Rab31, a receptor of advanced glycation end products (RAGE) interacting protein, inhibits AGE induced pancreatic β-cell apoptosis through the pAKT/BCL2 pathway

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Abstract. Receptor of advanced glycation end products (RAGE) mediates diverse signal transduction following ligand stimulation and plays an important role in diabetes complications and aging associated disease. We have previously verified that advanced glycation end products (AGE) bind to RAGE to cause pancreatic β-cell apoptosis through the mitochondrial pathway. However, the direct interacting protein(s) of RAGE in β cells has never been appreciated. In the present study, we utilized GST pull-down assay combined with mass spectrometry to identify the interacting proteins of the RAGE intracellular domain (C-terminal 43 amino acid of RAGE). Overall four RAGE interacting proteins, including Rab31, were identified with scores over 160. Rab31 was detected in three β-cell lines and confirmed to have interacted with RAGE via co-immunoprecipitation and immunostaining assays. This interaction was further enhanced by glycation-serum (GS) stimulation due to membrane distribution of Rab31 following treatment with GS. We further confirmed that Rab31 promoted RAGE endocytosis and inhibited GS-induced β-cell apoptosis through the pAKT/BCL2 pathway. These findings reveal a new RAGE interaction protein Rab31 that prevents AGE/RAGE-induced pancreatic β-cell apoptosis. Rab31 is therefore a promising therapeutic target for preserving functional β cells under diabetes conditions.

Key words: Receptor of advanced glycation end products, Rab31, Glycation-serum, Pancreatic β-cell apoptosis
signal transduction. Some reports have shown that low levels of sRAGE were significantly associated with an increased risk of diabetes, coronary heart disease, non-alcoholic fatty liver disease, and mortality [9-12]. On the contrary, sRAGE levels are increased in rosiglitazone-treated type 2 diabetic subjects and in atorvastatin-treated hypercholesterolemic subjects [13], raising the possibilities of capturing and eliminating AGE in circulation to prevent AGE-elicited tissue damage under diabetes conditions [14, 15]. In fact, administration of sRAGE results in shrinking of atherosclerosis plaques, lower infarct volumes, and prolonged allograft survival [16-18].

RAGE also functions as a pattern-recognition receptor and a pro-inflammatory molecular device for sensing danger signals [19]. Blocking all RAGE effects may weaken the ability of macrophages to clear pathogens, thereby rendering the body susceptible to infections [20]. This emphasizes the urgency of dissecting the intracellular signal transductions of the AGE/RAGE axis by identifying cTRAGE interacting proteins in specific cell types. At present, only a small set of cTRAGE interacting proteins have been verified. For example, cTRAGE interacts with diaphanous-1 to stimulate cell migration through activation of Rac1 and Cdc42 in glioma cells [21, 22]. The RAGE interactor PRAK also promotes the formation of the amyloid β-induced autophagosomes associated with Alzheimer’s disease [23]. Our previous studies have demonstrated that the AGE/RAGE axis triggers pancreatic β-cell apoptosis via the BCL2-dependent mitochondrial pathway and that activation of PPARγ protects β cells from impairments induced by AGE-containing GS [24, 25].

In the present study, our aim was to identify the interacting proteins of cTRAGE and to explore the contribution of these interactors to GS-induced β-cell apoptosis. Here, we demonstrated that Rab31 is a cTRAGE interacting protein and negatively affects GS-induced β-cell apoptosis, making it a promising therapeutic target for preserving functional β cells and treating diabetes.

Material and Methods

AGE-fetal bovine serum preparation
Glycation serum (GS) was prepared and the concentration of AGE within the GS was measured as described previously [8].

Primary islet and cell culture
Rat primary islets were isolated as previous description [25]. Primary islets were cultured in RPMI-1640 (GIBCO, USA) containing 11.1 mM D-glucose, 10% fetal bovine serum (FBS, GIBCO), 100 U/mL penicillin, and 100 µg/mL streptomycin. Rat insulinoma INS-1 cells were cultured in RPMI-1640 containing 11.1 mM D-glucose, 10% FBS, 50 µM β-mercaptoethanol (Sigma Aldrich, MO), 100 U/mL penicillin, and 100 µg/mL streptomycin [24]. Mouse MIN6 cells were cultured in Dulbecco’s modified Eagle medium (DMEM, GIBCO) containing 25 mM D-glucose, 15% FBS, 50 µM β-mercaptoethanol, 100 U/mL penicillin, and 100 µg/mL streptomycin [26]. Mouse βTC6 cells were cultured in DMEM medium containing 25 mM glucose, 15% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin [27]. All primary islets and cells were cultured at 37°C in a humidified incubator containing 5% CO₂ and 21% O₂. Non-glycation serum (NG) or GS was added to the appropriate experiments, and cells were incubated for indicated period.

Real-time RT-PCR
INS-1 cells (2 × 10⁶ cells per well) were seeded in 35 mm dishes and treated with NG or GS, as described above. Total RNA was extracted using Trizol reagent, according to the manufacturer’s protocol (Invitrogen, USA). First-strand cDNA synthesis was performed using 1 µg of total RNA and an avian myeloblastosis virus reverse transcription system. Quantitative real-time PCR was performed using the SYBR Green PCR Master Mix and Roche LightCycle480 II Sequence Detection System (Roche Diagnostics). The primers of rat genes were designed using the software Primer Express (Applied Biosystems, CA). Primer sequence (5’-3’): rat Rab31 forward: GACACGGGGGTGGGAAATC; reverse: ACAATCAGGTTTCAGCTA. Rat β-Actin forward: CCACTTATGAGGTTTGACCG; reverse: TTATGCAGCTTGAATC. β-Actin was used as an internal control for gene expression.

Western blot analysis and Co-IP
INS-1 cells were cultured and treated as described. Whole-cell extracts were prepared by lysing the cells with ice-cold lysis buffer: 50 mM Tris–HCl (pH 7.4), 1% NP-40; 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and complete proteinase inhibitor (one tablet per 10 mL; Roche Molecular Biochemicals). After protein content determination with a DC protein assay kit, western blotting was performed as previously described [24]. Individual immunobots were probed with antibodies to rabbit anti-Rab31 antibody (Bioworld), rabbit anti-BCL2 antibody (Santa Cruz Biotechnology) diluted to 1:500; mouse anti-RAGE (Abcam, USA) antibody, mouse anti-T-AKT antibody (Cell Signaling Technology), rabbit anti-P-AKT (S473) antibody (Cell Signaling Technology) diluted to 1:1,000; mouse antmyc antibody (Santa Cruz Biotechnology), and mouse
anti-EGFP antibody (Santa Cruz Biotechnology) diluted to 1:3,000. Target protein levels were quantified relative to the levels of control protein using mouse anti-α-tubulin antibody (Sigma Aldrich) diluted to 1:5,000.

For Co-IP, transiently transfected INS-1 cells in 60 mm dishes were lysed with lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM sodium orthovanadate, 1 mg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride), sonicated three times for 5 s each, and then centrifuged at 13,000 rpm for 20 min at 4°C. Immunoprecipitation was performed with an anti-EGFP or anti-myc antibody and Protein A/G Plus agarose beads. Immunoprecipitates and input were analyzed by western blotting, as described above.

Plasmid construction

The rat pCMV5-myc-Rab31 expression plasmid was constructed by inserting the full-length coding region sequence into pCMV5-myc vector, the rat RAGE expression plasmid was constructed by inserting the full-length coding region sequence into EGFP-N1 vector, and the RAGE intracellular domain (ctRAGE) prokaryotic expression plasmid was constructed by inserting C-terminal 43 amino acids of RAGE sequence into pGEX-4T vector. All constructions used here were sequenced and confirmed to be correct.

GST-pull down

The plasmid for pGEX-4T-ctRAGE was transformed into E. coli BL21 (DE3) competent cells. The fusion protein of GST-ctRAGE was expressed and purified using a prokaryotic expression system. Approximately 100 μg of GST or GST-ctRAGE fusion protein was immobilized with glutathione 4B beads in 50 μL reaction buffer, and then gently rolled at 4°C for 2 h. A portion of the mixture was centrifuged at 4°C for 5 min at 900 g, combined with 35 μL 1.25 × SDS buffer solution, and boiled for 8 min. The protein was subjected to polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue to determine the purification effect. The other portion of the sample was centrifuged for 5 min at 900 g, and the supernatant was decanted and added to the supernatant of a protein lysate of rat lung tissue and incubated at 4°C overnight. The slides were then incubated for 1 h with a donkey-anti-rabbit Delight 594 or a donkey-anti-mouse FITC-conjugated antibody (Bioworld). Finally, the slides were incubated with Hoechst33342 and mounted with Vectashield (Vector laboratories, Burlingame, CA). The fluorescence was visualized with an FV1200 laser scanning confocal microscope (Olympus). Images were processed with an FV10-ASW viewer (Olympus).

Flow cytometry analysis

INS-1 cells (0.5 × 10⁶ cells per well) were cultured in 35 mm plates and treated as described above. The cells were then harvested and fixed overnight in 1 mL 75 % ice-cold ethanol at −20°C. After fixation, the cells were washed in PBS and stained with 300 μL propidium iodide solution (PI: 50 μg/mL, Sigma) containing 25 μg/mL RNase. The cells were incubated at 37°C for 0.5 h in the dark and analyzed using a FACSCalibur flow cytometer and CellQuest Pro software (Becton-Dickinson Immunocytometry Systems, CA).

Statistical analysis

Comparisons between pairs of groups were performed using Student’s t test. The results are presented as mean ± SEM. A p value <0.05 was considered statistically significant.

Results

Identification of Rab31 as an interacting protein of RAGE

We identified proteins that interact with the RAGE
cytoplasmic domain (ctRAGE) by GST pull-down assays. Rat lung was used as a starting material for the purification of RAGE binding proteins due to its mainly expressed in the lung tissue [28]. Thus, the purified GST-ctRAGE and GST control proteins were incubated with protein lysate from rat lung tissue, and the interacting proteins were separated by SDS-PAGE. The gel was silver stained and four different bands associated with the GST-ctRAGE group were cut out for identification by mass spectrometry (MS) (Fig. 1A). Four proteins ranking first in each band were obtained by comparing and analyzing the peptide fingerprinting with Mascot software. These were nucleoporin P62, Rab31, low-quality protein G protein 4-like isoform x1 (GBP4-like isoform x1), and insulin-like growth factor mRNA binding protein 2 (IGF2BP2) (Fig. 1B).

Based on the spatial localization and the analysis of known biological functions, we chose Rab31 for further study of its role in RAGE function. Rab31 has a score of 162 and sequence coverage of 62.3% (Fig. 1C and D). These results suggest that Rab31 is a potential interaction protein of RAGE.

AGES potentiate Rab31 binding to its receptor RAGE

Rab31 has been reported to be abundant in the heart, adipose tissue, and parts of the neuroglia [29], but it has not been studied in pancreatic β cells. Therefore, we first compared the expression level of Rab31 between rat lung and primary islets, as we had identified Rab31 as an interaction protein in rat lung tissue. We found that the Rab31 gene was more abundantly expressed in rat primary islets than in rat lung tissue (Data not shown).

To further confirm the functionality of Rab31 in β cells, we detected the protein level of Rab31 in three different rodent β cell lines, and primary pancreatic islets from type 2 diabetic Goto-Kakizaki (GK) rats. Rab31 was abundantly expressed in rat INS-1, mouse βTC6, and mouse MIN6 cells (Fig. 2A). Interestingly, protein level of Rab31 in primary islets from diabetic GK rats was significantly increased compared to that from age-matched wistar rats (Fig. 2A). Immunofluorescence staining showed that Rab31 was distributed uniformly in the cytoplasm under the normal physiological state (Fig. 2B). After stimulation with GS, Rab31 became congregated in the cytoplasm (Fig. 2C), indicating a modulation of Rab31 by AGES in INS-1 cells. These results showed that Rab31 is expressed in β cells and could be functionally modulated by AGES.

We next constructed full-length RAGE (pEGFP-RAGE) and full-length Rab31 (pCMV5-myc-Rab31) plasmids to study their interaction. Co-transfected INS-1

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**Fig. 1** Rab31 was identified as an interaction protein of RAGE. (A) GST-ctRAGE fusion protein and GST control protein were incubated with rat lung tissue protein lysate for 12 h at 4°C. The GST-pull down protein complexes of GST-ctRAGE and GST control were stained by silver staining at 12% (left) and 10% (right) SDS-PAGE electrophoresis. (B) Four bands (1, 2, 3, and 4) were cut for mass spectrum analysis. The protein ranked first per band was listed. (C) Protein score of Rab31 using MASCOT software was shown. (D) Comparison of the obtained sequence with the Rab31 sequence. Matched amino acids of Rab31 were highlighted in bold.
cells were stimulated with GS for 15 min, followed by immunofluorescence staining. Confocal microscopy visualized a dot-shape colocalization of RAGE and Rab31 in the cytoplasm but not in the cell membrane (Fig. 2D). We used co-immunoprecipitation (co-IP) assay to further verify their interaction. Both EGFP-RAGE and myc-Rab31 

Fig. 2  AGE potentiate Rab31 binding to its receptor RAGE. (A) Western blot was used to detect the expression of Rab31 in rodent pancreatic β cell lines: mouse βTC6, rat INS-1, and mouse MIN6 cells, as well as in rat primary islets isolated from 14-week-old Wistar and type 2 diabetic Goto-Kakizaki (GK) rats. α-Tubulin was used as internal control. (B) Immunofluorescence staining of Rab31 in INS-1 cells was observed by confocal microscopy. Rab31: Red; nuclei: Blue. Scale bar: 20 μm. Zoom figure on the right. Scale bar: 4 μm. (C) INS-1 cells were transfected with pCMV5-myc-Rab31 for 24 h and then stimulated with GS for indicated time. Cells were fixed for immunofluorescence staining with myc-antibody and the distribution of myc-Rab31 fused protein was observed by confocal microscopy, myc-Rab31: Red; nuclei: Blue. Scale bar: 10 μm. Zoom figures were below. Scale bar: 4 μm. (D) INS-1 cells were transfected with pEGFP-RAGE or pEGFP-N1 control plasmid for 24 h, and then treated with GS for additional 15 min. Cells were co-stained with anti-EGFP and anti-Rab31 antibodies and representative images were recorded by confocal microscope. EGFP: Green; Rab31: Red; Nuclei: Blue. Scale bar: 10 μm. Zoom figures of EGFP-RAGE and Rab31 on the right. Scale bar: 1 μm. (E and F) INS-1 cells were co-transfected with pCMV5-myc-Rab31 with pEGFP-RAGE for 24 h, and then harvested for immunoprecipitation by using anti-EGFP antibody (E) or anti-myc antibodies (F), coimmunoprecipitates and input samples were detected by western blot. (G) INS-1 cells were treated with NG or GS for 15 min and co-stained with anti-RAGE and anti-Rab31 antibodies. RAGE: Green; Rab31: Red; Nuclei: Blue. Scale bar: 20 μm. (G') Enlarged images of figure G. Scale bar: 4 μm.
co-immunoprecipitated with each other following GS treatment (Fig. 2E and F), indicating that they formed a complex after GS stimulation. An endogenous interaction between RAGE and Rab31 was also observed by immunostaining (Fig. 2G and G'). Taken together, these results suggest that Rab31 interacts with RAGE in the presence of AGEs.

**Rab31 binds to RAGE to induce its degradation under GS treatment**

We investigated the biological function of Rab31 binding to RAGE after GS stimulation in INS-1 cells using RNA interference to knock down Rab31. Three siRNAs targeting Rab31 were introduced and siRab31-002 effectively suppressed Rab31 expression at both the mRNA and protein levels (Fig. 3A and B). Therefore, siRab31-002 was utilized in further experiments.

Rab31 is a small GTP-binding protein and mainly functions in early endosome formation and endosome-to-Golgi transport [30]. We therefore used EGFP-fused RAGE to monitor whether membrane-localized RAGE would be regulated by Rab31. In the siNC group, EGFP-RAGE was mainly expressed in the cell membrane under basal condition, while GS stimulation caused a time-dependent endocytosis of EGFP-RAGE. However, GS-stimulated RAGE endocytosis was largely inhibited by Rab31 knockdown, despite an observed lack of endocytosis under the basal condition (Fig. 3C). Green fluorescent spots were counted in both groups with or without GS treatment at the indicated minutes, and the total number of fluorescent spots following GS stimulation was less in siRab31 group than in the control group (Fig. 3D).

We also queried RAGE endocytosis for signaling transduction or for degradation by detecting the protein level of RAGE. Consistent with a previous result, GS stimulation triggered an increase in RAGE protein level. Knockdown of Rab31 further enhanced the amount of GS-stimulated RAGE protein (Fig. 3E and F), whereas overexpression of Rab31 slightly but significantly decreased the GS-stimulated RAGE protein level (Fig. 3G and H). These results indicated that Rab31 expression was negatively correlated with RAGE protein levels in GS treated INS-1 cells, suggesting that Rab31 is specifically involved in the internalization process of RAGE and contributes to GS-induced RAGE degradation.

**Inhibition of Rab31 expression promotes GS induced β-cell apoptosis partly through the pAKT/BCL2 pathway**

We showed that Rab31 can down-regulate RAGE protein levels under the stimulation of GS. We further clarified the role of Rab31 in GS-mediated β-cell apoptosis by first knocking down Rab31 and detecting GS-induced apoptosis in INS-1 cells. Knockdown of Rab31 significantly aggravated GS-induced β-cell apoptosis, as observed by pyknotic nuclei (Fig. 4A and B). The GS-inhibited protein expression of BCL2 was further decreased by Rab31 knockdown (Fig. 4C and D), suggesting that Rab31 was involved in β-cell apoptosis in a BCL2-dependent manner.

AGEs-RAGE signal transduction engages at least four classical signal pathways: PI3K/AKT, MAPK/ERK, MAPK/JNK, and MAPK/P38 [31]. Therefore, we used pathway-specific inhibitors to block signal transduction and determine which pathway was involved in GS-induced β-cell apoptosis. We found that the AKT-specific inhibitor LY294002 significantly protected INS-1 cells from GS-induced apoptosis (Fig. 4E), while three MAPKs inhibitors had no protective effects on β-cell apoptosis (Data not shown). However, knockdown of Rab31 had no effect on GS-provoked AKT activation, as shown by the measured P-AKT levels (Fig. 4F). These results suggest that the GS-stimulated recruitment of Rab31 is a downstream event of AKT signaling transduction and that Rab31 has a potential anti-apoptotic effect against GS in β cells via the BCL2-dependent pathway.

**Overexpression of Rab31 inhibits GS-induced apoptosis in INS-1 cells**

We examined the protective role of Rab31 by introducing a Rab31 overexpression plasmid. Elevation of Rab31 expression decreased the number of apoptotic INS-1 cells from 30.54% to 15.61% following GS treatment (Fig. 5A and B). Overexpression of Rab31 also reversed the decrease in BCL2 protein expression caused by GS (Fig. 5C and D). These results further confirmed that Rab31 largely alleviates GS-induced β-cell apoptosis that depends on the anti-apoptosis role of BCL2.

**Discussion**

RAGE mediates diverse signal transductions in different cell types following ligand stimulation [32]. One of the main RAGE ligand types, the AGEs, are enriched in diabetic patients and contribute to multiple diabetic complications [4]. We and other groups have shown that pancreatic β cells per se are targets of AGE and vulnerable to AGE-induced apoptosis due to the presence of the RAGE receptors in β-cell membranes [8, 24, 25]. However, we failed to identify the direct interacting partner of the RAGE cytosolic domain (ctRAGE) that is obligatory for AGE/RAGE mediated β-cell apoptosis. In this study, GST pull-down, combined with MS proteomics, were engaged to identify the ctRAGE interactors. We successfully disclosed that Rab31 is one candidate that reduces...
Fig. 3 Rab31 binds to RAGE to induce its degradation under GS treatment.

(A and B) qPCR (A) and western blotting (B) analysis of Rab31 in INS-1 cells transfected with siRab31-001, siRab31-002, siRab31-003 and negative control siRNAs (siNC) for 48 h. Actb was used as internal standard of gene expression. α-Tubulin was used as an internal control of protein levels. n = 3 per group. (C) INS-1 cells were cotransfected with siRab31 or siNC with EGFP-RAGE for 48 h, and then stimulated with GS at indicated minutes. The EGFP-fused RAGE was observed by confocal microscopy. Scale bar: 20 μm. Enlarged pictures with white circles showed RAGE endocytosis particles for quantification. Scale bar: 4 μm. (D) The green fluorescent dots larger than 10 pixels squared were counted by Image J software and measured in Pixel Square (PIX sq.). The ordinate value is the average number of fluorescent points per cell. About 100 cells per group were measured. (E) INS-1 cells were transfected with siRab31 or siNC for 48 h and then treated with NG or GS for additional 24 h. The whole protein was extracted and the protein expressions of RAGE and Rab31 were detected by western blot. (F) Gray level of RAGE in figure E was analyzed. n = 3 per group. (G) INS-1 cells were transfected with pCMV5-myc-Rab31 for 24 h and then treated with NG or GS for additional 24 h, western blot was used to detect the protein expressions of RAGE and Rab31. (H) Gray level of RAGE in figure G was analyzed. n = 3 per group. Values, mean ± SEM. * p < 0.05 or ** p < 0.01.
AGE/RAGE signaling and protects β cells from AGE-induced apoptosis via the P-AKT/BCL2 pathway, thereby exhibiting a therapeutic potential against β-cell failure.

The finding of Rab31 interaction with cRAGE was confirmed by co-IP and immunostaining assays. Rab31 is a member of Rab5 subfamily of small GTPases associated with cancers and metabolic diseases [33, 34]. It is mainly located in early endosomes and trans-Golgi vesicles, where it functions in early endosome formation and endosome transport to the Golgi [35, 36]. Irfan et al. have reported that Rab31 interacts with Gapex-5 to reduce the translocation of Glut4 vesicles to the cell surface and inhibit insulin-stimulated glucose uptake by adipocytes [37]. In addition, Rab31 interacts with epidermal growth factor receptor (EGFR) upon EGF stimulation and promotes EGFR internalization through EGF/EGFR-mediated endocytosis [38]. Rab31 is also phosphorylated by EGFR and sorts ligand-bound EGFR to multivesicular endosomes and controls exosome biogenesis [30].

Fig. 4  Inhibition of Rab31 promotes GS induced β-cell apoptosis partly through pAKT/BCL2 pathway. (A) INS-1 cells were transfected with siRab31 and siNC control for 24 h, and then treated with NG or GS for additional 72 h. Apoptosis cells were detected by Hoechst staining. Scale bar: 20 μm. (B) The cells with pyknosis of figure A were counted \((n > 1000\) per group). (C) INS-1 cells were transfected with siRab31 and siNC control for 48 h, and then treated with NG or GS for additional 24 h. The whole protein was extracted and the protein levels of BCL2 and Rab31 were detected by western blot. α-Tubulin was used as an internal control. (D) Gray intensity of BCL-2 in figure C was analyzed. \(n = 3\) per group. (E) INS-1 cells were pretreated with 10 μM LY294002 for 2 h, and then co-treated with GS for 60 h. The apoptotic cells in each group were detected by PI staining and flow cytometry (\(n = 3\) per group). (F) INS-1 cells were transfected with siRab31 or siNC for 48 h, and then treated with GS for 0’ and 15’. The whole protein was extracted and the protein levels of P-AKT(S473), T-AKT and Rab31 were detected by western blot. Values, mean ± SEM. * \(p < 0.05\); ** \(p < 0.01\).
Some reports have shown that RAGE could also be endocytically transported after ligand binding, including by AGE/RAGE complex endocytosis [39]. However, the underlying mechanisms remain undefined. Here, we have shown at least two lines of evidence that Rab31 can promote AGE/RAGE complex endocytosis: the first is that RAGE and Rab31 co-localized at both the membrane and cytoplasm, and the second is that knockdown of Rab31 increased membrane localization upon GS stimulation. These data indicate that Rab31 interacts with RAGE in the presence AGE stimulation and promotes AGE/RAGE complex endocytosis.

Receptor endocytosis has at least two outcomes. One is to stop signal transduction once receptors are internalized to regulate cellular phenotypes [40]. These processes include directing the receptor to intracellular compartments to reduce the number of cell surface receptors and movement of the receptor to lysosomes for degradation. Alternatively, receptor endocytosis can increase the rate of signal transduction and cargo movement [41]. Rab31 has been reported to enhance the degradation of EGFR via transportation from early endosomes to late endosomes after ligand stimulation and to result in a moderate decrease in astroglia cell proliferation [29]. On the contrary, elevated Rab31 expression stabilizes the levels of MUC1-C, a multifunctional oncoprotein, by reducing its lysosomal degradation and elicits MAPK and PI3K/AKT pathways to cause cancer progression [42, 43]. Rab31 also recruits the signaling adaptor APPL2 to promote FcγR-mediated phagocytosis by enhancing PI3K/AKT signaling and reducing p38 signaling [44]. Our data have shown that GS stimulation triggers Rab31 membrane trafficking and causes AGE/RAGE complex endocytosis. However, the net protein levels of RAGE and Rab31 were divergently expressed, resulting in an increase in RAGE and a decrease in Rab31 protein following GS stimulation. The protein levels of RAGE can also be decreased by over-expression of Rab31 and increased by knockdown of Rab31. Consequently, GS-induced BCL2 inhibition and...
β-cell apoptosis were inhibited by Rab31 overexpression and enhanced by Rab31 knockdown. The inhibition of AKT signaling transduction also protected β cells from GS-induced apoptosis. Thus, we conclude that Rab31 binds to RAGE to enhance AGE/RAGE complex endocytosis and induce RAGE degradation, probably by modulating the AGE-induced pAKT/BCL2 pathway to present an antiapoptotic effect, at least in the INS-1 β cell line.

The reason that overexpression of Rab31 did not completely reverse GS-mediated β-cell apoptosis is unclear. Although Rab31 has a negative regulatory effect on the AGE/RAGE signaling, this relies on AGE/RAGE complex endocytosis for RAGE degradation. During cellular processes, the AGE/RAGE complex may transduce oxidative stress signals to NF-κB activation [45], thereby promoting gene expressions of inflammatory factors, as well as RAGE gene and protein expressions. This positive feedback effect on RAGE expression results in extra damage and longer effects on the AGE-induced cells. Nonetheless, Rab31 does have the effect of counteracting the excessive upregulation of RAGE protein levels and has some protective effect on β cells stimulated by GS.

Focusing on ctRAGE to identify β-cell protectors is advisable. Pancreatic β cells are specialized to secret insulin, the only hormone in human body that is capable of lowering blood glucose levels. During the progression of diabetes, the number of β cells declines due to growth inhibition, trans/dedifferentiation, and apoptosis [46]. Factors associated with β-cell apoptosis include glucolipotoxicity, inflammation, amyloid deposition and AGE toxicity [47-49]. Preserving functional β-cell number and manipulating β-cell regeneration are important for the prevention and treatment of diabetes [50]. However, therapeutic targets are not locked in, as diabetes has complex internal environments. Chronic hyperglycemia initiates multiple reactions and generates numerous kinds of AGEs in vivo. Thus, GS is likely the most reasonable substance to mimic the in vivo formation of AGE in the body. We have confirmed that GS is highly enriched in AGEs and more toxic than high glucose to β cells [8]. Either antibody blockage or si-RNA-based knockdown of RAGE could significantly protect β cells from GS impairments through the BCL2/Trp53 axis mediated endogenous mitochondrial pathway [8, 24]. Thus, identification of ctRAGE interactors that promote RAGE degradation or inhibit AGE/RAGE signal transduction will enable the discovery of β-cell protectors under diabetes conditions. Rab31 is one such interacting protein for RAGE and a promising target for preserving functional β-cell numbers.

In conclusion, our study reveals that Rab31 is a cytosolic interacting protein of RAGE. Upon stimulation by GS, Rab31 decreases its level to promote β-cell apoptosis. Inhibition of Rab31 reduces AGE/RAGE complex endocytosis and causes membrane RAGE localization, leading to an enduring AGE/RAGE signaling. Overexpression of Rab31 strongly rescues the BCL2 protein level and suppresses GS-induced β-cell apoptosis. Thus, therapeutic strategies aimed at Rab31-mediated RAGE endocytosis may represent promising approaches for the prevention and treatment of diabetes.

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Disclosure

The authors declare that there is no conflict of interest.

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