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Mixed lineage kinase ZAK utilizing MKK7 and not MKK4 to activate the c-Jun N-terminal kinase and playing a role in the cell arrest☆

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Abstract

The leucine-zipper (LZ) and sterile-α motif (SAM) kinase (ZAK) belongs to the MAP kinase kinase kinase (MAP3K) when upon over-expression in mammalian cells activates the JNK/SAPK pathway. The mechanisms by which ZAK activity is regulated are not well understood. Co-expression of dominant-negative MKK7 but not MKK4 and ZAK significantly attenuates JNK/SAPK activation. This result suggests that ZAK activates JNK/SAPK mediated by downstream target, MKK7. Expression of ZAK but not kinase-dead ZAK in 10T1/2 cells results in the disruption of actin stress fibers and morphological changes. Therefore, ZAK activity may be involved in actin organization regulation. Expression of wild-type ZAK increases the cell population in the G_2/M phase of the cell cycle, which may indicate G_2 arrest. Western blot analysis shows that the decreased cyclin E level correlated strongly with the low proliferative capacity of ZAK-expressed cells. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: Leucine-zipper (LZ) and sterile-α motif (SAM) kinase (ZAK); Mixed lineage kinase (MLK); JNK/SAPK; Cell cycle.

The mixed lineage kinase (MLK) family comprises a group of highly related serine/threonine kinases that function as MAP3K [1–3]. Several members of this kinase family have been found to activate the c-Jun NH_2-terminal kinase/stress-activating protein kinase (JNK/SAPK) stress-signaling pathway [4,5]. The JNKs are activated by the dual specificity of MAP kinase kinases (MAP2Ks), MEK4/MKK4 and MEK7/MKK7) [6,7]. Upstream of the MAPKKs exist multiple MAPKK kinase (MAP3K) families that regulate JNK activity, including the MLK and MEKK family [8,9]. In a previous study, a novel gene that encodes a serine/threonine kinase, designed ZAK for leucine-zipper (LZ) and sterile-α motif (SAM) kinase [10]. The ZAK expression in mammalian cells leads specifically to the activation of the JNK/SAPK pathway as well as the transcription factor, NF-κB. The identified members of the MLK were thought to mediate JNK/SAPK activation in mammalian cells from the activation of a Rho-like small GTPase into intermediate protein kinases [11–14], including the identified MAP2Ks (MKK4/SEK and MKK7/JNKK2) [6,15]. It has been shown that MLK2, MLK3, DLK, and LZK activate JNK/SAPK when over-expressed in cultured cells [16,17]. Furthermore, MLK2 and MLK3 have been demonstrated to associate with, phosphorylate, and activate MKK4 [2,18,19], which in turn activates JNK/SAPK. A previous study demonstrated that ZAK expression induces JNK/SAPK activation and apoptotic cell death [10].

This study was undertaken to investigate the MAPK cascades involved in the sequential protein kinase reaction. ZAK expression preferentially activated the JNK/SAPK pathway by MKK7 stimulation but not SEK. This report also shows that ZAK expression induces the disruption of actin stress fibers, causing a dramatic change in cell morphology. Moreover, expression of wild-type ZAK, but not kinase-dead ZAK,

☆ *Abbreviations:* ZAK, leucine-zipper and sterile-α motif kinase; MLK, mixed lineage kinase; MAPK, mitogen-activated protein kinase; JNK/SAPK, c-Jun NH2-terminal kinase/stress-activating protein kinase.

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induces an increase in the G_2/M cell population. The report present here suggests a schematic pathway of ZAK > MKK7 > JNK > cell arrest. Furthermore, sustained ZAK expression significantly increases apoptotic cells compared to the control cell type. These findings indicate that ZAK plays a role in cell cycle checkpoint and apoptosis regulation.

Materials and methods

Cell culture and transfection. Rat6 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and antibiotics (25 U/ml penicillin and 25 U/ml streptonicillin). HepG2, 293T, and 10T1/2 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics (25 U/ml penicillin and 25 U/ml streptomycin transfection of the expression vectors was performed using the $Ca–PO_4$ method. The parental Rat6 Tet-on clone, which expresses the tetracycline-repressible transactivator, was used to generate stable lines expressing wild-type (pTRE-ZAK), catalytically inactive T-ZAK (pTRE-T-ZAK), and pTK-Hyg (Clontech), which carries the hygromycin resistance gene. Cells were transfected using the $Ca–PO_4$ method. Clone selection was carried out in the presence of 400 μg/ml hygromycin B (Calbiochem). Drug-resistant clones were further tested for expression of the transgenes after adding of doxycycline to induce ZAK gene expression.

Detection of the JNK/SAPK activities. Protein kinase assays were carried out using a fusion protein between glutathione-*S*-transferase (GST) and c-Jun (amino acids 1–79) as a substrate. The GST–Jun fusion proteins were bound to glutathione–Sepharose beads and incubated for 15 min on ice with the cellular extract that contained JNK in the presence of a kinase buffer (20 mM Hepes, pH 7.6, 1 mM EGTA, 1 mM dithiothreitol, 2 mM MgCl, 2 mM MnCl, 5 mM NaF, 1 mM NaVO, and 50 mM NaCl). The beads were pelleted and thoroughly washed with PBST (150 mM NaCl, 16 mM sodium phosphate, pH 7.5, 1% Triton X-100, 2 mM EDTA, 0.1%-MeOH, 0.2 mM phenylmethylsulfonyl fluoride, and 5 mM benzamidine), before they were incubated with [γ-^{32}P]ATP (50 cpm/fmol) in the presence of kinase buffer. These steps were undertaken to ensure that c-Jun phosphorylation was carried out by JNK, which is known to exhibit a high affinity to this c-Jun portion under these conditions. Following extensive washing, the phosphorylated GST–Jun was boiled in SDS sample buffer. The eluted proteins were run on a 15% SDS–PAGE. The gel was dried and phosphorylation of the Jun substrate was determined by autoradiography.

Cell staining. Cells were transfected with pEGFP-ZAK or pEGFP-T-ZAK then, fixed, and permeabilized. Actin filaments were visualized using Rhodamine-labeled phalloidin. Cells were examined and photographed using a Zesis Axioskop microscope.

Western blot analysis. ZAK- or empty vector-expressed cells were harvested in lysis buffer (50 mM Tris–HCl, pH 8.0/250 mM NaCl/1% NP-40, and 2 mM EDTA) containing 1 mM PMSF, 10 ng/ml leupeptin, 50 mM NaF, and 1 mM sodium orthovanadate. Total proteins were then separated on SDS–PAGE and the specific protein bands were visualized with an ECL chemiluminescent detection system (Amersham) performed as described [20,21].

Cell synchronization. To arrest cells near the G_1/S border of the cell cycle, a double drug block was used. An initial synchronization of cells near G_1/S phase was achieved by treating cells with 2 mM thymidine for 14 h. The cells were washed twice with PBS and further incubated in fresh medium for 9 h. The cultures were then treated with 300 μM mimosine and incubated for 16 h [22]. The cultures were then refed with fresh medium and harvested at different time points.

Cell cycle analysis. Cells were grown for 24 h or 72 h in the presence of doxycycline to induce the ectopic genes. Cells were then plated to 40% confluency (2.5×10^6/plate) on 100-mm plates. Cells were filtered to remove cell aggregates (Falcon filter top tubes) and analyzed for DNA content using fluorescence-activated cell sorting (FACS) analysis with a FACSCalibur flow cytometer (BD PharMingen). Data were analyzed using the Cell Quest (BD PharMingen) and ModFit (Verity) analysis software.

Results

ZAK protein is autophosphorylated

The over-expression of ZAK in 293T cells showed mobility retardation in SDS–PAGE. The in vitro treatment of GFP-ZAK with calf intestine alkaline phosphotase increased its mobility in SDS–PAGE (Fig. 1A). Thus, ZAK seems to be able to undergo autophosphorylation. To examine if autophosphorylation might occur through an inter-molecular reaction, different ZAK sequence deleted mutants were generated. 293T cells were transfected with different combinations of these GFP-tagged ZAK variants. As predicted, the wild-type ZAK showed a mobility shift, where the N-terminal 100 amino acid deleted ZAK (T-ZAK), which is a kinase-dead mutant, did not (Fig. 1B). In contrast to T-ZAK, the C-terminal 100 amino acid deleted ZAK (ZAK-100) showed a mobility shift (Fig. 1B). This result indicated that the autophosphorylation is required for the existence of kinase activity within ZAK. T-ZAK co-expressed with wild-type ZAK showed a relatively weak but significant mobility shift. ZAK-100 co-expressed with wild-type ZAK also showed a mobility shift. These results suggest that the intermolecular reaction contributes to ZAK autophosphorylation. Increasing all mobility retarded bands after the in vitro calf intestine alkaline phosphatase treatment, suggesting that the mobility retardation was due to the ZAK autophosphorylation reaction, induced mostly likely by the intracellular reaction.

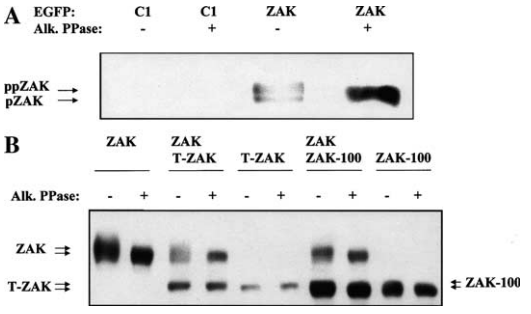

Fig. 1. Autophosphorylation reaction of ZAK. (A) 293T cells were transiently transfected with pEGFP-C1 or pEGFP-ZAK. The cell extracts were left untreated or incubated with calf intestine alkaline phosphatase. Cell extracts were subjected to immunoblotting with anti-GFP Abs. (B) 293T cells were transfected with the indicated constructs. The mobility in SDS–PAGE was analyzed as described in (A).

ZAK activate JNK pathway through MKK7

To closely examine the potential epistatic relationship between ZAK and MAP2K, two dominant-negative MAP2Ks, SEKdn and MKK7dn, were used to dissect the role that ZAK might play in JNK/SAPK activation. Hep G2 cells were co-transfected with either SEKdn or MKK7dn and ZAK, and then an in vitro kinase assay was performed to test whether SEKdn or MKK7dn could block JNK/SAPK activation under ZAK expression. The experiment demonstrated that MKK7dn, but not SEKdn, attenuated the ability of ZAK to activate JNK/SAPK in a dose-dependent manner (Fig. 2A). This result indicated that the ZAK-activated JNK/SAPK pathway might be mediated through MKK7. The JNK/SAPK signal has been implicated in AP-1-dependent gene expression because c-Jun, one component in the AP-1 complex, is a substrate of JNK/SAPK in vivo. To further confirm this linear relationship, HepG2 cells were co-transfected with a secreted alkaline phosphatase (SEAP) reporter construct containing an AP-1 binding site with various combinations of these genes. ZAK-stimulated AP-1 activity was blocked using the MKK7, but not SEK, mutant (Fig. 2B). Taken together, these experiments suggest that ZAK leads to the activation of JNK/SAPK through MKK7.

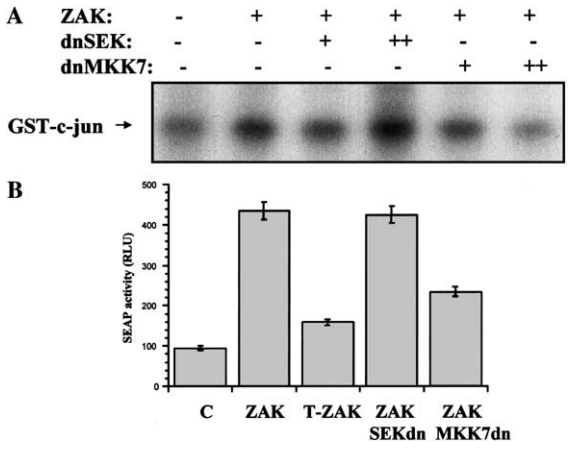

Fig. 2. JNK/SAPK pathway is blocked by the MKK7 mutant but not by the SEK mutant. (A) MKK7 mutant but not SEK mutant blocks ZAK-induced JNK/SAPK activation. HepG2 cells (5×10^5 cells in 100-mm plate) were transfected with pEGFP-C1 or pEGFP-ZAK (10 μg) alone or pEGFP-ZAK with various amounts [(+) 10 μg and (++) 20 μg] of either SEK or MKK7 mutants. Cell lysates were prepared at 48 h post-transfection. Cell lysates were subjected to performing the in vitro kinase assays. (B) ZAK-mediated the induction of AP1 activity in HepG2 cells. HepG2 cells 2.5×10^5) were seeded in six-well plates and transfected with indicated plasmids, ZAK or T-ZAK (4 μg); dominant-negative MKKs (8 μg) and 1 μg pAP1-SEAP. Samples were collected from the media 48 h after transfection and measured for SEAP activity using the Great EscAPe chemiluminescence assay. Experiments were performed four times; representative data are shown.

ZAK induces actin stress fiber destruction and cell morphological changes

A change in cell morphology was noticed when ZAK is expressed. This was therefore a reason to test whether the cytoskeleton might change when ZAK is expressed. GFP-ZAK or GFP-T-ZAK was expressed in 10T1/2 cells, actin filaments were stained with Rhodamine-labeled phaloidin. ZAK expression caused actin stress fiber disruption and cell shrinkage (Fig. 3). Conversely, T-ZAK expression did not cause actin stress fiber disruption and cell shrinkage (Fig. 3). This result suggested that ZAK kinase activity is essential for these cellular changes.

Effect on rat cell growth with and without ZAK or T-ZAK expression

To assess whether ZAK could influence the cell growth rate, stable Rat6 cell lines that expressed wild-type or kinase-dead ZAK (T-ZAK) were generated under tetracycline-responsible transactivator control. In this system, by adding a tetracycline analog, doxycycline induces the expression of the recombinant proteins. A set of 5000 cells that expressed the ZAK or T-ZAK protein were cultured in six-well dishes. The cell growth rate was determined every 24 h. The ZAK expressing cells showed a decreased growth rate in comparison with parental cells. However, the T-ZAK expressing cells increased significantly in number (Fig. 4A). To determine the cell cycle phase in which ZAK-expressed cells grew slower than the parental and T-ZAK cells, asynchronous cultures were trypsinized and their cell cycle distributions were analyzed using flow cytometry. ZAK-expressed cells had a significantly higher percentage of cells in the G_2/M phase of the cell cycle stage (39.79%) compared with the control cells (9.62%) and T-ZAK-expressed cells (5.58%) (Fig. 4B). Moreover, ZAK-expressed cells decreased in the G_1 phase (49.28%) of the cell cycle compared with the control (79.19%) and T-ZAK-expressed cells (82.23%). This observation suggested that ZAK activation might be involved in the regulation of the G_2 checkpoint control. After sustained ZAK expression for 72 h, a small but significant proportion of the sub-G_1 phase (2.86%) was determined compared with the control cells (0.54%) (data not shown). These results showed that the ZAK activation might regulate the G_2 arrest and apoptosis cell cycle events.

Effects of ZAK on cell cycle-regulatory protein expression

The expression levels of cyclin D, E, and A are each rate-limiting for cell cycle progression [22–26]. Therefore, the expression of any of these cyclins was tested. Asynchronous cultures of each cell type were harvested,

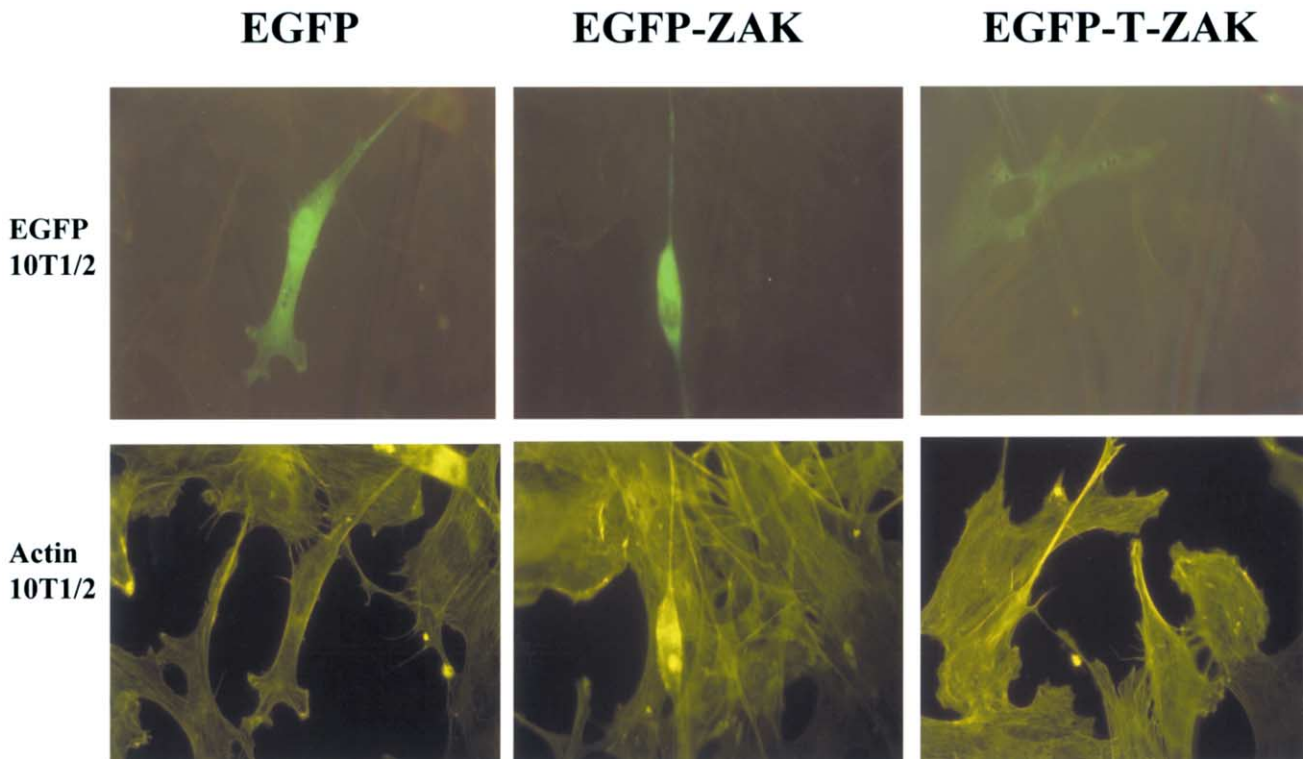

Fig. 3. ZAK induces the disruption of actin stress fibers and changes in cell morphology. 10T1/2 cells were transfected with pEGFP-C1, pEGFP-ZAK, or pEGFP-T-ZAK and then fixed 16 h after transfection. The cells were stained with Rhodamine-conjugated phalloidin to detect actin filaments. Experiments were performed three times and gave similar results.

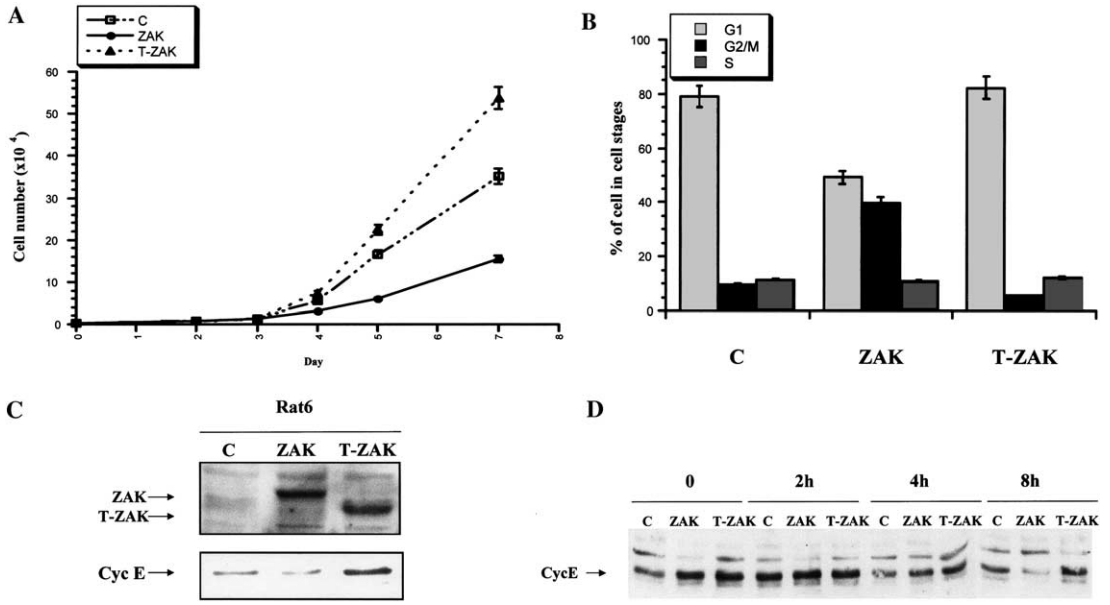

Fig. 4. ZAK expression induced cell cycle arrest in G_2/M phase and decreased the expression level of cyclin E. (A) Growth curves of Rat6 cells with or without expression of ZAK or T-ZAK. Stable Rat6 cell lines, which expressed wild-type or kinase-dead ZAK (T-ZAK) under the control of a tetracycline-responsible transactivator, were seeded at a original density of 5000 cells per six-well dish in the presence of 1 μg/ml doxycycline and cell number was counted at the indicated time. (B) Stable Rat6 cell lines were induced to express the indicated genes for 24 h and then analyzed by FACE. The percentage of cells in the cell cycle phases was calculated by the ModFit software. Experiments were performed three times and gave similar results. (C) Steady-state levels of cyclin E protein in these stable Rat6 cell lines. Stable Rat6 cell lines were induced to express the indicated genes for 24 h and then 50 μg total-cell extract was analyzed by Western blotting and probing with specific antibodies for ZAK or cyclin E. (D) Expression levels of cyclin E protein in these G_1/S-synchronized Rat6 cell lines. Stable Rat6 cell lines were synchronized by double thymidine block followed by mimosine block and then cells were released. Cell extracts were collected at the indicated times and 50 μg total-cell extract was analyzed by Western blotting and probing with specific antibody for cyclin E.

lysed, and analyzed using Western blotting. Decreasing the cyclin E level correlated strongly with the low proliferative capacity of ZAK-expressed cells (Figs. 4A and C). Conversely, the cyclin E level was elevated in the T-ZAK-expressed cells, which correlated with their faster growth rate. These results indicated that the ZAK activation might exert its effects on cyclin E expression (Figs. 4A and C). The decreasing levels of cyclin E in ZAK-expressed cells might explain the G_2/M arrest in such cells. To confirm that the cyclin E level might be regulated by ZAK, cells were synchronized and arrested near the G_1/S border via drug treatment. Fig. 4D demonstrates that the level of cyclin E decreased in both control and ZAK-expressed cell types 8 h after being released from the block. Moreover, the level of cyclin E in ZAK-expressed cells decreased further at a faster rate than the control cells. Conversely, T-ZAK-expressed cells exhibited a higher level of sustained cyclin E. Cyclin E production in synchronized cultures, closely mirroring that observed in asynchronized cultures. These results suggested that the expression of cyclin E could be a crucial aspect of ZAK-mediation, resulting from regulating the cell cycle checkpoint.

Discussion

Reversible protein phosphorylation is important in regulating the physiological process in cells. ZAK forms dimers when it was over-expressed in cells. Thus, ZAK may undergo autophosphorylation for regulating its activity upon upstream stimulation. In this case, autophosphorylation is similar to receptor tyrosine kinase activation. Upon ligand binding, these receptors form homodimers and phosphorylate their dimerized partner, resulting in kinase activation. Similar autophosphorylation mechanisms have been investigated for such MAP3Ks as MLK3 and ASK1. The data present that over-expressed ZAK in cells shows a mobility shift in SDS–PAGE, which seems to be caused by autophosphorylation and a presumably non-phosphorylated form was obtained using phosphatase treatment. A mutant of ZAK (T-ZAK) that lacks 100 amino acids in the kinase domain is not phosphorylated, indicating that ZAK phosphorylation is due to autophosphorylation. Furthermore, autophosphorylation of ZAK-100, which has 100 amino acids deleted in the C-terminal, occurs. In addition, T-ZAK is phosphorylated when co-expressed with wild-type ZAK. It therefore appears that the autophosphorylation of ZAK may be mediated by an intermolecular reaction. However, an intramolecular reaction cannot be ruled out with the present data.

In this study, wild-type ZAK was found to mediate the disruption of actin polymerization and activate the stress-activating protein kinase pathway. The expression of wild-type ZAK causes cell cycle arrest in the G_2/M phase. The results shown here are similar to Gachet et al.'s [27] proposal that actin polymerization might be involved in the cell cycle checkpoint. Gachet et al. proposed that a novel actin-dependent checkpoint exists that delays the separation of sister chromatids without activating the spindle-assembly checkpoint. ZAK expression in 10T1/2 cells causes actin stress fiber disruption whereas the ZAK kinase-dead mutant did not. This suggests that the ZAK kinase activity is essential for this cellular change. The cell cycle effect caused by wild-type ZAK suggests that the kinase activity mediates the signals, leading to G_2 arrest. ZAK expression in Rat6 cells is able to decrease the cyclin E protein level, which is correlated to the proliferation rate. Conversely, the kinase-dead ZAK, which might play the role of an interference mutant, increases the cyclin E protein level and cell division. It is not known whether the cyclin E expression level depends on actin polymerization or JNK/SAPK pathway activation. The mechanism that regulates the cyclin E expression by ZAK is underinvestigated.

It has been reported that the small GTPase family regulates the polymerized actin structures. The study from yeast two-hybrid system revealed that ZAK was able to interact with RhoGDI (J.J. Yang unpublished data). It is possible that ZAK modulates the RhoGDI function for actin cytoskeleton organization. It is unclear if the relationship between RhoGDI and ZAK is involved in causing actin stress fiber disruption; thus, further detailed experiments are needed to clarify this relationship.

In conclusion, ZAK expression causes G_2 arrest in the cell cycle and ZAK activity is necessary for the cell cycle checkpoint regulation in cell. The mechanism of this cell cycle arrest is due to ZAK activity that exerts its effect on the decreasing levels of cyclin E expression. Further studies will be needed to elucidate the downstream targeting molecules that are dependent on the activated ZAK for the regulation of the cyclin E expression.

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