Molecular Modeling and Biochemical Studies of Transglutaminase 2 Mutation Found in Patients with Early-onset Type 2 Diabetes

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Transglutaminase 2 is a calcium-dependent multi-functional protein that catalyzes the formation of Nε-(γ-glutamyl)lysine isopeptide bond between lysine and glutamine residues.[1] Transglutaminase 2 induces Nuclear Factor-κB (NF-κB) activation through the polymerization of I-κBα, which results in anti-apoptotic cellular function.[2,3] It is highly associated with inflammatory diseases and various cancers.[4,5]

Recently, three types of missense mutations (M330R, I331N, N333S) in transglutaminase 2 were found in patients with early-onset type 2 diabetes. Mutated residues, moreover, were located near the catalytic site, and the mutations resulted in loss of the transamidation activity of transglutaminase 2 from the *in vitro* analysis.[6] To explain why these mutants do not have transamidation activity *in vitro*, we analyzed the structural basis of functional loss in mutants using the molecular modeling method. The *in vivo* analysis of functional loss of mutants was studied by monitoring the substrate I-κBα level in cytosolic fraction of PANC-1 cells.

Structural basis of mutation and loss of function by molecular modeling. Poirzio *et al*. reported that three mutations in early-onset type 2 diabetes lacked their transamidation activity.[6] The structural basis of the loss of function was analyzed by the molecular modeling method. A five-amino-acid sequence around the glutamine 182 (SQ[182]SKV) of β-casein was used as substrate for modeling.[7]

The crystal structure of the active transglutaminase 2 complex with the peptide inhibitor has been reported.[8] The docking models were determined using the inhibitor (Ac-P(DON)LPF-NH2) and X-ray crystal structure of transglutaminase 2 (2Q3Z) as a template (Figure 1A).

We also derived several transglutaminase 2:I-κBα complex models using the IQ[266]QQL, QQ[267]QLG and GQ[313]RLT sequences, respectively.[9]

The length between the M330 of the transglutaminase 2 and the proline of the inhibitor (Ac-P(DON)LPF-NH2) is about 4.3 Å, as shown in the open form of the transglutaminase 2 crystal structure (2Q3Z; Figure 1A). The M330 residue forms a proline interaction pocket and inter-

acts with the inhibitor. In our model using β-casein, the Q+2 position residue of the substrate can interact with the M330 residue, and a change of the M330 residue by another polar amino acid can affect the function of transglutaminase 2 (Figure 1B).

I331 residue is one of the residues at the hydrophobic pocket, which is also related to inhibitor binding. The phenyl residue of the inhibitor (Ac-P(DON)LPF-NH2) binds to the hydrophobic pocket formed by the A304, L312, I313, F316, I331 and L420 residues.[8] Transglutaminase 2 preferred the hydrophobic residue at the Q+3 position from the glutamine residue of substrates.[10] Thus, this pocket is strongly related
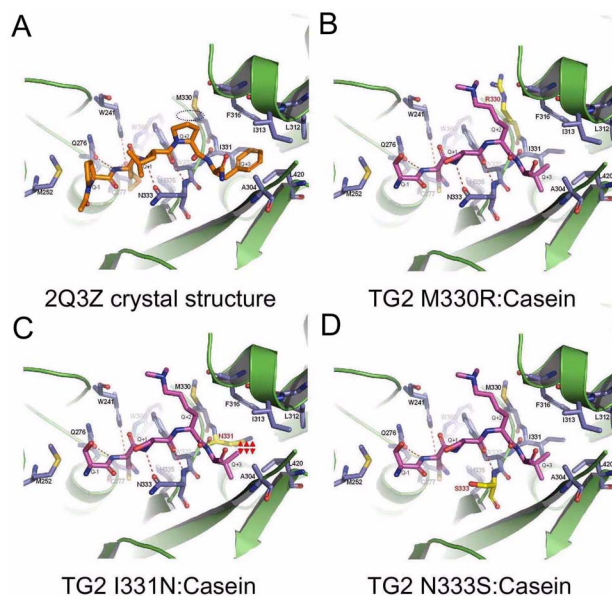

Figure 1. Crystal structure complex with inhibitor and substrate binding model for three types of mutants. (A) Active sites of transglutaminase 2 (TG2) complex with inhibitor (Ac-P(DON)LPF-NH2).[8] M330's interaction with the proline residue of the inhibitor is shown in the circle. (B) M330R mutant model of transglutaminase 2 with *N,N*-dimethyl β-casein. (C) β-casein binding model in I331N mutant showing that valine residue of β-casein did not interact with N331. (D) N333S mutant loses hydrogen-bonding with backbone 'NH' of substrate. The peptide inhibitor of the crystal structure is represented by the orange stick, and the SQ[182]SKV sequence of β-casein, by the magenta stick.

to the specificity for the substrate. In the I331N mutation, the interaction between β-casein and transglutaminase 2 was disrupted by substitution of the hydrophobic residue to polar residue (Figure 1C).

The crystal structure of the transglutaminase 2: inhibitor complex shows the hydrogen bonding between the inhibitor and the backbone 'NH' of N333.[8] The carbonyl oxygen of the side chain of the N333 also can interact with the inhibitor. Also, the side chain of Q276 forms a hydrogen bond with the backbone 'NH' of the inhibitor. These two residues are near the acyl-acceptor tunnel of transglutaminase 2. So, these interactions allow the glutamine residue of the substrate to access the tunnel. In the binding model, the backbone 'NH' of the substrate Q+1 position residue interacts with the side chain of N333, and the interaction is disrupted by substitution of serine (Figure 1D).

Transglutaminase 2 activates NF-κB through polymerization of I-κBα and NF-κB was down-regulated when transglutaminase 2 was inhibited.[2,3] Therefore, the loss of function by the mutations may be related to the NF-κB down-regulation. We derived models of the transglutaminase 2 complex with three five-amino-acid sequences of I-κBα (Figure 2).

There was no specific interaction between the Q+2 position of the IQ[266]QQL sequence and the M330 residue (Figure 2A). But in the I331N mutation, the interaction between the Q+3 position of the substrate was disrupted by substitution to the polar residue, as in the β-casein model. In the transglutaminase 2:QQ[267]QLG and transglutaminase 2:GQ[313]RLT models, there were no specific interactions between the Q+3 position of the substrate and the I331 residue (Figures 2B, C). However, repulsive interaction between the Q+2 position of the substrate and the arginine residue at the M330 position was found in both models (Figures 2B, C). The disruption of the hydrogen bonding was shown in the N333S mutation for all three I-κBα models (Figures 2A, B, C). The interaction between the side chain of N333 and the backbone 'NH' of the substrate at the Q+1 position was disrupted by the substitution of serine.

Taking these results together, the residues at the mutation sites strongly contribute to substrate binding in the model.

Depletion of I-κBα by transglutaminase 2 in cells. The previous results are based on the *in vitro* transamidation activity of transglutaminase 2 using the β-casein substrate. We also tested the polymerization function of transglutaminase 2 in cells in order to compare the *in vitro* and *in vivo* conditions. Transglutaminase 2 induces NF-κB activation through the polymerization of I-κBα.[2] And the depletion of free I-κBα implies the activation of NF-κB. To test whether transglutaminase 2 mutants lack the I-κBα polymerization function, we analyzed the I-κBα level in the cytosolic fraction of transglutaminase 2-transfected PANC-1 cell lines. When the wild-type transglutaminase 2 was expressed in PANC-1 cells, the free I-κBα level was reduced, whereas the M330R, I331N, and N333S mutants could not deplete the I-κBα like positive-control C277S mutant (Figure 2). These results are consistent with a the previous report on the
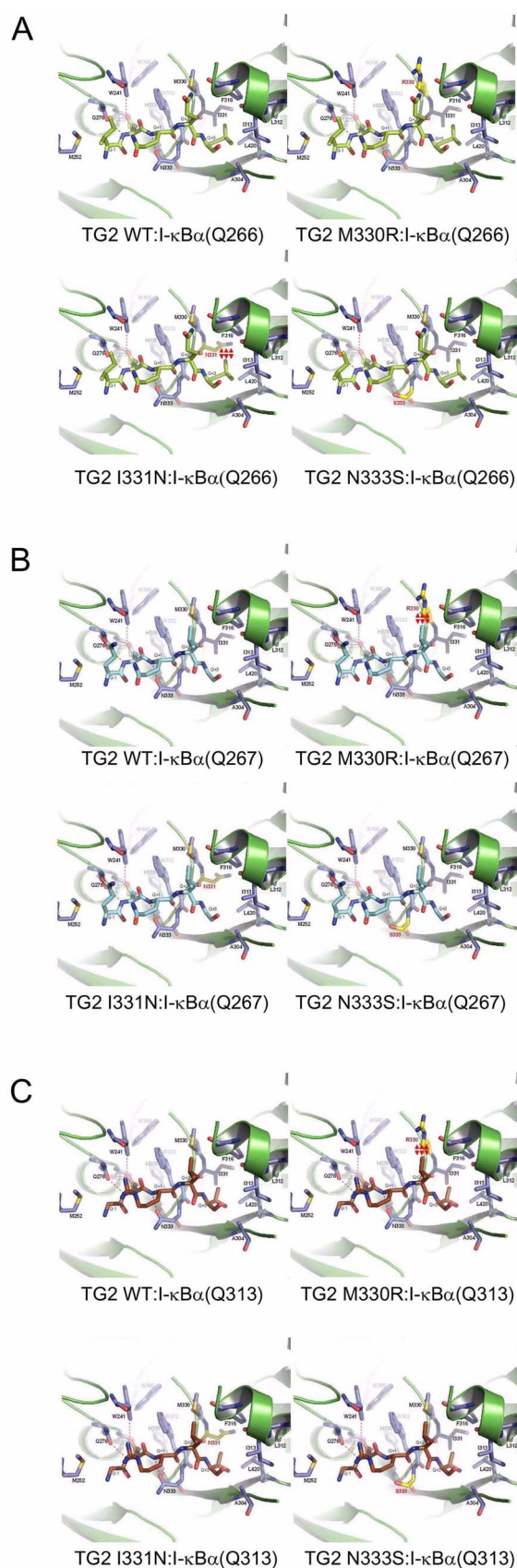

Figure 2. I-κBα binding model for three types of mutants. (A) Models for IQ[266]QQL sequence of I-κBα. (B) Models for QQ[267]QLG sequence of I-κBα. (C) Models for GQ[313]RLT sequence of I-κBα.
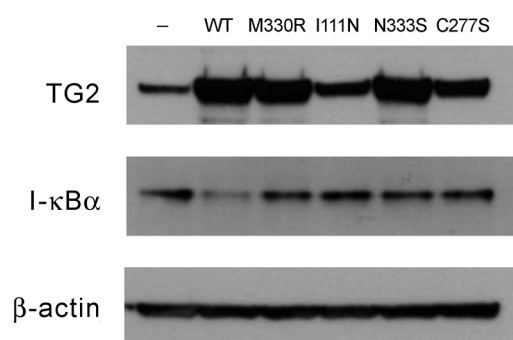
Figure 3. Western blot analysis of I-κBα and transglutaminase 2 mutants in PANC-1 cells. The decrease of the I-κBα level in cytosol implies the polymerization of I-κBα by transglutaminase 2. Lane 1: pcDNA 3.0 as mock; lane 2: wild-type transglutaminase 2; lane 3: M330R mutant; lane 4: I331N mutant; lane 5: N333S mutant; lane 6: C277S mutant as a positive control. The free I-κBα level was decreased when the wild type was expressed, whereas the three mutants found in early-onset type 2 diabetes could not polymerize I-κBα.

transamidation activity of mutants *in vitro*.[6] This means that all three transglutaminase 2 mutants in early-onset type 2 diabetes lose their ability for polymerization of I-κBα, which results in NF-κB down-regulation in cells.

In conclusion, transglutaminase 2 mutations in early-onset type 2 diabetes lack protein polymerization activity. The loss of activity by theses mutants was mainly due to the disruption of interaction with the substrate. These mutants do not have the I-κBα polymerization function *in vivo* that they have *in vitro*. The mutants' loss of function can result in down-regulation of NF-κB in pancreatic cells. NF-κB down-regulated pancreatic cells might be susceptible to apoptosis, which can cause early-onset type 2 diabetes.

Experimental Section

Molecular modeling. The reactive glutamine residue of β-casein was identified from the Transdab data base server (http://genomics.dote.hu/wiki/index.php/Main_Page).[7] The IQ^{266}QQL, QQ^{267}QLG and GQ^{313}RLT sequences of I-κBα were also used for modeling.[9] The conformations of the mutated residues were generated by Rotamer module in InsightII package and the conformation of substrate sequence was optimized with Discover 2000 using the CVFF force field (Accelrys, Inc.). Figure 1 and Figure 2 were made using PyMol program (http://pymol.sourceforge.net/). The docking models were determined using the inhibitor (Ac-P(DON)LPF-NH2) of X-ray crystal (2Q3Z) structure as a template.[8] In the substrate sequence (SQ^{182}SKV) of β-casein, the lysine residue at the Q+2 position was *N,N* dimethylated to explain the *in vitro* assay.[6]

Cloning, cell culture and transient transfection of trans-glutaminase 2. Wild-type and mutant human transglutaminase 2 genes were subcloned into pcDNA 3.0 vector (Invitrogen) using the HindIII/XhoI restriction sites. The human pancreatic cancer PANC-1 cell line was grown in DMEM (Hyclone) supplemented with 10% fetal bovine serum (Hyclone) and 100 units/mL penicillin-streptomycin (Gibco), and maintained in a humidified 5% CO_2 environment. Transient transfection of pcDNA3.0 containing the full-length human transglutaminase 2 wild type and the three DNA mutants was performed using the LipofectAMINE 2000 (Invitrogen), according to the manufacturer's instructions. Briefly, the cells were seeded at a density of 2×10^5 per well in a six-well plate and incubated overnight. The media was replaced by 1 mL Opti-MEM (Invitrogen) supplemented with DNA-LipofectAMINE mixture. After 6 hours of incubation, the transfection medium was replaced with fresh culture medium, and the cells were further incubated for 48 hours in a humidified 5% CO_2 environment.

Depletion of I-κBα by transglutaminase 2 in cells. We monitored the I-κBα level in the cytosol fraction by Western blotting to test the function of the transglutaminase 2 in the cells.[2] The cytosolic fraction of the samples were prepared using a CelLytic NuCLEAR Extract kit (Sigma). And anti-I-κBα (Cell Signaling), anti-transglutaminase 2 (clone CUB 7402, NeoMarkers) and anti-β-actin (Abcam) antibodies were used for Western blotting.

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