Transglutaminase 2 Mediates Polymer Formation of I-κBα through C-terminal Glutamine Cluster*

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Recently we reported that transglutaminase 2 (TGase 2) activates nuclear factor-κB (NF-κB) independently of I-κB kinase (IKK) activation, by inducing cross-linking and protein polymer formation of inhibitor of nuclear factor-κBα (I-κBα). TGase 2 catalyzes covalent isopeptide bond formation between the peptide-bound-glutamine and the lysine residues. Using matrix-assisted laser desorption ionization time-of-flight mass spectra analysis of I-κBα polymers cross-linked by TGase 2, as well as synthetic peptides in an in vitro competition assay, we identified a glutamine cluster at the C terminus of I-κBα (amino acids 266–268) that appeared to play a key role in the formation of I-κBα polymers. Although there appeared to be no requirement for specific lysine residues, we found a considerably higher preference for the use of lysine residues at positions 21, 22, and 177 in TGase 2-mediated cross-linking of I-κBα. We demonstrated that synthetic peptides encompassing the glutamine cluster at amino acid positions 266–268 reversed I-κBα polymerization in vitro. Furthermore, the depletion of free I-κBα in EcR/TG cells was completely rescued in vivo by transfection of mutant I-κBαs in glutamine sites (Q266G, Q267G, and Q313G) as well as in a lysine site (K177G). These findings provide additional clues to the mechanism by which TGase 2 contributes to the inflammatory process via activation of NF-κB.

NF-κB² belongs to a family of transcription factors that contribute to the progress of inflammatory disease and the development of cancer through the regulation of specific gene expression (1). Under normal cellular conditions, NF-κB is retained in an inactive state in the cytosol through its association with I-κBα (2). Exposure to certain stresses, such as lipopolysaccharide or tumor necrosis factor-α (TNF-α), activates signaling pathways that ultimately result in ubiquitination of I-κBα, through a mechanism that involves IKK-dependent phosphorylation of I-κBα (3). Proteosome-mediated degradation of ubiquitinated I-κBα releases NF-κB, which then translocates to the nucleus and becomes active (4). The development of therapeutics that block the degradation of I-κBα represents a promising approach to treating many of the diseases in which NF-κB plays a role (5). For example, IKK-dependent stress signaling has been shown to deplete I-κBα through phosphorylation of serine 32 and serine 36 (6–8), and administration of peptides carrying the same amino acid sequences as the I-κBα phosphorylation sites selectively inhibits I-κBα phosphorylation and degradation and subsequent activation of NF-κB (9).

Recently, we identified an IKK-independent mechanism of NF-κB activation through TGase 2-mediated cross-linking of I-κBα (10, 11). TGase 2 (EC 2.3.2.13) is an enzyme that catalyzes the formation of a strong covalent bond between peptide-bound glutamine and lysine residues, resulting in protein cross-linking and the formation of protein polymers (12). TGase 2-mediated polymerization of I-κBα depletes free I-κBα from the cytosol, resulting in the activation of NF-κB (11). We have previously shown, using rationally designed peptides, that administration of peptide inhibitors for TGase 2 reverses inflammatory conjunctivitis and lipopolysaccharide-induced injury in the lung and brain, respectively (11, 13, 14). These results suggest a model in which peptide inhibitors of TGase 2 mediate their anti-inflammatory effects through the inhibition of free I-κBα depletion. In the current study, we examined whether TGase 2 targeted specific glutamine and lysine residues of I-κBα. I-κBα contains many glutamine residues throughout its secondary structure, whereas lysine residues cluster in the N-terminal domain of the protein. To identify residues of I-κBα that were involved in the formation of glutamyl-lysine cross-links, I-κBα polymers cross-linked in vitro by TGase 2 were analyzed by MALDI-TOF MS. Candidate residues were identified using proteolytic digestion of full-length I-κBα polymers, and their identity was confirmed using synthetic peptides containing candidate glutamine and lysine residues. The results of an in vitro competition assay using full-length I-κBα and synthetic peptides carrying putative cross-linking sites and in vivo rescues of I-κBα using mutant I-κBα carrying each mutated putative cross-linking site confirmed that the residues we identified by MALDI-TOF contributed to TGase 2-mediated I-κBα polymerization.

EXPERIMENTAL PROCEDURES

I-κBα Polymerization by TGase 2—To obtain purified I-κBα, DNA sequences encoding full-length human I-κBα were subcloned into the pET-30 Ek/LIC vector (Novagen) using PCR and the full-length I-κBα cDNA as a template (pCMV-1κBα, BD Biosciences). Protein was expressed and purified using a HiTrap IMAC FF column (Amersham Biosciences) according to the manufacturer’s instructions. For the in vitro polymerization reactions,
10 μg of purified I-κBα was incubated with 1 milliunit of TGase 2 (guinea pig liver, Sigma) in a reaction mixture containing 50 mM Tris-HCl, pH 7.5, and 10 mM CaCl₂. Polymerization was analyzed by 4–12% NuPAGE gel electrophoresis (Invitrogen). High molecular weight I-κBα polymers were separated for further analysis using a centrifugal filter device (Centricon, molecular size cut-off 100 kDa, Millipore).

Proteolysis of I-κBα Polymers—Filter-purified I-κBα polymer (10 μg) was denatured in 4 M urea containing 10 mM dithiothreitol at 55 °C, and then the concentration of urea was diluted to 1 M using 100 mM NH₄HCO₃. The polymer was digested to completion with trypsin (Promega) or endoproteinase Glu-C (V8, Roche Applied Science) at an enzyme to substrate ratio of 1:100 (w/w) overnight at 37 °C.

MALDI-TOF MS Analyses—Peptide masses were determined using a MALDI-TOF mass spectrometer (4700 Proteomics Analyzer, Applied Biosystems) with a matrix of 5 mg/ml α-cyano-4-hydroxy-cinnamic acid (Sigma) in 0.1% aqueous trifluoroacetic acid/acetonitrile (1:1, v/v). Samples were desalted using Zip-Tips C18 (Millipore), eluted in matrix solution directly onto the target, and air-dried. Analyses was performed in positive ion reflectron mode, and spectra were calibrated at 50 ppm tolerance using the [M+H]⁺ ion from 4700 Cal Mix (Applied Biosystems): des-Arg¹-bradykinin (Mr 904.4681), angiotensin 1 (Mr 1296.6853), Glu¹-fibrinopeptide B (Mr 1570.6774), ACTH 1–17 clip (Mr 2093.0867), ACTH 18–39 clip (Mr 2465.1989), and ACTH 7–38 clip (Mr 3657.9294). For data processing, Data Explorer software (Applied Biosystems) was used. After removing peptide masses corresponding to keratins and proteases (trypsin or V8), measured masses were matched with the peptide masses from an ideal digest at 100 ppm tolerance using Paws (Genomic Solutions).

Identification of Cross-linking Sites Using Synthetic Peptides—Synthetic peptides were obtained by solid-phase synthesis and purified to >95% purity by preparative high-performance liquid chromatography (Peptron Co., Daejeon, Korea). Peptides (Table 1) were dissolved in double distilled water. For the in vitro cross-linking reaction, each peptide containing candidate glutamine residues (PQ-1, PQ-2, PQ-3, or PQ-4, 5 pmol) and a mixture of PK-1, PK-2 and PK-3, which contained candidate lysine residues, were incubated with TGase 2 (guinea pig liver, Sigma). To determine which glutamine residues served as acyl donors, the derivatives of PQ-4 (PQ-5–PQ-9) were tested for cross-linking with PK-3. The cross-linked dipeptides were analyzed by MALDI-TOF MS. The reaction mixture was desalted using Zip-Tips C18 and eluted in matrix solution containing internal standards (4700 Cal Mix). The quantities of the dipeptides were calculated relative to the peak area of ACTH (1–17 clip).

Competition Assay for I-κBα Polymerization Using Synthetic Peptides in Vitro—I-κBα (2 μg) was incubated with 0.5 milliunit of TGase 2 in a reaction mixture containing 50 mM Tris, pH 7.5, and 10 mM CaCl₂ with or without each of the indicated...
peptides (2 nmol). After 10 min at 37 °C, the reactants were separated by SDS-PAGE and visualized by Coomassie staining. The relative intensities of the bands corresponding to intact I- and H9260B/H9251 were calculated by densitometry.

**Competition Assay for I- and H9260B/H9251 Polymerization by Transfection of I- and H9260B/H9251 Mutants in Vivo**—To identify critical residues of I- and H9260B/H9251 in cross-linking, point mutated full-length cDNAs of I- and H9260B/H9251 (K177G, Q255G, Q266G, Q267G, Q313G) were constructed by the recombinant PCR method using cDNA of full-length I- and H9260B/H9251 (pCMV-I- and H9260B/H9251; BD Biosciences). The PCR products of the mutants were designed to be inserted into pcDNA 3.0 (Invitrogen) at HindIII and XbaI sites. The mutations were confirmed by DNA sequencing after cloning. The wild type or mutated I- and H9260B/H9251 DNAs (1 μg) were transfected to EcR293/TG cell lines (11) as per the manufacturer’s instruction using Lipofectamine™ 2000 (Invitrogen) in a 6-well tissue culture dish. After a 24-h incubation, TGase 2 was induced by treatment with 1 μg/ml tetracycline for 24 h. The cytosolic fraction of cells was prepared using a nuclear extraction kit (Sigma). The level of I- and H9260B/H9251 in the cytosolic fraction was examined by Western blot.

**RESULTS**

It was previously reported that overexpression of TGase 2 in cells causes a reduction in free I-κBα and the concomitant appearance of a discrete pattern of high molecular weight species of I-κBα (11). When I-κBα and TGase 2 were incubated in vitro, we observed the formation of high molecular weight polymers of I-κBα (Fig. 1A). The size of the polymer complex was such that it was unable to migrate into the 4% polyacrylamide gel and remained trapped in the loading well at the top of the gel. However, we were able to purify the polymer complex by centrifugation on a filtering device.

To understand the mechanism of I-κBα polymerization, the filter-purified I-κBα polymer complex was subjected to proteolytic digestion using two different proteases, trypsin and V8, and the resultant peptides were analyzed by MALDI-TOF MS. Peaks corresponding to contaminants (keratins and autodigestion) were identified by comparison with a negative control that did not contain I-κBα polymers. I-κBα-specific peaks were then matched to theoretical peptides derived from digestion of intact I-κBα (Fig. 1, B and C). Approximately 88.3% of the full-length I-κBα sequence was represented by the matching peptides, indicating that the sequences in the remaining 11.7% of I-κBα were involved in TGase 2 mediated cross-linking. The regions of I-κBα that were not retrieved by proteolytic digestion included amino acid (aa) residues 141–143, 265–287, and 307–317 (Fig. 2). The absence of the first region, aa 141–143, appeared to be an experimental artifact, given that it contained no glutamine or lysine residues. The second and third regions contained 6 glutamine residues, aa 266, 267, 268, 271, 278, and 313, but did not contain any corresponding lysine residues. Interestingly, these regions of I-κBα overlapped the glutamine- and leucine-rich region (QL-rich region; amino acids 264–276) located between aa 263 and 277. This region has been shown to be required for both inducible degradation and inhibition of RelA function (15). Our data suggested that there was no specificity for lysine residues in the cross-linking reaction, resulting in the saturation of identifiable peptides.

A series of peptides containing candidate glutamines and lysines were synthesized (Table 1) and tested for their ability to undergo cross-linking. A peptide containing a glutamine residue from one of the recovered regions (aa 165) was selected as a negative control for cross-linking (PQ-1). Because there

![Figure 1](image-url)
appeared to be a lack of specificity for lysine residues, lysine-containing peptides were designed ad hoc. We ruled out lysine residues in the ankyrin repeat region (aa 73–275), with the exception of one that we used as a negative control (PK-2, aa 83–91), because the ankyrin domains are known to bind to NF-κB and lysines in this region were considered inaccessible by TGase 2. The two regions of IκB-α that were selected were the bridge sequence between ankyrin domains 3 and 4 (PK-3, aa 173–181) and the N terminus of IκB-α containing target lysines for ubiquitination (PK-1, aa 17–26). Interestingly, there were no lysine residues in the C-terminal region of IκB-α (aa 245-C terminus).

Peptides containing candidate glutamine residues (PQ-1, -2, -3, or -4) were added to a peptide mixture containing candidate lysine residues (PK-1, -2, and -3), incubated with TGase 2, and then analyzed by MALDI-TOF MS (Fig. 3). The mass spectra obtained following the cross-linking reaction showed that cross-linked dipeptides of PQ-3 (Fig. 3B) or PQ-4 (Fig. 3C) were generated, whereas PQ-1 and PQ-2 did not appear to undergo a cross-linking reaction (data not shown). The result suggested that glutamine 313 in PQ-3 and one or more of the glutamine residues in PQ-4 were involved in TGase 2-mediated cross-linking. PQ-4 contained two potential glutamine regions, a glutamine cluster at aa 266–268 and glutamine 271. PQ-4 was further divided into two peptides, PQ-5 and PQ-9, and these two peptides were incubated with the mixture of peptides con-
TGase 2 Targets C-terminal Glutamines of I-κBα

We observed that glutamine residues in PQ-3 and PQ-4 were simultaneously deaminated following incubation with TGase 2, as well as cross-linked to PK peptides. Peptide deamination was not detected in PQ-1, PQ-2, or PQ-9 (data not shown). TGase 2 catalyzes a two-step reaction process in which a peptide-bound glutamine residue in a target protein first forms a thiol ester with the active site cysteine of TGase 2, releasing ammonia and resulting in the formation of an acyl group. In the second step, the acyl group is transferred to an acyl acceptor (amine donor) lysine residue, and an isopeptide bond is formed. However, when there is no amine donor available, the thiol ester bond can be also hydrolyzed, resulting in deamination of glutamine and a corresponding mass increase of Δ1 as glutamine is converted to glutamate. In the absence of a lysine donor, the glutamine residue of PQ-3 was deaminated by TGase 2 in a dose-dependent manner (Fig. 4A). To identify the glutamine residues preferred by TGase 2, we compared the peak areas of the monoisotopic masses of the synthetic peptides with TGase 2 treatment. We found that the molecular ratio of intact PQ-3 to deaminated PQ-3 was 1:3. After incubation of PQ-4 with 0.001 unit TGase 2 for 1 h in the absence of a lysine donor, we observed that glutamine residues 266 and 267 were completely deaminated and that glutamine 268 was partially deaminated (Fig. 4B). We found no evidence for deamination of glutamine 271 (data not shown). Therefore, glutamine residues at positions 266 and 267 may play an important role for initiation of TGase cross-linking.

To identify the glutamine residue(s) within the glutamine cluster (aa 266–268) involved in TGase 2-mediated cross-linking of I-κBα, three additional peptides were synthesized (PQ-6, PQ-7, and PQ-8) and tested in the in vitro cross-linking reaction using PK-4 as the donor lysine (Fig. 5). Each of the peptides contained glutamine to glutamate substitutions at two of the three positions in the glutamine cluster based on the data in Fig. 4. After incubation with PK-4, cross-linked dipeptides of PQ-6 (Q267E,Q268E) and PQ-7 (Q266E,Q268E) were evident, whereas PQ-8 (Q266E,Q267E) showed no evidence of cross-linking (Fig. 5). Interestingly, the intensity of PQ-7 was higher than that of PQ-6. The calculated peak areas of each of the mutant peptides in relation to PQ-5 were 0.44 (PQ-7) and 0.21 (PQ-6). These results suggested that TGase 2 was most selective for the second glutamine of the cluster, residue 267, whereas the third glutamine residue did not appear to participate in cross-linking.
To determine the cross-linking efficiency of the putative TGase 2 targets identified thus far (glutamines 266, 267, and 313 and lysine 177), an I-/H9260B/H9251 polymerization competition assay was performed using synthetic peptides carrying each of the target glutamine and lysine residues (Fig. 6). PQ-4 and PQ-5, which contained the entire glutamine cluster (aa 266–268), completely blocked I-/H9260B/H9251 polymerization. PQ-3 also had an inhibitory effect on I-/H9260B/H9251 polymerization (Fig. 6). Of note, PK-1, which contained the ubiquitination sites lysine 21 and lysine 22, had an inhibitory effect on I-/H9260B/H9251 polymerization similar to that of PK-3 (Fig. 6). Based on the relative intensity of the I-/H9260B/H9251 bands generated in the in vitro cross-linking reaction, the peptides containing glutamines at positions 266 and 267 (PQ-4 and PQ-5) showed a higher inhibitory effect on I-/H9260B/H9251 polymerization than any of the mutant peptides (PQ-6, PQ-7, and PQ-8) of PQ-5 (Fig. 6).

In addition to in vitro test, we determined whether I-/H9260B/H9251 polymerization by TGase 2 could be rescued in vivo by treatment of synthetic peptides containing TGase 2 substrate domains of I-/H9260B/H9251. I-/H9260B/H9251 (2 μg) and each of the indicated peptides (2 nmol) were incubated with 0.5 milliunit of TGase 2. After 10 min at 37 °C, the reaction was resolved by SDS-PAGE and visualized by Coomassie Blue staining. The relative amounts of intact I-/H9260B/H9251 were quantitated by densitometry and are depicted in the bar graphs below each gel. Data represent the means ± S.D. of three independent experiments.

24-h transfection, TGase 2 was induced for 24 h by tetracycline treatment. Interestingly, the depletion of free I-/H9260B was almost rescued by transfection of mutant I-/H9260B in glutamine sites (Q266E, Q267E) as well as in the lysine site (K177G) (Fig. 7). Densitometry analysis showed that the differential depletion of each mutation occurs in I-/H9260B cross-linking in vivo. The data suggest that Lys-177 and Gln-267 of I-/H9260B are preferentially selected sites for TGase 2 cross-linking in vivo. Mutation of a non-substrate residue (Q255G) in this region of the molecule was employed for a specificity control, which showed no comparable effect on TGase 2.
DISCUSSION

In the current study, we demonstrated that TGase 2 catalyzes the cross-linking of I-κBα monomers by preferentially targeting specific glutamine residues in the C terminus of I-κBα. In contrast, we found that there was no apparent specificity for lysine donor residues. However, based on analysis of peptides containing Lys-177, which is located between the third and the fourth ankyrin repeats of I-κBα, and lysines 21 and 22 in the N terminus, it appeared that certain lysines functioned as good acyl acceptors in the TGase 2-catalyzed acyl transfer reaction (Fig. 3). Interestingly, peptide PK-1 blocked I-κBα polymerization in an in vitro competition assay as efficiently as the PK-3 peptide (Fig. 6). Based on our current results, we cannot rule out the possibility of heteropolymerization between I-κBα and other TGase 2 substrates containing better lysine donors than I-κBα. However, TGase 2-catalyzed I-κBα homopolymerization in vitro was very efficient, suggesting that I-κBα homopolymerization may also occur in vivo.

Upon activation of IKK by various stimuli, I-κBα binds to IKK and is phosphorylated on serine residues 32 and 36 (16). Phosphorylation is followed by ubiquitination and rapid proteasome-dependent degradation of I-κBα (17). Interestingly, phosphorylation of I-κBα does not lead to dissociation of I-κBα from NF-κB but, rather, targets free I-κBα for rapid degradation (6–8). This mechanism of regulation of NF-κB activity is not universal, however, as it has been shown that proteasome-mediated degradation of I-κBα does not play a role in the constitutive activation of NF-κB in early B cells in response to oxidative stress (18). Under conditions of oxidative stress, I-κBα is phosphorylated on tyrosine 42. Rather than mediating degradation however, tyrosine phosphorylation appears to play a role in inhibiting the association between NF-κB and free I-κBα (19). In addition to phosphorylation-mediated regulation of I-κBα, there is evidence that other pathways of activation of NF-κB exist in tumors and contribute to the acquisition of resistance to chemotherapy. Depletion of free I-κBα induced by doxorubicin does not require classical phosphorylation, or the PEST domain of I-κBα (20). In fact, the mechanism of activation of NF-κB upon prolonged anticancer chemotreatment has yet to be fully characterized. TGase 2 is highly up-regulated in chemoresistant breast cancers (21), and depletion of free I-κBα by TGase 2-mediated polymerization does not require kinase activation, making this pathway an attractive candidate for the regulation of NF-κB during tumorigenesis. As we showed previously (11) and in the current study (Fig. 1), TGase 2 efficiently polymerizes I-κBα in vivo and in vitro. TGases are Ca$^{2+}$-dependent enzymes, so in the absence of activated kinase-dependent signaling pathways, the process of TGase-mediated NF-κB activation could be triggered by any type of stimuli that results in an increase in calcium uptake. However, TGase 2 can be induced by NF-κB (22), suggesting that kinase-dependent NF-κB activation and TGase 2-mediated NF-κB activation may be related.

Our data show that TGase 2 specifically targets glutamine residues in the C-terminal region of I-κBα (Fig. 2). This region also contains the QL-rich region (15) as well as a PEST sequence that is associated with rapid protein turnover (23). Interestingly, it was the QL-rich region, rather than the PEST sequence, that appeared to be involved in the inducible degradation of I-κBα observed by Sun et al. (15). Deletion of the 13 amino acids that constituted

![FIGURE 7. I-κBα polymerization by TGase 2 was rescued in vivo by human I-κBα constructs containing mutations at TGase 2 targeting sites.](image)

The C-terminal region of I-κBα contains two ubiquitination sites (lysine 21 and lysine 22), two serine phosphorylation sites (serine 32 and serine 36), and a tyrosine phosphorylation site (tyrosine 42). The C-terminal region contains PEST sequences, and the QL-rich region. The six ankyrin repeats are represented by cross-hatched boxes. The cross-linking sites identified in this study are indicated above the map with bold characters.
the QL-rich region (and that constitute the TGase 2 targeting region) in the C terminus of I-κBα completely abolished TNF-α-induced degradation of I-κBα (15). Our results suggest that the depletion of I-κBα by TNF-α may be due to TGase 2-mediated polymerization of I-κBα.

Inhibition of NF-κB represents an attractive therapeutic approach to the treatment of many diseases, including inflammatory disease and cancer. Several strategies for reversing NF-κB activation have been employed, including inhibition of tyrosine phosphorylation, NF-κB translocation to nucleus, degradation, IKK-I-κBα complex formation, IKK activation, and cyclooxygenase-2 activity. NF-κB translocation into the nucleus can be blocked by a synthetic compound, dehydroxyethylpoxyquinomicin, which is a derivative of 2,5-dimethyl-3-thoxyaniline (24). Proteasome inhibition has broad application, and is well tolerated in phase I/II clinical trials in patients with multiple myeloma (27). The tripeptide aldehydes proteasomal inhibitor (PSI: N-benzoyloxycarbonyl-Ile-Glu-(O-t-Bu)-Ala-leucinal) and MG-132 reversibly inhibit the proteasome and is well tolerated in phase I/II clinical trials in patients with multiple myeloma (27). The tripeptide aldehydes proteasomal system inhibitor (PSI: N-benzoyloxycarbonyl-Ile-Glu-(O-t-Bu)-Ala-leucinal) and MG-132 reversibly inhibit the proteasome complex and are able to sensitize cancer cells to antitumor agents through the inhibition of NF-κB (28). Nonsteroidal anti-inflammatory drugs (NSAIDs), including cyclooxygenase-2 inhibitors, are well known for their ability to prevent the development of various cancers. Interestingly, NSAIDs also inhibit the activity of IKKβ and prevent NF-κB activation (29). Therefore NSAIDs also appear to sensitize cancer cells to chemotherapeutic agents through inhibition of NF-κB activation. A synthetic peptide, IKKβ 644–756, which inhibits the interaction of I-κBα and NF-κB essential modulator (NEMO), has been shown to inhibit cytokine-induced NF-κB activation in a dose-dependent manner (30). Synthetic peptides that prevent phosphorylation of I-κBα by IKK have been shown to completely abolish lipopolysaccharide-induced activation of NF-κB (9). In the current study, we identified specific glutamine targets of TGase 2, glutamines 266 and 267 in I-κBα, and demonstrated that a synthetic peptide in which the sequence encompasses aa 264 to 270 of I-κBα (PQ-5) dramatically blocked I-κBα polymerization by TGase 2 (Fig. 8). These peptides also demonstrated the potential to block free I-κBα depletion in vitro (Fig. 6). Furthermore, transfection of mutant I-κBα constructs containing TGase 2 targeting sites effectively reversed the TGase 2-mediated loss of I-κBα in vivo. Interestingly, the mutation at lysine 177 reversed I-κBα depletion as efficiently as the mutation at glutamine targets (Q266G, Q267G, and Q313G) did in vivo. Therefore, lysine 177 may have an important role for TGase cross-link in vivo, although in vitro competition assay shows about 50% rescue effect of I-κBα depletion. The physiological impact of TGase 2 inhibitors mimicking TGase 2 targeting domains of I-κBα is the subject of ongoing and future studies.

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