1/mTOR interaction is decreased (Fig. 1C).
mitophagy, the phosphorylation of ULK1 at Ser-555 and Ser-317 is increased, which may be due to the strengthened binding of AMPK α1 with ULK1 (Fig. 1C). In contrast, under normoxic conditions, the activity of AMPK is inhibited by mTORC1 binding, as shown by the absence of phosphorylation at Ser-757 of ULK1 (Fig. 1A and C).

ULK1 translocates to dysfunctional mitochondria during mitophagy initiation, yet the upstream ULK1 activator has not been reported. In our study, inhibition of AMPK α1 activation or silencing of AMPK α1 results in loss of ULK1 phosphorylation at Ser-555 and disturbs the translocation of ULK1 to mitochondria, suggesting
that AMPK α1 may be an ULK1 activator during hypoxia-induced mitophagy (Fig. 2A and B). Deletion of ULK1 retards mitophagy induction upon hypoxia treatment, while ULK1 (S555D), which mimics constitutively phosphorylated ULK1, rescues mitophagy induction in ULK1 knockdown (Fig. 4). In conclusion, we found that AMPK α1 phosphorylases and activates ULK1, and the activated form of ULK1 translocates to the mitochondria in hypoxia-induced mitophagy. Moreover, the ULK1 (S555D) mutant, which mimics constitutive phosphorylation of ULK1 at Ser-555 by AMPK α1, is able to restore the impaired mitophagy resulting from AMPK α1 silencing or inhibition. Thus, our results revealed that AMPK and ULK1 work co-operatively in mitophagy induced by hypoxia.

Disclosure of potential conflicts of interest

The authors declare that they have no conflict of interest.

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