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Author(s)	Sato, Noriko; Kamada, Nobuyuki; Muromoto, Ryuta et al.
Citation	Immunology Letters, 103(2), 127-134 https://doi.org/10.1016/j.imlet.2005.10.015
Issue Date	2006-03-15
Doc URL	https://hdl.handle.net/2115/8407
Type	journal article
File Information	P-ZIPK-Imm-Lett.pdf



Title: Phosphorylation of threonine-265 in Zipper-interacting protein kinase plays an important role in its activity and is induced by IL-6 family cytokines

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Keywords: ZIPK, IL-6, LIF, apoptosis, phosphorylation

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Abbreviations: ZIPK, Zipper-interacting protein kinase; STAT, signal transducer and activator of transcription; LIF, leukemia inhibitory factor

Abstract

Zipper-interacting protein kinase (ZIPK) is a widely expressed serine/threonine kinase that has been implicated in cell death and transcriptional regulation, but its mechanism of regulation remains unknown. Here, we identified threonine-265 (Thr265) in ZIPK as a major autophosphorylation site. Mutational analyses revealed that autophosphorylation of Thr265 was essential for its full catalytic activity toward an exogenous substrate as well as for cell death induction. Furthermore, leukemia inhibitory factor (LIF) stimulated Thr265 phosphorylation of ZIPK, thereby leading to phosphorylation and activation of signal transducer and activator of transcription (STAT3). Taken together, our findings demonstrate that ZIPK is positively regulated through Thr265 phosphorylation and that this phosphorylation is essential for its function.

1. Introduction

Zipper-interacting protein kinase (ZIPK) was originally identified as a binding partner of activating transcription factor 4 (ATF4), a member of the activating transcription factor/cyclic AMP-responsive element binding family of transcription factor proteins [1,2]. ZIPK aggregates through its C-terminal leucine zipper (LZ) structure, thereby becoming an active enzyme. Ectopic expression of ZIPK in NIH 3T3 cells induced apoptosis. In contrast, a kinase-inactive mutant protein, ZIPK K42A, failed to induce apoptosis, indicating that ZIPK stimulates apoptosis via its catalytic activity [1]. The kinase domain of ZIPK shows strong homology to that of death-associated protein kinase (DAPK). ZIPK and DAPK constitute members of a family of related kinases that includes DAPK2/DRP-1, DRAK1 and DRAK2 [3-8], all of which have been implicated in the execution of apoptosis. Introduction of a DAPK antisense was shown to block interferon (IFN)- γ -induced apoptosis in HeLa cells [9], indicating that DAPK is required for IFN- γ -induced cell death. Furthermore, evidence that the DAPK may function as a tumor suppressor was provided [10,11]. Previous studies showed that ZIPK, in collaboration with Daxx and Par-4, induced apoptosis

from nuclear promyelocytic leukemia protein (PML) oncogenic domains (PODs) [12]. However, the mechanisms responsible for the activation of these kinases and the downstream substrates that mediate their apoptotic activities remain unknown. Recently, we have demonstrated that ZIPK interacts physically with STAT3 [13]. ZIPK specifically interacted with STAT3, and did not bind to STAT1, STAT4, STAT5a, STAT5b or STAT6. ZIPK phosphorylated STAT3 on serine-727 (Ser727) and enhanced STAT3 transcriptional activity. Small-interfering RNA-mediated reduction of ZIPK expression decreased LIF- and IL-6-induced STAT3-dependent transcription. Furthermore, LIF- and IL-6-mediated STAT3 activation stimulated ZIPK activity.

In the present study, we identified threonine-265 (Thr265) as an autophosphorylation site of ZIPK, and found that Thr265 phosphorylation was critical for both kinase activation and cell death induction. Furthermore, mutation of Thr265 suppressed ZIPK-mediated enhancement of IL-6-induced STAT3 activation. In addition, LIF stimulated Thr265 phosphorylation, suggesting that IL-6/LIF signaling mediates ZIPK activation through phosphorylation of Thr265.

2. Materials and Methods

2.1. Reagents, antibodies, Glutathione S-transferase (GST)-fusion proteins and peptides.

Human recombinant IL-6 was a gift from Ajinomoto (Tokyo, Japan). Human recombinant LIF was purchased from INTERGEN (Purchase, NY). Expression vectors for epitope-tagged ZIPK, ZIPK kinase domain(KD), DAPK2, DRAK1, DRAK2 and DAPK KD were described previously[1, 4, 7, 13]. Expression vectors for STAT3-LUC [14] was kindly provided by Dr. T. Hirano (Osaka Univ. Med. Sch., Osaka, Japan), respectively. Epitope-tagged ZIPK mutant constructs (ZIPK KD T265A, ZIPK KD S269A, ZIPK KD S273A and ZIPK T265A) were generated by PCR and sequenced (primer sequences are available upon request). Anti-Myc and anti-GST antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-FLAG M2 monoclonal antibody was purchased from Sigma (St Louis, MO). To obtain recombinant GST -fusion ZIPK proteins, the full-length or mutant cDNA of ZIPK was inserted in frame into pGEX4 vector. Recombinant proteins were expressed in

Escherichia coli (E. coli) DH5 α . The cells were grown for 6 h at 37 °C, and the protein was induced with 1 μ M isopropyl- β -D-thiogalactopyranoside (IPTG) at 30 °C for 15 h. E. coli were harvested and lysed by sonication. GST- fusion ZIPK proteins were purified by affinity chromatography on glutathione-Sepharose 4B (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's instructions. Synthetic peptides listed in Fig. 2B, were synthesized and obtained from Operon Biotechnologies (Tokyo, Japan). The phosphopeptide used as immunogen for anti-phosphoZIPK Thr265 (anti-pZIPK Thr265) was KRRMpTIAQSLEH, corresponding to residues 261 to 272 of murine ZIPK. Rabbits were injected with a mixture of peptide and Freund's complete adjuvant and then boosted three times at monthly intervals with peptide in Freund's incomplete adjuvant, before blood was collected and immune serum was obtained

2. 2. Cell culture, transfection, and luciferase assays.

Rat hepatoma cell line H35 was maintained in DMEM containing 10 % FCS [13]. Stable H35 transformants expressing empty vector (pcDNA3), Myc-ZIPK WT or Myc-ZIPK T265A were established as described previously [1,13] and comparable

protein expression of Myc-ZIPK was confirmed by immunoblotting by anti-Myc antibody (data not shown). H35 cells were maintained in the presence of G418 (0.5 mg/ml) and transfected with STAT3-LUC using Metafectene (Biontex Laboratories GmbH, München, Germany) according to the manufacturer's instructions. Thirty hours after transfection, the cells were treated with IL-6 for 12 h. The cells were harvested and assayed for the luciferase activity using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's instructions. At least three independent experiments were carried out for each assay. Human embryonic kidney carcinoma cell line, 293T, was maintained in DMEM containing 10 % FCS and transfected by the standard calcium precipitation protocol [15].

2. 3. Immunoprecipitation, immunoblotting and *in vitro* phosphorylation.

The immunoprecipitation and immunoblotting were performed as described previously[16]. The cells were harvested and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1 % NP-40, 1 μ M sodium orthovanadate, 1 μ M phenylmethylsulfonyl fluoride and 10 μ g/ml each of aprotinin, pepstatin and leupeptin).

The immunoprecipitates from cell lysates were resolved on SDS-PAGE and transferred to Immobilon filter (Millipore; Bedford, MA). The filters were then immunoblotted with relevant antibodies. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). *In vitro* kinase reactions were performed as described [1, 17]. Briefly, immune complex of ZIPK was washed in kinase buffer (10 mM HEPES, pH7.4, 50 mM NaCl, 0.1 μ M sodium orthovanadate, 5 mM MnCl₂, 5 mM MgCl₂) and mixed with 5 μ Ci/ μ l [γ -³²P]-ATP and the respective peptide at 25 °C for 30 min. The products of these reactions were separated by SDS-PAGE.

2. 4. Cell death assay.

Cell death assay was performed as described previously [1]. Briefly, mouse NIH 3T3 cells were seeded at a density of 10⁶ cells/100-mm dish in DMEM containing 10% FCS. Cells were transfected with various plasmids in combination with pEGFP (Clontech). After 48 h of culture, the percentage of apoptotic cells that showed membrane blebbing was determined by fluorescence microscopy, counting GFP-positive cells. Alternatively,

both floating and attached cells were collected, fixed, and incubated in 1.0 $\mu\text{g/ml}$ of DAPI. The percentage of cells with fragmented nuclei or condensed chromatin was determined by fluorescence microscopy, counting a minimum of 300 cells. Similarly, cell death assay using human cervix carcinoma cell line, HeLa was performed. HeLa cells in 6-well plate were maintained in DMEM containing 10% FCS. As described the above, after 48 h of transfection, cells were fixed, and incubated in 1.0 $\mu\text{g/ml}$ of DAPI. The percentage of apoptotic cells was determined by fluorescence microscopy, counting more than 200 GFP-positive cells. Cells with intact nuclei were counted as nonapoptotic, and cells with condensed chromatin or completely disintegrated nuclei were counted as apoptotic.

3. Results and Discussion

3. 1. Identification of autophosphorylation sites in the kinase domain of ZIPK

ZIPK has been shown to undergo autophosphorylation, but the phosphorylation sites and their functions have not yet been determined [1]. Therefore, we attempted to identify phosphorylation sites in ZIPK and clarify their significance. To this end, we first performed *in vitro* kinase assays for a series of DAPK family proteins. Myc-tagged full-length DAPK2, DRAK1, DRAK2 or ZIPK, or the DAPK kinase domain, was transiently expressed in 293T cells, and immunocomplexes were subjected to *in vitro* kinase reactions in the presence of [γ - ^{32}P]-ATP followed by resolution by SDS-PAGE. As shown in Fig. 1A, strong ^{32}P incorporation was observed in full-length DAPK2 and ZIPK, but not in the kinase domain of DAPK or full-length DRAK2. Full-length DRAK1 showed weak ^{32}P incorporation. These results indicate that ZIPK successfully undergoes autophosphorylation in 293T cells.

Next, we examined the autophosphorylation activity of purified GST-ZIPK fusion proteins (Fig. 1B). As shown in Fig. 1C and D, GST-full-length ZIPK and its kinase

domain (GST-ZIPK KD 1-275) showed ^{32}P incorporation in a dose-dependent manner, whereas the N-terminal region of the GST-ZIPK kinase domain (GST-ZIPK KD 1-156) showed no ^{32}P incorporation (Fig. 1E). These results suggest that there are major autophosphorylation sites between residues 157 and 275 in the C-terminal region of the ZIPK kinase domain.

To further narrow down the candidate autophosphorylation sites, we performed *in vitro* kinase assays on synthetic peptides of sequences present in the kinase domain. A total of 10 peptides (Pep#1-Pep#10) that contained serine (Ser) or Thr residues in the kinase domain were synthesized (Fig. 2A and B). All the synthetic peptides were successfully separated by SDS-PAGE and silver-stained (Fig. 2C). The synthetic peptides were then *in vitro* phosphorylated in the presence of GST or GST-ZIPK and separated by SDS-PAGE (Fig. 2B), followed by autoradiography. Notably, Pep#9, which contained one threonine residue (Thr265) and two serine residues (Ser269 and Ser273), was markedly phosphorylated by GST-ZIPK *in vitro*.

3. 2. Thr265 in ZIPK is critical for its kinase activation

To confirm that Thr265 and Ser269/273 are autophosphorylation sites in ZIPK, we tested the kinase activity of ZIPK using deletion mutants that only encoded the kinase domain (ZIPK KD) with specific substitutions of each of these residues (T265A, S269A and S273A) (Fig. 3A). ZIPK KD, T265A, S269A or S273A was transiently expressed in 293T cells, and immunocomplexes of ZIPK were subjected to *in vitro* kinase assays in the presence of [γ -³²P]-ATP. As shown in Fig. 3B, the T265A substitution resulted in disappearance of the autophosphorylation, indicating that Thr265 is a candidate autophosphorylation site in the ZIPK kinase domain.

Next, we examined the effect of this substitution in full-length ZIPK on its kinase activity. Wild-type ZIPK or ZIPK T265A was transfected into 293T cells, and immunocomplexes of ZIPK were subjected to *in vitro* kinase assays in the presence of [γ -³²P]-ATP. As shown in Fig. 3C, ZIPK T265A demonstrated a marked reduction of the autophosphorylation, suggesting that there are other phosphorylation sites in the C-terminal domain of ZIPK. We further tested whether the T265A substitution has any effect on the kinase activity. Wild-type ZIPK or ZIPK T265A was transfected into 293T

cells, and immunocomplexes of ZIPK together with Pep#9 were subjected to *in vitro* kinase assays. As shown in Fig. 3D, strong phosphorylation of Pep#9 by wild-type ZIPK was observed. However, ZIPK T265A showed a marked reduction in the Pep#9 phosphorylation. These results indicate that Thr265 in ZIPK is critical for its kinase activity.

3. 3. The T265A substitution in ZIPK reduces its cell death activity in the NIH 3T3 and HeLa cell lines

It has been demonstrated that ectopic expression of ZIPK causes cell death. Expression vectors for wild-type ZIPK or ZIPK T265A together with a plasmid containing EGFP were transiently transfected into NIH 3T3 cells. The GFP-positive cells transfected with wild-type ZIPK exhibited the morphological changes of apoptotic cells, as characterized by their shrunken appearance, condensed chromatin and membrane blebbing (data not shown). As shown in Fig. 3E, wild-type ZIPK positively regulated cell death. Importantly, the ZIPK T265A transfectants showed an approximately 50% reduction in dead cells, compared to the wild-type ZIPK

transfectants. Similarly, the ZIPK T265A but not wild-type ZIPK failed to induce apoptosis in HeLa cells by as characterized by chromatin condensation (Fig. 3F). These results suggest that Thr265 in ZIPK is required for its ability to mediate cell death, and strongly suggest that autophosphorylation of Thr295 of ZIPK plays an important role in the induction of cell death.

3. 4. Thr265 in ZIPK is important for ZIPK-mediated enhancement of STAT3 activation

Next, we measured the effect of Thr265 on ZIPK-mediated enhancement of STAT3 activation using reporter assays. The STAT3-mediated transcriptional responses were measured by using STAT3-LUC, in which the α 2-macroglobulin promoter [13, 14] drives expression of the LUC gene. H35 hepatoma transfectants (H35/vector, H35/ZIPK WT and H35/ZIPK T265A) were transiently transfected with STAT3-LUC and treated with IL-6. As shown in Fig. 4A, IL-6 induced STAT3-LUC activation in H35 cells. Overexpression of wild-type ZIPK markedly enhanced STAT3-mediated transcriptional activity compared with overexpression of a control mock vector. Importantly, overexpression of ZIPK T265A failed to enhance IL-6-induced STAT3 activation. These

data suggest that Thr265 in ZIPK plays an important role in the modulation of IL-6-induced STAT3 activation.

3. 5. LIF stimulates phosphorylation of Thr265 in ZIPK

To verify that Thr265 is phosphorylated in response to ligand activation, we raised a phospho-specific antibody directed against Thr265 in ZIPK. Since this antibody, designated anti-pZIPK Thr265, had not yet been characterized, we initially confirmed its specificity using cell lysates prepared from FLAG-tagged wild-type ZIPK- or ZIPK T265A-transfected 293T cells. Cell lysates were immunoprecipitated with an anti-FLAG antibody, and blotted with preimmune serum or the anti-pZIPK Thr265 antibody. The expressions of wild-type ZIPK and ZIPK T265A were comparable when confirmed by immunoblotting with an anti-FLAG-antibody (Fig. 4B, bottom panels). When the lysates were blotted with the anti-pZIPK Thr265 antibody, wild-type ZIPK, but not ZIPK T265A, was recognized (Fig. 4B, upper panels).

Next, we examined whether Thr265 is phosphorylated in response to stimuli. Wild-type ZIPK- or ZIPK T265A-transfected 293T cells were stimulated with LIF. The

cell lysates were analyzed by immunoblotting with the anti-pZIPK Thr265 or anti-FLAG antibodies. Phosphorylation of Thr265 was only detected in wild-type ZIPK-transfected 293T cells (Fig. 4C). Furthermore, its phosphorylation was remarkably enhanced by LIF stimulation. Blotting with the anti-FLAG antibody confirmed equal loading. These results clearly demonstrate that ZIPK is phosphorylated at Thr265 in response to LIF. IL-6 also stimulated ZIPK autophosphorylation (data not shown).

3. 6. Concluding remarks

During the preparation of this manuscript, a report describing the identification of several ZIPK autophosphorylation sites was published [18]. In that report, Thr180, Thr225, Thr265, Thr299, Ser306 and Ser311 in ZIPK were phosphorylated *in vivo*. These data support that the C-terminal domain of ZIPK T265A is still phosphorylated in 293T cells as shown in Fig. 3C. Furthermore, phosphorylation of three sites within the kinase domain (Thr180, Thr225 and Thr265) was shown to be required for its kinase activity, which is consistent with our data on Thr265. Similarly, these ZIPK mutants

T180A, T225A, and T265A have been also shown to lose ZIPK cell death activity.

Our present study provides evidence that Thr265 in ZIPK is an autophosphorylation site that is essential for its full kinase activity. Phosphorylation of Thr265 is also important for ZIPK-mediated cell death and the enhancement of IL-6-induced STAT3 activation. We have further clarified that LIF induces phosphorylation of Thr265 in 293T cells using an anti-pZIPK Thr265 antibody. STAT3 is mainly activated by the IL-6 family of cytokines, epidermal growth factor and leptin [19-21]. Similar to other members of the STAT family, STAT3 is tyrosine-phosphorylated by Janus kinases (Jaks), which induce its dimerization and translocation into the nucleus where it activates its target genes [22, 23]. STAT3 is known to play crucial roles in early embryonic development as well as in other biological responses including cell growth and apoptosis [20,24]. Furthermore, it is constitutively activated in oncogenic tyrosine kinase *v-src*- or *v-abl*-transformed cells and various primary tumors and cell lines [25,26]. Recently, we demonstrated that IL-6/LIF-mediated STAT3 activation enhanced ZIPK activity [13]. This finding may suggest that IL-6/LIF/STAT3 signaling mediates apoptotic activity by stimulating ZIPK. Indeed, co-expression of ZIPK with STAT3

induced a massive cell death in 293T and HeLa cells (data not shown). Further detailed work requires stable ZIPK transformants. However, we were unable to generate 293T cells stably expressing ZIPK due to a massive cell death after ZIPK transfection. It is well known that establishing cell lines overexpressing DAP kinase family is hard due to induction of a massive cell death. It would be very attractive to know the contribution of ZIPK in IL-6/LIF-induced apoptosis *in vivo* using ZIPK knockout mice.

The present results provide novel clues toward identifying the physiological roles of ZIPK and developing a new therapeutic strategy for a variety of cancers mediated by dysregulated STAT3 activation.

Acknowledgements

This work was supported in part by grants from Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government, AstraZeneca Research Grant 2003, the Naito Foundation, the Akiyama Foundation and theSasakawa Scientific Research Grant from the Japan Science Society.

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Figure legends

Fig. 1 Autophosphorylation of ZIPK. (A) Kinase activity of DAP family kinases. Myc-tagged DAP family kinase (DAPK KD, DAPK2, DRAK1, DRAK2 and ZIPK) (10 μ g) were transiently transfected into 293T cells. Cell lysates were immunoprecipitated with anti-Myc antibody and assayed for in vitro kinase activity (upper panel). The amounts of DAP family kinase proteins were shown to be the same by Western blotting with anti-Myc antibody (lower panel). Relative molecular mass standards are indicated on the left. (B) Schematic view of GST-fused ZIPK and its deletion mutants. (C) Kinase activity of GST-ZIPK, GST-ZIPK KD and GST-ZIPK KD (1-156). Recombinant GST-ZIPK, GST-ZIPK KD or GST-ZIPK KD (1-156) (1-5 μ g) was subjected on in vitro kinase assay (upper panels). The amount of GST-fusion proteins was shown by immunoblotting with anti-GST antibody (lower panels).

Fig. 2. In vitro ZIPK synthetic peptide phosphorylation. (A) Schematic view of ZIPK and location of ZIPK synthetic peptides. (B) Peptides (#1-#9) synthesized with

seine/threonine in the kinase domain. (C) Silver staining pattern of synthetic peptides (4 μ g each) on SDS-PAGE 15-25% gradient gel. The asterisk indicates the migration position and a faint staining of Peptide#7. (D) Synthetic peptides (4 μ g each) were 32 P labeled with GST or GST-ZIPK, separated on SDS-PAGE 15-25% gradient gel.

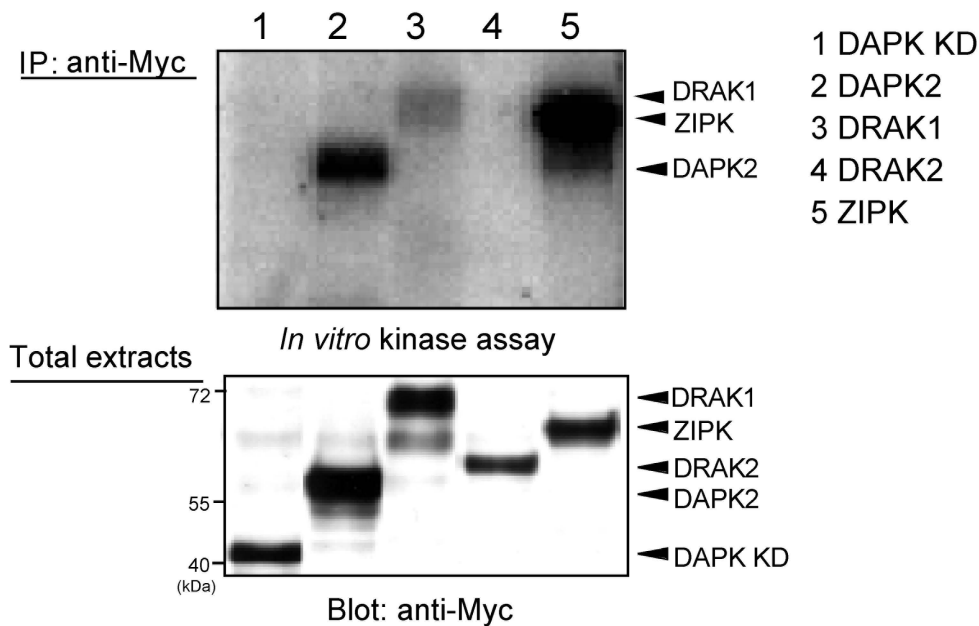
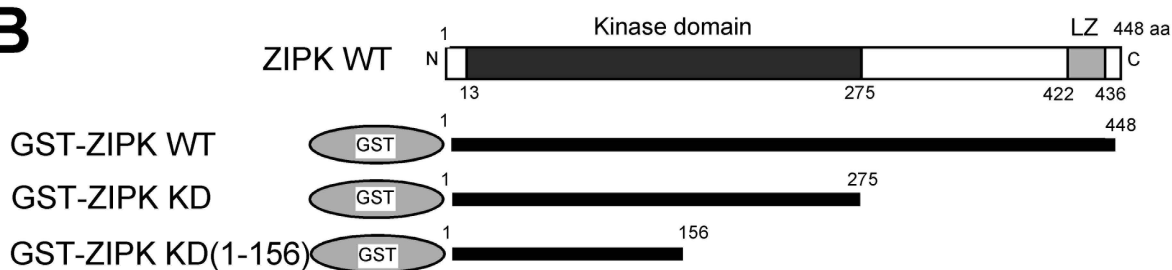
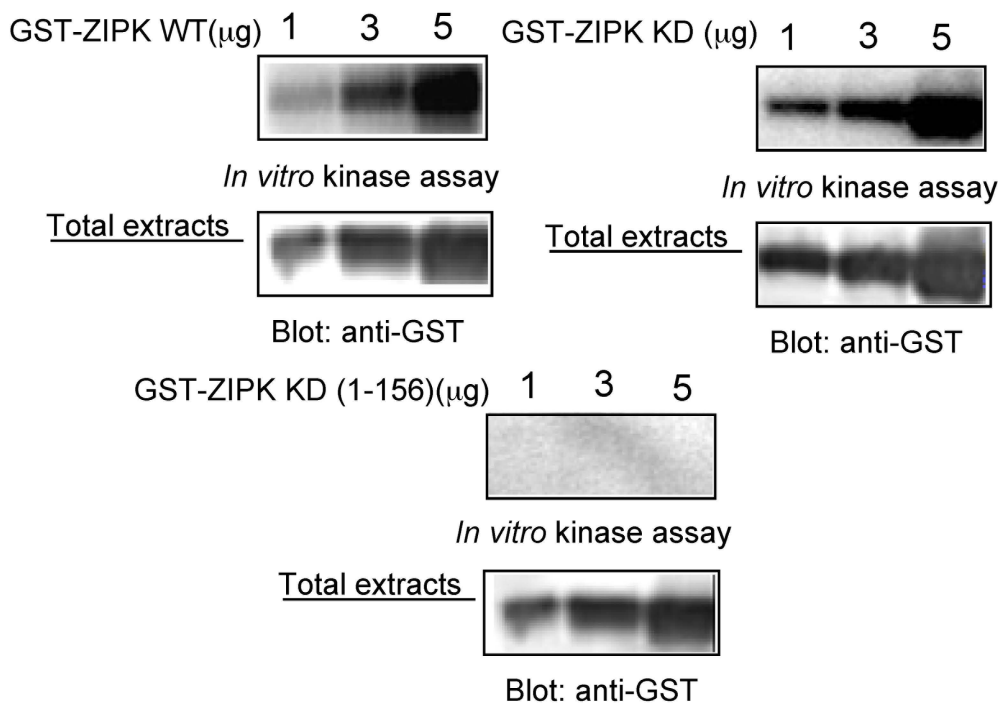
Fig. 3. ZIPK Thr265 is an autophosphorylation site and critical for its kinase activity and cell death activity. (A) Schematic view of ZIPK and its truncated mutants. (B) Kinase activity of ZIPK mutants. Myc-tagged ZIPK mutants (wild-type ZIPK KD, ZIPK KD T265A, ZIPK KD S269A and ZIPK KD S273A) (5 μ g) were transiently transfected into 293T cells. Cell lysates were immunoprecipitated with anti-Myc antibody and assayed for in vitro kinase activity (upper panel). The amounts of ZIPK KD proteins were shown to be the same by immunoblotting with anti-Myc antibody (lower panel). (B) Kinase activity of ZIPK mutants. Myc-tagged ZIPK mutants (wild-type ZIPK KD, ZIPK KD T265A, ZIPK KD S269A and ZIPK KD S273A) (5 μ g) were transiently transfected into 293T cells. Cell lysates were immunoprecipitated with anti-Myc antibody and assayed for in vitro kinase activity. (C) Kinase activity of ZIPK

mutants. Myc-tagged ZIPK mutants (wild-type ZIPK and ZIPK T265A) (5 μ g) were transiently transfected into 293T cells. Cell lysates were immunoprecipitated with anti-Myc antibody and assayed for in vitro kinase activity. (D) Transphosphorylation of peptide #9 by ZIPK mutants. Myc-tagged ZIPK mutants (empty vector, wild-type ZIPK and ZIPK T265A) (5 μ g) were transiently transfected into 293T cells. Cell lysates were immunoprecipitated with anti-Myc antibody and assayed for in vitro kinase activity in the absence or presence of peptide #9 (4 μ g). (E) An expression plasmid of wild-type ZIPK or ZIPK T265A was transiently cotransfected together with a plasmid expressing EGFP into NIH 3T3 cells. After 48 h of transfection, cells with the apoptotic phenotype are counted. The data presented shows the percentage of GFP-positive cells with the apoptotic phenotype relative to the total number of GFP-positive cells counted. The results are from three independent experiments. (F) An expression plasmid of wild-type ZIPK or ZIPK T265A was transiently cotransfected together with a plasmid expressing EGFP into HeLa cells. After 48 h of transfection, cells with the apoptotic phenotype are counted. The data presented shows the percentage of GFP-positive cells with the

apoptotic phenotype relative to the total number of GFP-positive cells counted. The results are from three independent experiments.

Fig. 4. ZIPK Thr265 plays important roles in enhancement of STAT3 activation, and its phosphorylation is induced by LIF stimulation. (A) Stable H35 transfectant expressing pcDNA3, wild-type ZIPK or ZIPK T265A was established. Total extracts of each transfectant were examined by Western blot using anti-FLAG antibody (left panels). H35 transfectant cells in a 12-well plate were transfected with STAT3-LUC (0.4 μ g). Twenty-four hours after transfection, the cells were stimulated with IL-6 (100 ng/ml) and sIL-6R α (100 ng/ml) for 12 h. The cells were then harvested, and luciferase activities were measured. The results are indicated as fold induction of luciferase activity from triplicate experiments, and the error bars represent the S.D. (B) 293T cells transfected with empty vector, FLAG-tagged wild-type ZIPK or ZIPK T265A (5 μ g). Thirty-six hours after transfection, the cells were lysed, immunoprecipitated with anti-FLAG antibody and immunoblotted with pre-immune control IgG (left panels) or

anti-pZIPK Thr265 (right panels). (C) 293T cells transfected with empty vector, FLAG-tagged wild-type ZIPK or ZIPK T265A (5 μ g). Twenty-four hours after transfection, the cells were stimulated with LIF for 30 min. Cells were then lysed, immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-pZIPK Thr265 (upper panel) or anti-FLAG antibody (middle panel).

A**B****C****Fig. 1**

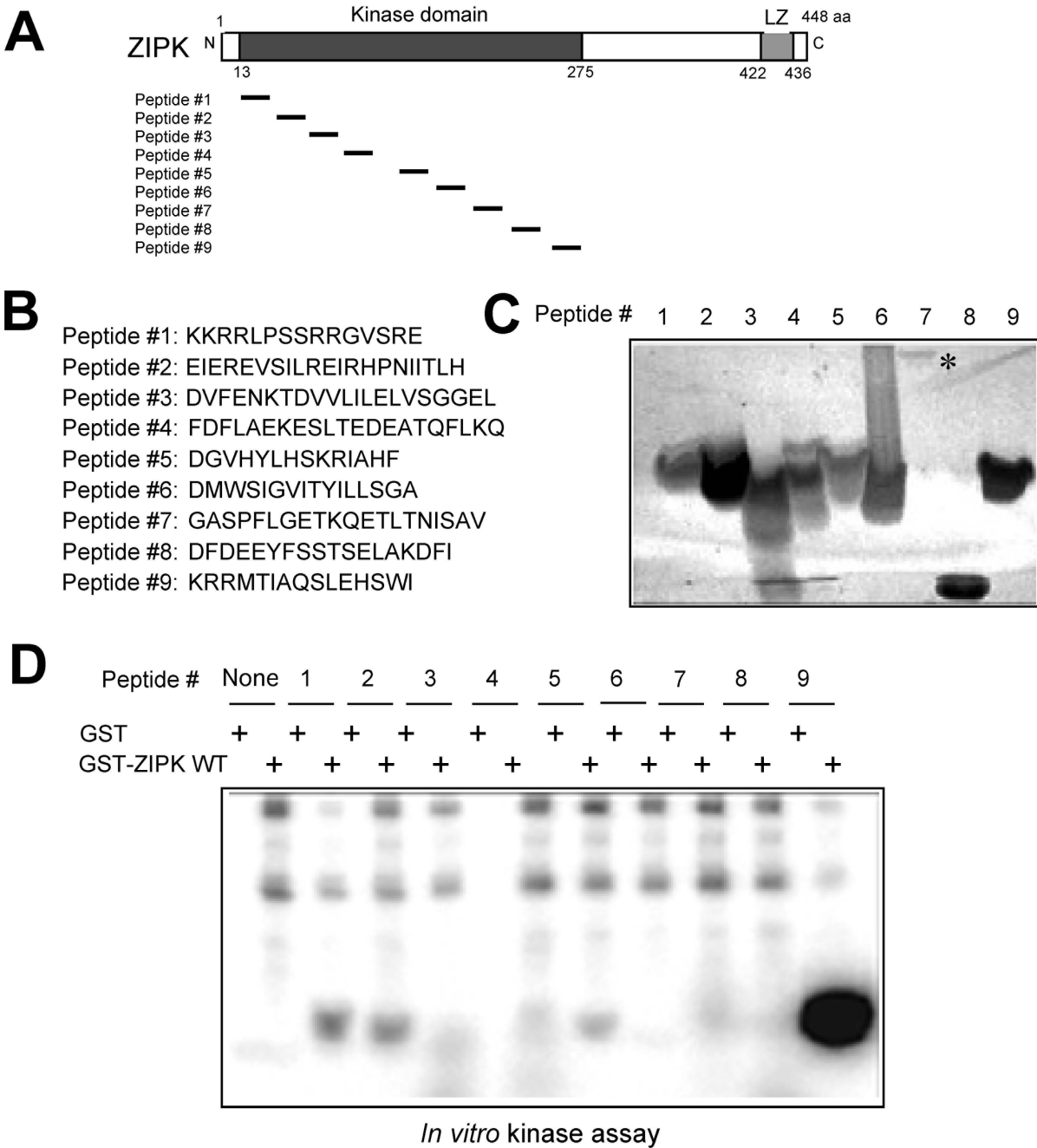


Fig. 2

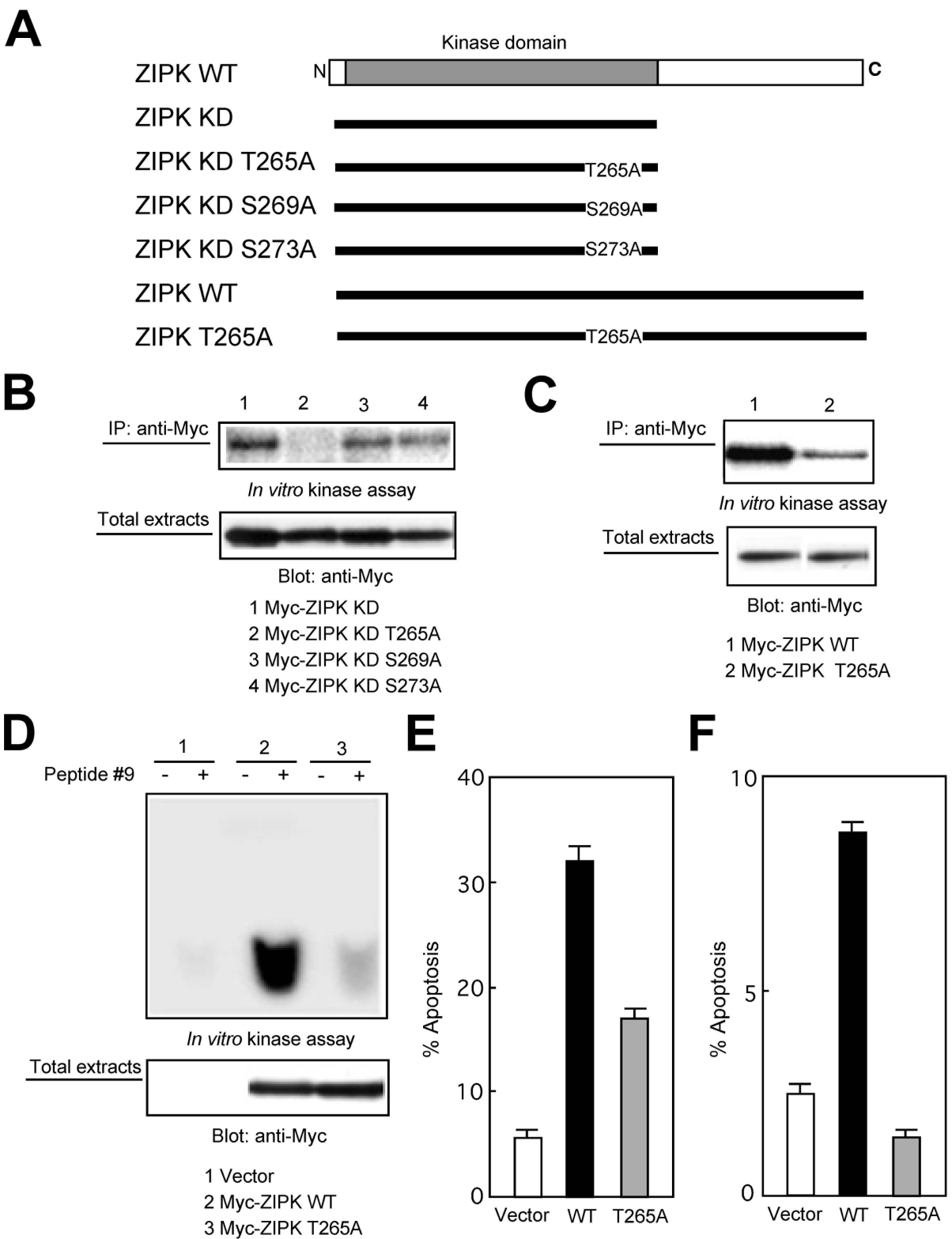
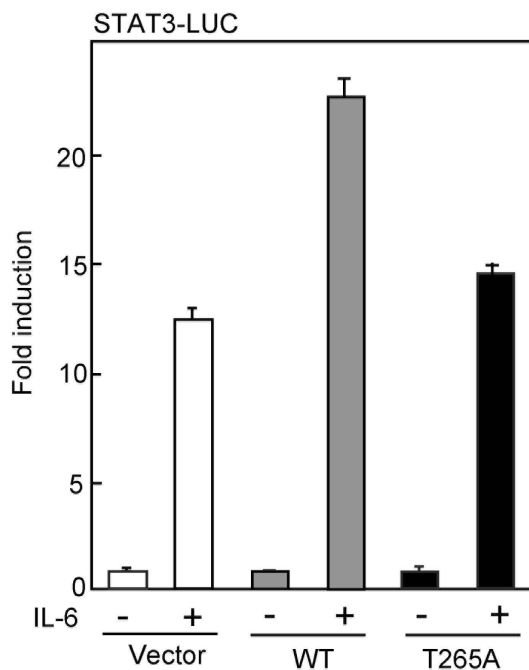
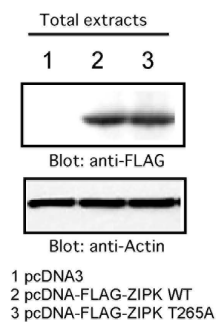
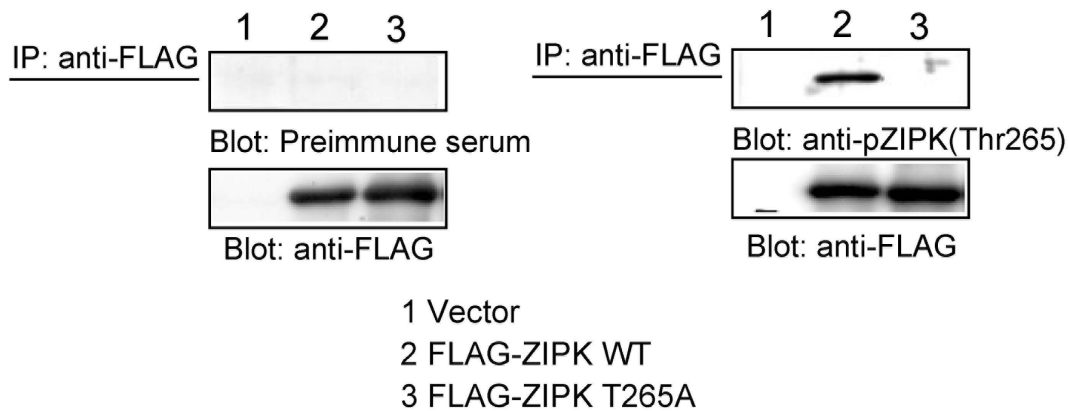
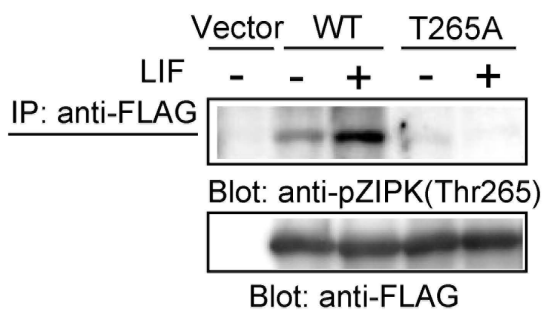


Fig. 3

A**B****C****Fig. 4**