Supporting Information

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Methods

Cell Culture, DNA Transfection, and Plasmids. NFκB p65 knockout mouse embryonic fibroblasts cell lines (*rela*$^{-/-}$-2) (1) were kindly provided by Alexander Hoffmann (University of California at San Diego, La Jolla, CA). Rat vascular smooth muscle cells (2, 3) and A549 cells (human lung carcinoma, American Type Culture Collection) were maintained in DMEM (Invitrogen) supplemented with 10% inactivated FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in 5% CO_2. DNA transfection was performed by lipofection according to the manufacturer's instructions (Lipofectamine Plus; Invitrogen). Expression constructs encoding the human NFκB p65 wild-type and various C-terminal deletion mutant genes were prepared by PCR and cloned into the pcDNA3.1-His mammalian expression vector (Invitrogen). The human *O*-GlcNAcase (OGA) gene was cloned into the pRK5-FLAG mammalian expression vector (Genentech), and the human O-GlcNAc transferase (OGT) gene was cloned into the p3X FLAG-CMV7.1 mammalian expression vector (Sigma–Aldrich). The human IκBα gene, the luciferase reporter plasmid, and the β-galactosidase control plasmid were kindly provided by Tae Ho Lee (Yonsei University, Seoul, Korea). NFκB p65 Ser or Thr to Ala substitution mutants, which included NFκB p65 T322A (Thr-322 mutated to Ala), T352A, and the double mutants T322A/T352A, T322A/S353A, T322A/S354A, T322A/S356A, and T322A/T357A were created by site-directed mutagenesis (Stratagene) as described in ref. 4. The mutations were confirmed by DNA sequence analysis (COSMO).

Reagents and Antibodies. Streptozotocin (STZ) was purchased from Sigma. *O*-(2-acetamido-2-deoxy-D-glucopyroanosylidene)-amino-*N*-phenylcarbamate (PUGNAc) was purchased from Toronto Research Chemicals. Antibodies against NFκB p65 (A and F-6), IκBα (C-21), VCAM-1 (H-276), histone H2A (H-124), α-tubulin (TU-02), and actin (C-2) were purchased from Santa Cruz Biotechnology. The CTD110.6 mouse monoclonal antibody for *O*-GlcNAc detection was purchased from Covance. Antibodies against OGT (DM-17), phospho-Ser (mouse monoclonal), phospho-Thr (mouse monoclonal), FLAG (rabbit polyclonal), and FLAG conjugated to FITC were purchased from Sigma. The antibody against OGA (rabbit polyclonal) was purchased from Ab Frontier.

Immunoblotting, Immunoprecipitation, and Immunostaining. For immunoblotting, cells were lysed in RIPA buffer [150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and 50 mM Tris·HCl (pH 8.0)] supplemented with protease inhibitor mixture (Roche) on ice. Protein concentrations were determined by using the Bio-Rad protein assay. Cellular extracts were subjected to reducing SDS/PAGE and transferred to nitrocellulose membranes (GE Healthcare). Immunoblotting bands were quantified by densitometry. For immunoprecipitation, cell lysates were mixed gently with specific antibodies and protein A beads (GE Healthcare) for 3 h at 4 °C. Immunoprecipitates were washed with lysis buffer, eluted with SDS sample buffer, and subjected to SDS/PAGE. For immunostaining, cells were plated onto coverslips and treated as described. Cells were fixed, permeabilized, and sequentially incubated with anti-NFκB p65 antibody followed by anti-mouse or anti-rabbit Ig covalently conjugated to FITC or rhodamine (R) (Santa Cruz Biotechnology). FLAG-OGT or OGA was detected by using an anti-FLAG antibody covalently conjugated to FITC (Sigma). Immunostaining was examined by using an Axioplan 2 multipurpose microscope (Carl Zeiss MicroImaging) as described in ref. 5.

Luciferase Assays. Luciferase assays were performed as described in ref. 6. Cells were cotransfected with 100 ng of a multimerized κB-luciferase reporter gene plasmid and a β-galactosidase control plasmid and then incubated for 12 h. Cells were exposed to high glucose for 24 h or treated with STZ, PUGNAc, or OGT for 12 h. Cell extracts were prepared, and the levels of luciferase activity were determined by using a luminometer (Turner Designs).

Electrophoretic Mobility Shift Assay (EMSA). EMSA experiments were performed as described in ref. 7. The specificity of the assay was examined by supershift assays with antibodies against NFκB p65. Gel images were detected by using the BAS-2500 (Fuji Film).

Mapping *O*-GlcNAc Sites with ESI-MS/MS. Mapping of *O*-GlcNAc sites by using electrospray ionization tandem mass spectrometry (ESI-MS/MS) was performed as described (8, 9).

Diabetic Mouse Models. Seven- to 8-week-old male C57BL/6 mice were injected i.p. once a day for 4 sequential days with 50 mg of STZ per kg of body weight (10). Blood glucose concentrations of mice were measured daily, and mice were considered diabetic if the blood glucose concentrations were ≥400 mg/dL. Mice were killed and immediately dissected. Tissues were obtained and homogenized in lysis buffer as described in ref. 10. Nuclear extracts of the tissues were harvested as described in ref. 11).

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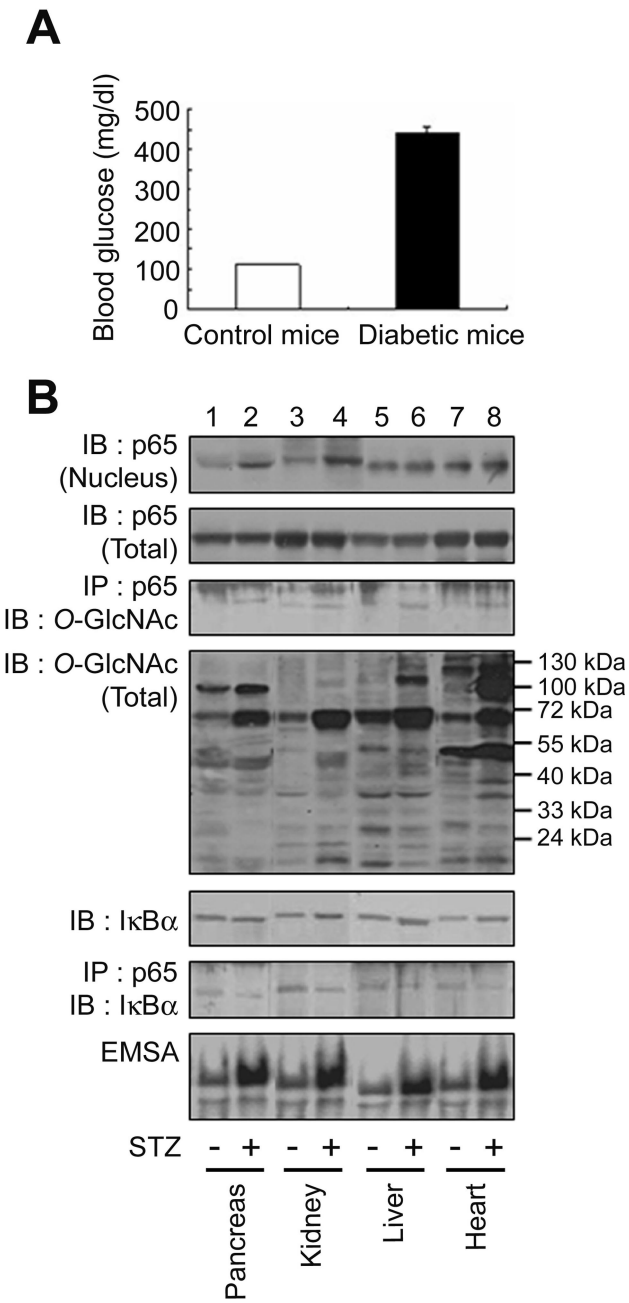

Fig. S1. *O*-GlcNAcylation and the transcriptional activity of NFκB p65 are increased in a STZ-induced diabetic mouse model. (*A*) Mice were injected with STZ (200 mg/kg) to induce diabetic conditions, and after 1 week, blood glucose concentrations of mice were measured. The blood glucose concentration of control mice and STZ-induced diabetic mice was ≈100 mg/dL and 400 mg/dL, respectively. The data shown represent the mean ± SD ($n = 4$). (*B*) The indicated tissues of control and diabetic mice were homogenized in lysis buffer, and the extracts were analyzed for total NFκB p65, *O*-GlcNAc, and IκBα levels by using immunoblotting (IB) (2nd, 4th, and 5th panels, respectively). NFκB p65 immunoprecipitates from the tissue extracts were analyzed for *O*-GlcNAc and IκBα using immunoblotting (3rd and 6th panels, respectively) IP, immunoprecipitation. Nuclear extracts from the tissues were used for immunoblotting of NFκB p65 and EMSA (1st and 7th panels, respectively).

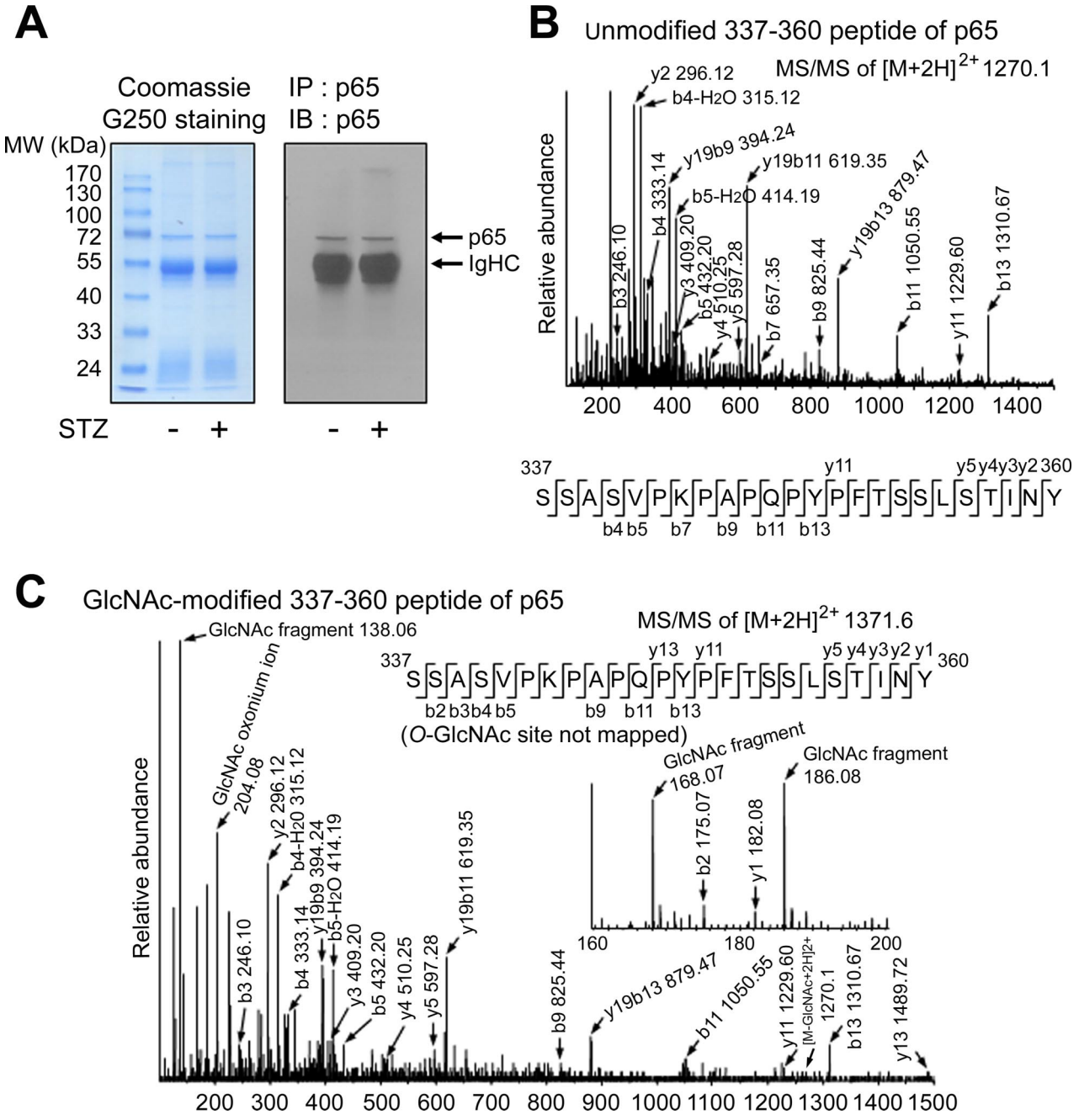

Fig. S2. Mass spectrometry analysis by using quadrupole time-of-flight (Q-TOF) MS shows the existence of *O*-GlcNAcylation in NFκB p65 in the peptide fragment encoding amino acids 337–360. (*A*) Endogenous NFκB p65 was immunoprecipitated (IP) from A549 cell lysates that had been untreated (−) or treated (+) with 2 mM STZ for 12 h. The NFκB p65 immunoprecipitates were then subjected to SDS/PAGE, detected by Coomassie blue G250 staining, and analyzed by immunoblotting (IB) for NFκB p65. The migration of NFκB p65 and immunoglobin heavy chain (IgHC) is indicated by arrows. Immunoprecipitated NFκB p65 was alkylated, and in-gel digestion was performed with AspN and trypsin at the same time, because trypsin alone gave peptides containing Thr-352 that were too large in size. *O*-GlcNAcylation sites were mapped by using Q-TOF MS analysis. (*B*) The Q-TOF spectrum and sequencing results of an unmodified peptide obtained from AspN + tryptic digests of NFκB p65 are shown. These peptides correspond to residues 337–360 (SSASVPKPAPQPYPFTSSLSTINY) at $[M + 2H]^{2+}$ *m/z* 1,270.1. Several major peaks on the Q-TOF MS spectrum were assumed to be generated by an internal cleavage (12). (*C*) The Q-TOF spectrum and sequencing results of a GlcNAc-modified peptide corresponding to residues 337–360 at $[M + 2H]^{2+}$ *m/z* 1,371.6. The GlcNAc oxonium ion and GlcNAc fragments ions are observed, but *O*-GlcNAc sites are not precisely mapped.

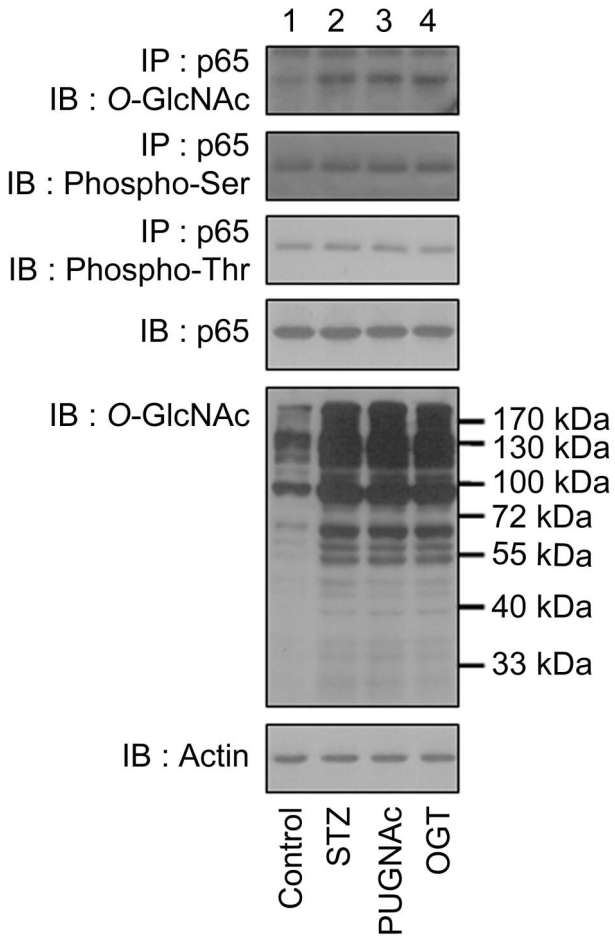

Fig. S3. The NFκB p65 subunit is modified by *O*-GlcNAc. A549 cells were incubated with STZ (2 mM), PUGNAc (100 μM), or transfected with a plasmid expressing FLAG-tagged OGT (2 μg/mL) for 12 h. NFκB p65 immunoprecipitates (IP) obtained from the cellular extracts were analyzed by immunoblotting (IB) for *O*-GlcNAc, phospho-Ser, and phospho-Thr (1st, 2nd, and 3rd panels, respectively. Total levels of NFκB p65 (4th panel) and *O*-GlcNAc (5th panel) in these extracts were also examined. Actin was included as a loading control (6th panel).

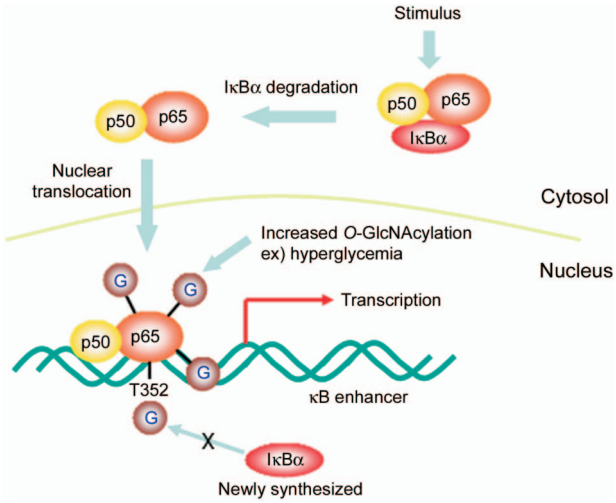

Fig. S4. Model for the role of *O*-GlcNAcylation in NFκB transcriptional activation. *O*-GlcNAcylation of NFκB p65 at Thr-352 prevents the binding of IκBα to NFκB and increases NFκB nuclear translocation. Consequently, NFκB transcriptional activity increases.