

Protein-Binding Function of RNA-Dependent Protein Kinase Promotes Proliferation through TRAF2/RIP1/NF- κ B/c-Myc Pathway in Pancreatic β cells

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Double-stranded RNA-dependent protein kinase (PKR), an intracellular pathogen recognition receptor, is involved both in insulin resistance in peripheral tissues and in downregulation of pancreatic β -cell function in a kinase-dependent manner, indicating PKR as a core component in the progression of type 2 diabetes. PKR also acts as an adaptor protein via its protein-binding domain. Here, the PKR protein-binding function promoted β -cell proliferation without its kinase activity, which is associated with enhanced physical interaction with tumor necrosis factor receptor-associated factor 2 (TRAF2) and TRAF6. In addition, the transcription of the nuclear factor kappa-light-chain-enhancer of activated B cell (NF- κ B)-dependent survival gene *c-Myc* was upregulated significantly and is necessary for proliferation. Upregulation of the PKR protein-binding function induced the NF- κ B pathway, as observed by dose-dependent degradation of I κ B α , induced nuclear translocation of p65 and elevated NF- κ B-dependent reporter gene expression. NF- κ B-dependent reporter activity and β -cell proliferation both were suppressed by TRAF2-siRNA, but not by TRAF6-siRNA. TRAF2-siRNA blocked the ubiquitination of receptor-interacting serine/threonine-protein kinase 1 (RIP1) induced by PKR protein binding. Furthermore, RIP1-siRNA inhibited β -cell proliferation. Proinflammatory cytokines (TNF α) and glucolipotoxicity also promoted the physical interaction of PKR with TRAF2. Collectively, these data indicate a pivotal role for PKR's protein-binding function on the proliferation of pancreatic β cells through TRAF2/RIP1/NF- κ B/c-Myc pathways. Therapeutic opportunities for type 2 diabetes may arise when its kinase catalytic function, but not its protein-binding function, is downregulated.

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INTRODUCTION

Double-stranded RNA-dependent protein kinase (PKR) is well known as a pathogen recognition receptor against virus infection and a tumor suppressor in cell growth (1–6). During virus infection, PKR is activated and blocks viral

protein synthesis through phosphorylation of eIF2 α , thus leading to antiviral defense (1,3,7). Recently, it was observed that PKR is involved in insulin resistance in peripheral tissues (8–11) and antiproliferation activities in pancreatic β cells (12), indicating a novel role of PKR in

metabolism regulation and type 2 diabetes mellitus (T2DM). These functions of PKR are attributed to its kinase catalytic activity and an effective therapeutic strategy of pharmacologically targeting PKR was confirmed using small-molecule inhibitors of PKR kinase activity that improved insulin sensitivity and glucose clearance in a mouse model of obesity and insulin resistance (13).

Notably, PKR can function as an adaptor protein via its protein-binding domain, but not via its regulatory dsRNA-binding domain (14,15). Structurally, there are two tumor necrosis factor receptor-associated factor (TRAF)-binding domains with one TRAF-interacting motif P/S/A/T \times Q/E E in the regulatory domain and another, P \times Q \times S/T/D, in the kinase do-

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main (14). In unstimulated cells, the two motifs are embedded in the closed conformation of PKR that is mediated by inhibitory intramolecular interactions (16,17). Upon ligand binding, dimerization of PKR is induced and conformation occurs to expose the TRAF-binding domains. TRAF family members, including TRAF2, TRAF5 and TRAF6, combine with PKR upon dimerization in some cells (14). Despite the existence of TRAF-interacting motifs on PKR, physical interaction between PKR and TRAF proteins in pancreatic β cells remains elusive.

TRAF family members originally were identified as signaling adaptors, with a TRAF domain at the C-terminus to interact with upstream recruiter molecules (such as TLR, TNFR, CD40, PKR and a really interesting new gene [RING] finger and several zinc finger motifs in the N-terminal domain) to combine with and activate downstream molecules, including receptor-interacting serine/threonine-protein kinase 1 (RIP1) and other TRAF family members (14,15). TRAF family members play a critical role in signaling transduction forward to the nuclear factor kappa-light-chain-enhancer of activated B cell (NF- κ B) pathway and other transcriptional modulations (18–20). For example, TNF α mediates constitutive NF- κ B activation and proliferation in human head and neck squamous cell carcinoma through the TRAF signaling pathway (21).

The aims of this investigation were to determine the effect of the protein-binding domain of PKR on pancreatic β cells and to analyze the underlying mechanism. Under the conditions where the kinase catalytic activity was defective, the protein-binding function of PKR was shown to promote β -cell proliferation, suggesting a role of the PKR protein-binding domain distinct from its kinase activity. If small pharmacological molecules could agonize the protein-binding function to counteract the kinase activity of PKR *in vivo*, then therapeutic opportunities in T2DM may arise.

MATERIALS AND METHODS

Reagents

Roswell Park Memorial Institute (RPMI) 1640, Dulbecco modified Eagle medium and fetal bovine serum (FBS) were Gibco products (Thermo Fisher Scientific Inc., Waltham, MA, USA). TRIzol and Lipofectamine 2000 reagent were Invitrogen products (Thermo Fisher Scientific). The Reverse Transcription Kit and the SYBR Green PCR Master Mix were bought from Takara (Otsu, Shiga, Japan). Type V collagenase, Histopaque-1077, MTT and coumermycin were obtained from Sigma-Aldrich (St. Louis, MO, USA). TNF α was purchased from Pepro-Tech (Rocky Hill, NJ, USA). The Cell-LightEdU DNA Cell Proliferation Kit was purchased from RiboBio (Guangzhou, China). TRAF2-siRNA, TRAF6-siRNA, RIP1-siRNA, *c-Myc*-siRNA and antibodies against β -actin, PKR, eIF2 α , p-eIF2 α , TRAF2, TRAF6, RIP1 and ubiquitin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against Cyclin D1, Cyclin D2, Cdk2, Cdk4 and c-Myc were from Cell Signaling Technology (Boston, MA, USA). Antibodies against P21, P27 and P53 were purchased from Bioss (Beijing, China). The NF- κ B-dependent reporter construct pGMNF κ B-Lu was purchased from Genomeditech (Shanghai, China). Bay11-7082, *ortho*-Nitrophenyl- β -galactoside, RIPA lysis buffer, nuclear and cytoplasmic protein extraction kit and BCA kit were obtained from Beyotime Inc (Shanghai, China). Plasmids encoding PKR-K296R, GyrB-PKR, GyrB-PKR-K296H and pSG5 (22) were provided by Tom Dever (National Institutes of Health, Bethesda, MD, USA).

Cell Culture and Transfection

The MIN6 cell line was grown in Dulbecco modified Eagle medium (4.5 g/L glucose) containing 15% (v/v) FBS, 121 mol/L 2-mercaptoethanol (23). Lipofectamine 2000 (Invitrogen [Thermo Fisher Scientific]) was used for transfection of siRNAs and plasmid constructs, according to the manufacturer's instructions.

Islet Isolation, Culture and *In Vitro* Transfection

All animal studies were performed according to guidelines established by the Research Animal Care Committee of Nanjing Medical University (Nanjing, China). Ten-week-old (20–25 g) male imprinting control region (ICR) mice were purchased from the Model Animal Research Center of Nanjing University. All animals were handled in accordance with the *Guide for the Care and Use of Laboratory Animals* (24). Islet isolation and culturing techniques have been described previously (25).

At 2-d postisolation, the isolated islets were transferred to and cultured in serum-free transfection medium (Ca²⁺-containing Krebs-Ringer-HEPES medium) and *in vitro* transfection was conducted as described previously (26).

Protein Isolation and Western Blotting

The protein concentrations were determined using a BCA kit (Beyotime Inc., China). Denatured samples were prepared for Western blot analysis using various primary antibodies as indicated. Protein signals were detected using secondary antibodies against rabbit or mouse IgG.

Coimmunoprecipitation

The same amounts (400 μ g) of cell lysates were incubated with 1 to 2 μ g antibody overnight at 4°C. Protein A/G-agarose spheres (Santa Cruz Biotechnology) were added to the samples and stored at 4°C. After 2 h, the samples were centrifuged at 14,000g for 2 min at 4°C. The samples were then washed three times with lysis buffer and 20 μ L 5 \times SDS loading buffer was added before boiling for 10 min. Denatured samples were kept at –20°C for Western blotting (27).

Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) Analysis

Total RNA was isolated using the TRIzol reagent. By using a reverse transcription kit, 1 μ g of total RNA was con-

verted into first-strand cDNA. SYBR Green and the 7300 Real-Time PCR system (Applied Biosystems [Thermo Fisher Scientific]) were used to carry out the qRT-PCR analysis. All data were analyzed using β -actin gene expression as an internal standard.

Cell Viability

For MTT measurement, MIN6 cells were seeded in 96-well plates at a density of 1×10^4 cells/well and then subjected to the indicated treatments. Thereafter, 20 μ L of 5 mg/mL MTT was added to each well and incubated for 4 h. The supernatant was removed and the formazan crystals were dissolved in dimethyl sulfoxide. Cell viability was assessed by measuring the absorbance at 490 nm using a microplate reader (12).

Cell Proliferation Assay by 5-Ethynyl-2'-Deoxyuridine EdU Labeling

For the EdU incorporation assay, MIN6 cells were cultured in 24-well plates on coverslips. After treatment, EdU was added to the culture medium (50 μ mol/L) for 2 h and cell proliferation was determined according the manufacturer's instructions. For the isolated mouse islets, media supplemented with 20 μ mol/L EdU was added to the plates. Isolated mouse islets are not adherent and could not easily be made adherent, therefore, an alternative protocol for suspended cells was used and centrifugation (5 min, 4°C, 268 \times g) was required for every step.

Immunofluorescence Assay (IFA)

IFA was used to observe changes in the localization of NF- κ B (p65) or the level of PKR, as described previously (12). After transfection and pharmaceutical treatment, MIN6 cells or isolated mouse islets were subjected to IFA. Antibodies against insulin were applied to mark pancreatic β cells of islets. Isolated mouse islets in suspension were subject to centrifugation (5 min, 4°C, 268 \times g) for every step (23).

Flow Cytometric Analysis

MIN6 cells were digested in a trypsin-EDTA solution, collected by centrifugation at 500g for 5 min, washed three times with PBS and at last fixed in cold 75% ethanol at 4°C overnight. The percentages of cells in G0/G1, S and G2/M phases were determined by flow cytometry following propidium iodide (PI) staining.

Luciferase Reporter Assay

The luciferase reporter construct pGMNF- κ B-Lu was cotransfected transiently with pSG5 or GyrB-PKR-K296H into MIN6 cells grown in 24-well plates, using the lipofectamine 2000 reagent according to the manufacturer's instructions. A plasmid expressing the gene-encoding β -galactosidase driven by the cytomegalovirus (CMV) promoter (Clontech Laboratories, Palo Alto, CA, USA) was cotransfected simultaneously as an internal control. The medium was replaced 6 h after transfection. Twenty-four hours after transfection, the cells were treated with the specific ligand coumermycin for an additional 24 h and harvested for luciferase reporter assays, as described previously (28). Similar protocol for PKR-K296R-induced MIN6 cells was conducted.

Statistics

All data were representative of at least three experiments. Results are expressed as the mean \pm SEM. Comparisons were performed using the Student *t* test for two groups or analysis of variance (ANOVA) for multiple groups. *P* values < 0.05 were considered statistically significant.

All supplementary materials are available online at www.molmed.org.

RESULTS

A Model of PKR with Its Protein-Binding Function but without Its Kinase Catalytic Activity Was Built into Pancreatic β cells

To explore the function of the PKR protein-binding domain in pancreatic

β cells, a plasmid encoding a GyrB-PKR-K296H fusion protein was used to overexpress a kinase-defective mutant in pancreatic β cells. This fusion protein comprises the N-terminal 220 aa of *Escherichia coli* GyrB and the kinase domain (aa 258 to 551) of PKR with a mutation at position 296 from K to H. The presence of *E. Coli* GyrB permits the chimeric protein to dimerize and conformationally change when treated with the specific inducer, coumermycin (22). To examine whether coumermycin would interfere with the potential activation of endogenous PKR, both the PKR inhibitor 2-AP and the agonist BEPP (12,29,30) were applied as a negative and positive control, respectively. As shown in Figure 1A, the protein levels of p-eIF2 α did not change upon coumermycin stimulus among the four transfected groups. On the other hand, the levels in coumermycin groups were obviously lower than those in the BEPP group (*P* < 0.01), despite no significant difference with mock or 2-AP groups. This observation implied that coumermycin could not trigger endogenous PKR kinase activity. A significant increase in the amount of PKR protein (about 3.5 times more than that in endogenous PKR, Figure 1C) was detected by Western blotting after GyrB-PKR-K296H overexpression. To examine the feasibility of PKR combining with TRAF proteins, qRT-PCR was carried out to detect the mRNA levels of *TRAF2*, *TRAF3*, *TRAF5* and *TRAF6* in MIN6 cells. Interestingly, except for *TRAF5*, the other three *TRAF* members were detected in pancreatic β cells, and the mRNA level of *TRAF3* was the highest (Figure 1B). Western blotting was then performed to detect TRAF2, TRAF3 and TRAF6 after immunoprecipitation of PKR with PKR-specific antibodies. Under conditions of PKR overexpression, coumermycin induced coimmunoprecipitation of PKR with TRAF2 and TRAF6, while TRAF3 was not detected as a negative control (see Figure 1C). Additionally, IFA with anti-PKR antibodies (red fluorescence) was carried out to detect the alterations

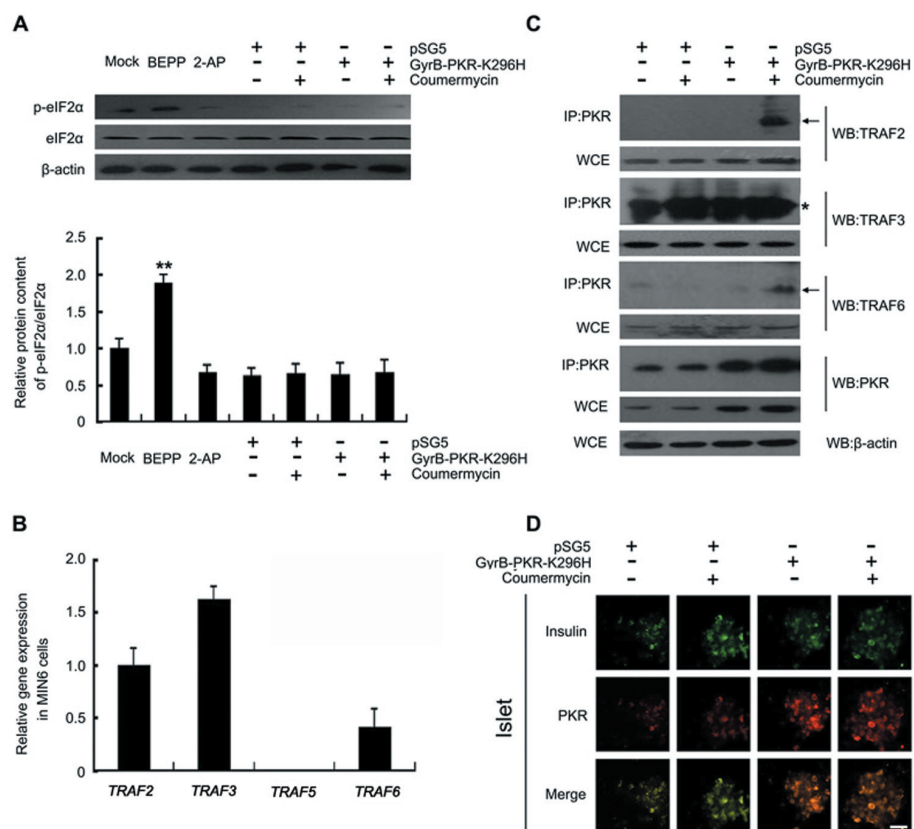


Figure 1. A model of PKR with its protein-binding function but lacking kinase catalytic activity was built in pancreatic β cells. MIN6 cells were transfected with plasmids encoding GyrB-PKR-K296H or the empty vector as indicated. Twenty-four hours after transfection, cells were treated with coumermycin for 24 h and cell extracts were collected. (A) The first three lines are mock, 2-AP and BEPP and others were treated as indicated. Cell extracts were analyzed by Western blotting using antibodies against PKR, p-eIF2 α and eIF2 α . (B) qRT-PCR performed to detect the mRNA levels of TRAF2, -3, -5 and -6 in MIN6 cells. (C) Extracts prepared as described for panel A (the last four lines) were immunoprecipitated with antibodies against PKR. Immunocomplexes or whole cell extracts (WCE) were analyzed by Western blotting with antibodies against TRAF2, -3 and -6. Asterisks denote IgGs, and arrows indicate the specific proteins. (D) Isolated mouse islets were administered as MIN6 cells. IFA was performed with antibodies directed against PKR (red) and insulin (green); DAPI was used for nuclear staining (blue). Insulin with green staining was used to identify β cells (scale bar = 100 μ m). β -actin was detected as an internal control. Data are means \pm SEM of three separate experiments. * $P < 0.05$ versus control.

of PKR in primary islets. As shown in Figure 1D, PKR overexpression in primary islets was obtained after transfection with plasmid GyrB-PKR-K296H and coumermycin did not interfere with this overexpression. These data suggested that a model with upregulated PKR protein-binding function, but defective in kinase catalytic activity, has been constructed successfully in pancreatic β cells.

Protein-Binding Function of PKR Improved Both Cell Viability and Proliferation of Pancreatic β cells

To evaluate the functional impact of the PKR protein-binding domain in pancreatic β -cells' growth, coumermycin was used in the experiment at different concentrations (0, 0.1, 1, 10 and 100 ng/mL) for different time periods (6 h, 12 h and 24 h) after transfection in MIN6 cells. MTT assays indicated that

coumermycin could effectively enhance the viability of MIN6 in a dose- and time-dependent manner in the groups transfected with plasmid GyrB-PKR-K296H, whereas there were no obvious changes among the groups transfected with vector pSG5 (Figure 2A). Moreover, cell viability reached its peak when MIN6 cells were treated with 10 ng/mL coumermycin for 24 h after transfection with plasmid GyrB-PKR-K296H. The condition of 10 ng/mL coumermycin for 24 h was used in the experiments where coumermycin would be applied. Accordingly, we then investigated whether PKR protein-binding-mediated enhancement in β -cell viability was derived from increased proliferation. After coumermycin treatment, the percentage of EdU-positive β cells in GyrB-PKR-K296H-induced MIN6 cells was elevated as compared to the other groups (Figure 2B). Meanwhile, flow cytometry showed a decreased percentage of MIN6 cells in the G1 phase (Figure 2C, $P < 0.05$) and an increased percentage in the S phase (Figure 2D, $P < 0.05$) in the group with elevated PKR protein-binding function. By contrast, coumermycin-dependent dimerization of GyrB-PKR had a similar biological function to activated PKR in MIN6 cells. That is, it could induce persistent phosphorylation of eIF2 α and inhibit cell proliferation through cycle arrest at G1 phase (Supplementary data). Similar EdU staining results were observed in primary islets (Figure 2E, $P < 0.05$), indicating that PKR protein binding could effectively increase the proliferation of β cells. The data suggested that the PKR protein-binding function improves both cell viability and proliferation in pancreatic β cells, separate from its kinase function.

Protein-Binding Function of PKR Upregulated Transcription of *c-Myc* in MIN6 Cells

Many molecules that govern cell cycle progression from the G1 to S phase play a pivotal role in pancreatic β -cell proliferation (12,23,31–35). In the

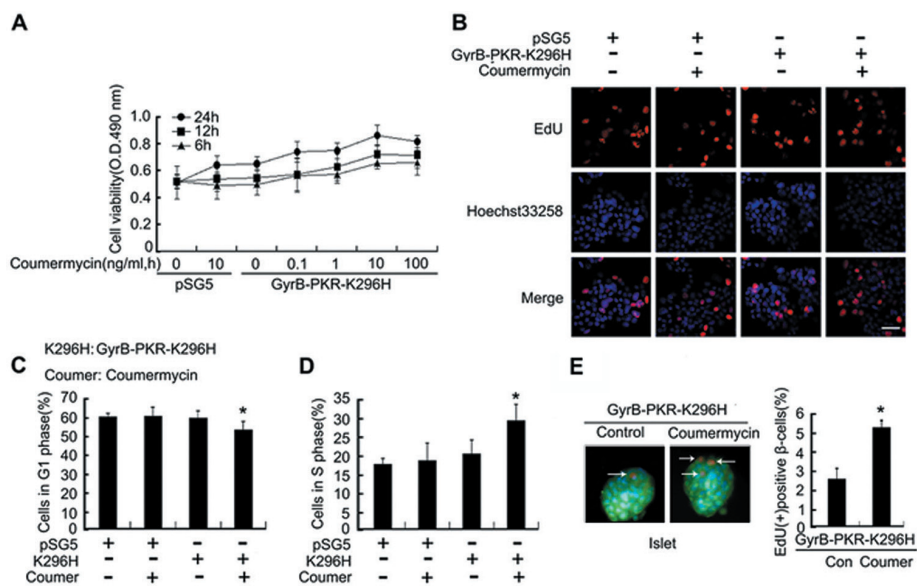


Figure 2. The PKR protein-binding function improves both cell viability and proliferation in pancreatic β cells. Twenty-four hours after transfection, MIN6 cells were treated with the indicated concentrations of coumermycin for the indicated times. (A) Cell viability was assessed by the MIT assay. (B) DNA synthesis was analyzed using EdU labeling assays. Representative micrographs of EdU labeling assays in MIN6 cells are shown (scale bar = 120 μ m). Flow cytometric assays were performed with percentages of MIN6 cells at the (C) G1 phase and (D) S phase. (E) Isolated mouse islets were administered as MIN6 cells and DNA synthesis was analyzed using EdU labeling assays. In primary islets, insulin with green staining was used to mark β cells (scale bar = 100 μ m), and the percentage of EdU-positive β cells was quantified (using six islets for each group). Arrows indicate the EdU-positive β cell. Data are means \pm SEM of three separate experiments. * P < 0.05 versus control.

present study, positive regulators such as *cyclin D1*, *cyclin D2*, *CDK2*, *CDK4* and *c-Myc*, and negative regulators such as *p21*, *p27* and *p53*, were analyzed by qRT-PCR and Western blotting. After MIN6 cells were transfected with plasmid GyrB-PKR-K296H and treated with coumermycin (10 ng/mL, 24 h), only the RNA level of *c-Myc* was increased significantly compared with the control group without coumermycin treatment (Figure 3A, P < 0.01), implying a transcriptional upregulation of *c-Myc* upon PKR binding. Consistently, Western blotting showed a significant increase of c-Myc protein in the same group (Figure 3B, P < 0.01), whereas there were no remarkable changes for the other regulators (Figures 3B, C). To get deep insight into the role of c-Myc in the PKR protein-binding function-mediated β -cell proliferation, si-*c-Myc* was used

to get gene silence of *c-Myc*. By comparison with the si-NC group, si-*c-Myc* was shown to greatly inhibit MIN6 cell proliferation (Figure 3D, P < 0.05), and disturb cycle progression by increasing the percentage of cells at G1 phase (Figure 3E, P < 0.05) and decreasing the percentage at S phase (see Figure 3E, P < 0.05). Taken together, these data suggested that the PKR protein-binding function could upregulate the c-Myc protein level by increasing its transcription in MIN6 cells and c-Myc was essential for this proliferative effect.

NF- κ B Is Required for PKR Protein-Binding Domain-Induced c-Myc Upregulation and Cell Proliferation in MIN6 Cells

c-Myc is thought to be modulated by NF- κ B-dependent transcriptional activ-

ity (33,36–38). Therefore, we explored the possible involvement of the NF- κ B pathway in PKR binding-triggered signals in pancreatic β cells. After transfection of GyrB-PKR-K296H, MIN6 cells were pretreated with the NF- κ B-specific inhibitor Bay11-7082 (39,40) (30 μ mol/L) 2 h before coumermycin administration. Compared with the control group, Bay11-7082 remarkably abolished the increase of *c-Myc* mRNA level (Figure 4A, P < 0.01) and induced a significant decline of the c-Myc protein level (Figure 4B, down to 50%). Based on the negative effect of Bay11-7082, NF- κ B was implicated in the PKR protein-binding-induced c-Myc upregulation. Meanwhile, the percentage of EdU-positive β cells in MIN6 cells (Figure 4C, P < 0.01) and primary islets (Figure 4F, P < 0.01) decreased substantially upon Bay11-7082 stimulus. Consistently, Bay11-7082 was shown to increase the percentage of MIN6 cells in G1 phases (Figure 4D, P < 0.05) and to decrease the percentage in the S phase (Figure 4E, P < 0.05). Taken together, these data support the requirement of NF- κ B in the PKR protein-binding domain-induced MIN6 cell proliferation through transcriptional upregulation of the *c-Myc* gene.

NF- κ B Was Activated by PKR Protein Binding in MIN6 Cells

We next sought to explore whether PKR protein binding would activate NF- κ B. The translocation of p65 α from the cytoplasm to the nucleus, preceded by proteolytic degradation of I κ B, is a necessary step for NF- κ B transcriptional activity (41,42). As shown in Figure 5A, coumermycin substantially induced I κ B α degradation (P < 0.01) and nuclear translocation of p65 (P < 0.01) in a dose-dependent manner in GyrB-PKR-K296H-induced MIN6 cells. Correspondingly, obvious translocation of p65 into the nucleus in MIN6 cells was observed in the group with elevated protein-binding function of PKR, using IFA with an anti-p65 antibody (green fluorescence, Figure 5B). Demonstration of

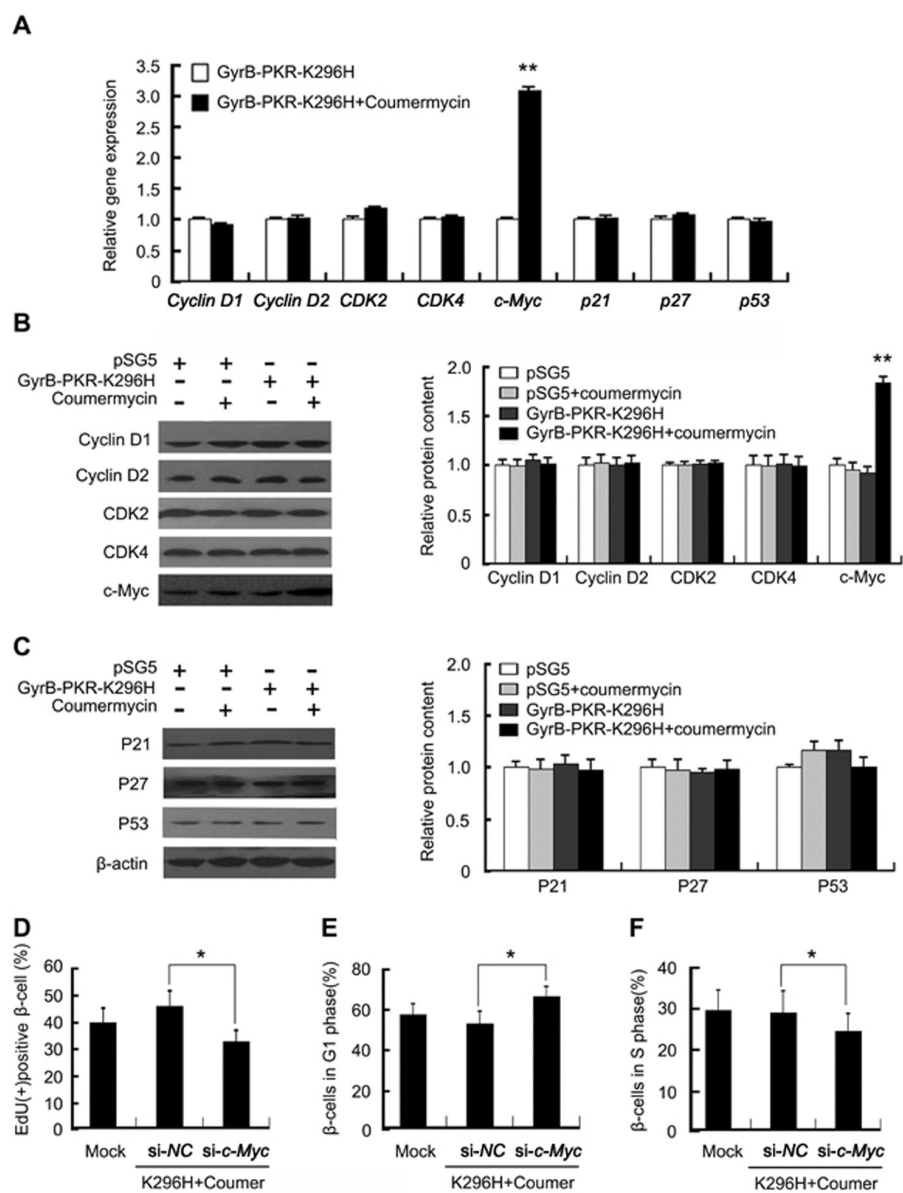


Figure 3. The PKR protein-binding function promotes cell cycle progression via upregulation of c-Myc transcription in MIN6 cells. Twenty-four hours after transfection, MIN6 cells were treated with coumermycin as indicated. (A) The mRNA levels of the indicated genes, including positive cell cycle regulators *cyclin D1*, *D2*, *CDK2*, *CDK4* and *c-Myc* and negative regulators *p18*, *p27* and *p53* were determined by qRT-PCR. Cell extracts were analyzed by Western blotting with antibodies against (B) positive and (C) negative cell cycle regulators. si-c-Myc was cotransfected with GyrB-PKR-K296H into MIN6 cells, followed by treatment of coumermycin. (D) Quantification of EdU-positive β cell and percentages of MIN6 cells at the (E) G1 phase and (F) S phase were shown. Data are means ± SEM of three separate experiments. * $P < 0.05$ versus control.

nuclear translocation of p65 is not sufficient to prove increased NF-κB transcriptional activity, therefore, a luciferase reporter assay was performed

to determine the role of PKR protein binding in NF-κB-dependent reporter gene expression. An NF-κB-dependent reporter construct, pGMNF-κB-Lu, was

cotransfected into MIN6 cells with plasmid GyrB-PKR-K296H or pSG5. As shown in Figure 5C, a significant increase in NF-κB activity (up to five-fold) was observed under conditions of elevated PKR protein binding. These observations implied that NF-κB activation was induced by PKR protein binding in MIN6 cells.

TRAF2, but Not TRAF6, Plays a Critical Role in PKR Protein-Binding-Induced NF-κB Activation and Proliferation in MIN6 Cells

TRAF proteins are universal adapters linking NF-κB activation (19,43), therefore, we investigated the integration of TRAF in PKR protein-binding-induced NF-κB activation. In view of the binding of PKR to TRAF2 and TRAF6 in MIN6 cells (see Figure 1B), specific siRNAs for *TRAF2* and *TRAF6* were applied to examine their exact roles. MIN6 cells were transfected with each siRNA before cotransfection with plasmid GyrB-PKR-K296H and the NF-κB-dependent reporter construct pGMNF-κB-Lu and coumermycin stimuli. Compared with the control group, the luciferase activity was reduced greatly in the group subjected to *TRAF2* gene silencing, however, the si-*TRAF6* group was barely affected (Figure 6A, $P < 0.01$). These results indicate that TRAF2 was involved in PKR protein-binding-induced NF-κB activation. Consistently, the upregulation of *c-Myc* diminished after *TRAF2* gene silencing at the mRNA (Figure 6B, $P < 0.01$) and protein levels (Figure 6C, down to 30%). To gain deeper insight into the impact of TRAF2 on the biological function of the PKR protein-binding domain, EdU labeling and flow cytometric analysis were performed after gene silencing of *TRAF2* or *TRAF6*. Compared with the control group, *TRAF2* gene silencing obviously reduced the percentage of EdU-positive β cells (Figure 6D, $P < 0.05$), but increased the percentage of β cells in the G1 phase (Figure 6E, $P < 0.05$), along with a decreased percentage in S phase (Figure 6F, $P < 0.05$). Mean-

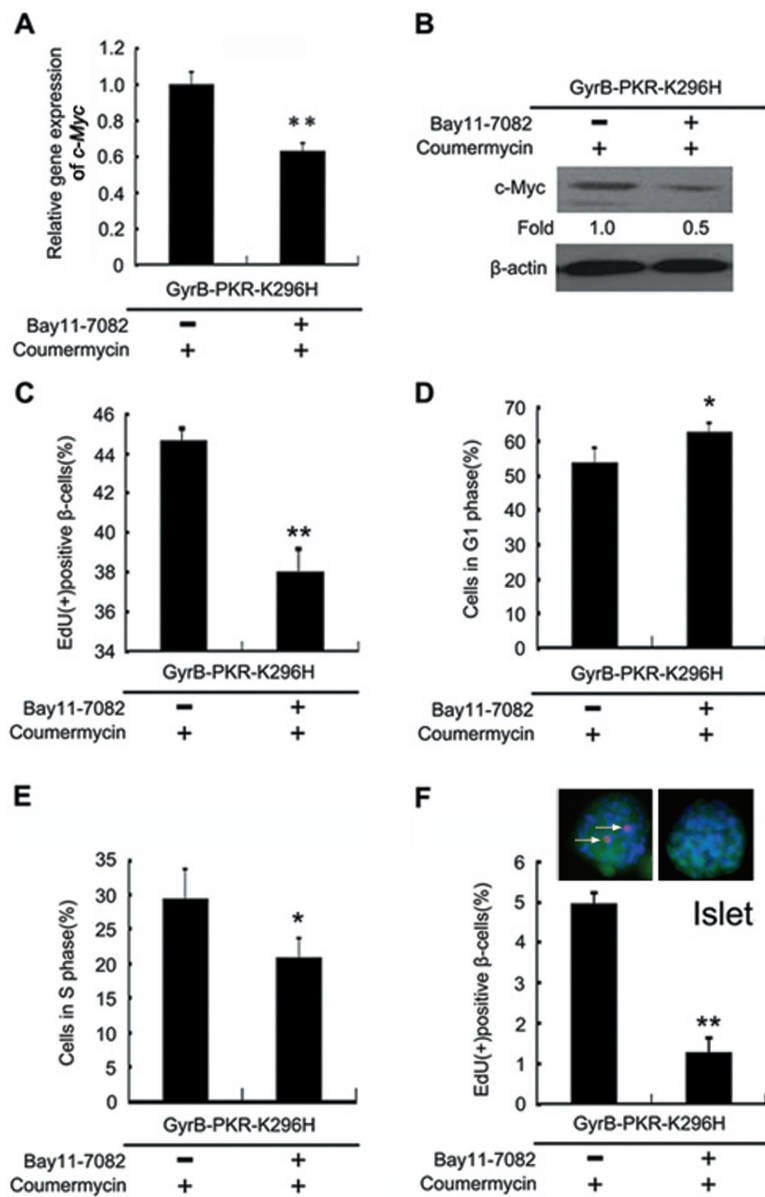


Figure 4. NF- κ B is required for PKR protein-binding-induced c-myc upregulation and cell proliferation in pancreatic β cells. After transfection, MIN6 cells were pretreated with Bay11-7082 (30 μ mol/L, 2 h) and subsequently treated with coumermycin for 24 h. mRNA and protein levels of c-Myc were determined by (A) qRT-PCR and (B) Western blotting. (C) Quantification of EdU-positive β cell and percentages of MIN6 cells at the (D) G1 phase and (E) S phase were shown. (F) Isolated mouse islets were administered as panel C and DNA synthesis was analyzed using EdU labeling assays. The percentage of EdU-positive β cells in islets was quantificated (using six islets for each group). Arrows indicate the EdU-positive β cell. β -actin was detected as an internal control. Data are means \pm SEM of three separate experiments. * P < 0.05 versus control.

while, gene silencing of *TRAF6* had no effect on PKR protein-binding-induced β -cell proliferation (Figures 6D–F). Taken together, the results indicated that

TRAF2, but not TRAF6, plays a critical role in PKR protein-binding-induced NF- κ B activation and proliferation in MIN6 cells.

RIP1 Is Located Downstream of TRAF2 and Implicated in PKR Protein-Binding-Mediated NF- κ B Activation and Proliferation in MIN6 Cells

TRAF2 can function as an E3 ubiquitin ligase in the Ubc133/Uev1A ubiquitin conjugation enzyme system, catalyzing the assembly of K63-linked polyubiquitin chains on target proteins, including TRAFs, RIP1 and IKK γ /NEMO, which in turn activates the IKK complex (21,44,45). As a result, RIP1 was targeted for subsequent investigation. After transfection and coumermycin treatment, MIN6 cells were collected for immunoprecipitation of RIP1 using an antibody against RIP1. Western blotting was carried out to detect ubiquitin-RIP1 using an antibody against ubiquitin. As expected, ubiquitin-RIP1 was found only in the group where PKR strongly interacted with TRAF2. To further elucidate whether the ubiquitination of RIP1 was attributed to recruitment of TRAF2 by PKR, si-*TRAF2* was used to silence the *TRAF2* gene in MIN6 cells. Compared with the control group, ubiquitin disappeared from the coimmunoprecipitate after gene silencing of *TRAF2* (Figure 7B). Additionally, si-*RIP1* interfered with NF- κ B reporter gene expression, as observed by impaired luciferase activity (Figure 7C, left panel, P < 0.05) and abolished c-Myc protein upregulation (Figure 7C, right panel, down to 40%). As depicted in Figure 7D, PKR protein-binding-induced β -cell proliferation of MIN6 was remarkably disturbed by si-*RIP1*. Based on the above data, we suggested that RIP1 acted as a downstream molecule of TRAF2 signaling and participated in PKR protein-binding-triggered signaling and MIN6 cell proliferation.

PKR Protein Binding, Which Can Remit the Deleterious Effect of TNF α on MIN6 Cell Proliferation Also Was Evoked in Response to Glucolipotoxicity and TNF α

Glucolipotoxicity and proinflammatory cytokines have been identified as two critical components in the development of insulin resistance and T2DM

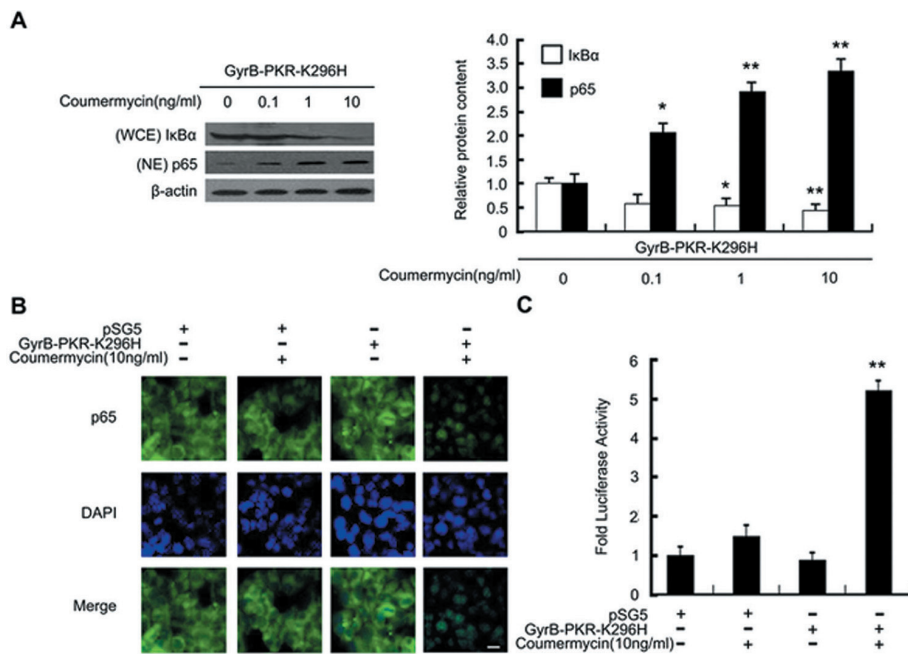


Figure 5. NF- κ B was activated by the PKR protein-binding function in MIN6 cells. Twenty-four hours after transfection, MIN6 cells were treated with coumermycin as indicated. (A) Whole cell extracts (WCE) and prepared nuclear extracts (NE) were collected and analyzed by Western blotting with antibodies against I κ B α and p65, respectively. (B) Administered as Figure 1B, p65 localizations in cytoplasm and nuclear of MIN6 cells were determined by IFA (scale bar = 100 μ m). (C) Luciferase activity was measured and shown in fold. β -actin was detected as an internal control. Data are means \pm SEM of three separate experiments. * P < 0.05 versus control.

(12,13,23,46–52). Therefore, we examined PKR protein binding in response to glucolipotoxicity and TNF α . MIN6 cells were transfected with plasmids encoding PKR-K296R or the empty vector pSG5 and then stimulated with glucolipotoxicity (glucose 16.7 mmol/L and 0.4 mmol/L palmitate) for 6 h. As expected, in PKR-K296R-induced MIN6 cells, glucolipotoxicity induced coimmunoprecipitation of TRAF2 and TRAF6, followed by immunoprecipitation of PKR (Figure 8A). Similar results were obtained under conditions of 80 nmol/L proinflammatory cytokine TNF α after transfection of the PKR-K296R plasmid (see Figure 8A). Interestingly, NF- κ B-dependent luciferase activity was not altered between the control and glucolipotoxicity groups, but was remarkably increased in the TNF α groups where PKR protein binding seemed to induce a more significant ele-

vation (Figure 8B). Furthermore, *c-Myc* upregulation did not always correlate with NF- κ B activation, because there was no significant increase of *c-Myc* at the mRNA level in the group with robust NF- κ B activation mediated by PKR protein binding (Figure 8C). Still, suppressed cell viability (Figure 8F, P < 0.01) and proliferation (Figure 8E, P < 0.05) in response to TNF α both were found to be relieved in PKR-K296R-induced MIN6 cells, presenting a similar result as the control groups without TNF α stimuli. These data indicated that PKR protein binding was indeed evoked in response to glucolipotoxicity and TNF α , which remitted the deleterious effect of TNF α on MIN6 cells proliferation.

DISCUSSION

Considering that T2DM is characterized by relative insufficiency of insulin

production by β cells (12,23,27,30,53,54), how to increase the β -cell mass by expanding β cells and enhancing insulin secretion are of great importance. In adult mammals, proliferation of mature β cells plays a critical role in the maintenance of the β -cell mass (55). Hence, inducible proliferation of pancreatic β cells in pathophysiological settings is proposed to improve T2DM. Our investigations suggested that pancreatic β -cell proliferation would be promoted by elevating the protein-binding function of PKR in the absence of its kinase catalytic activity.

PKR acts as an adaptor protein via its protein-binding domain, whose biological function may be quite different from its catalytic activity (14,56,57). A pitfall in this investigation is that the β -cell line MIN6 and primary islet β cells used were not devoid of PKR; therefore, endogenous PKR potentially would be activated upon stimulus by poly(I:C), BEPP, glucolipotoxicity or proinflammatory cytokines (8,12,29,30,58) when kinase-defective mutant PKR-K296R was utilized to establish a model of elevated PKR protein-binding function. Fortunately, GyrB-PKR-K296H was able to avoid such a pitfall, as demonstrated by the fact that the specific activator coumermycin (14,22) had no influence on endogenous PKR activity (see Figure 1A). As depicted in Figure 1B, the PKR binding function was greatly upregulated after treatment by coumermycin in GyrB-PKR-K296H-induced β -cell line, accompanied by enhanced physical interactions between PKR and TRAF2 and TRAF-6. These observations indicated prospective roles of TRAF2 and -6 in PKR protein-binding-mediated signaling pathways.

In contrast to the kinase activity of PKR, the PKR protein-binding function exhibited a proliferative role on pancreatic β cells, indicating a kinase-free biological function of PKR. Analogous to PKR, other kinases such as RIP, IRAK, JNK1 and Tyrk2 also are able to mediate certain biological functions independent of their kinase activity when their binding domains are ready for alignment

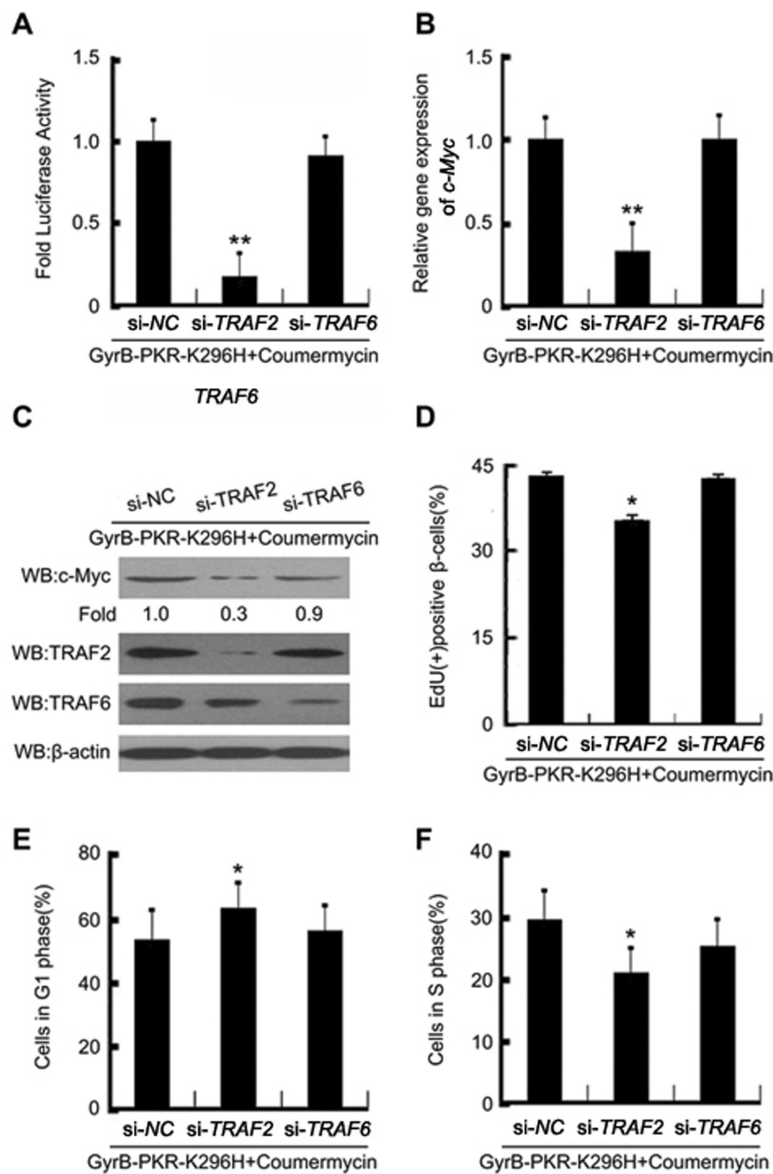


Figure 6. TRAF2 plays a critical role in PKR protein-binding-induced NF- κ B activation and proliferation in MIN6 cells. MIN6 cells were transfected with si-NC, si-TRAF2 or si-TRAF6 before GyrB-PKR-K296H plasmids. After 48 h of gene silencing, cells were treated with coumermycin as indicated. (A) Luciferase activity was measured and shown in fold. mRNA and protein levels of c-myc were determined by (B) qRT-PCR and (C) Western blotting. Antibodies against TRAF2 and TRAF6 were used to examine the protein levels after gene silencing. (D) Quantification of EdU-positive β cell was shown. Flow cytometric assays were carried out, with percentages of MIN6 cells at the (E) G1 phase and (F) S phase displayed. β -actin was detected as an internal control. Data are means \pm SEM of three separate experiments. * P < 0.05 versus control.

with associated partners and trigger signals (56,57,59,60). Therefore, the exact biological function of PKR is determined mainly by the predominance of related

domains in certain settings. In fact, the proliferative role of PKR in cells is not infrequent, with high expression and activation levels of PKR found in various

cancers (4,61–63). Further support for our data comes from the evidence that PKR participates in TNF α -induced proliferation in mouse embryo fibroblast (MEF) (33). The impact of PKR in cellular processes of different cells or even of the same cell type, from antiproliferation to proliferation, might be separate from its kinase catalytic activity and protein-binding function.

In the present study, the PKR protein-binding domain-dependent proliferation seemed to correlate with reduced β cells at G1 phase and augmented levels at S phase in pancreatic β -cell line (Figure 2). This was associated with the substantial increase in the positive regulator c-Myc and was further confirmed by the antiproliferative role of c-Myc-siRNA (Figure 3). Generally, c-Myc hyperactivates cyclin/Cdk or antagonizes the activity of cell cycle inhibitors, such as P21 and P27, to drive cell cycle progression and promote cell proliferation (64). As demonstrated, modest overexpression of c-Myc is able to drive proliferation in normal rat and human β cells (34). In addition, c-Myc mRNA was upregulated and contributed to adaptive proliferation of pancreatic islets during rat pregnancy (55). Hence, it is feasible that PKR protein-binding-induced proliferation might be closely linked with elevated levels of c-Myc proteins in pancreatic β cells.

Correlating with previous data that c-Myc is modulated by NF- κ B transcriptional activity (38,42), there was a significant boost in NF- κ B-dependent luciferase activity after increased PKR protein-binding function, as well as proteolytic degradation of I κ B α and nuclear translocation of p65 in pancreatic β -cell line (Figure 5). Most importantly, activated NF- κ B in this investigation obviously promoted β -cell proliferation in pancreatic β cells (see Figure 5). Notably, the proliferative role of NF- κ B was observed previously in NIT-1 cells when stimulated with a low dosage of LPS (40). Furthermore, the PKR-facilitated pro-survival NF- κ B pathway in cholangiocarcinoma cell lines is significantly

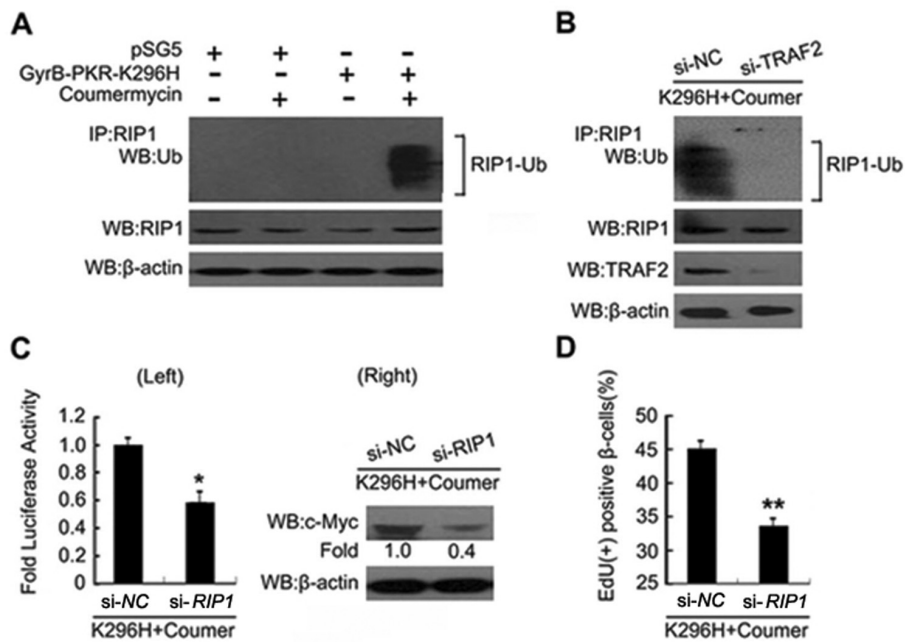


Figure 7. RIP1, located downstream of TRAF2, is implicated in PKR protein-binding-mediated NF- κ B activation and β -cell proliferation in MIN6 cells. (A) Administered as Figure 1B, MIN6 cell extracts were collected for immunoprecipitation with antibodies against RIP1 and thoroughly washed. Immunocomplexes were analyzed by Western blotting with antibodies against ubiquitin and whole cell extracts (WCE) with anti-RIP1 antibody. (B) si-NC or si-TRAF2 was transfected into MIN6 cells before GyrB-PKR-K296H plasmids. Immunoprecipitation and Western blotting were performed as in panel A. (C) Twenty-four hours after transfection of si-RIP1, luciferase reporter assay was conducted as in Figure 6A, and fold luciferase activity is shown (left panel); Western blot to detect c-myc protein level is described (right panel). (D) Quantification of EdU-positive β cell was shown. β -actin was detected as an internal control. Data are means \pm SEM of three separate experiments. * $P < 0.05$ versus control.

associated with neoplastic progression in human cancers (61,63). However, previous studies also suggested that the PKR-triggered NF- κ B pathway could cause cell death or apoptosis when eIF2 α was phosphorylated and activated by PKR (65). The data was in line with a previous hypothesis that PKR-mediated activation of NF- κ B has two distinctive functions decided by the presence of p-eIF2 α and inhibition of protein synthesis (29).

As well-characterized adaptor proteins, TRAFs have an important role in assembling active NF- κ B signaling scaffolds in response to extracellular or intracellular stimuli (19,43). To date, only TRAF2 and TRAF6 have been identified in pancreatic β cells (66). In the immunoprecipitation experiments, these

two proteins could bind with dimerized PKR (see Figure 1B), implying a possible association of TRAF2 and TRAF6 with the PKR protein-binding-dependent NF- κ B pathway. Human PKR interaction with TRAFs in HeLa and 293T cells after treatment with IFN- α/β reinforces our hypothesis (14). Nevertheless, PKR protein-binding function-accelerated β -cell proliferation was reversed only by si-TRAF2, whereas si-TRAF6 produced no effect in β -cell line. We also observed abrogated NF- κ B activation and reduced c-Myc expression after gene silencing of TRAF2 (Figure 6). Thus, we concluded that TRAF2, but not TRAF6, links PKR with the proliferative pathway of NF- κ B when recruited by PKR via the TRAF-interacting motif at the C-terminus (14). This conclusion was in

accordance with previous evidence showing that an increase in the TRAF2 level correlated with improved cell viability of MIN6 (67), whereas TRAF6 was reported to participate in the early stages of cytokine-induced pancreatic β -cell death (68).

TRAF2-mediated NF- κ B pathways contain canonical and alternative routes (69–71). However, in the alternative NF- κ B pathway, downregulation of TRAF2 was shown to increase activity of the p52/RelB NF- κ B complex through stabilization and activation of NF- κ B-inducing kinase (NIK) (72). In this sense, TRAF2 appeared to participate in the assembly of protein complexes necessary for canonical NF- κ B activation, where TRAF2 would form multimeric complexes and subsequently facilitate Lys63 ubiquitination of downstream molecules, including cIAP1, RIP1, TANK and TAK1 (73,74). TRAF2-mediated Lys63 ubiquitination of RIP1 is a prerequisite for IKK complex activation and the removal of the central inhibitor I κ B α from NF- κ B complexes (21,44,45,74,75). In the present study, RIP1 was ubiquitinated by the PKR protein-binding function, and gene silencing of TRAF2 abolished such ubiquitination in β -cell line (Figures 7A, B), confirming the involvement of RIP1 downstream of TRAF2 in PKR protein-binding-triggered signaling. Additionally, Jackson-Bernitsas *et al.* clearly demonstrated that proliferation in human head and neck cancer cells was in part attributed to RIP1-associated constitutive NF- κ B activation (21). In our study, a similar role of RIP1 on proliferation in a pancreatic β -cell line was verified.

We also sought to examine whether PKR protein binding initiated and affected β -cell growth during progression of T2DM. Glucolipotoxicity and TNF α are closely associated with the compensatory capacity of *in vivo* β -cell mass and the development of T2DM (12,13,23,46–52). The catalytic activity of PKR is promoted by glucolipotoxicity and TNF α and implicated in β -cell pro-

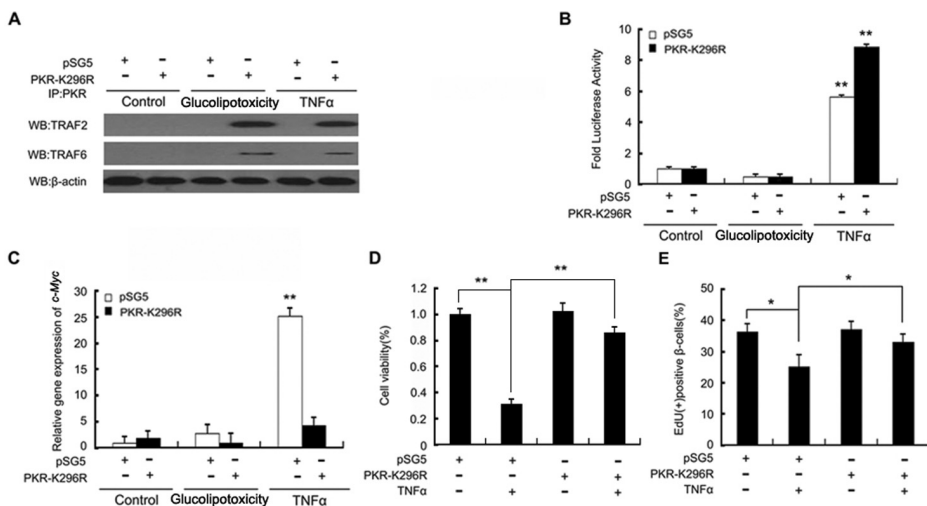


Figure 8. PKR protein binding also was evoked in response to glucolipototoxicity and TNF α , which remitted the deleterious effect of TNF α on MIN6 cell proliferation. Twenty-four hours after transfection with PKR-K296R or pSG5, cells were stimulated with or without glucolipototoxicity (glucose 16.7 mmol/L and 0.05 mmol/L palmitate) or proinflammatory cytokines TNF α (80 ng/mL) for 6 h. (A) Immunocomplexes of PKR were analyzed by Western blotting with antibodies against TRAF2 and TRAF6. (B) Luciferase activity was measured and shown in fold. (C) mRNA and protein levels of c-myc were determined by qRT-PCR (upper panel) and Western blotting (down panel). After transfection, MIN6 cells were stimulated with TNF α . (D) Cell viability and (E) quantification of EdU-positive β cell of MIN6 cells were shown. β -actin was detected as an internal control. Data are means \pm SEM of three separate experiments. * P < 0.05 versus control.

liferation inhibition (12). In PKR-K296R-induced β -cell line, PKR binding with TRAF2 and -6 was evoked in response to glucolipototoxicity and TNF α . However, PKR protein binding could not activate NF- κ B or c-Myc upregulation under conditions of glucolipototoxicity. Glucolipototoxicity also can trigger endoplasmic reticulum stress, which plays an important role in insulin resistance and the decline in pancreatic β cell mass during T2DM (34,76,77). It is possible that endoplasmic reticulum stress-mediated signals would interfere with the PKR protein-binding domain-induced NF- κ B pathway. In a PKR-K296R-induced β -cell line, NF- κ B activation did not result in a remarkable increase of c-Myc mRNA level (Figures 8B, C). Since overexpressed PKR-K296R was still able to alleviate the negative impact of TNF α on cells viability (Figure 8D) and proliferation (Figure 8E) of β -cell line, we presumed that a kinase-defective

mutant may protect β cells from TNF α -triggered deleterious effect through downregulating c-Myc mRNA level to a reasonable extent. As a vivid example, moderate expression of c-Myc can promote cell proliferation, whereas immoderate overexpression of c-Myc can exert a negative effect on cell growth through apoptosis (34,78,79). The protective effect of overexpressed mutant PKR-K296R may be partially attributed to the PKR protein-binding function. Of course, its powerful competitive capacity against endogenous PKR activity elicited by TNF α also may play a role.

Based on our studies (12), both kinase-catalytic and protein-binding activities of PKR would be induced in the development of type 2 diabetes, despite their opposite effects. While the proliferative effort of the PKR binding function in pancreatic β cells is highlighted, its side effect should not be overlooked. For instance, tumorigenesis might occur if the

PKR protein-binding function is elevated remarkably, leading to uncontrollable cell proliferation, and potential issues remain to be further verified.

CONCLUSION

This study shows that the PKR protein-binding function was inducible upon dimerization and could promote modest β -cell proliferation through the TRAF2/RIP1/NF- κ B/c-Myc pathway when its kinase-catalytic activity was suppressed. These results indicated a therapeutic opportunity in T2DM through pharmacological maintenance of the PKR protein-binding function after suppressing kinase activity, since inducible proliferation of β cells plays a pivotal role in the mass increase and compensatory capacity of islets for insulin resistance.

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DISCLOSURE

The authors declare they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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