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DAPK 抑制 T 細胞中 PKCθ 活化機轉之研究

Study on how DAPK inhibits the activation of PKCθ in T cells

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本論文係張沛昀君（學號 R00449011）在國立臺灣大學醫學院免疫學研究所完成之碩士學位論文，於民國 102 年 06 月 27 日承下列考試委員審查通過及口試及格，特此證明

口試委員：賴明宗 （簽名）

（指導教授）

鄧尚樑　　李秀香

系主任、所長 江伯倫

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又快樂地順利完成厲害的研究真是太棒了。謝謝子軒，身為康樂股長真是十分稱職，一起舉辦活動以及玩樂真是太開心了。謝謝琮皓，你半夜打卡常常讓我感到溫馨，但是騎車一定要小心身體。謝謝宗霖，雖然許多活動你都無法參加實在可惜，但是以後還有很多機會的。謝謝高高高賢能，雖然你身為系學會會長好像有點辛苦，但又好像蠻輕鬆的。謝謝冠雄，希望你早日找到意中人，然後繼續深造好像也不錯。

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摘要

DAPK 是一個已知的抑癌蛋白。實驗室先前的研究發現，DAPK 能在 T 細胞受體刺激之下活化，並且藉由抑制 NF-κB 路徑而阻礙 T 細胞活化。在更進一步的研究中指出，DAPK 只會特定地抑制由 T 細胞受體所引發的 NF-κB 活化，並不影響 TNFα 或 IL-1β 所引發的 NF-κB 活化。DAPK 抑制 T 細胞受體所引發的 NF-κB 活化是透過影響 T 細胞受體所引發的 PKCθ 磷酸化；然而，DAPK 抑制 PKCθ 活化過程的機制目前仍然未被探討。在本研究中，我們探討 DAPK 如何抑制 T 細胞受體所引發的 PKCθ 磷酸化。最近的研究中指出，MAP4K3/GLK 是在 T 細胞受體活化之下，負責磷酸化 PKCθ 的激酶。我們發現在 HEK293T 細胞及 Jurkat T 細胞之中，DAPK 與 PKCθ 及 MAP4K3/GLK 皆具有交互作用。我們也發現 DAPK 與 PKCθ 及 MAP4K3/GLK 之間會以許多不同蛋白區位進行交互作用。在體外激酶實驗中，我們發現 DAPK 並不會磷酸化 PKCθ 及 MAP4K3/GLK，但是 DAPK 會抑制 MAP4K3/GLK 活化所必需的自體磷酸化作用及其激酶活性。有研究中指出，T 細胞受體所引發的 MAP4K3/GLK 活化過程必須經過 SLP-76 與 MAP4K3/GLK 進行交互作用才能達成。我們發現在 HEK293T 細胞及 Jurkat T 細胞之中，DAPK 與 SLP-76 具有交互作用。在免疫沉澱實驗中，我們發現 DAPK 抑制 SLP-76 與 MAP4K3/GLK 在 T 細胞受體刺激之下所引發的交互作用。我們的結果顯示 DAPK 可能是利用不同的機制調控 T 細胞受體刺激所引發的 PKCθ 活化，一是透過降低 MAP4K3/GLK 激酶的活性，二是藉由抑制 MAP4K3/GLK 與上游鷹架蛋白 SLP-76 之間的連結而使 MAP4K3/GLK 無法活化，進而間接抑制其下游受質 PKCθ 的活化。DAPK 如何抑制 T 細胞受體刺激所引發的 NF-κB 訊息，並專一性抑制 PKCθ 活化之詳細機制尚待更進一步的實驗探討。

Abstract

Death-associated protein kinase (DAPK) is well known as a tumor suppressor. Previous studies from our lab have shown that DAPK is activated after TCR stimulation. DAPK inhibits T cell activation through suppression of NF-κB signaling. Furthermore, DAPK specifically inhibits the TCR-induced NF-κB activation but not TNFα- or IL-1β-induced NF-κB activation. Results from our lab also demonstrated that DAPK inhibits TCR-induced PKCθ phosphorylation, but the exact biochemical process targeted by DAPK to inhibit PKCθ activation remains unclear. The specific aim of this study is to delineate the molecular mechanism on how DAPK inhibits TCR-induced PKCθ phosphorylation. MAP4K3/GLK is recently identified as the kinase that phosphorylates and activates PKCθ after TCR stimulation. We found that DAPK interacted with both PKCθ and MAP4K3/GLK in HEK293T cells and Jurkat JE6.1 T cells. We also identified several domains of DAPK that mediate the binding to PKCθ and MAP4K3/GLK. Using *in vitro* kinase assay, we found that DAPK did not phosphorylate PKCθ. DAPK did not phosphorylate MAP4K3/GLK, but DAPK inhibited MAP4K3/GLK auto-phosphorylation and kinase activity. We also found that DAPK bound SLP-76, the adaptor protein which binds MAP4K3/GLK and is required for MAP4K3/GLK activation. *In vitro* binding analysis demonstrated that the presence of DAPK decreased the binding between SLP-76 and MAP4K3/GLK.

DAPK also inhibited the association between SLP-76 and MAP4K3/GLK in Jurkat JE6.1 T cells. Therefore, DAPK uses at least two different mechanisms to inhibit TCR-induced PKCθ activation: one by suppressing MAP4K3/GLK kinase activity, the other by reducing the binding of MAP4K3/GLK to SLP-76. Further characterization on the processes underlying the inactivation of MAP4K3/GLK and the reduced association of MAP4K3/GLK-SLP-76 by DAPK may help to understand the exact inhibitory mechanism of DAPK in TCR-induced NF-κB signaling.

List of Abbreviations

BSA	Bovine serum albumin
CaM	Calmodulin
Bisacrylamide	N', N'-Methylene bisacrylamide
CCLR	Culture cell lysis reagent
DAPK	Death-associated protein kinase
DMEM	Dulbecco's modified eagle medium
ECL	Enhanced chemiluminescence
EDTA	Ethylene diaminetetraacetic acid
FBS	Fetal bovine serum
HA	Hemagglutinin
His	Histidine
IB	Immunoblot
IP	Immunoprecipitation
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LAR	Luciferase assay reagent
MAP4K3/GLK	Germinal center kinase-like kinase
MBP	Myelin basic protein
MLC	Myosin light chain
NF-κB	Nuclear factor kappa beta
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PKCθ	Protein kinase C θ
PMSF	Benzylsulfonyl fluoride

SLP-76	Src homology 2 (SH2) domain-containing leukocyte protein of 76 kDa
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCR	T cell receptor
WCE	Whole cell extract

Content

Chapter I Introduction

1.1 Death-associated protein kinase (DAPK)

Death-associated protein kinase is a tumor suppressor, and its expression is downregulated in many cancer types. DAPK was identified from the γ-interferon induced cell death in HeLa cells, where DAPK is one of the positive regulators[1].Further evidences have shown that DAPK is required for cell death induction by various death signals.

DAPK is a 160 kDa protein with multiple domains, and the kinase domain locates at the N-terminus part of the protein. Like other kinds of CaM kinases, the kinase domain is followed by a calcium/calmodulin regulatory domain, which acts as a pseudo-substrate to suppress catalytic activity by binding to the kinase region[2, 3].Upon the binding of $Ca^{2+}$to calmodulin, the affinity between calcium/calmodulin regulatory domain and kinase domain is reduced, which results in the activation of DAPK. Thecalcium/calmodulin regulatory domain-deletedform of DAPK,DAPK-ΔCaM, acts as a constitutively active kinase.In contrast, with the mutation of a conserved lysine in the kinase domain to alanine, DAPK-K42A, loses kinase activity and acts as a dominant negative form of DAPK[4]. The kinase activity of DAPK is affected by additional posttranslational modifications. The

auto-phosphorylation ofSer308 located in calcium/calmodulin regulatory domain interferes with the activation of DAPK[3].

The ankyrin repeats and the death domain at C-terminus of DAPK interact with other proteins to transduce signals. The cytoskeleton domain of DAPK serves to bind to cytoskeleton. The function of P-loop motif of DAPK is still not clear.

1.2 T cell activation

1.2.1 The signal transduction of T cell activation

T cells are a subset of lymphocytes that play essential roles in the cell-mediated immunity. The activation of T cells leads to clonal expansion and differentiation. Upon the engagement of T cell receptor and MHC-antigenic peptide complex presented by antigen presenting cells, the Src family PTKs (protein tyrosine kinases) will be activated by CD45 tyrosine phosphatase. The activated Src PTKs then phosphorylate the tyrosine residue at immunoreceptor tyrosine-based activation motifs (ITAMs) on CD3ε, CD3δ, CD3γ and CD3ζ subunits of T cell receptor complex[5, 6]. Following the phosphorylation of ITAMs on T cell receptor, another Syk family PTK, ZAP-70 (ζ-associated protein of 70kDa), is recruited to bind to the phosphorylated ITAMs within its SH2 (Src-homology 2) region. ZAP-70 is phosphorylated by Src PTKs to become active.ZAP-70 phosphorylates adaptor

protein LAT (linker for activation of T cells), and the phosphorylated LAT links a variety of adaptor proteins and enzymes to form a signalosome for transducing activation signals[7-9].ZAP-70 also phosphorylates and activates SLP-76 (SH2 domain containing leukocyte protein of 76kDa)[5]. PLC-γ1, Grb2 and Gads are recruited to specific phosphorylated tyrosines on LAT. SLP-76 is then become associated with LAT through binding to Gads. SLP-76 accommodates Itk, and Itk phosphorylates the PLC-γ1within signalosome. The activated PLC-γ1 is responsible for hydrolyzing phosphatidylinositol-4,5-bisphosphate (PIP_2) in the plasma membrane into second messengers diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3)[5]. After the cleavage, DAG activates numbers of proteins, including different isoforms of PKC (protein kinase C), for further downstream signal transduction.Another second messenger, IP_3, stimulates the efflux of Ca^{2+} from endoplasmic reticulum to the cytosol and the influx of Ca^{2+} from outside of the cell[10]. As a result, increased Ca^{2+} bindscalmodulin and leads to the activation of calcineurin, which consequently dephosphorylates nuclear factors of activated T-cells NF-AT1 (NFATc2 or NFATp), NF-AT2 (NFATc1 or NFATc), NF-AT3 (NFATc4) and NF-AT4 (NFATc3). The dephosphorylated NFATsare then translocated into the nucleus, where they cooperate with other transcription factors and promote the transcription of specific genes[11, 12].

The binding of phosphorylated LAT to adaptor protein Grb2 results in recruitment of SOS (Son of sevenless homolog 1 and 2), SOS in turns activates H-Ras (v-Ha-Ras Harvey rat sarcoma viral oncogene homolog). Ras activation leads to sequential activation of c-Raf-1 (v-Raf-1 murine leukemia viral oncogene homolog 1), MEK1/2 (mitogen-activated protein kinase 1/2 and ERK1/2 (mitogen-activated protein kinases 1/2. The phosphorylated ERK1/2enter the nucleus and activate the c-Fos, and the activated JNK phosphorylates c-Jun. c-Fos and c-Jun are dimerized in the nucleus to form the transcription factor AP-1 (activator protein 1).

Together with the activation of NF-κB, transcription factors NFAT and AP-1 serve to turn on the gene expression of IL-2 and other kinds of cytokines, leading to the growth and differentiation of T cells[13].

1.2.2 Src homology 2 (SH2) domain-containing leukocyte protein of 76 kDa (SLP-76)

SLP-76 is an adaptor protein originally identified as a downstream substrate of ZAP-70. TCR engagement leads to ZAP70,then phosphorylates SLP-76 and LAT. Activated LAT recruits SLP-76 to the membrane, where LAT and SLP-76 nucleate a complex composed of the guanine-nucleotide-exchange factor VAV, PLCγ1, non-catalytic region of tyrosine kinase (NCK), TEC-family kinase interleukin T-cell

kinase (ITK), adhesion- and degranulation-promoting adaptor protein (ADAP) and haematopoietic progenitor kinase 1 (HPK1)[14]. SLP-76 has three distinct domains. NH2-terminal domain is rich in tyrosine residues. Phosphorylated tyrosine residues bind SH2-domain containing proteins such as Vav, Nck and the Tek kinase ITK [15-17]. The central proline-rich domain binds to SH3-domain containing proteins that include PLCγ1 and Gads. The C-terminal SH2 domain associates with haematopoietic progenitor kinase 1 (HPK1) and adhesion- and degranulation-promoting adaptor protein (ADAP)[18].

SLP-76 is essential for thymocyte development. SLP-76 is involved in the signaling pathway initiated by TCR engagement. Recent study indicated that SLP-76 is a crucial adaptor protein in the TCR-induced PKCθ activation[19].

1.2.3 Germinal center kinase-like kinase (MAP4K3/GLK)

The conserved serine/threonine-specific protein kinase, germinal center kinase-like kinase (GLK, also termed MAP4K3), belongs to the ste20-like serine/threonine kinases family, which serve to respond to the extracellular stimuli and regulate a number of gene transcription, cell mitosis, cell differentiation, cell apoptosis and immune inflammation[20, 21].Recentstudy reveals that MAP4K3/GLKdirectly activates PKCθ in the TCR signaling-induced T cell

activation[19]. Furthermore, the enhanced expression of MAP4K3/GLK is shown to be involved in autoimmune disease such as systemic lupus erythematosus[21].

1.2.4 Protein kinase C θ (PKCθ)

Protein kinase C (PKC) is a family of serine/threonine specific protein kinases. The PKC family consists of fifteen isoforms in humans [22], and can be divided into three groups. The first group of PKC family is Ca^{2+}-dependent type, containing PKCα, PKCβ and PKCγ. The second group of PKC family is Ca^{2+}-independent type, containing PKCδ, PKCε, PKCη, PKCθ and PKCμ. The third group of PKC family is unconventional type, containing PKCζ and PKCι/λ [23]. PKCθ belongs to the Ca^{2+}-independent type conventional PKC, in which the activation requires DAG but notCa^{2+}. In the activation of T cells, PKCθ is localized in the cSMAC (central region of the supra-molecular activation complex) of the immunological synapse formed by T cells and APC, suggesting that PKCθ plays an essential role in the T cell activation [24]. SLP-76 is important for recruiting PKCθ. Activated PKCθ phosphorylates CARMA1 and enhances the formation of complex composed of Bcl-10, MALTI and CARMA1, leading to activation of the IKK complex, followed by IκB protein to undergo phosphorylation and degradation. The transcription factor NF-κB will be translocated into nucleus and hence activate the expression of specific downstream

genes [25].

In addition to regulating the T cell activation, PKCθ is also important for promoting the viability of T cells. By activating the NF-κB pathways, PKCθ induces the expression of Bcl-2, Bcl-xL and c-FLIP, the critical anti-apoptotic molecules. In addition, PKCθ directly phosphorylates BAD and inactivates BAD, a pro-apoptotic protein, to promote the viability of T cells [13].

1.2.5 DAPK inhibits T cell activation at the stage on or upstream of PKCθ

While DAPK is a well-known tumor suppressor, the role it plays in the T cell activation is barely defined. Previous studies from our lab have shown that after TCR stimulation, DAPK is activated immediately. DAPK inhibits T cell activation through down-regulating the NF-κB pathway. Furthermore, DAPK specifically inhibits the TCR-induced NF-κB activation but not TNFα- or IL-1β-induced NF-κB activation. Results from our lab also demonstrated that DAPK inhibits the phosphorylation of PKCθ [13], but whether DAPK targets to PKCθ or a step upstream of PKCθ remains unclear. In this study, our goal is to investigate how DAPK inhibits the phosphorylation of PKCθ, based on recent identification that MAP4K3/GLK directly activates PKCθ [19].

1.3 Rationale

DAPK negatively regulatesTCR-induced PKCθ phosphorylation.A logic first step to delineate the mechanism of inhibition is to investigate whether DAPK directly phosphorylates and inactivates PKCθ.Based on the direct phosphorylation of PKCθ by MAP4K3/GLK, we will also examine whether DAPK affects PKCθ activation through direct phosphorylation and inhibition of MAP4K3/GLK.We will also explore the potential interaction between DAPK, MAP4K3/GLK and PKCθ in HEK293T cells, and in Jurkat JE6.1 T cells expressing DAPK.

The specific aims of the present study are:

(1) To investigate whether DAPK inhibits TCR-induced PKCθ activation through direct phosphorylation and inhibition of PKCθ.

(2) To determine whether DAPK suppresses PKCθ activation through direct phosphorylation and inhibition of MAP4K3/GLK.

(3) To delineate the exact biochemical mechanism for the inhibition of PKCθ or MAP4K3/GLK by DAPK.

Chapter II Materials and Methods

2.1 Celllines and cell culture

2.1.1 Cell lines

Jurkat E6.1 is human T lymphoma cells. EL4 is mouse thymus lymphoma cells. HEK293T is primary human embryonic kidney cells transfected with SV40 large T antigen. 293 Phoenix cell is transformed human kidney cells that express proteins essential for retroviruses, the cell line is derived from Dr. Garry Nolan (Stanford University. CA).

2.1.2 Cell culture

Jurkat E6.1 cell and EL4 cell were cultured in completed RPMI 1640 with 5% FBS, 10 μM L-glutamine, 10 unit/ml Penicillin/streptomycinand 20 mMβ-Mercaptoethanol, and incubated in 5% CO2 incubator at 37℃. HEK293T cell and 293 Phoenix cell were cultured in 10% FBS contained DMEM medium in 5% CO2 incubator at 37℃.

2.2 Antibodies

Anti-DAPK antibody (clone DAPK-55), anti-MAP4K3/GLK antibody (766-780) and anti-Flag-M2 antibodywerepurchased from Sigma (St. Louis, MO).Anti-PKCθ antibody (clone C-19), anti-SLP-76 antibody (clone H-300), anti-MBP (clone C-16)

and anti-PLC-γ1 antibody (clone E-12) werepurchased from Santa Cruz Biotech (Santa Cruz, MA).Anti-phospho-PKCθ antibody (T538) and anti-GLK/MAP4K3 antibody werepurchased from Cell Signaling (Beverly, MA).Anti-β-tubulin antibody was purchased from Upstate (Lake Placid, NY). HRP-conjugated rabbit-anti-mouse immunoglobulin and HRP-conjugated gout-anti-rabbit immunoglobulinwere purchased from Amersham Bioscience (Buckinghamshire, UK). Anti-GLK/MAP4K3 antibody was derived from Dr. Tse-Hua Tan (National Health Research Institutes). Anti-HA antibody was derived from Dr. Soo-Chen Cheng (Academia Sinica).

2.3 Chemicals and reagents

Phenol chloroformand Polybrenewere purchased from Calbiochem (Darmstadt, Germany). Phenylmethanyl sulfonyl fluoride (PMSF), 1,4-dithiothreitol (DTT) and β-Mercaptoethanolwere purchased from Merck (Darmstadt, Germany). PVDF membrane was purchased from Millipore (Massachusetts, US). [^{32}P]phosphate was purchased from Amersham Bioscience (Buckinghamshire, UK).Maestrofectin transfection reagentwas purchased from Omics Bio (Taipei, Taiwan).ECL reagent was purchased from Advansta (California, US). PageRuler pre-stained protein adderand stripping buffer were purchased from Thermo (Rockford, US). Acrylamide/bis 30% solution (29:1) was purchased from Bio Basic Inc. (Ontario, Canada). Albumin, from

bovine serum was purchased from Sigma (Missouri, US). Protein G Mag Sepharose Xtrawas purchased from GE Healthcare (Uppsala, Sweden). Protein assay dye reagent concentrate was purchased from Bio-Rad (California, US).Ethanol, EDTA, glycerol and isopropanol were purchased from Mallinckrodt Baker Inc (New Jersey, US).Complete Protease Inhibitor Cocktail Tablet was purchased from Roche (Indianapolis, US).

2.4 Plasmid construction and preparation

2.4.1 Construction of pcDNA4-hPKCθ-6xHA

For over-expressing the human PKCθ-6xHA protein with the expression of HA tag, we subcloned humanPKCθ from pcDNA4-hPKCθ-mycto pcDNA4-6xHA vector.The pcDNA4-hPKCθ-mycand pcDNA4-6xHA were digested with XhoI and PmeI, and digested human PKCθwas subcloned into pcDNA4-6xHAvector to express pcDNA4-hPKCθ-6xHA.

2.4.2 Construction of pcDNA4-hMAP4K3/GLK-myc

For over-expressing the human MAP4K3/GLK protein in HEK293 T cell and Jurkat JE6.1 T cell, we purchased human pBluescriptR-MAP4K3/GLK clone from Transomic Technologies. pBluescriptR-MAP4K3/GLK and pcDNA4-mycwere

digested with XhoI and BamHI, anddigested human MAP4K3/GLKand pcDNA4-myc vector were ligated to express pcDNA4-MAP4K3/GLK-myc.

2.4.3 Construction of pcDNA4-hMAP4K3/GLK-6xHA

For over-expressing the human MAP4K3/GLK-6xHA protein with the expression of HA tag, we subcloned humanMAP4K3/GLK from pcDNA4-hMAP4K3/GLK-mycto pcDNA4-6xHA vector.The pcDNA4-hMAP4K3/GLK-mycand pcDNA4-6xHA were digested with XhoI and PmeI, and digested human MAP4K3/GLKwas subcloned into pcDNA4-6xHAvector to express pcDNA4-hMAP4K3/GLK-6xHA.

2.4.4 Construction of truncated forms of human MAP4K3/GLK

Human MAP4K3/GLK-6xHAis composed of kinase domain, proline-rich repeats and CNH domain. All of the truncated forms were generated by PCR using Jurkat JE6.1 cDNAas a template. For kinase domain the sense primer (5’-AGGATCCATGAACCCCGGCTTCGATTTG-3’) introduced a BamHI site, and an antisense primer (5’-TCTCGAGTACAAAAGGATGCTGTAATAA-3’)introduced aXhoI site.For proline-rich repeats the sense primer (5’-AGGATCCATGACACAACATTTGACACGGTC-3’) introduced a BamHI site,

and an antisense primer (5’-TCTCGAGTTTAGGTGTTGGAGGAAGAC-3’) introduced aXhoI site.For C-terminal citron homology domain the sense primer (5’-AGGATCCATGGTGCATATGGGTGCATG-3’) introduced a BamHI site, and an antisense primer (5’-TCTCGAGGTAACTGTTTTCATGACCCG-3’) introduced aXhoI site.Each amplified fragment was ligated into the BamHI and XhoI sites of pcDNA4-6xHA.

2.4.5 Construction of pcDNA4-hSLP-76-6xHA

Human SLP-76 was generated by PCR using Jurkat JE6.1 cDNA as a template. Sense primer (5’- AGGATCCATGGCACTGAGGAATGTGCCC -3’) introduced a BamHI site, and antisense primer (5’-TCTCGAGTGGGTACCCTGCAGCATGCGTTA -3’) introduced a XhoI site. Amplified fragment was ligated into the BamHI and XhoI sites of pcDNA4-6xHA.

2.4.6 Construction of kinase-dead form of human PKCθ

The kinase activity of human PKCθis activated after the phosphorylation of Lys409. Kinase-dead K409A PKCθ was constructed from wild-type PKCθ by site-directed mutagenesis. Mutagenesis was performed with sense primer (5’-CAATTTTTCGCAATAGCGGCCTTAAAGAAAGATG) and antisense primer

(5’-CATCTTTCTTTAAGGCCGCTATTGCGAAAAATTG-3’) by polymerase.

2.4.7 Construction of kinase-dead form of human MAP4K3/GLK

The kinase activity of human MAP4K3/GLKis activated after the phosphorylation of Lys45.Kinase-dead K45A MAP4K3/GLK was constructed from wild-type MAP4K3/GLK by site-directed mutagenesis. Mutagenesis was performed with sense primer (5’- GAATTAGCAGCAATTGCAGTAATAAAATTGGAAC) and antisense primer (5’-GTTCCAATTTTATTACTGCAATTGCTGCTAATTC-3’)by polymerase.

2.5 Transfection of plasmid DNA

2.5.1 Calcium phosphate transfection

HEK293T cells were maintained to 80% confluent, and media were replaced on each plate with 8ml of completed DMEM.10μg plasmid DNA was added into sterile ddH_2O in total volume of 450μl, and 50μl 2.5M $CaCl_2$was added and mixed in each tube immediately.DNA/$CaCl_2$ mixture was slowly (drop-wise) added into 0.5 ml of 2x HBS with bubbling.Transfection mix was added to plates in drop-wise fashion, and mixed by rocking back and forth and put in 37℃, 5% CO_2 incubator. Media were changed the next day with fresh completed medium.

2.5.2 Retrovirus infection

HEK293T cellswere transfectedwith pGC-YFP and pGC-DAPK-YFP by calcium phosphate transfection. After 24 hours, mediawere change with 10% FBS RPMI-1640. After 48 hours, supernatant of HEK293T cells culture mediumwas collected, containing infective retrovirus particles.5μg/ml polybrene was added into supernatant. $1x10^6$ Jurkat JE6.1 T cells were suspendedwith the supernatant, and were transferred into 6-well plate. Infected Jurkat JE6.1 T cells were centrifugedin 1650 rpm at room temperature for 45 minutes, then were incubated in 37℃, 5% CO_2 incubator for 48 hours.

2.5.3 Electroporation

$8.8x10^6$ EL4 T cellspellets were resuspendedin 100μl Neon™ R buffer, and were mixed with plasmid DNA. Adequate program of electroporator (Neon™) was chose to transfect plasmids. Electroporated cellswere resuspended withRPMI-1640 containing 10% FBS, then were incubated in 37℃, 5% CO_2 incubator for at least 24 hours before experimental analysis.

2.6 T cell activation

Jurkat JE6.1 T cells were activated in 6-well plate. 6-well plate was coated with

5μg/ml anti-CD3 and 2.5μg/ml anti-CD28 antibodies 16 hours before the activation. Jurkat JE6.1 T cells were rested with 1% FBS-containing RPMI-1640 for 30 minutes, and were seeded into anti-CD3/anti-CD28-coated plate and cultured in the incubator at 37℃. Activated Jurkat JE6.1 T cells were collected at specific time point, and were lysed to perform western blotting.

2.7 Western Blotting

Protein samples were boiled for 5 minutes in SDS sample buffer (50 mM Tris-HCl pH 6.8, 5% glycerol, 2.5% β-Mercaptoethanol, 2% SDS, 0.0025% bromophenol blue), and were separated with 10% SDS-PAGE then transferred to nitrocellulose membrane. Membrane was blockedwith 5% skim milk dissolved in TBST and was incubated with primary antibody at 4℃ overnight. Secondary antibody was added on next day for 1 hr, and the signals were revealed by ECL.

2.8 Immunoprecipitation

Sample cells were collected and washedby PBS once and lysed with WCE lysis buffer. Cell lysates were immunoprecipitated with specific antibody swirling overnight, and were mixed with 20μl protein G magnetic beads for 1 hour at 4℃. Unbound antibodies were washed out withWCE buffer for 4 times. Protein G

magnetic beads with sample bufferwere resuspendedand boiled at 100℃ for 10 min. Cell lysates were collected to perform SDS-PAGE and western blotting.

2.9 Luciferase assay

pNF-κB-Luc (App. 9) is the reporter of the analysis. There are five NF-κB binding sites at the enhancer element before *luciferase* gene, and the luminescent enzyme is activated after the binding of NF-κB.

pNF-κB-Luc was transduced into EL4 T cellsby electrophoresis. 24 hours afterthe transduction, EL4 T cells were activated with anti-CD3/anti-CD28, and were collected at specific time point. Collected cells were rinsed with PBS twice, and were lysed with CCLR (culture cell lysis reagent, 0.1 M K-phosphate pH 7.8, 0.1 mM EDTA, 10% glycerol, 1% Triton X-100, 7mM 2-mercaptoethanol). Lysed cells were vortexed for 5 minutes, and centrifuged in 14000rpm for 6 minutes at 4 ℃. Supernatant was collected as cell extraction to perform the luciferase assay.

Fresh LAR (Luciferase assay reagent, 25 mM Tricine pH 7.8, 15mM K-phosphate pH 7.8, 15mM $MgSO_4$, 4mM EGTA, 2mM ATP, 1mM DTT) was prepared before performing the luciferase assay, and 180 μl LAR was mixed with 50μl 0.2mM Luciferin. 50μl cell extraction was mixed with 200μl LAR/Luciferin mixture in 96-well plate. Analysis of the luminescent signal was performed with

Perkin Elmer Life Science VICTOR.

2.10 *In vitro* kinase assay

Recombinant proteins were incubated with kinase buffer, 1μM CaM, 0.5mM $CaCl_2$, 50μM cold ATPand 1μCiγ^{32}P-ATP at 25℃ for 30 minutes. The reaction mixture was resolved by SDS-PAGE and the phosphorylation was determined by autoradiograghy. 1/10 volume of reaction mixture withoutγ^{32}P-ATP was collectedto perform SDS-PAGE and western blotting as loading control.

2.11 *In vitro* binding assay

Recombinant proteins were incubated with BSA at 4℃ for 2hours, and antibodies were added to incubate for 16 hours. The reaction mixture was resolved by SDS-PAGE and the binding quantity was determined by antibodies. 1/10 volume of reaction mixture was collected to perform SDS-PAGE and western blotting as loading control.

Chapter III Results

3.1Establishmentof Jurkat JE6.1 T cell line stably expressing DAPK

To study the role of DAPK in the NF-κB pathway in T cells, we subcloned *dapk* gene into pGC-YFP vector. We transfected pGC-YFP and pGC-DAPK-YFP plasmids into 293 Phoenix cells forretrovirus package. After 72 hours, we collected the virus supernatant and infected JurkatJE6.1 T cells to establish stable clone. 48~72 hours after virus infection,the YFP-expressingJurkat JE6.1 T cells were sorted by FACS (Fig.1A). The DAPK expression in pGC-DAPK-YFP transducedJE6.1 cells was confirmed by Western blotting(Fig.1B).

3.2 DAPK inhibits TCR-stimulated NF-κB activation

3.2.1 Expression of DAPK mutant forms affects the activation of NF-κB reporter

Previous studies from our lab have shown that DAPK inhibits the TCR-induced NF-κB activation[13]. To re-examine the effect of DAPK on NF-κB activation,κB-Luc was transfected with RK5F (vector only), DAPK-K42A (dominant negative form) or DAPK-ΔCaM (constitutively active form) into EL4 T cells by electroporation. EL4 cells were then stimulated by anti-CD3/anti-CD28 TCR stimulation, and the luciferase activity is determined 6 hours and 9 hours after activation. Result shows that TCR-inducedκB-Lucactivity was enhanced by

DAPK-K42A and was inhibited by DAPK-ΔCaM (Fig.2).

3.2.2 DAPK suppresses TCR-induced phosphorylation of PKCθ

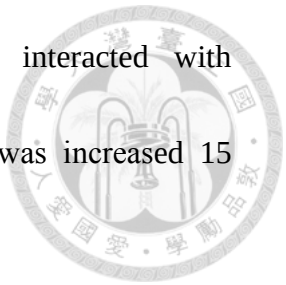
To further confirm the stage of TCR-induced NF-κB pathway regulated by DAPK, control and DAPK-expressing Jurkat JE6.1 cells were activated by anti-CD3/anti-CD28.PKCθ and the phosphorylation of PKCθ at Thr538 were analyzed in control and Jurkat JE6.1 T cells stably expressing DAPK by Western blotting. Result shows that over-expression of DAPK decreased TCR-induced PKCθ phosphorylation (Fig. 3).

3.3 Interaction of DAPK with PKCθ

3.3.1 Immunoprecipitation analysis of DAPK and PKCθ

To determine how DAPK regulates the activation of PKCθ, we first investigated whether DAPK interacts with PKCθ. DAPK-FLAG and PKCθ-6xHAwere transfected into HEK293T cells, cell lysates were immunoprecipitated with anti-FLAG or anti-HA, and the presence of DAPK-FLAG and PKCθ-6xHAwere determined. Result shows that DAPK-FLAG was associated with PKCθ-6xHA in HEK293T after overexpression (Fig. 4). We also investigated whether DAPK interacts with PKCθ in T cells after TCR stimulation. pGC-DAPK-YFP-JE6.1 cells were activated by anti-CD3/anti-CD28, and the association of DAPK and endogenous PKCθ was

analyzed by immunoprecipitation. Result shows that DAPK interacted with endogenous PKCθ in Jurkat JE6.1 T cells, and the interaction was increased 15 minutes and 45 minutes after TCR stimulation (Fig. 5).



3.3.2 Determination of the interaction domains between DAPK and PKCθ

To determine which domain of DAPK is responsible for interacting with PKCθ, we transfected FLAG-tagged DAPK fragments with PKCθ-6xHA into HEK293T cells and performed immunoprecipitation analysis. Result shows that ankyrin repeats and death domain of DAPK were the regions on DAPK that interacted with PKCθ (Fig. 6). We similarly transfected FLAG-tagged PKCθ fragments with DAPK-Myc into HEK293T cells to determine the DAPK-interacting domain of PKCθ. Result shows that both N-terminus and C-terminus of PKCθ interacted with DAPK (Fig. 7).

3.4 Interaction of DAPK with MAP4K3/GLK

3.4.1 Immunoprecipitation analysis of DAPK and MAP4K3/GLK

To determine whether DAPK regulates the activation of MAP4K3/GLK, we examined whether DAPK interacts with MAP4K3/GLK. After the transfection of DAPK-FLAG and MAP4K3/GLK-6xHA into HEK293T cells, cell lysates were immunoprecipitated with anti-FLAG or anti-HA, and the presence of DAPK-FLAG and MAP4K3/GLK-6xHA were examined. Result shows that DAPK-FLAG was

associated with MAP4K3/GLK-6xHA in HEK293T after over-expression (Fig. 8). We further investigated whether DAPK interacts with MAP4K3/GLK in T cells after TCR stimulation. Jurkat JE6.1 T cells stably expressing DAPK-FLAG were activated by anti-CD3/anti-CD28, and the association of DAPK and the endogenous MAP4K3/GLK was determined by immunoprecipitation with anti-FLAG. Result shows that DAPK interacted with endogenous MAP4K3/GLK in Jurkat JE6.1 T cells, and the interaction was enhanced 15 minutes after TCR stimulation (Fig. 9).

3.4.2 Determination of the interaction domains of DAPK and MAP4K3/GLK

To determine the domain of DAPK that is responsible for interacting with MAP4K3/GLK, we transfected FLAG-tagged DAPK fragments with MAP4K3/GLK-6xHA into HEK293T cells and performed immunoprecipitation analysis. Result shows that cytoskeleton binding domainand death domain of DAPK interacted with MAP4K3/GLK (Fig. 10). We further constructed 6xHA-tagged MAP4K3/GLK fragments containing kinase (K) domain, proline-rich repeats (PR) domain and C-terminal citron homology (CNH) domains of human MAP4K3/GLK. 6xHA-tagged MAP4K3/GLK fragments were transfected into HEK293T cells with DAPK-FLAG and immunoprecipitation with anti-HA was used to examine the DAPK-binding region of MAP4K3/GLK. Result shows that kinase domain of

MAP4K3/GLK was the DAPK-interacting domain on MAP4K3/GLK (Fig. 11).

3.5 To determine whether PKCθ or MAP4K3/GLK is the substrate of DAPK

3.5.1 Preparation of human PKCθ recombinant protein

Previous report has shown that DAPK-K42A (dominant negative form)promotes the activation of TCR-induced NF-κB pathway[13]. We speculated that kinase activity of DAPK may play an important role in the inhibitory process. We subcloned human PKCθ into pET21a vector (App. 7), after transformation into E. coli; PKCθ expression was induced with 0.5mM IPTG.PKCθ recombinant protein was purified with anti-6xHis antibody-coated beads. Fig. 12 shows the quantity of human PKCθ recombinant protein compared with various concentration of BSA.

3.5.2 Preparation of human MAP4K3/GLK recombinant protein

We subcloned human MAP4K3/GLK into pcDNA4/6xHA vector (App. 8) and transfected into HEK293T cells.The human MAP4K3/GLK recombinant protein was purified from HEK293T cell lysates with anti-6xHA antibody-coated beads. Fig. 13shows the quantity of human MAP4K3/GLK recombinant protein compared with various concentration of BSA.

3.5.3 Kinase assay performed with DAPK and PKCθ

We then investigated whether PKCθ is a direct substrate of DAPK. We incubated recombinant DAPKwith PKCθ-6xHA and γ^{32}P-ATP in kinase buffer at 25℃ for 30 minutes. The reaction mixture was resolved by SDS-PAGE and the phosphorylation was determined by autoradiograghy. Result shows that the phosphorylation level of PKCθ was elevated in the presence of DAPK (Fig. 14). However, the auto-phosphorylation of PKCθ was detected in the *in vitro* kinase assay in the absence of DAPK. Hence we generated recombinant PKCθ-K409A,the kinase-dead PKCθ,to further examine the phosphorylation phenomenon.

3.5.4 Preparation of PKCθ and GLK kinase-dead recombinant proteins

We use site-direct mutagenesis to generate PKCθ-K409A and MAP4K3/GLK-K45A kinase-dead form plasmids. We subcloned PKCθ-K409A and MAP4K3/GLK-K45A into pcDNA4/6xHA vector (App. 8),and transfected into HEK293T cell. The PKCθ-K409A-6xHA and MAP4K3/GLK-K45A-6xHArecombinant proteins were purified with anti-6xHA antibody-coated beads. Fig. 15 shows the quantity of PKCθ-K409A-6xHA and MAP4K3/GLK-K45A-6xHArecombinant proteins compared with various concentration of BSA.

3.5.5 DAPK does not phosphorylates PKCθ *in vitro*

We incubated recombinant DAPK with PKCθ-K409A-6xHA and γ^{32}P-ATP in kinase buffer at 25℃ for 30 minutes. The reaction mixture was resolved by SDS-PAGE and the phosphorylation was determined by autoradiograghy. Result shows that DAPK did not phosphorylate PKCθ in contrast to DAPK-mediated phosphorylation of MLC (Fig. 16).

3.5.6 DAPK does notphosphorylatesMAP4K3/GLK *in vitro*

We also incubated recombinant DAPK with MAP4K3/GLK-K45A-6xHA and γ^{32}P-ATP in kinase buffer at 25℃ for 30 minutes. The reaction mixture was resolved by SDS-PAGE and the phosphorylation was determined by autoradiograghy. Result shows that DAPK did not phosphorylate MAP4K3/GLK in contrast to DAPK-mediated phosphorylation of MLC(Fig. 17).

3.6 To determine whether DAPK affect the kinase activity of MAP4K3/GLK

Our results suggestedthat PKCθ andMAP4K3/GLK are not the direct kinase substrates of DAPK. We next examined whether DAPK affects the kinase activity of MAP4K3/GLK.

3.6.1 Kinase assay performed with MAP4K3/GLK and MBP

To determine whether MAP4K3/GLK kinaseactivity is affected by DAPK, we incubated recombinant MAP4K3/GLK-6xHA and its substrate myelin basic protein (MBP) with or without DAPK in kinase buffer and γ^{32}P-ATP at 25℃ for 30 minutes. The reaction mixture was resolved by SDS-PAGE and the phosphorylation was determined by autoradiograghy. Result shows that the MAP4K3/GLK auto-phosphorylation and the phosphorylation of MBP were both diminished in the presence of DAPK, ina dose-dependent manner (Fig. 18), suggesting a possibility that DAPK inhibits the kinase activity of MAP4K3/GLK to MBP.

3.6.2 Preparation of human SLP-76 recombinant protein

Recent report has shown that the interactionbetween SLP-76 and MAP4K3/GLK is mediated through tyrosine phosphorylation of SLP-76[19]. Since GLK acts as a kinase, we investigated whether SLP-76 is one of the substrates of GLK. To analyze whether SLP-76 is direct substrate of GLK, human SLP-76 were subcloned into pcDNA4/6xHA vector (App. 8), and transfected into HEK293T cells. The human SLP-76 recombinant protein was purified with anti-6xHA antibody-coated beads. Fig. 19 shows the quantity of human SLP-76 recombinant protein compared with various concentration of BSA.

3.6.3 Kinase assay performed with MAP4K3/GLK and SLP-76

To determine whether MAP4K3/GLKdirectly phosphorylates SLP-76, we incubated recombinant MAP4K3/GLK-6xHA with SLP-76-6xHA and γ^{32}P-ATP in kinase buffer at 25℃ for 30 minutes. The reaction mixture was resolved by SDS-PAGE and the phosphorylation was determined by autoradiograghy. Result shows that MAP4K3/GLK directly phosphorylatesSLP-76 (Fig. 20).

To determine whether the MAP4K3/GLK-mediatedphosphorylation of SLP-76 is affected by DAPK, we incubated recombinant MAP4K3/GLK-6xHA and SLP-76-6xHA with or without DAPK in kinase buffer and γ^{32}P-ATP at 25℃ for 30 minutes. The reaction mixture was resolved by SDS-PAGE and the phosphorylation was determined by autoradiograghy. Result shows that the MAP4K3/GLK auto-phosphorylation and the phosphorylation of SLP-76 were both diminished in the presence of DAPK, in a dose-dependent manner (Fig. 20), suggesting a possibility that DAPK inhibits the kinase activity of MAP4K3/GLK to SLP-76.

3.7 To determine whether DAPK interferes with the association of MAP4K3/GLK and SLP-76

Previous report has shown that the association with SLP-76 is necessary for the activation of MAP4K3/GLK [19]. We examined another possibility that DAPK may inhibit the kinase activity by interfering with the binding of MAP4K3/GLK to

SLP-76.

3.7.1 DAPK interacts with SLP-76

To determine whether DAPK inhibits the association of MAP4K3/GLK and SLP-76, we examined whether DAPK interacts with SLP-76. After the transfection of DAPK-FLAG and SLP-76-6xHA into HEK293T cells, immunoprecipitation and immunoblot were used to analyze the association of DAPK-FLAG and SLP-76-6xHA in HEK293T cells. Result shows that DAPK-FLAG was associated with SLP-76-6xHA in HEK293T after overexpression (Fig. 21).

3.7.2 The endogenous MAP4K3/GLK-SLP-76 association is reduced by DAPK

To determine whether DAPK interferes with the association of the endogenous MAP4K3/GLK and SLP-76, control and DAPK-expressing Jurkat JE6.1 cells were activated by anti-CD3/anti-CD28, SLP-76 was immunoprecipitated and the presence of endogenous MAP4K3/GLK was determined. Result shows that the association of MAP4K3/GLK and SLP-76 wasdiminished in Jurkat JE6.1 T cell stably expressing DAPK (Fig. 22).

3.7.3 DAPK inhibits the *in vitro* binding betweenMAP4K3/GLK and SLP-76

To further investigate the effect of DAPK in the association of MAP4K3/GLK

and SLP-76, we used recombinant SLP-76-6xHA to perform the *in vitro* binding assay.MAP4K3/GLK-6xHA and SLP-76-6xHA were incubated with or without DAPK-FLAG in the binding buffer for 2 hours,and anti-SLP-76 antibodywas used to capture the recombinant SLP-76. The reaction mixture was resolved by SDS-PAGE. Result shows that the presence of DAPK-FLAG inhibited the binding of MAP4K3/GLK-6xHA and SLP-76-6xHA*in vitro* (Fig. 23).

Chapter IV Discussion

In this study, we investigated how DAPK regulates the activation of PKCθ in TCR-induced NF-κB activation. The expression of DAPK inhibited the NF-κB activation in T cell after TCR stimulation (Fig.2), and DAPK specifically down-regulated the activation and phosphorylation of PKCθ after TCR stimulation (Fig. 3), similar to previous report from our lab[13].

4.1 DAPK is associated with SLP-76, MAP4K3/GLK, and PKCθ

After TCR stimulation, SLP-76 recruits MAP4K3/GLK and other molecules to form early T cell activation complex. MAP4K3/GLK thenphosphorylates SLP-76, and becomes fully activated with enhanced binding to SLP-76.Activated MAP4K3/GLK phosphorylates PKCθ. We observed that DAPK interacted with SLP-76, MAP4K3/GLK, and PKCθ (Fig. 4, 5,8, 9, 21), suggestingthe possibility that DAPK may inhibit TCR-induced NF-κB activation byits binding to molecules in the early T cell activation complex.

The interactions of DAPK with MAP4K3/GLK and PKCθinvolve several domains on DAPK(Fig. 6, 7, 10, 11). Whether this suggests the presence of multiple routes for DAPK to inhibit TCR-induced NF-κB activation, may deserve further exploration.

4.2 DAPK kinase activity and inhibition of NF-κB activation

We observed that the expression of DAPK-K42A promotes NF-κB activation and the expression of DAPK-ΔCaM inhibits the NF-κB activation in T cell after TCR stimulation (Fig.2). These results suggest that the kinase activity of DAPK is involved in the down-regulationof TCR-induced NF-κB. However, we found that DAPK didnot phosphorylateMAP4K3/GLK and PKCθ (Fig. 16 and 17).Therefore, DAPK inhibits TCR-induced NF-κB activation not through direct phosphorylation of GLK or PKCθ. Whether DAPK phosphorylates other molecules that lead to inactivation of NF-κB remains to be determined.

4.3 DAPK inhibits the kinase activity of MAP4K3/GLK

Recent study illustrates that MAP4K3/GLK phosphorylates PKCθ after TCRengagement[19]. The auto-phosphorylation of MAP4K3/GLK atSer170 is necessary for its kinase activity[26].We observed that DAPK inhibits the auto-phosphorylation of MAP4K3/GLK (Fig. 18 and20).By using myelin basic protein (MBP)asa substrate of MAP4K3/GLK in the *in vitro* kinase assay, we observed that the kinase activity of MAP4K3/GLK is reduced by the presence of increasing amounts of DAPK (Fig. 18).

SLP-76 recruits MAP4K3/GLK and other molecules to form complex after

TCR stimulation in T cell[19]. We found that MAP4K3/GLK directly phosphorylates SLP-76, and the SLP-76 phosphorylationby MAP4K3/GLK is decreased by DAPK in a dose dependent manner in *in vitro* kinase assay (Fig. 18). Together, these results suggest that DAPK directly interferes with MAP4K3/GLK kinase activity. To further examine the direct effect of DAPK on MAP4K3/GLK kinase activity, we will use DAPK mutants with reduced MAP4K3/GLK-binding to determine whether the inhibitory ability is proportional to the binding of DAPK to MAP4K3/GLK.

4.4 DAPK inhibits the association of MAP4K3/GLK and SLP-76

MAP4K3/GLK is recruited by SLP-76 after TCR stimulation in T cell,theassociation with SLP-76 is required for fully activation of MAP4K3/GLK [19]. To further investigate the role of DAPK in the TCR-induced NF-κB activation, we examined the possibility that DAPK may physically interfere with the association of SLP-76 and MAP4K3/GLK.

We observed that the interaction of SLP-76 and MAP4K3/GLK was increased after anti-CD3/anti-CD28 activation in pGC-YFP Jurkat JE6.1 T cell.However, the enhanced SLP-76-MAP4K3/GLK associationwas inhibited in the pGC-DAPK-YFP Jurkat JE6.1 T cell (Fig. 22). This result suggests that over-expression of DAPK interferes with TCR-induced association of SLP-76 and MAP4K3/GLK.

We next examined that if the association of SLP-76 and MAP4K3/GLK is affected by DAPK in cell-free system. In *in vitro* binding assay, we observed that the interaction of SLP-76 and MAP4K3/GLK was blocked by the presence of increased amount of DAPK (Fig. 23). The results further validate that DAPK physically interferes with the association of SLP-76 and MAP4K3/GLK.

In summary, we confirmed that DAPK inhibits the TCR-induced NF-κB activation. DAPK specifically inhibits the PKCθ phosphorylation downstream of TCR ligation. We identified at least two possible different mechanisms for DAPK to inhibit PKCθ phosphorylation stimulated by TCR. DAPK inhibits the kinase activity of MAP4K3/GLK on MBP and SLP-76 (Fig. 24A). DAPK blocks the association of SLP-76 and MAP4K3/GLK, which prevents the fully activation of MAP4K3/GLK (Fig. 24B).Future experiments are required to fully validate these two inhibitory processes by DAPK. There remains the possibility that DAPK may inhibit TCR-induced PKCθ activation through additional mechanism. Further investigation may help to identify these suppressive pathways mediated by DAPK.

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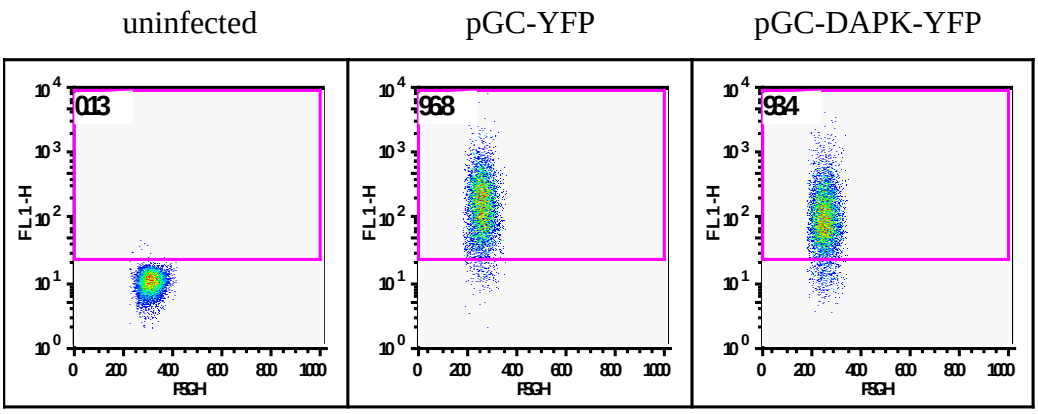
Chapter VI Figures

A



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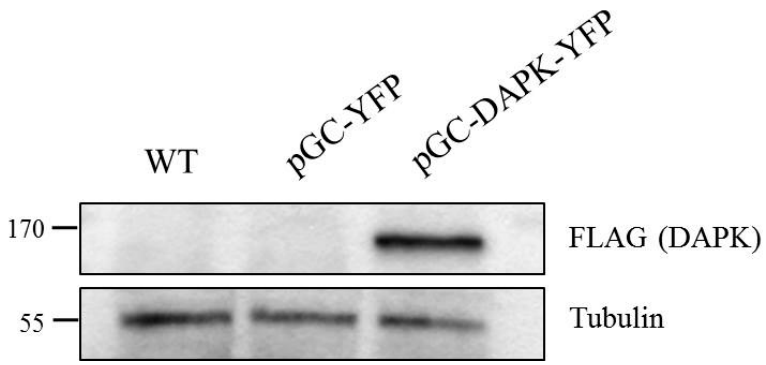


Figure 1. Over-express DAPK in Jurkat JE6.1 T cell

pGC-YFPor pGC-DAPK-YFP was transfected into 293 Phoenix cells forvirus package. Virus supernatant was collected to infect Jurkat JE6.1 T cells. Jurkat JE6.1 T cells transduced with pGC-YFP or pGC-DAPK-YFP were sorted by YFPexpression on FACS. The purity of YFP-expressing Jurkat JE6.1 T cells is as shown by FACS analysis (A).(B) Cells from A were lysed and the expressionof DAPK-FLAG is determined by Western blotting.

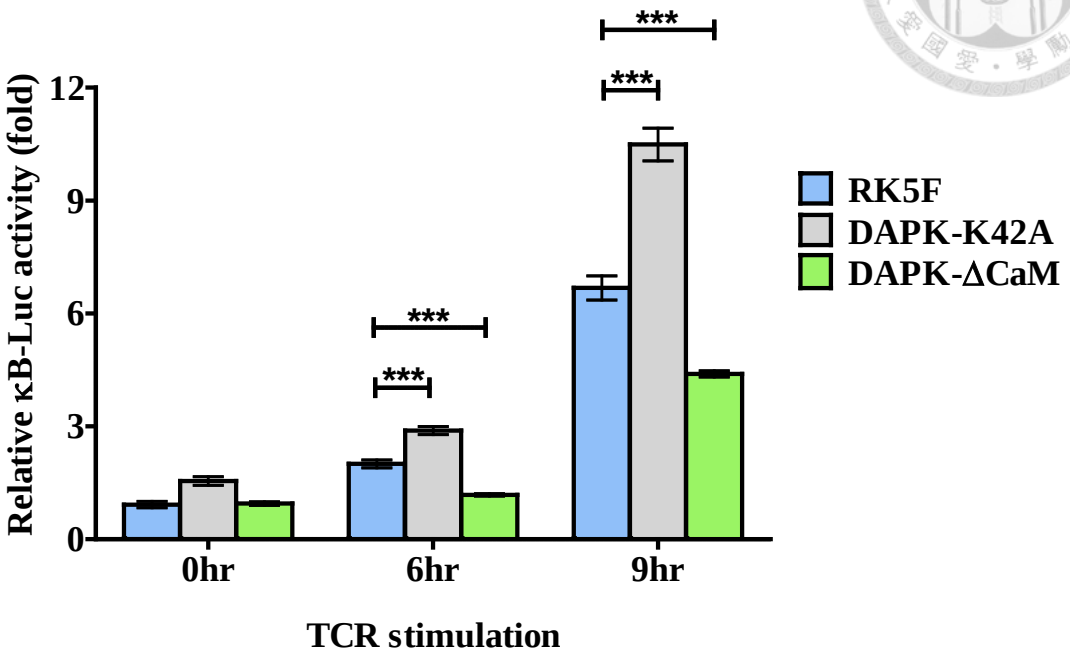


Figure 2. DAPK inhibits TCR-stimulated NF-κB activation

TCR-induced NF-κB pathway was increased by DAPK-K42A and inhibited by DAPK-ΔCaM. EL4 T cells were transfected with κB-Luc togetherwith RK5F (vector only), DAPK-K42A (dominant negative form) or DAPK-ΔCaM (constitutively active form).One day after transfection, EL4 cells were activated by anti-CD3/anti-CD28, and luciferase activity is determined at 6, 9 hours after stimulation.***P< 0.001 for paired t test.

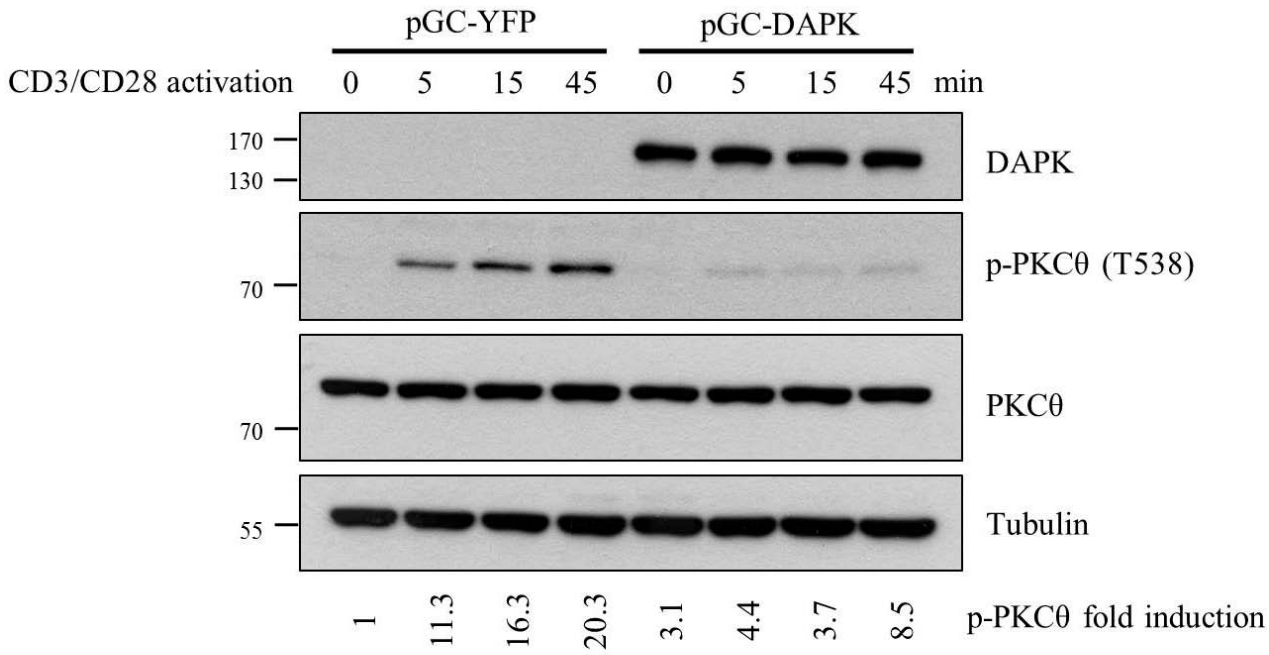


Figure 3. DAPK inhibits TCR-stimulated PKCθ phosphorylation

Over-expression of DAPK decreases TCR-induced PKCθ phosphorylation. Control and DAPK-overexpression Jurkat JE6.1 T cells were activated by anti-CD3/anti-CD8, and the contents of DAPK, PKCθ, p-PKCθ were determined by immunoblot. The extent of p-PKCθ induction was quantified by densitometry and normalized by PKCθ levels.

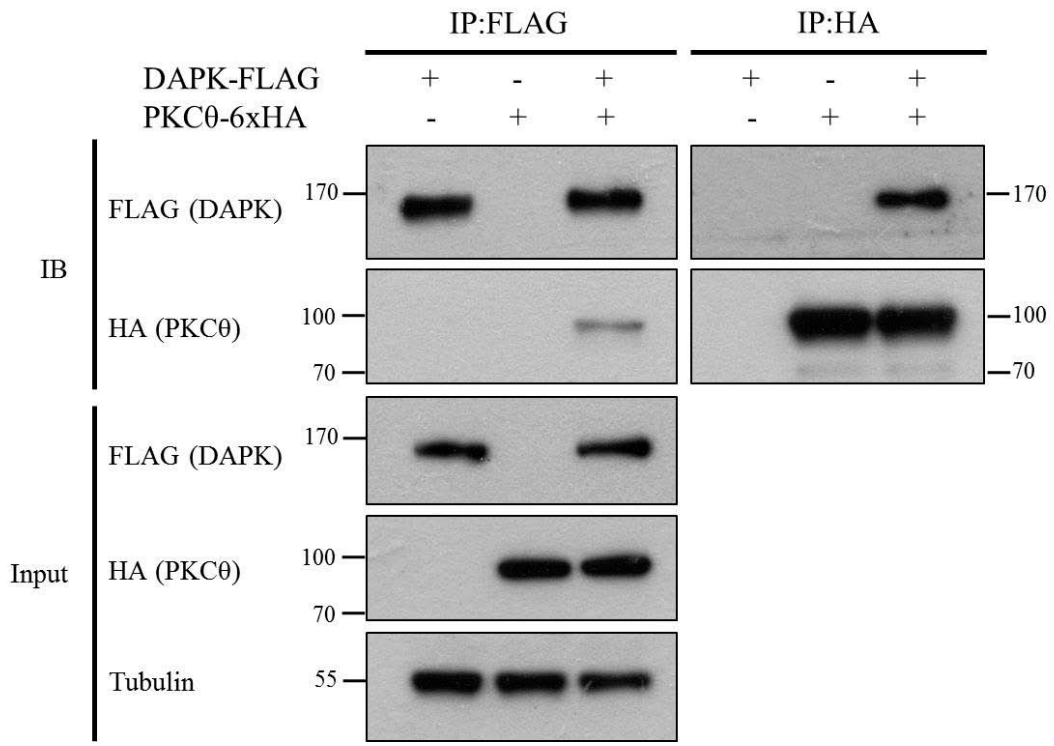


Figure 4. Interaction of DAPK with PKCθ in HEK 293T cell

HEK293T cells were transfected with DAPK-FLAG and PKCθ-6xHA as indication. Cell lysates were prepared 48 hours later, and were precipitatedwith anti-FLAG or anti-HA. The presence of DAPK and PKCθ was detected by anti-FLAG and anti-HA respectively.

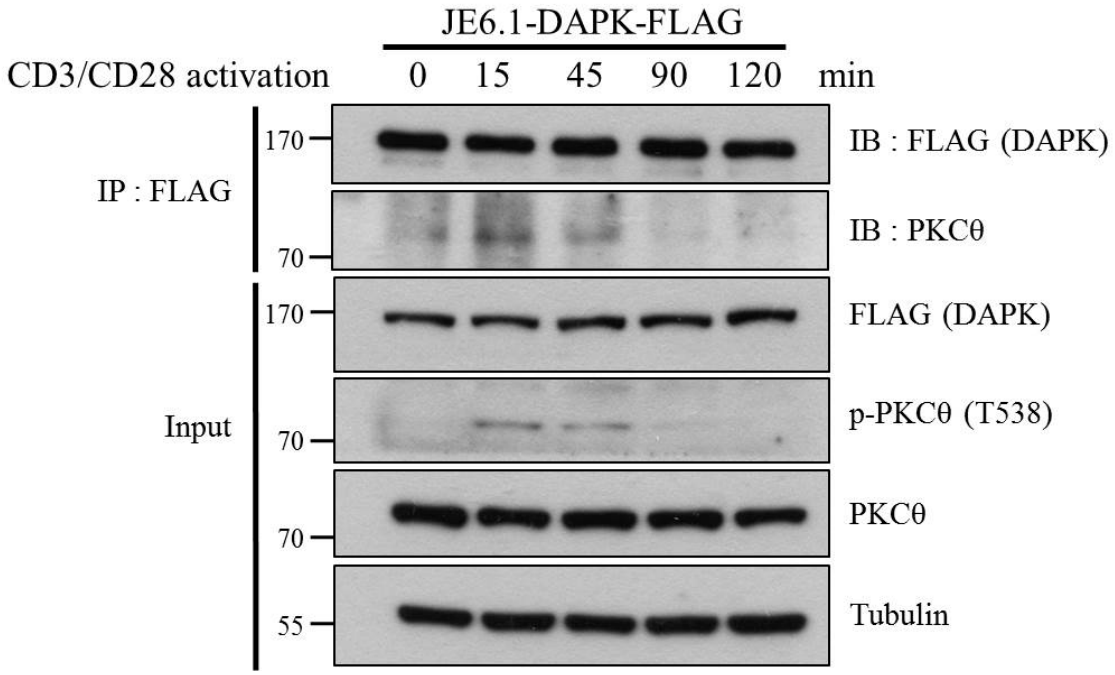


Figure 5. Interaction of DAPK with PKCθin Jurkat JE6.1 T cell

Jurkat JE6.1 T cell expressing DAPK-FLAG (Fig.1) were activated by anti-CD3/anti-CD28, and cell lysates were prepared at the indicated time points. Cell lysates were precipitated with anti-FLAG, and the presence of PKCθ was determined by anti-PKCθ. Input indicates the levels of DAPK-FLAG, PKCθ and p-PKCθ (T538) in cell lysates.

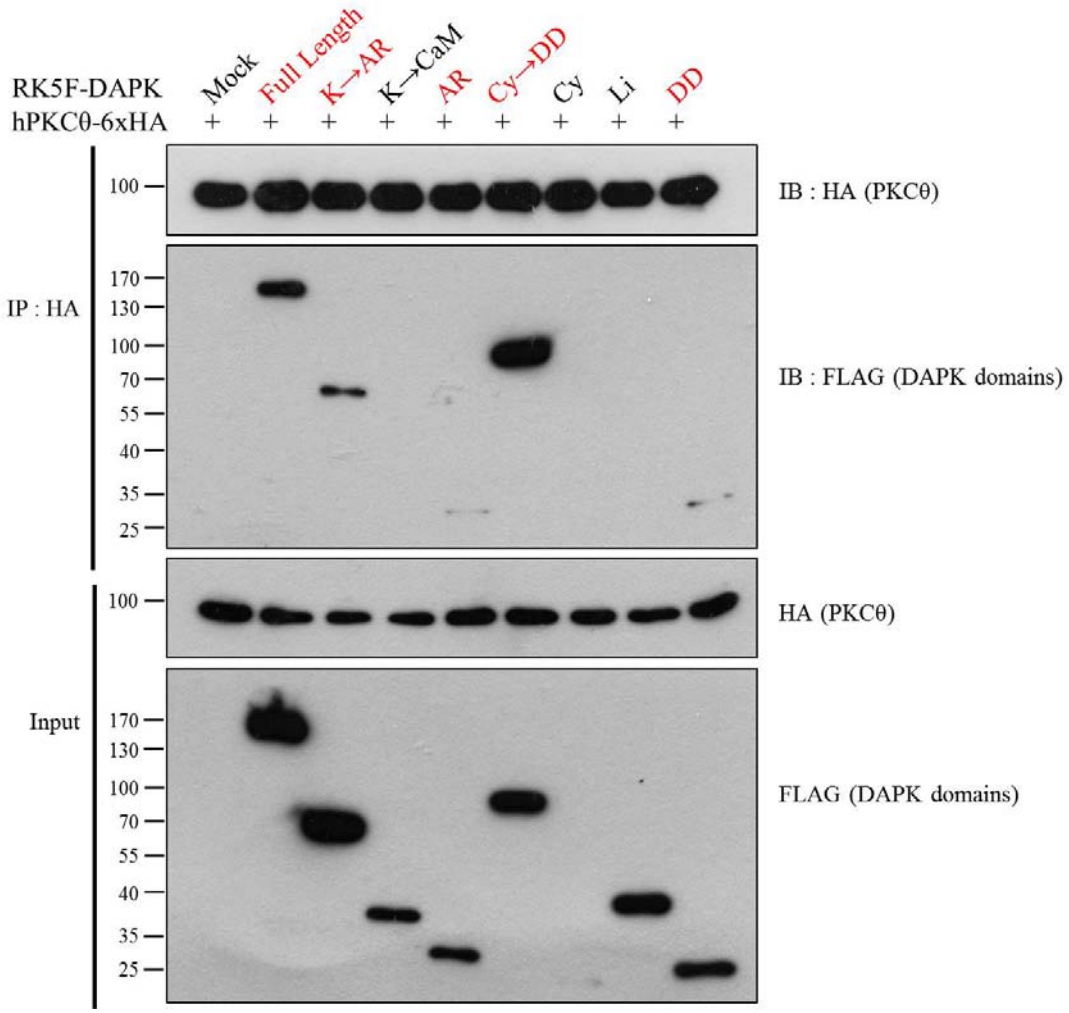


Figure 6. The interaction between DAPK and PKCθ is mediated by multiple domains.

HEK293T cells were transfected with Flag-tagged DAPK fragments containing kinase domain (K), calcium/calmodulin regulatory domain (CaM), ankyrin repeats domain (AR), cytoskeleton binding domain (Cy), linking domain (Li), or death domain (DD) (App. 1), in addition to PKCθ-6xHA. Cell lysates were prepared 48 hours later, and were immunoprecipitated with anti-HA, the presences of DAPK fragments were detected by anti-FLAG. Input indicates the contents of PKCθ-HA and DAPK fragments in cell lysates.

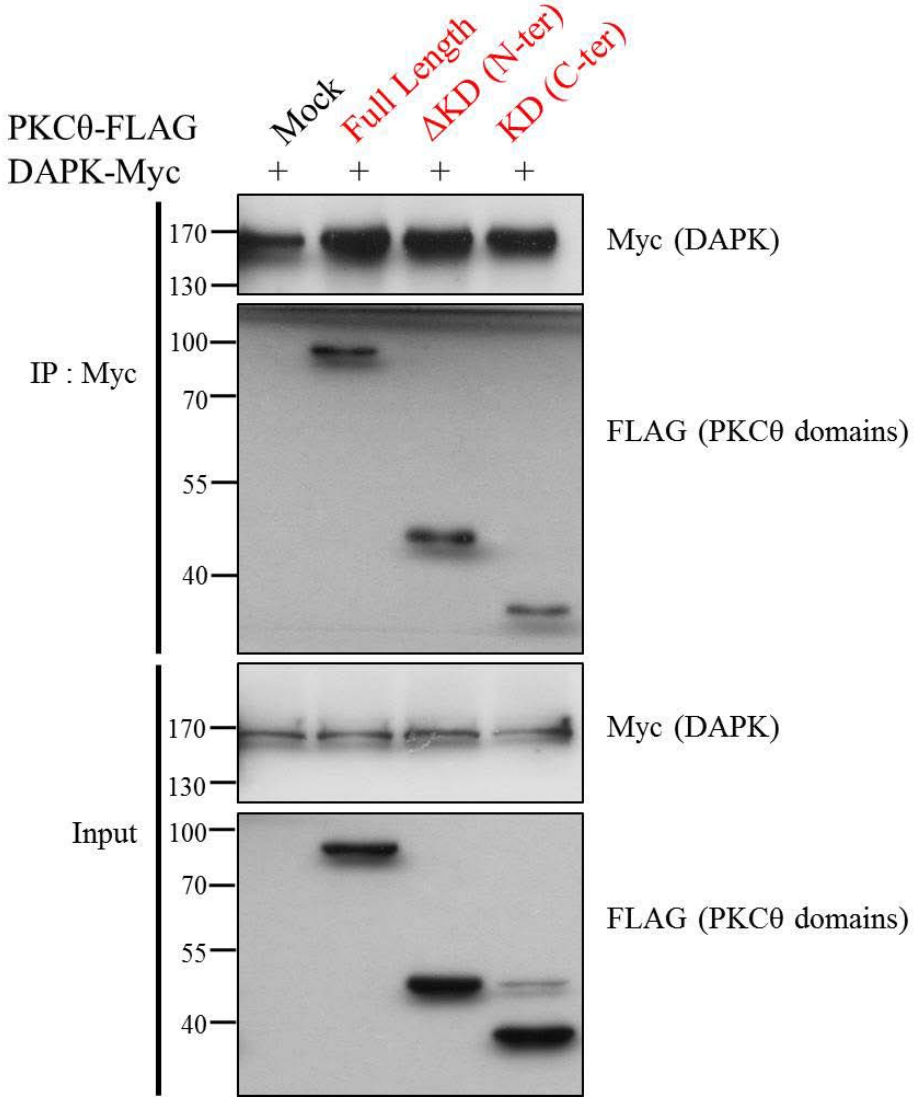


Figure 7. The interaction between PKCθ and DAPK is mediated by multiple domains.

HEK293T cells were transfected with Flag-tagged PKCθ fragments containingkinase-deletion domain (N-terminus) and kinase domain (C-terminus) (App. 3), in addition to DAPK-myc. Cell lysates were prepared 48 hours later, and were immunoprecipitated with anti-myc. The presences of PKCθ fragments were detected by anti-FLAG. Input indicates the contents of DAPK-myc and PKCθ fragments in cell lysates.

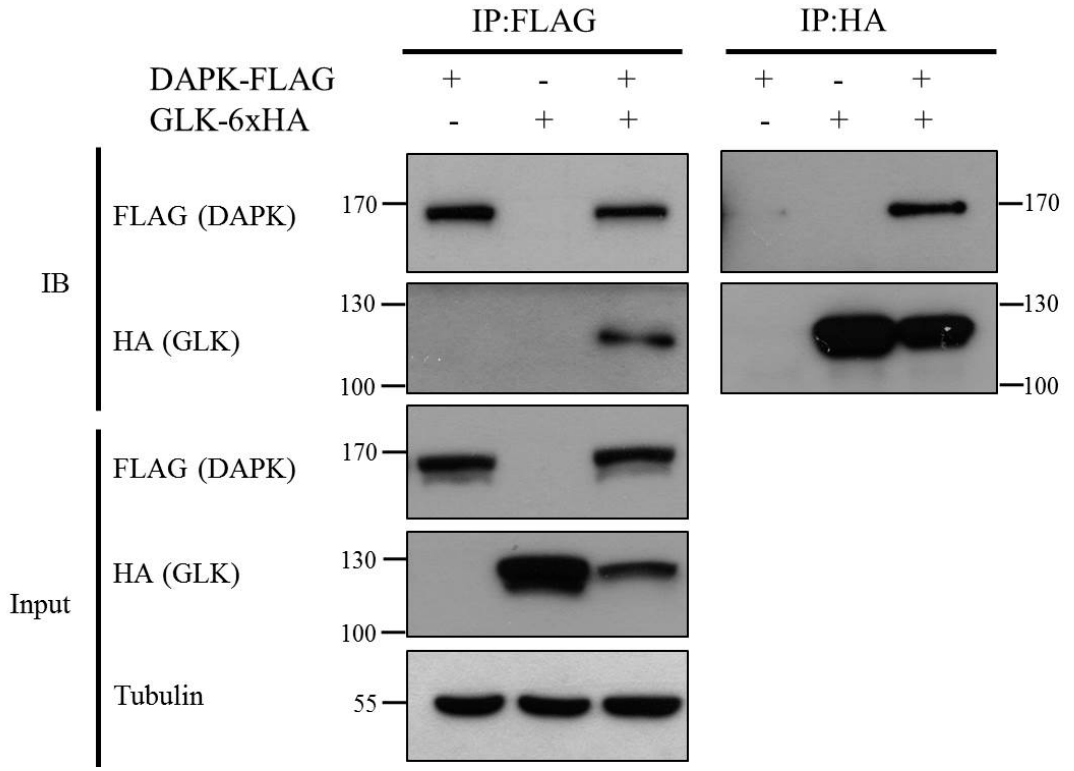


Figure 8. Interaction of DAPK with MAP4K3/GLK in HEK 293T cell

HEK293T cells were transfected with DAPK-FLAG and MAP4K3/GLK-6xHA as indication. Cell lysates were prepared 48 hours later, and were precipitated with anti-FLAG or anti-HA. The presence of DAPK and MAP4K3/GLK was detected by anti-FLAG and anti-HA respectively.

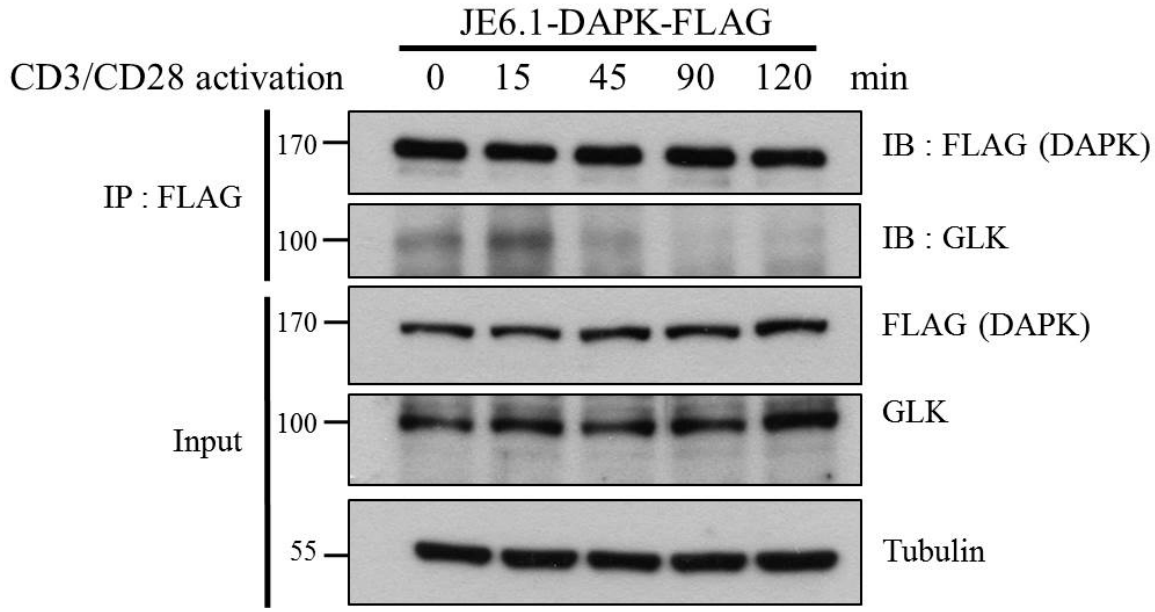


Figure 9. Interaction of DAPK with MAP4K3/GLK in Jurkat JE6.1 T cell

Jurkat JE6.1 T cell expressing DAPK-FLAG (Fig.1) were activated by anti-CD3/anti-CD28, and cell lysates were prepared at the indicated time points. Cell lysates were precipitated with anti-FLAG, and the presence of MAP4K3/GLK was determined by anti-GLK. Input indicates the levels of DAPK-FLAG and MAP4K3/GLK in cell lysates.

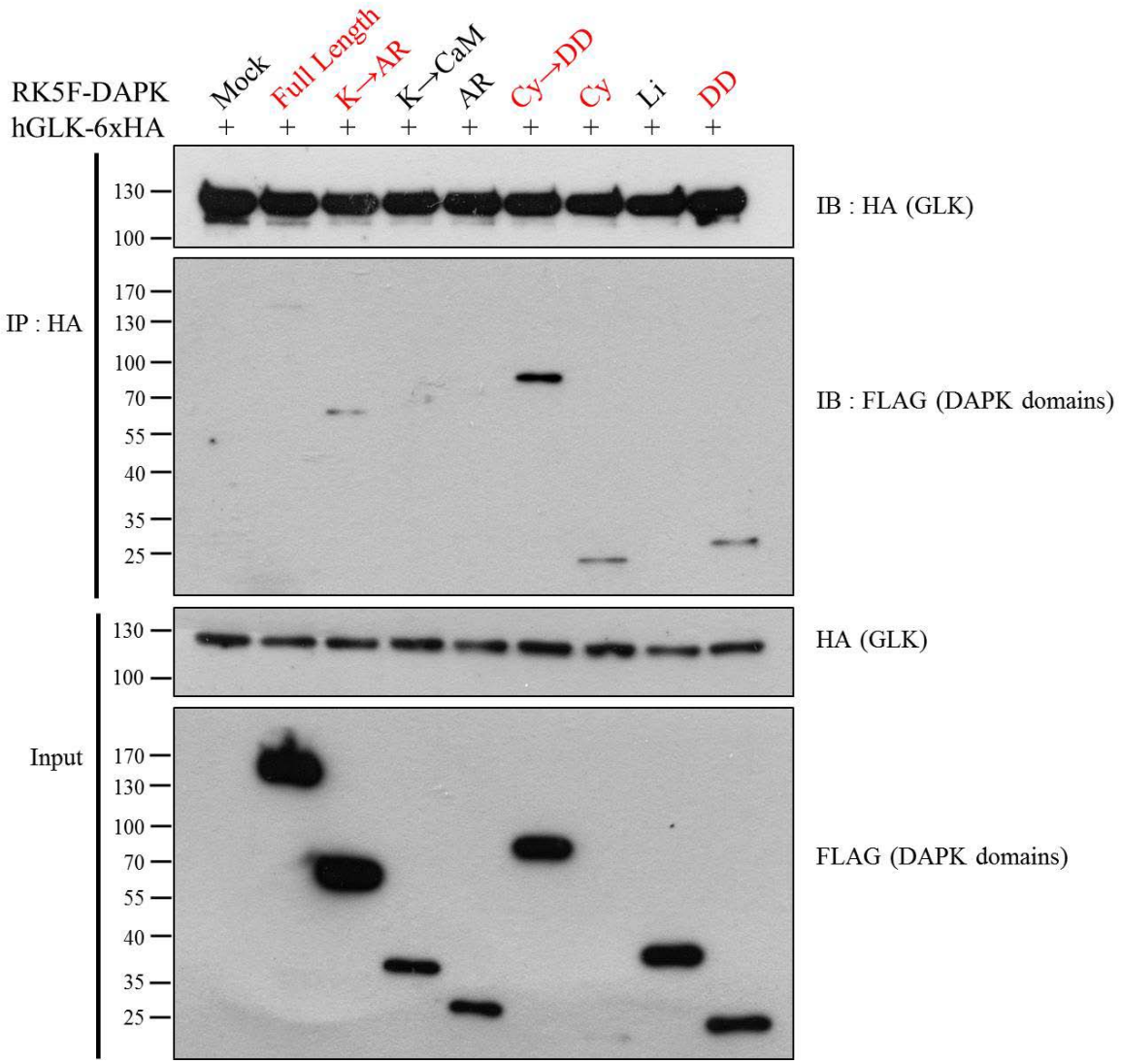


Figure 10. The interaction between DAPK and MAP4K3/**GLK is mediated by multiple domains.**

HEK293T cells were transfected with Flag-tagged DAPK fragments containing kinase domain (K), calcium/calmodulin regulatory domain (CaM), ankyrin repeats domain (AR), cytoskeleton binding domain (Cy), linking domain (Li), or death domain (DD) (App. 1), in addition to MAP4K3/GLK-6xHA. Cell lysates were prepared 48 hours later, and were immunoprecipitated with anti-HA. The presences of DAPK fragments were detected by anti-FLAG. Input indicates the contents of GLK-HA and DAPK fragments in cell lysates.

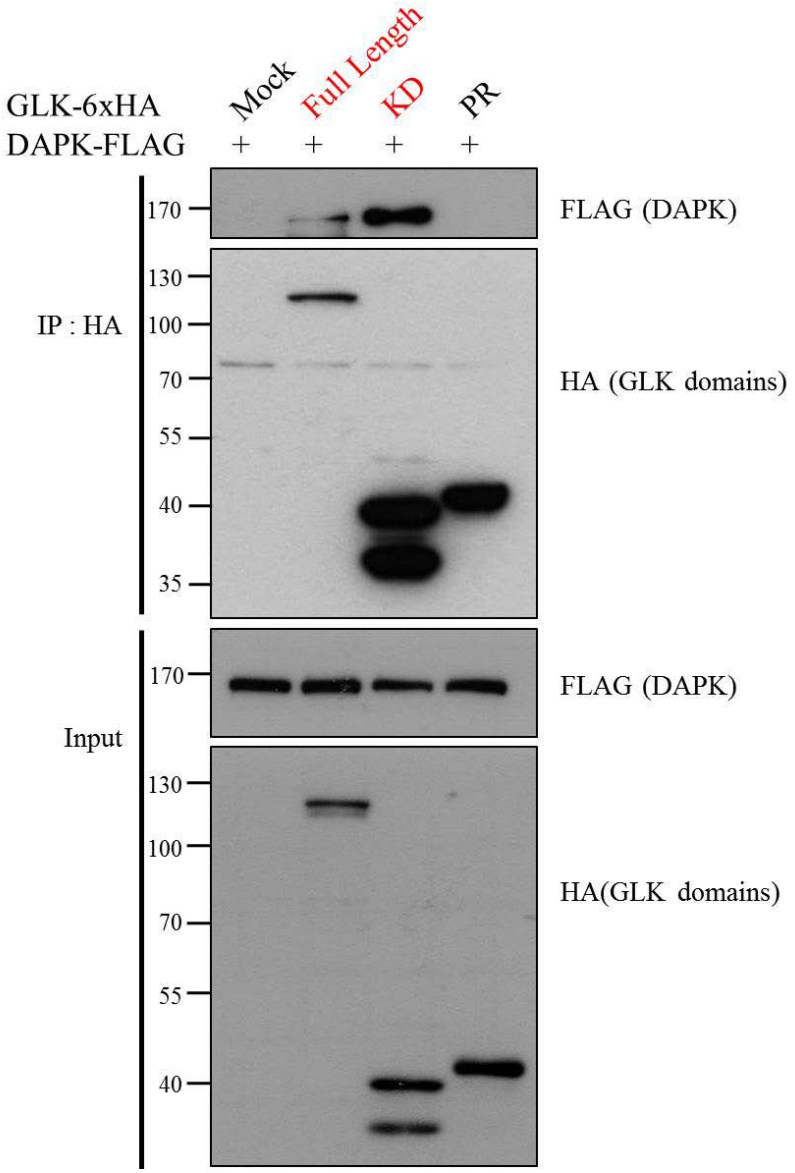


Figure 11. The interaction between MAP4K3/GLK and DAPK is mediated by multiple domains.

HEK293T cells were transfected with HA-tagged MAP4K3/GLK fragments containing kinase domain (KD) and proline-rich domain (PR)(App. 4) in addition to DAPK-FLAG. Cell lysates were prepared 48 hours later, and were immunoprecipitated with anti-HA. The presences of DAPK were detected by anti-FLAG. Input indicates the contents of DAPK-FLAG and MAP4K3/GLK fragments in cell lysates.

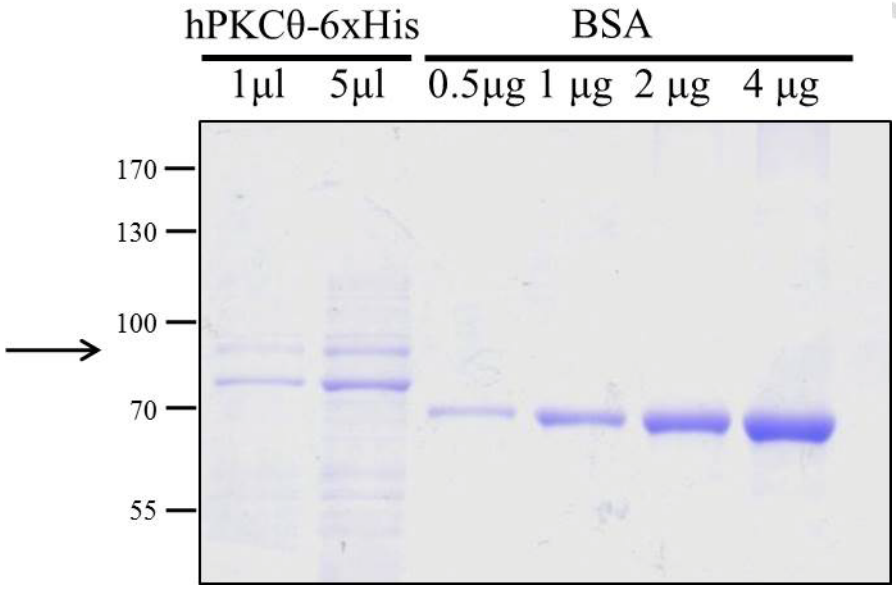


Figure 12. Generation of the recombinant protein of PKCθ

The recombinant PKCθprotein was purified with anti-6xHis antibody coated beads.

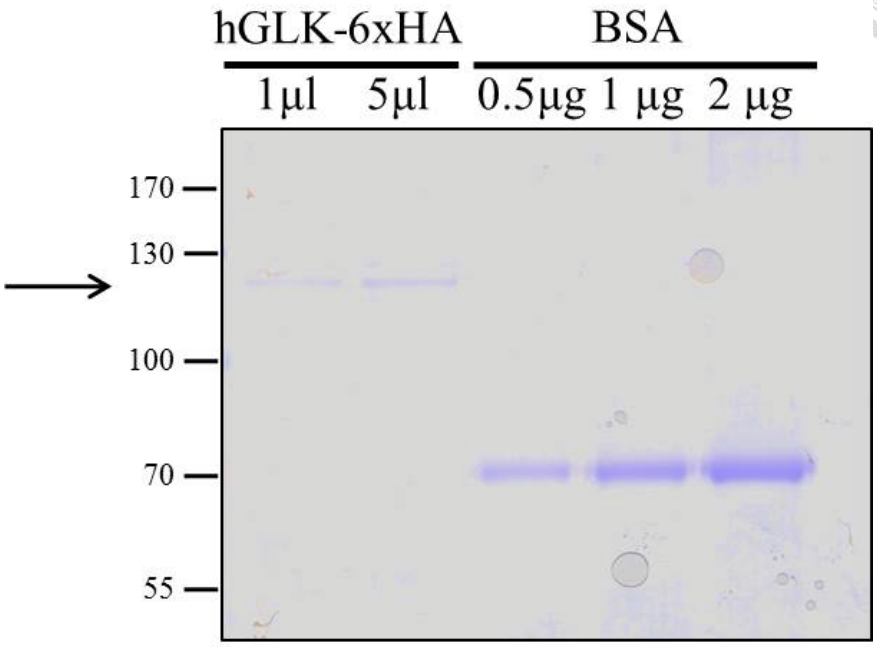


Figure 13. Generation of the recombinant protein of MAP4K3/GLK

The recombinant MAP4K3/GLK protein was purified from cell lysate by anti-6xHA antibody coated beads.

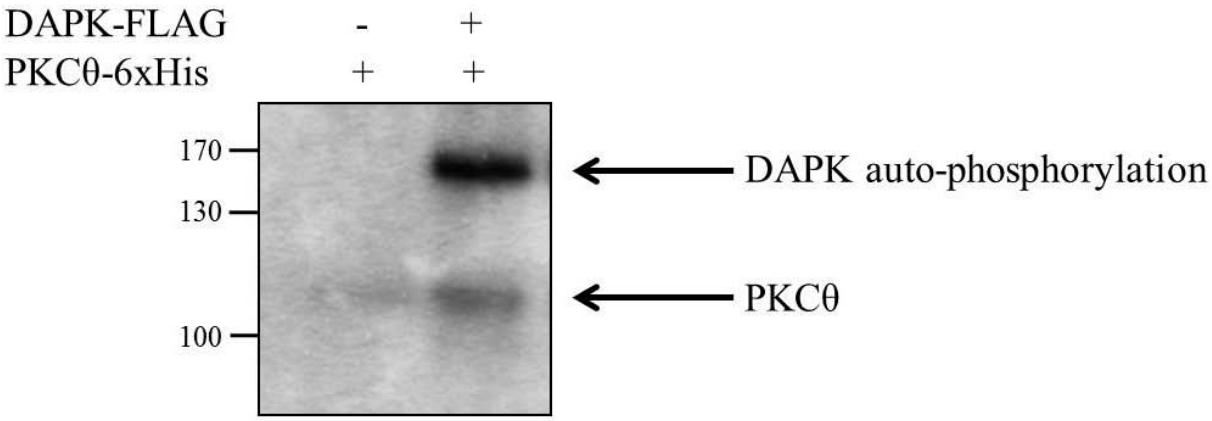


Figure 14. Auto-phosphorylation of PKCθ

Recombinant DAPK was incubated with PKCθ-6xHis in kinase buffer and γ^{32}P-ATP. The kinase reaction was proceeded for 30 minutes at 25℃. The reaction mixture was resolved by SDS-PAGE and the phosphorylation was determined by autoradiography.

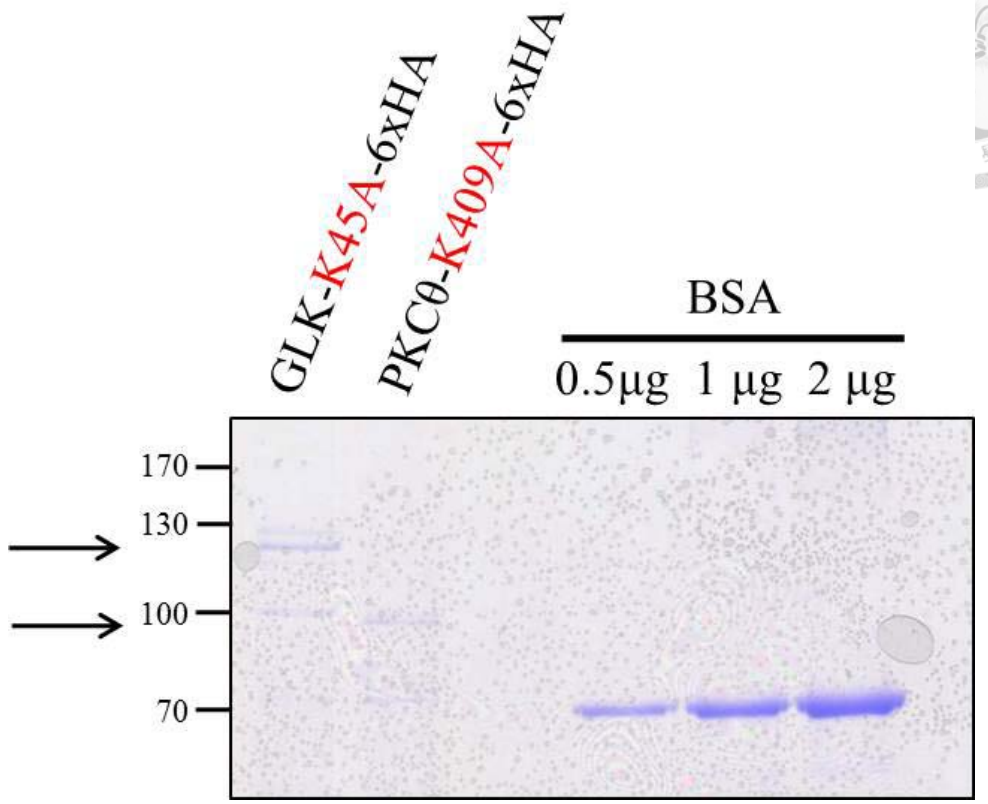


Figure 15. Generation of the kinase-dead recombinant protein of MAP4K3/GLK and PKCθ

The kinase-dead recombinant protein of MAP4K3/GLK and PKCθwere purified from cell lysate by anti-6xHA antibody coated beads.

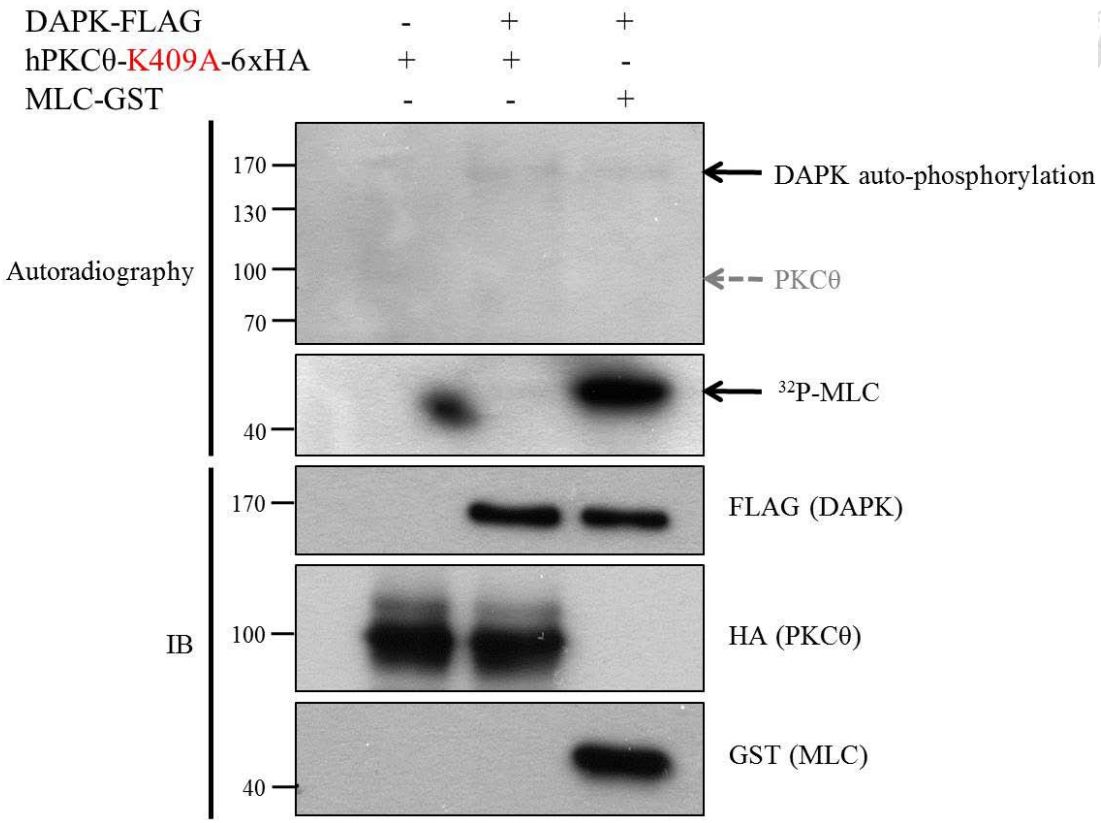


Figure 16. PKCθ is not a substrate of DAPK.

Recombinant DAPK was incubated with PKCθ-K409A-6xHA or myosin light chain (MLC) in kinase buffer and γ^{32}P-ATP. The kinase reaction was proceeded for 30 minutes at 25℃. The reaction mixture was resolved by SDS-PAGE and the phosphorylation was determined by autoradiography. The Western blotting of the reaction mixture was used as loading control.The quantity of DAPK was detected by anti-FLAG, and the quantity of PKCθ is detected by anti-HA, and the quantity of MLC is detected by anti-GST.

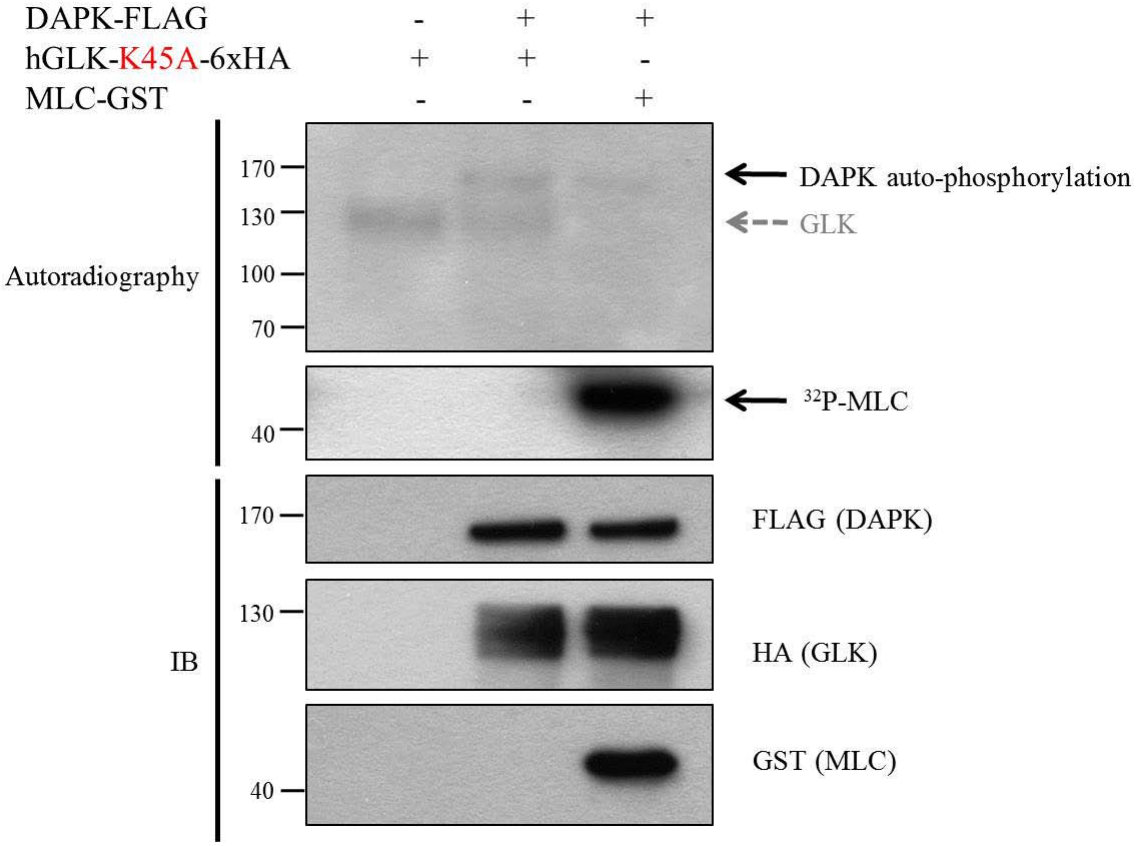


Figure 17. MAP4K3/GLK is not a substrate of DAPK.

Recombinant DAPK was incubated with MAP4K3/GLK-K45A-6xHA or myosin light chain (MLC)in kinase buffer and γ^{32}P-ATP. The kinase reaction was proceeded for 30 minutes at 25℃. The reaction mixture was resolved by SDS-PAGE and the phosphorylation was determined by autoradiography. The Western blotting of the reaction mixture was used as loading control.The quantity of DAPK was detected by anti-FLAG, and the quantity of MAP4K3/GLK is detected by anti-HA, and the quantity of MLC is detected by anti-GST.

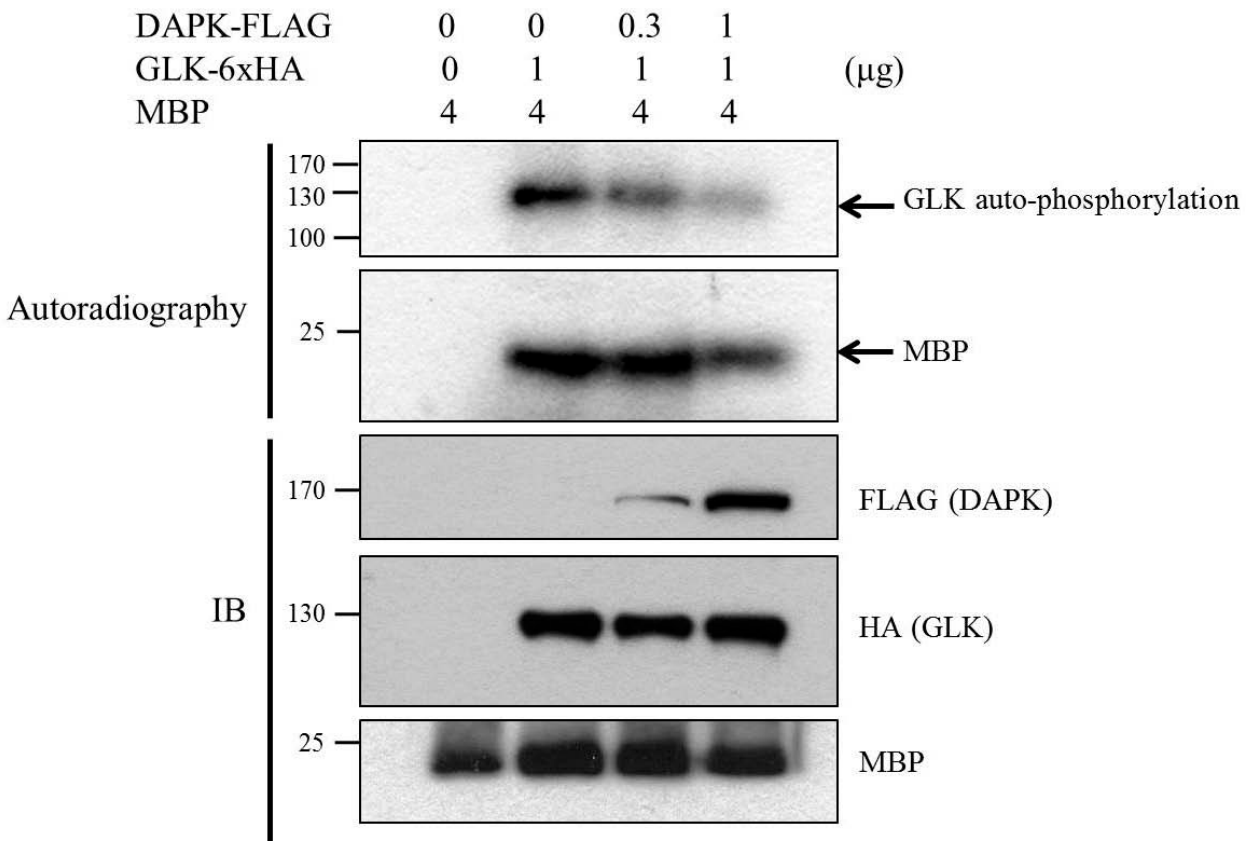


Figure 18. The presence of DAPK reduces the kinase activity of MAP4K3/GLK

Recombinant MAP4K3/GLK-6xHA and myelin basic protein (MBP) were incubated with 0μg, 0.3μg or 1μg DAPK in kinase buffer and γ^{32}P-ATP at 25℃ for 30 minutes. The reaction mixture was resolved by SDS-PAGE and the phosphorylation was determined by autoradiograghy.The quantity of DAPK was detected by anti-FLAG, and the quantity of MAP4K3/GLK is detected by anti-HA, and the quantity of MBP is detected by anti-MBP.

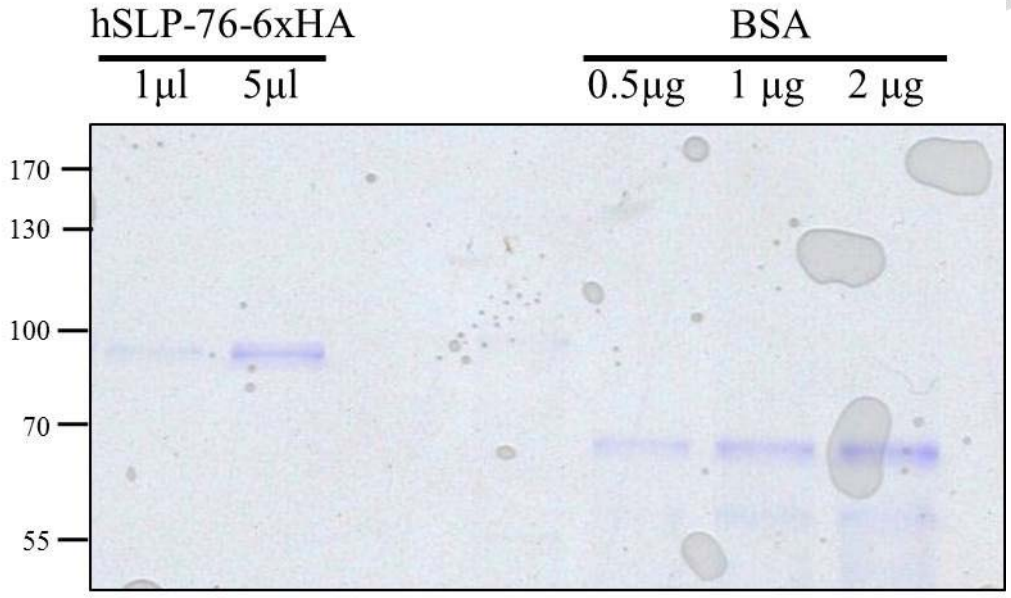


Figure 19. Generation of the recombinant protein of SLP-76

The recombinant SLP-76 protein was purified from cell lysate by anti-6xHA antibody coated beads.

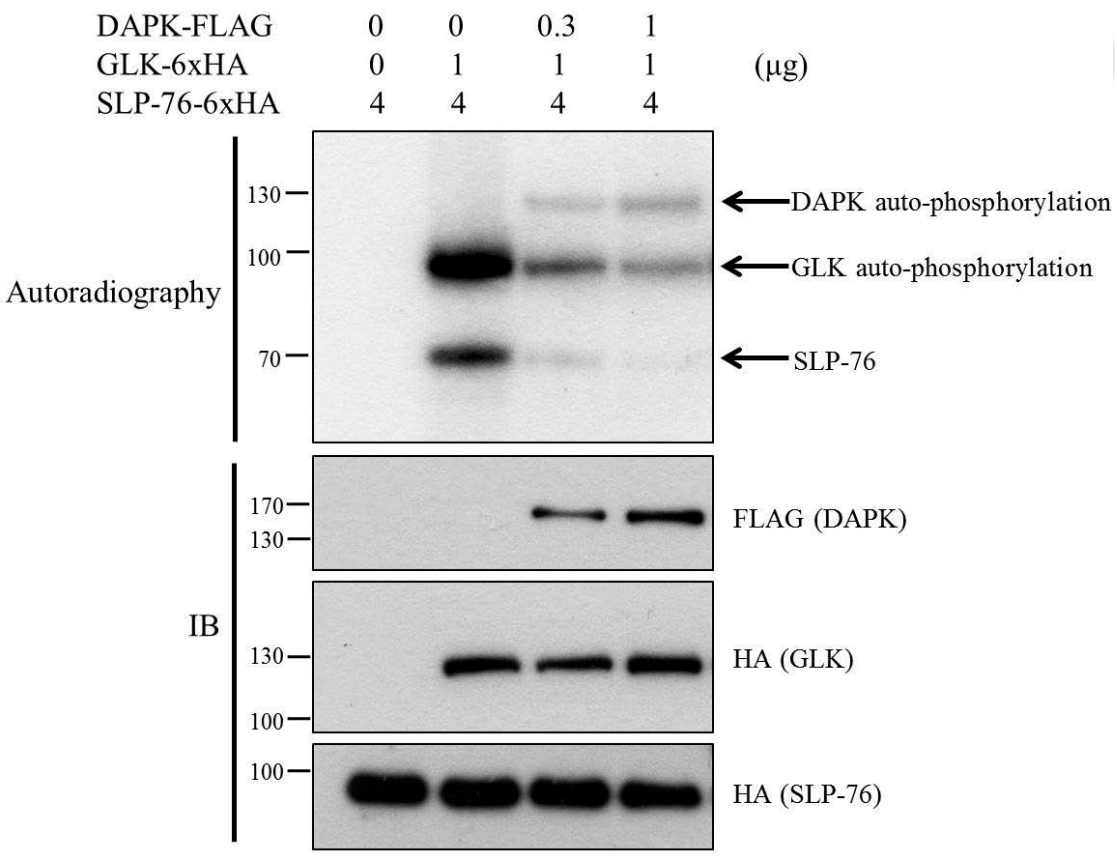


Figure 20. The presence of DAPK reduces the kinase activity of MAP4K3/GLK.

Recombinant MAP4K3/GLK-6xHA and SLP-76-6xHA were incubated with 0μg, 0.3μg or 1μg DAPK in kinase buffer and γ^{32}P-ATP at 25℃ for 30 minutes. The reaction mixture was resolved by SDS-PAGE and the phosphorylation was determined by autoradiograghy.The quantity of DAPK was detected by anti-FLAG, and the quantity of MAP4K3/GLK and SLP-76 are detected by anti-HA.

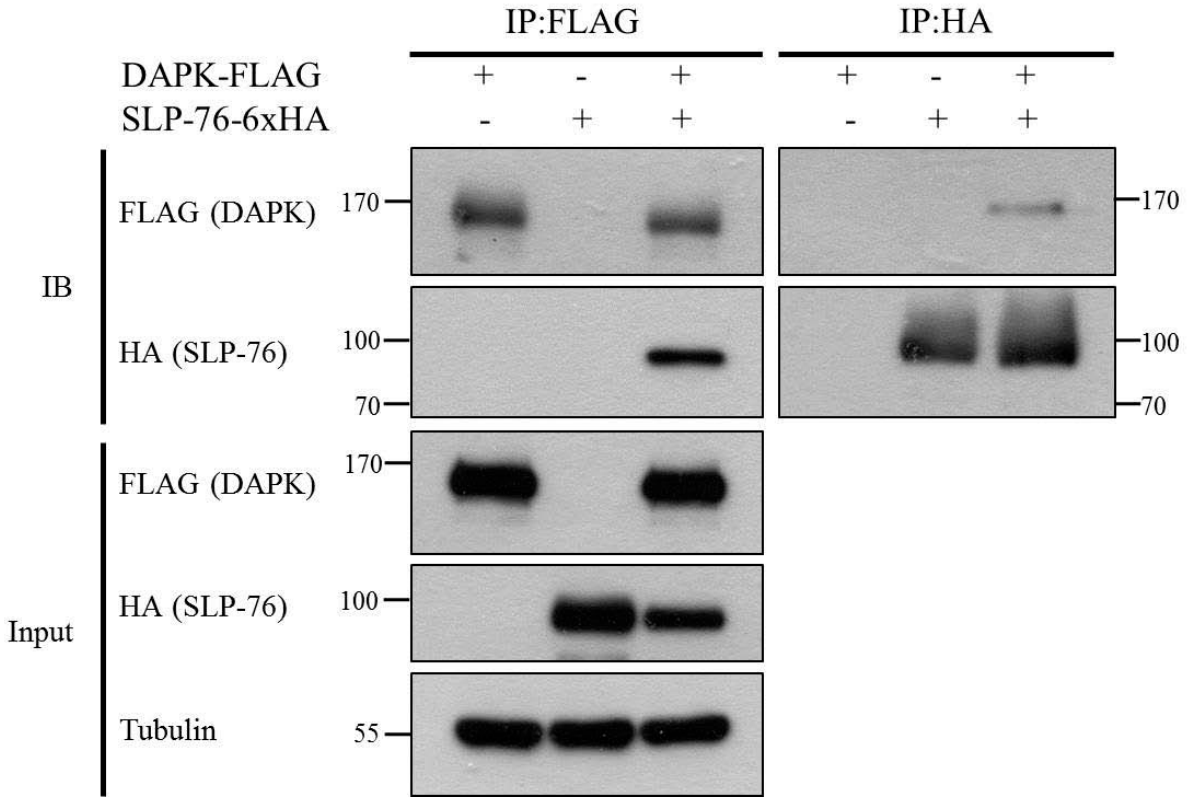


Figure 21. Interaction of DAPK with SLP-76

HEK293T cells were transfected with DAPK-FLAG and SLP-76-6xHA as indication. Cell lysates were prepared 48 hours later, and were precipitated with anti-FLAG or anti-HA. The presence of DAPK and SLP-76 was detected by anti-FLAG and anti-HA respectively.

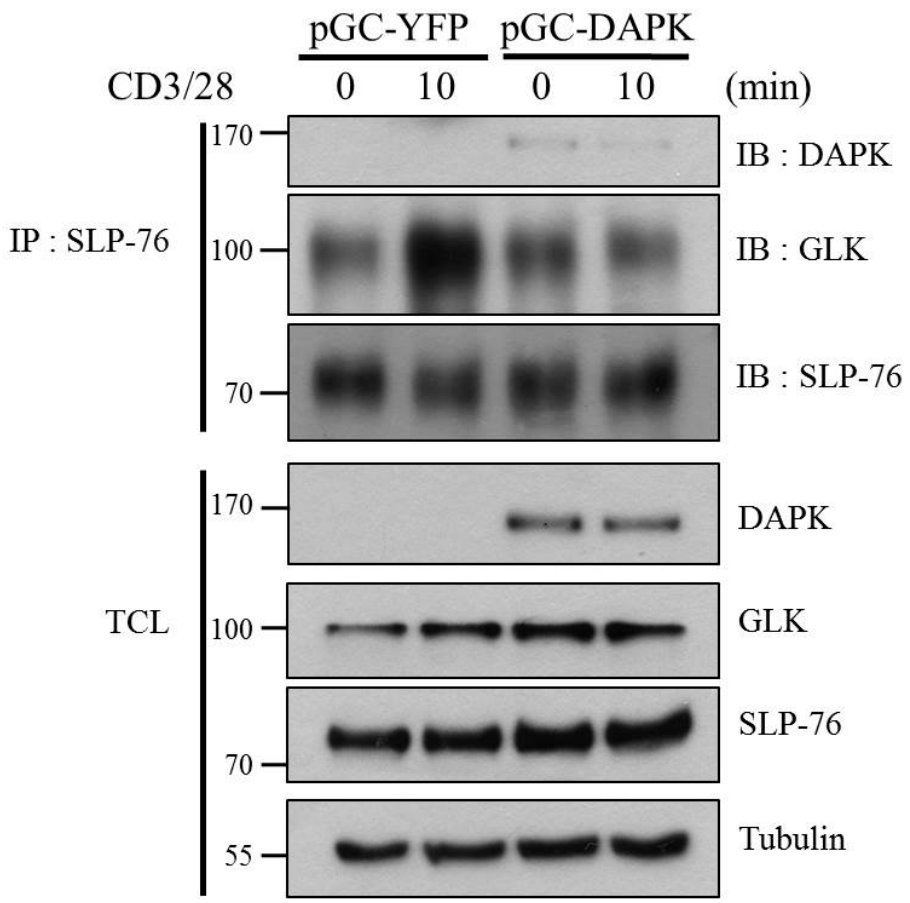


Figure 22. The association of MAP4K3/GLK and SLP-76 is attenuated by DAPK in Jurkat JE6.1 T cells.

Control and DAPK-overexpression Jurkat JE6.1 T cells (Fig. 1) were activated by anti-CD3/anti-CD28, and cell lysates were prepared at the indicated time points. Cell lysates were precipitated with anti-SLP-76. The presence of MAP4K3/GLK was determined by anti-GLK, and the presence of SLP-76 was determined by anti-SLP-76. Total cell lysate indicates the levels of DAPK, MAP4K3/GLK, and SLP-76 in cell lysates, and the contents of DAPK, MAP4K3/GLK, and SLP-76were determined by immunoblot.

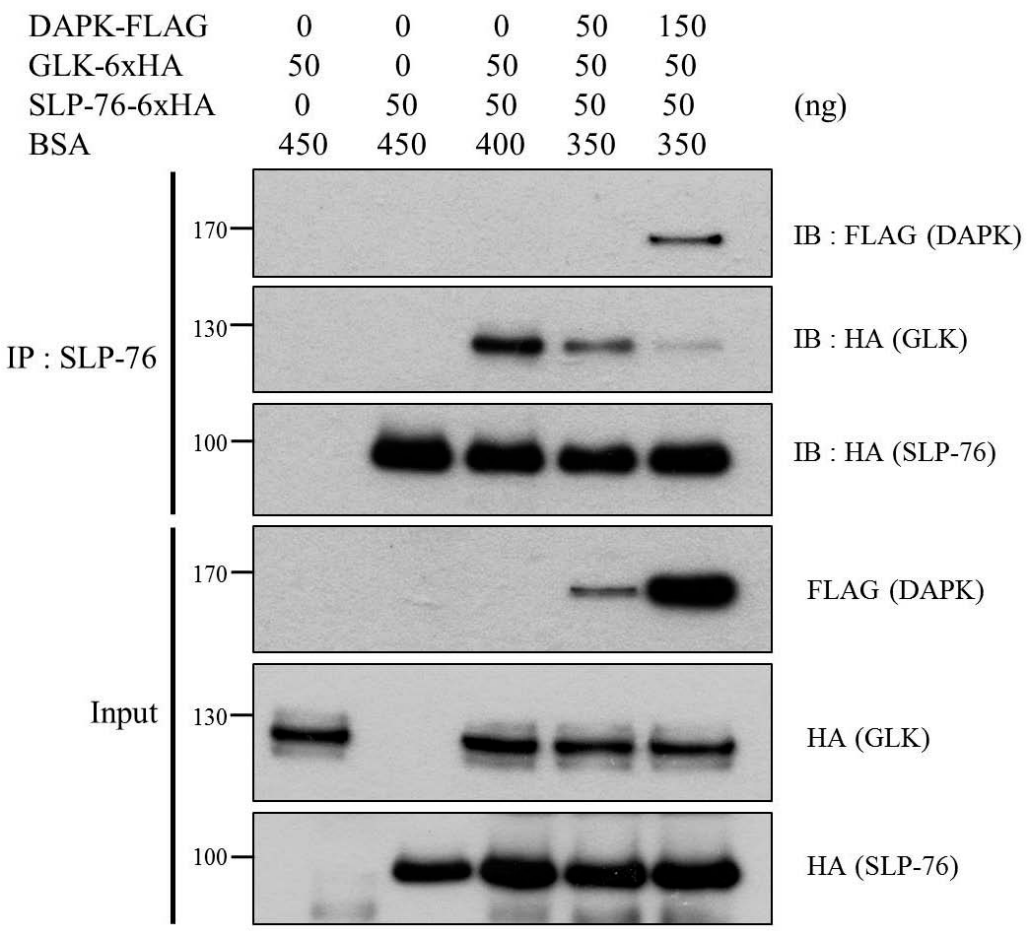


Figure 23. The association of MAP4K3/GLK and SLP-76 is inhibited by DAPK *in vitro*.

Recombinant MAP4K3/GLK-6xHA and SLP-76-6xHA were incubated with or without the presence of recombinant DAPK-FLAG in binding buffer. The reaction mixture was incubated for 2hours at 4℃, and anti-SLP-76 antibody was added to incubate for 16 hours. The reaction mixture was resolved by SDS-PAGE.Input indicates the levels of DAPK-FLAG, MAP4K3/GLK-6xHA, and SLP-76-6xHA in the reaction mixture, and the contents of DAPK-FLAG, MAP4K3/GLK-6xHA, and SLP-76-6xHA were determined by immunoblot.

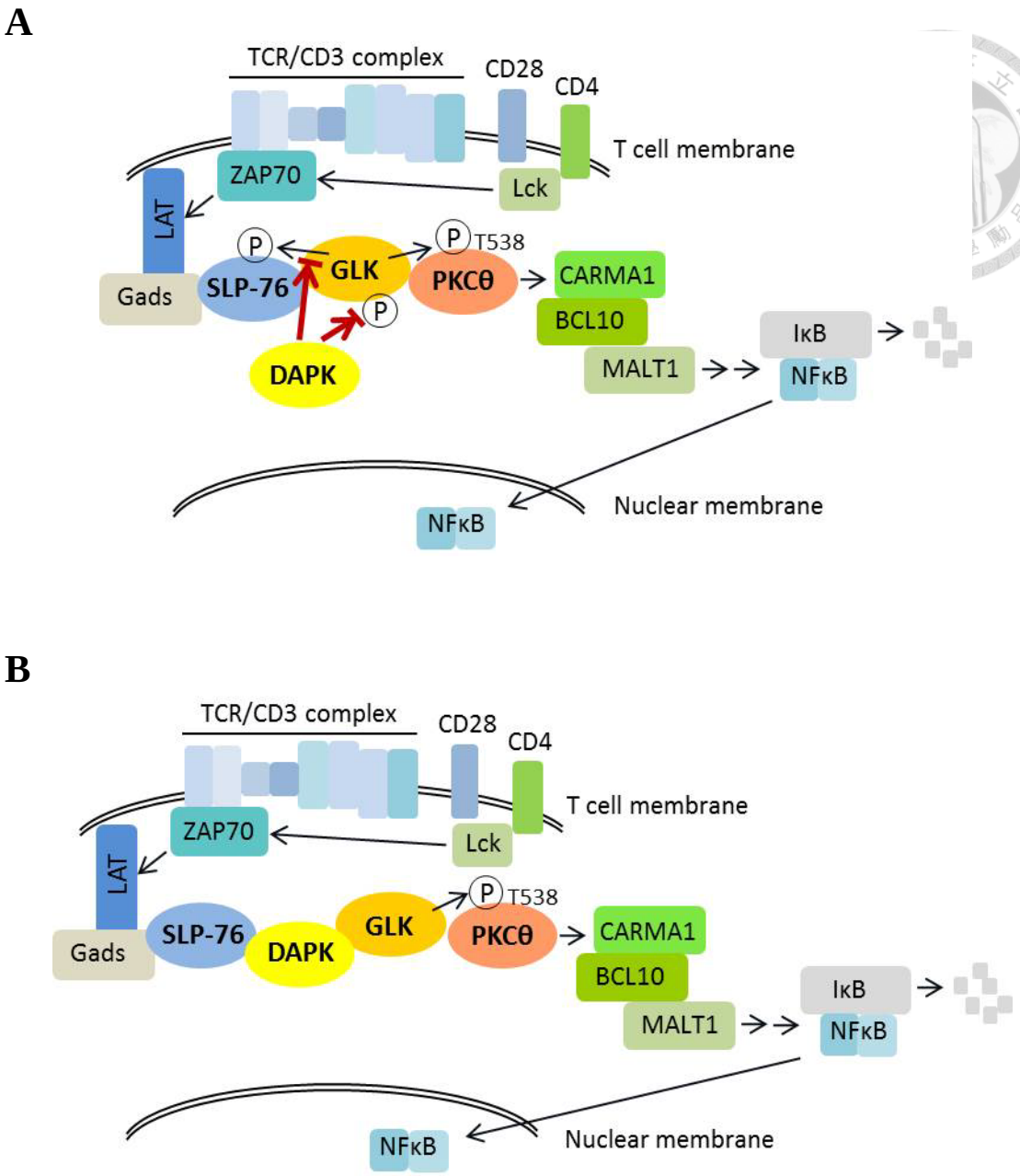
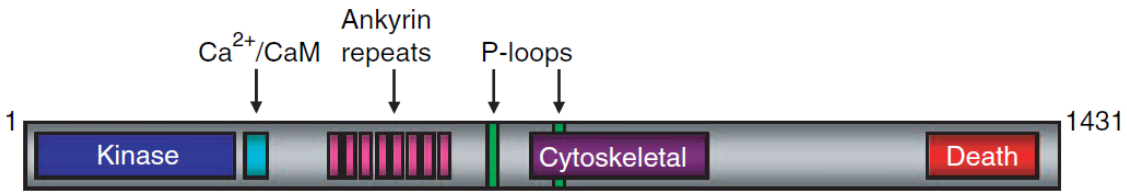


Figure 24. Model on DAPK-mediated inhibition of PKCθ activation

Results from this study suggest two different mechanisms that DAPK inhibits TCR-induced PKCθ activation. First, DAPK inhibits the kinase activity of MAP4K3/GLK (A),second, DAPK blocks the association of SLP-76 and MAP4K3/GLK, which prevents the fully activation of MAP4K3/GLK (B).

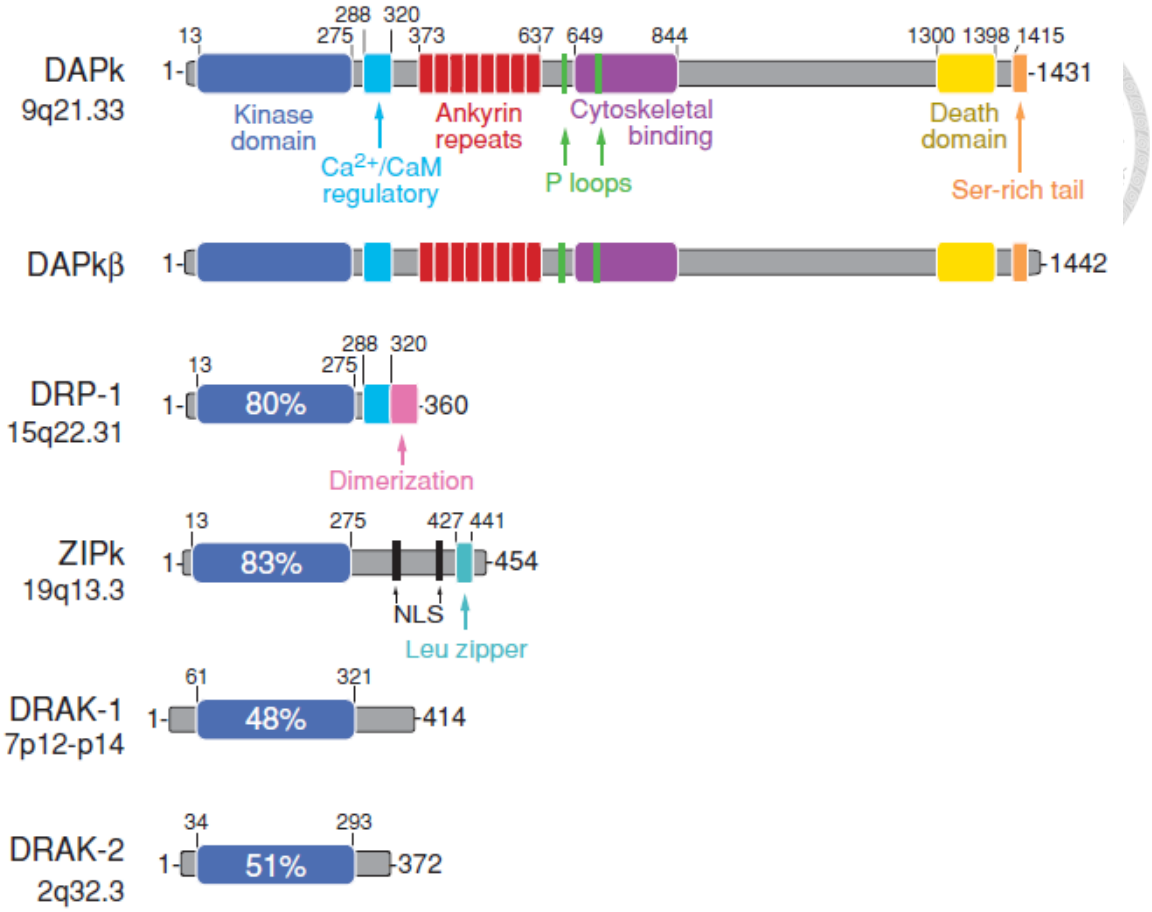
Chapter VII Appendix



Appendix 1. DAPK protein structure

DAPK is protein composed of multiple domains. Kinase domain locates at the N-terminus, and following with a CaM-regulated domain, eight ankyrin repeats, two P-loop motifs, a cytoskeleton domain, and a death domain.

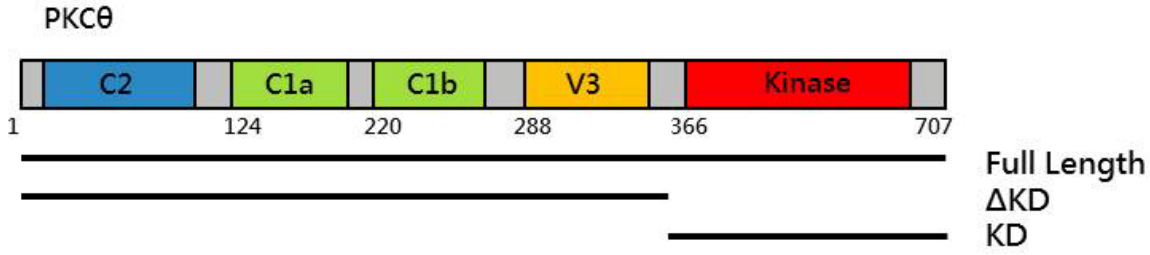
(From FEBS J. 2010 Jan;277(1):48-57)



Appendix 2. DAPK family

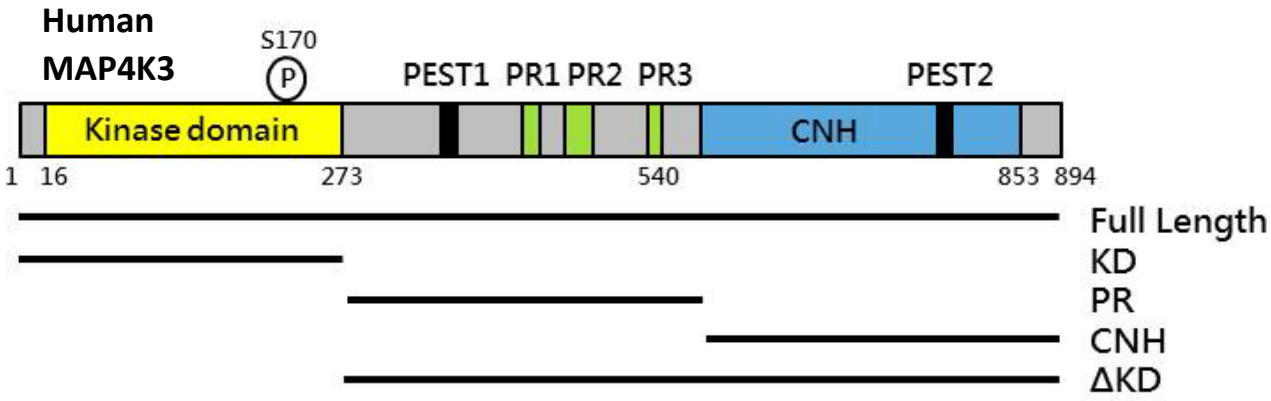
DAPK family is composed of death associated protein kinase (DAPK), death associated protein kinase related protein 1/ death associated protein kinase 2 (DRP-1/ DAPK2), DAP like kinase/ Zipper interacting protein kinase (Dlk/ ZIP-kinase), DAP kinase related apoptosis inducing protein kinase 1 (DRAK1), and DAP kinase related apoptosis inducing protein kinase 2. The sequence consistent among the five members is limited in the kinase domain located at N terminus.

(From Annu Rev Biochem. 2006;75:189-210)



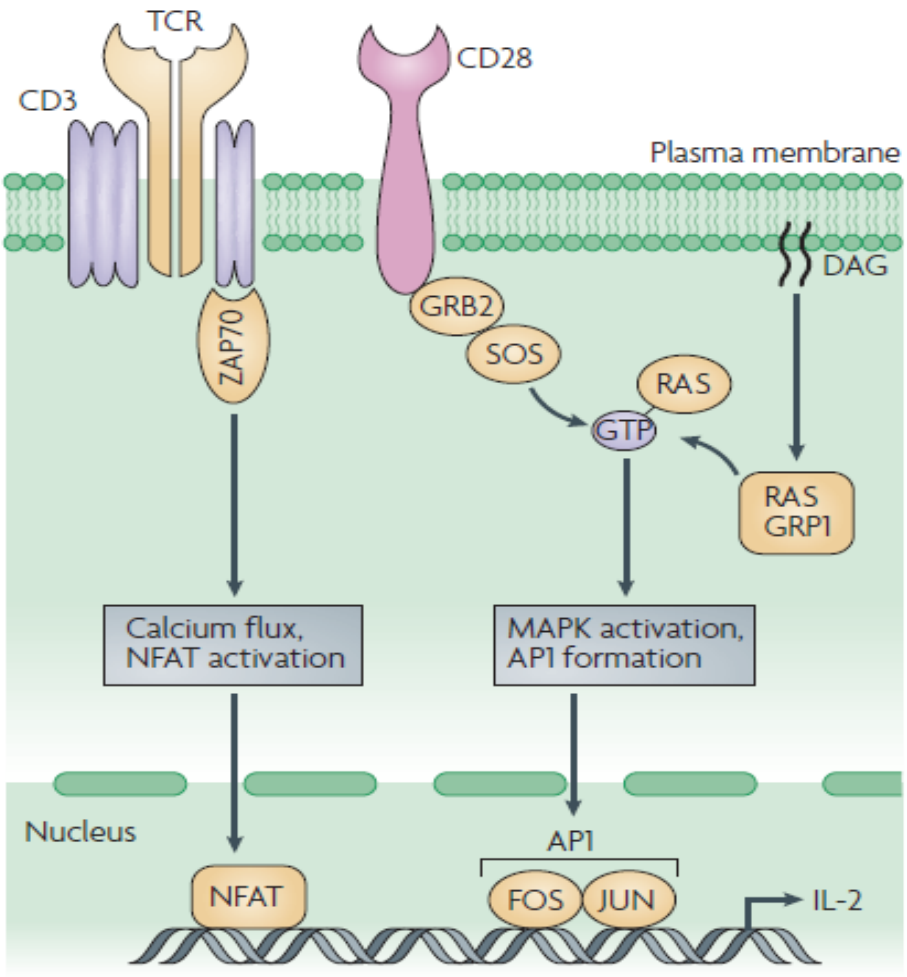
Appendix 3. PKCθ protein structure

PKCθ is a protein composed of multiple domains. C2 domain locates at the N-terminus, and following with C1a domain, C1b domain, V3 domain, and the kinase domain locates at the C-terminus.



Appendix 4. MAP4K3/GLK protein structure

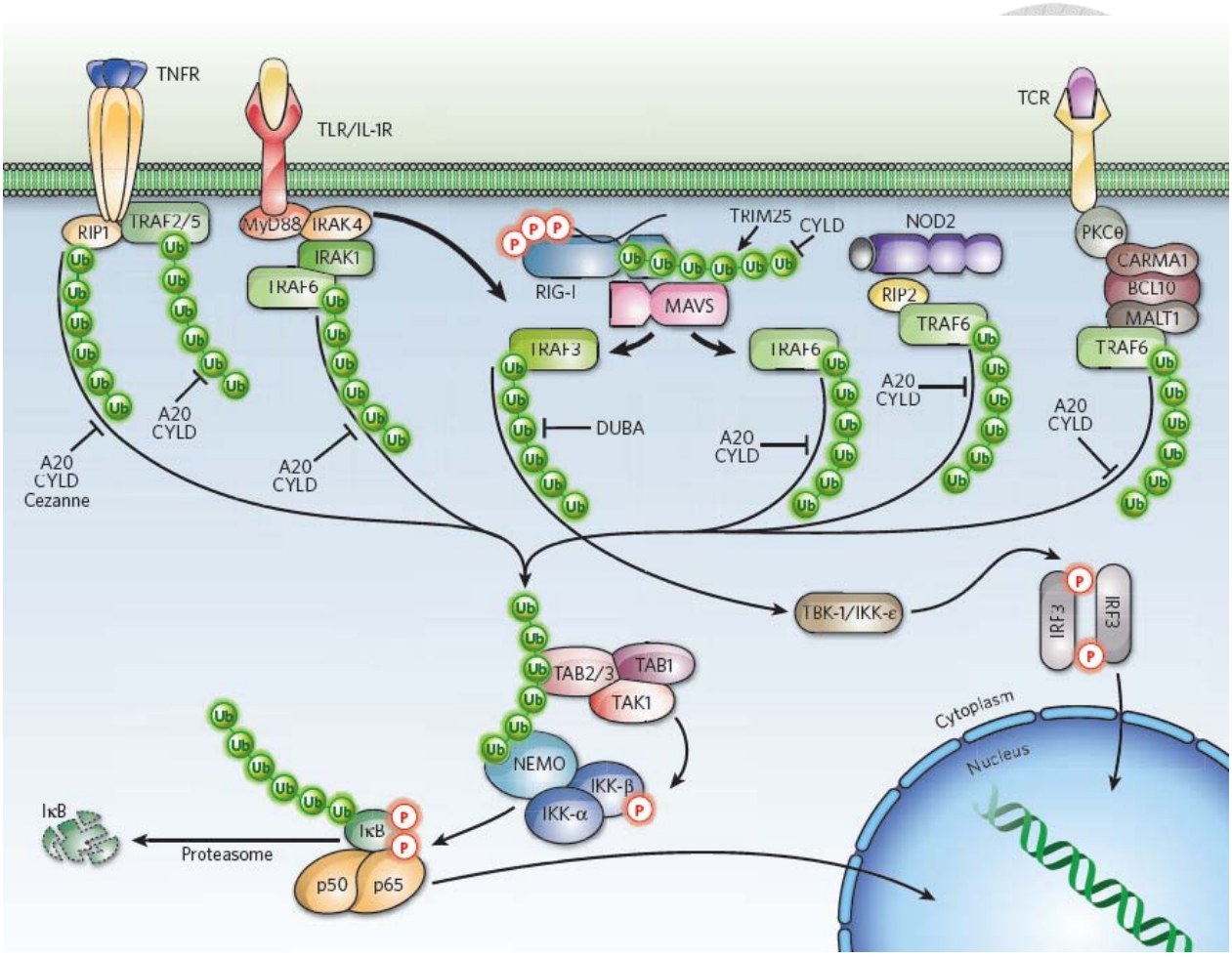
MAP4K3/GLK is a protein composed of multiple domains. Kinase domain locates at the N-terminus, and following with three proline-rich domainsand a C-terminal citron homologydomain.



Appendix 5. Signal transduction of TCR-induced T cell activation

The stimulation of cell surface receptors TCR and CD28 induces the activation of Ca^{2+} cation, PKC and Ras, which consequently activates transcriptional factors NF-κB, NFAT and AP-1. Then the *il2* gene expression is drove and the production of IL-2 is promoted.

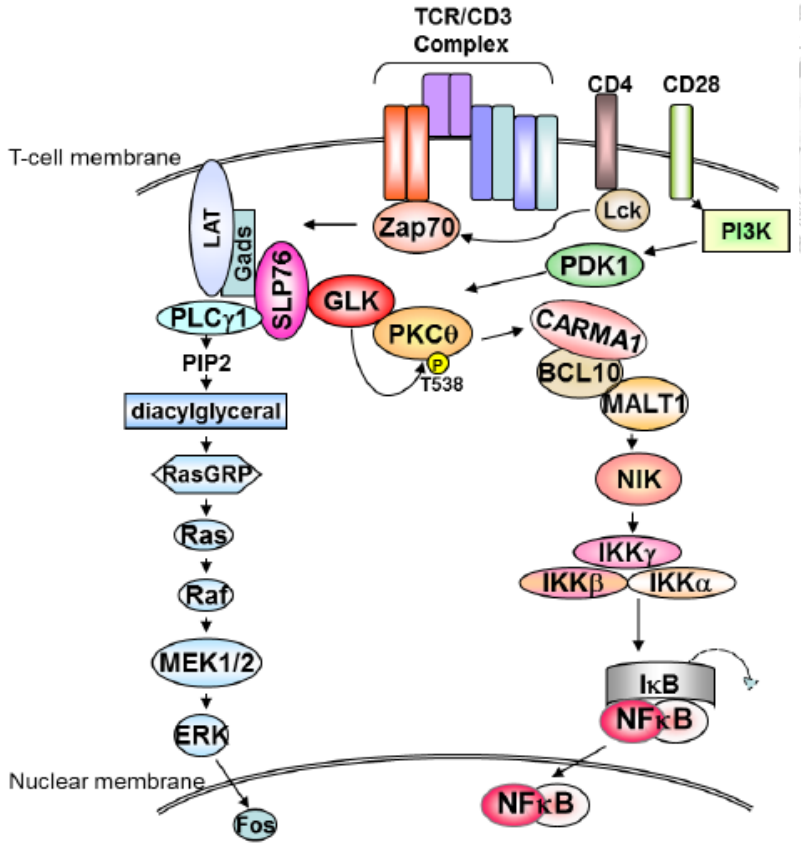
(From Nat Rev Immunol. 2007 Aug;7(8):599-609)



Appendix 6. NF-κB pathway

There are three distinct pathways to activate NF-κB pathway in T cells. The activation of TCR stimulates the activation of PKCθ, and the signal is transduced to Bcl-10, MALT1 and CARMA1 complex, and then the downstream IKK complex is activated consequently. Activated TNF receptor recruits TRADD, and binds to TRAF2 to transduce signals to RIP, which result in the activation of IKK. Activated IL-1 receptor or TLRs recruits MyD88, and MyD88 binds to IRAK. Activated IRAK phosphorylates TRAF6, and transduces signals to TAK-1, TAB-1, and TAB-2, and then activates the IKK complex. Activated IKK complex phosphorylates IκBα, and leads to the activation of NF-κB.

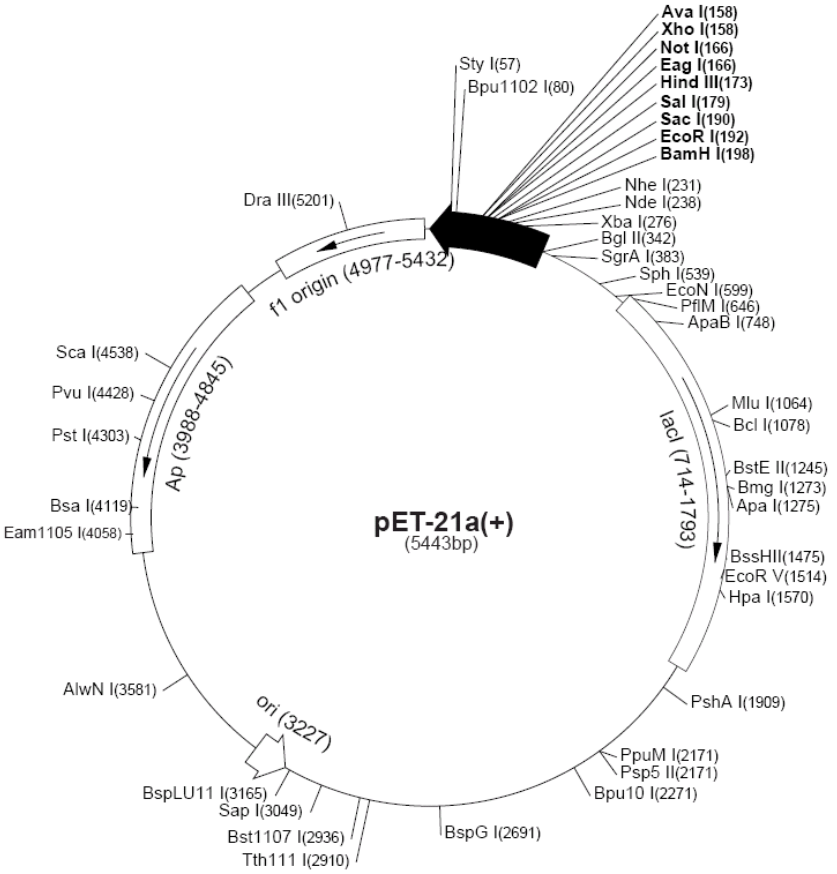
(From Nature. 2009 Mar 26;458(7237):430-7)



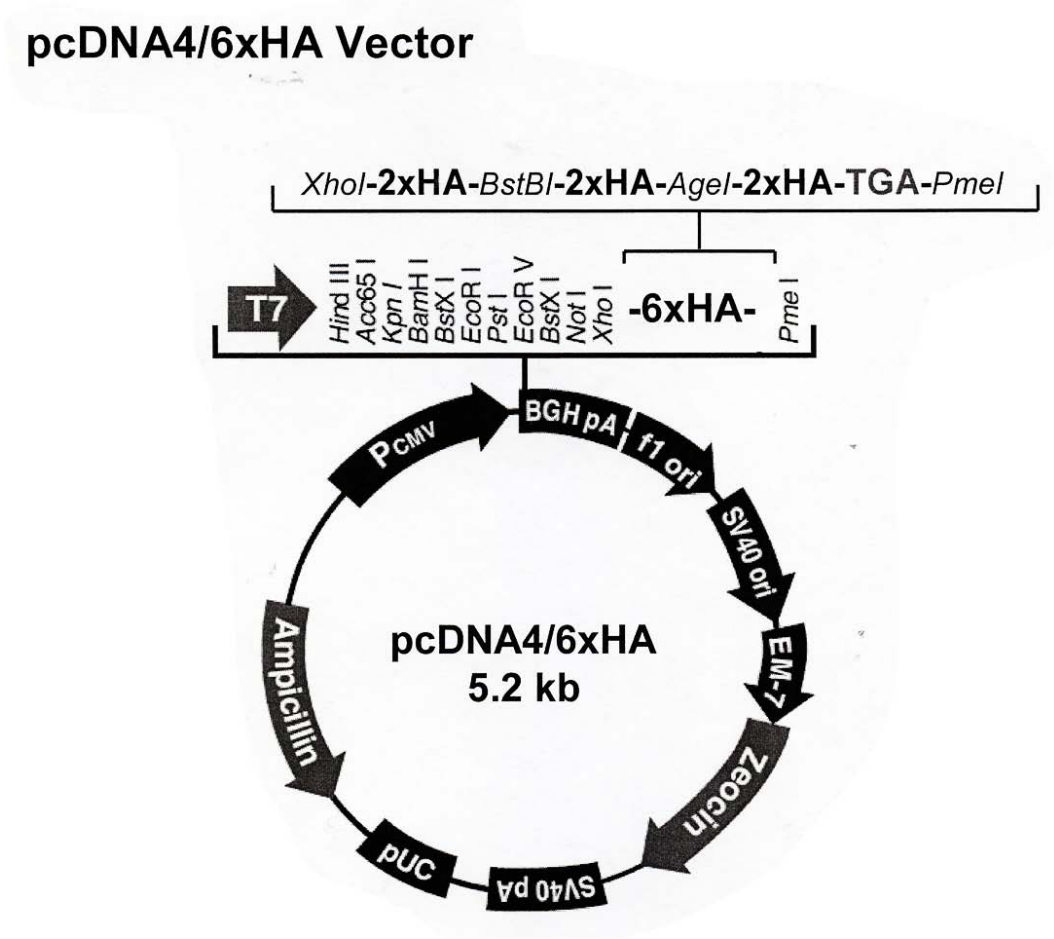
Appendix 7. Diagram of GLK-induced PKCθ/NF-κB activation during TCR signaling

After TCR stimulation, activated Lck is recruited to TCR complex and phosphorylates ITAMs of CD3, resulting in Zap70 recruitment and activation. Zap70 activation induces the assembly of the proximal SLP-76 signaling complex. SLP-76 directly interacts with GLK and is required for GLK kinase activation. The activated GLK directly interacts with and phosphorylates PKCθat Ter538, resulting in PKCθmembrane translocation and kinase activation. The activated PKCθ binds to and phosphorylates the signaling scaffold protein CARMA1, resulting in the activation of CARMA1 and the subsequent assembly of CARMA1-BCL10-MALT1 (CBM) complex. The CBM complex in turn induces the activation of the IKK/NF-κB signaling cascade.

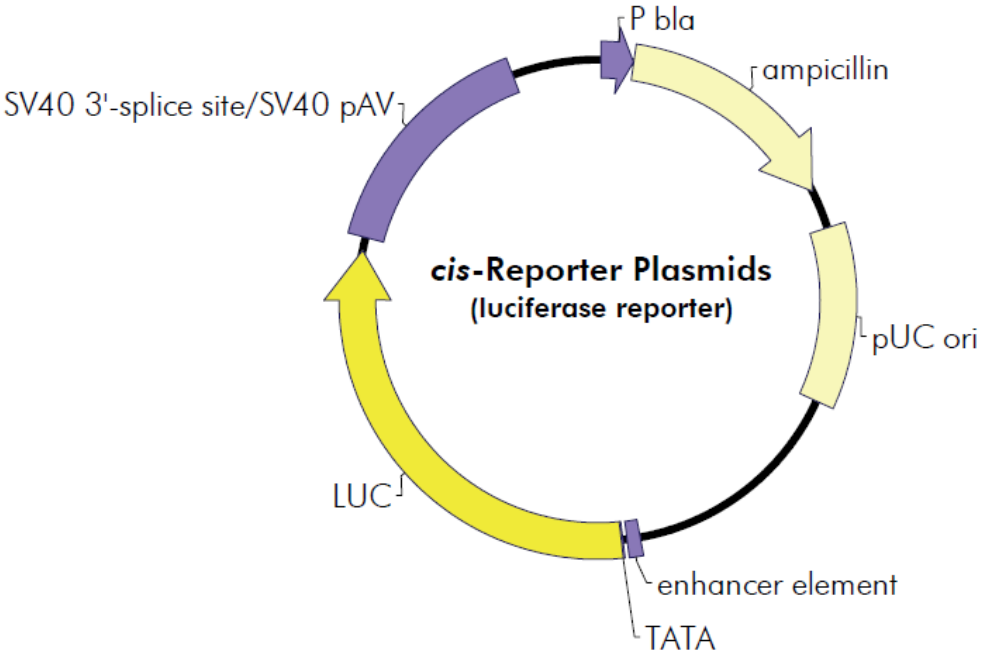
(From Nat Immunol. 2011 Oct 9;12(11):1113-8)



Appendix 8. pET-21a map



Appendix 9. pcDNA4/6xHA map



Appendix 10. κB-Luc schematic diagram

enhancer element : 5x (TGGGGACTTTCCGC)

(From PathDetect in Vivo signal transduction pathway *cis*-reporting system instruction manual, Agilent technologies)