AMPK \(\gamma\) is Required for Maintaining Epithelial Cell Structure and Polarity

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Received January 27, 2011 / Accepted February 9, 2011

AMP-activated protein kinase (AMPK), a heterotrimeric complex comprising a catalytic \(\alpha\) subunit and regulatory \(\beta\) and \(\gamma\) subunits, has been primarily studied as a major metabolic regulator in various organisms, but recent genetic studies discover its novel physiological functions. The first animal model with no functional AMPK \(\gamma\) subunit gene was generated by using Caenorhabditis elegans genetics. AMPK \(\gamma\) null flies demonstrated lethality with severe defects in cuticle formation. Further histological analysis found that deletion of AMPK \(\gamma\) causes severe defects in cell polarity in embryo epithelia. The phosphorylation of nonmuscle myosin regulatory light chain (MRLC), a critical regulator of epithelial cell polarity, was also diminished in AMPK \(\gamma\) null embryo epithelia. These defects in AMPK \(\gamma\) mutant epithelia were successfully restored by over-expression of AMPK \(\gamma\). Collectively, these results suggested that AMPK \(\gamma\) is a critical cell polarity regulator in metazoan development.

**Key words**: Drosophila, AMPK \(\gamma\), MRLC, epithelia, cell polarity

Introduction

AMP-activated protein kinase (AMPK), a heterotrimeric complex comprising a catalytic \(\alpha\) subunit and regulatory \(\beta\) and \(\gamma\) subunits, is well conserved from yeast (Saccharomyces cerevisiae), worm (Caenorhabditis elegans) and fruit fly (Drosophila) to human [2,6,8]. During metabolic stress, when cellular AMP/ATP ratios rise, AMPK senses increased AMP level with its cystathionine beta-synthase (CBS) domains in its regulatory \(\gamma\) subunit and is activated by phosphorylation of Thr172 in the activation loop of its catalytic \(\alpha\) subunit [2,6,8]. This activated AMPK down-regulates ATP-consuming anabolic pathways, and up-regulates ATP-generating catabolic pathways to maintain energy homeostasis in the cell [2,6,8]. Although the biochemical characteristics of AMPK were extensively studied by cell line-based studies, there were few genetic data on in vivo function of metazoan AMPK, due to the existence of multiple AMPK subunit isoforms encoded by different genes [8]. Because Drosophila has no redundancy in AMPK subunit genes [13], AMPK signaling was successfully nullified in the Drosophila system [10,12]. All AMPK \(\alpha\) -null mutant flies are lethal and fail to develop to adulthood even in the presence of sufficient nutrients [10,12]. Surprisingly, loss of AMPK \(\alpha\) induces disruption of cell polarity accompanying with disorganized actin cytoskeleton in embryonic and wing epithelial cells [10,12]. These abnormalities in epithelial cell polarity are highly similar to those of the mutants of LKB1, the upstream kinase of AMPK [10]. Moreover, constitutive activation of AMPK restores these defects in LKB1-null mutants, demonstrating AMPK as a novel regulator of cell polarity [10]. These genetic studies using Drosophila successfully discovered novel physiological functions of AMPK, and also provide valuable tools to dissect its in vivo signaling mechanisms.

In this report, the first AMPK \(\gamma\) null Drosophila mutant was generated and characterized. The deletion of AMPK \(\gamma\) induced lethality and the severe defects in cuticle formation. Further analysis showed that AMPK \(\gamma\) has an important role in maintaining epithelial cell polarity. These data strongly suggest that AMPK \(\gamma\) is critical for in vivo AMPK signaling.

Materials and Methods

Fly Strains

The GS100 fly line with a P-element in the AMPK \(\gamma\) locus was obtained from GenExel (Taejon, Korea). The deletion mutants were generated from P-element excision experiments. To generate the over-expression lines for AMPK \(\gamma\), a HA-tagged entire AMPK \(\gamma\) open reading
frame was subcloned into pUAST vector. The fly lines for FLP-DIP (autosomal flipase-dominant female sterile) technique and hsGAL4 were obtained from the Bloomington Stock Center (Bloomington, IN, USA).

Production of AMPK γ null embryos

The germ line clones of AMPK γ 1D9 were generated using the autosomal FLP-DIP technique. In detail, 82AR/P AM PK γ 1D9/TM6B females were crossed with yw hsFLP/Y; 82AR/P[w, ovdP] males. Their progeny larvae were heat-shocked for 2 hr at 37°C at the first instar larval stage. yw hsFLP; 82AR/P[w, ovdP]/82AR/P AM PK γ 1D9 females (3 day-old) were selected and crossed with AM PK γ 1D9/TM3 GFP males to obtain AM PK γ 1D9 null embryos. To produce AM PK γ null embryos expressing AM PK γ, yw hsFLP; 82AR/P[w, ovdP]/82AR/P AM PK γ 1D9 females were crossed with hs-Gal4 UAS-AM PK γ /CyO Act-GFP; AM PK γ 1D9/TM3 GFP males. For expression of UAS-AM PK γ in AM PK γ null embryos, eggs were collected and aged at 30°C.

Cuticle preparation

For the cuticle preparations, embryos were collected and dechorinated as previously described [10]. Dechorinated embryos were immersed in a solution containing acetic acid and glycerol at a 3:1 ratio and incubated overnight at 65°C. Embryos were then mounted in Hoyer’s medium and incubated 24 hr at 65°C.

Immunostaining

I used anti-phospho MRLC (1:50, Cell Signaling Technology, Danvers, MA, USA), anti-aPKC (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-Discs large (4F3, 1:200, DSHB, Iowa City, IA, USA) antibodies as primary antibodies. Texas red and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (Molecular Probes, Eugene, OR, USA) were used at a 1:200 dilution. DNA was visualized by DAPI (Sigma, St. Louis, MO, USA). Drosophila tissues were fixed in 4% formaldehyde for 5 min. After the standard immunostaining procedures [10], tissues were observed with a laser scanning confocal microscope LSM700 (Carl Zeiss, Göttlingen, Germany).

Results and Discussion

Drosophila AMPK γ subunit is highly homologous to its mammalian counterparts and Saccharomyces cerevisiae SNF4, especially in its CBS domains [17]. Drosophila has 6 AMPK γ subunit isoforms encoded by a single gene (17, Fig. 1), but the null mutant which nullified the expression of all

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**Fig. 1.** Genomic map of AM PK γ. P-element insertion (triangle), exons (rectangles and arrow heads) and introns (lines) are shown. AM PK γ 1D9 contains an about 17 kb deletion encoding whole CBS domains.
AMPK γ isoforms was not available. From an extensive searching of the GenExel library (~20,000 independent EP lines), we isolated AMPK γ^D39^ (G5100), an EP line with a P-element insertion near exons encoding the CBS domains shared by all AMPK γ subunit isoforms (Fig. 1). Subsequently, I have generated an AMPK γ deficient line, AMPK γ^D39^ by imprecise excision of the P-element from G5100. PCR-based molecular analyses demonstrated that the exons containing the CBS domains were totally deleted in this mutant (Fig. 1). RT-PCR clearly demonstrated that AMPK γ^D39^ is a genuine null allele (data not shown).

This null mutant displayed a larval lethality, demonstrating that AMPK γ is essential to complete development. Then, I investigated role of AMPK γ in early development by generating germ line clones (GLC) of AMPK γ null mutants to eliminate the maternal effect. Interestingly, AMPK γ^D39^ null embryos completely failed to hatch, demonstrating that AMPK γ is indispensable for the completion of embryogenesis. Extensive examination of AMPK γ mutant embryos revealed almost complete loss of the cuticle structure (Fig. 2).

Because the structure of embryonic cuticle highly reflects the organization of underlying epidermis that secretes it, I supposed that the epithelial cell structures of AMPK γ mutant embryos would be also severely impaired. Wild-type *Drosophila* embryonic epidermis contain two distinct membrane domains—an apically localized cell-cell adhesive junction known as zonula adherens (ZA) and a more basal junctional complex known as septate junction (SJ) [9]. However, in AMPK γ mutant embryos, localization of atypical PKC (aPKC), a component of the apical complex which regulates the formation of ZA [9], was found severely disrupted (Fig. 3). Discs-large (Dlg), normally localizing at or below SJ [9], was also mislocalized in AMPK γ mutant embryos (Fig. 3). When AMPK γ was re-introduced in AMPK γ mutants using GAL4-UAS system, the defected epithelial structures and mislocalized polarity determinants were successfully restored (Fig. 3). These results strongly supported that AMPK γ is critical for maintaining epithelial structures in *Drosophila* development.

In previous reports, extensive biochemical and genetic analyses demonstrated that AMPK regulates cell polarity by phosphorylating myosin regulatory light chain (MRLC; also known as MLC2), a critical molecule for cell polarity establishment [3,7,10,15]. The regulatory phosphorylation site of MRLC is directly phosphorylated by activated AMPK *in vitro* and *in vivo* [10]. After this phosphorylation, MRLC induces the actin cytoskeleton structural change which has a critical role in the regulation of cell polarity [10]. To test the role of AMPK γ *in vitro* MRLC phosphorylation, AMPK γ mutant embryos were stained with phospho-specific MRLC antibodies. Although phosphorylated MRLC was specifically localized to apical region of wild-type epithelia, the deletion of AMPK γ almost completely suppressed MRLC phosphorylation (Fig. 4). Moreover, over-expression of AMPK γ completely restored MRLC phosphorylation in the AMPK γ null epithelia (Fig. 4). Collectively, these data demonstrated that

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![Fig. 2. Cuticle formation defects in AMPK γ null embryos. Wild type (Con) and AMPK γ null (AMPK γ^D39^) embryo cuticles were analyzed by dark field (DF) and phase contrast (PH) microscopy. Yellow scale bar: 50 μm.](image)

![Fig. 3. AMPK γ is required for maintaining epithelial cell polarity.](image)
$\text{AMPK} \gamma$ is essential for in vitro MRLC phosphorylation, suggesting the critical role of AMPK $\gamma$ in AMPK-mediated cell polarity regulation.

In genetic analyses during decades, mutations in $\text{AMPK} \gamma$ isoforms induce various symptoms in various animals. An autosomal dominant mutation in $\text{AMPK} \gamma$3 induces a dramatic increase in skeletal muscle glycogen content in pigs [11]. After this discovery, several groups identified AMPK $\gamma$2 gene mutations associated with familial cardiac hypertrophy [1,5]. The most patients with these $\gamma$2 mutations also demonstrated severe defects in electrical conductance, similar to the conduction abnormalities observed in Wolf-Parkinson-White syndrome [1,5]. In addition, a deletion of first exon of an $\text{AMPK} \gamma$ isoform induced progressive neurodegeneration and neuronal cell death in Drosophila [17]. Because loss of cell polarity is strongly correlated with more aggressive and invasive growth of malignant cells [16], the cell polarity controlling roles of AMPK $\gamma$ suggest that AMPK $\gamma$ mediates the tumor suppressing function. A small scale case study shows that metformin, an AMPK activating anti-diabetic drug, reduces the risk of cancer in diabetic patients [4]. Moreover, metformin suppresses carcinogen-induced cancers in hamsters [14]. These data support the tumor suppressing role of AMPK, and raise the possibility that metformin and other AMPK activating agents can be used for the treatment of AMPK-related cancers. Collectively, the $\text{AMPK} \gamma$ mutant and $\text{AMPK} \gamma$ transgenic models generated in this study will provide valuable tools and insights into investigating various AMPK $\gamma$-related diseases and abnormalities.

Acknowledgement

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (MEST) (331-2008-1-C00225).

References

초록: AMPK γ 유전자와의 표피세포극성 유지기능 규명

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AMPK는 catalytic α subunit과 regulatory β 및 γ subunit으로 구성된 인산화 효소로, 그 동안 생체 내 중요한 역할을 하는 것으로 알려져 왔다. 최근 유전학 연구를 통해 그 기능이 더욱 복잡하고 기능이 있는 것이 밝혀졌다. 본 연구에서 초파리 유전학 기법을 활용하여 AMPK γ subunit 유전자가 결손된 모델 초파리를 제작하여 연구한 결과, AMPK γ 유전자 결손 시 초파리 epidermal 형성이 심각하게 저해됨을 발견하였고, 조직학적 실험을 통해 표피세포의 극성이 AMPK γ 유전자 결손 초파리에서 손상되어 있음을 확인하였다. 또한 세포극성을 조절하는 중요한 분자인 MRLC의 인산화 또한 AMPK γ 유전자 결손 시 저해되었으며, AMPK γ 유전자 결손 시 MRLC의 인산화와 표피세포의 극성이 모두 흑백화 되어, 초파리 표피세포의 극성유지에 AMPK γ 유전자가 필수적임을 확인하였다.
