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ZAK re-programs atrial natriuretic factor expression and induces hypertrophic growth in H9c2 cardiomyoblast cells

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Abstract

Various intracellular or intercellular stimuli have been associated with the development of cardiac cell hypertrophy. However, the mechanisms underlying this association are not completely understood. In a previous study we determined that ZAK mRNA expression is abundant in heart. ZAK is a mitogen-activated protein kinase kinase kinase (MAP3K) that activates the stress-activated protein kinase/c-jun N-terminal kinase pathway and activates NF-κB. We, therefore, investigated the potential involvement of ZAK (which in cultured H9c2 cardiomyoblast cell is a positive mediator of cell hypertrophy). Our results showed that the expression of a wild-type form of ZAK induces the characteristic hypertrophic growth features, including increased cell size, elevated atrial natriuretic factor expression, and increased actin fiber organization.

Keywords: ZAK; Atrial Natriuretic factor; Hypertrophic growth; Cardiomyblast cells

Cardiac hypertrophy is a multi-step pathological process that occurs in response to a variety of mechanical, hemodynamic, hormonal, and pathologic stimuli. Under these stimuli, the heart adapts to increased demands for cardiac work by increasing its muscle mass through cardiac cells hypertrophic growth [1–5]. The characteristic features of cardiac hypertrophy include increased cell size, induced sarcomere organization, and elevated cardiac gene expression, including atrial natriuretic factor (ANF) [3,6,7]. In humans, sustained cardiac hypertrophy is a key factor in the development of heart failure [8–11]. To understand the molecular cardiac hypertrophy mechanisms, it is important to define the extracellular stimuli and specific signaling molecules that elicit the hypertrophic phenotype.

In a previous study [12], we cloned a novel gene that encodes a serine/threonine kinase, designated ZAK for leucine-zipper (LZ) and sterile-α motif (SAM) kinase. ZAK belongs to the mixed lineage kinase (MLK) family, which comprises a group of closely related serine/threonine kinases that function as MAP3K. Northern blot analysis revealed that ZAK is the most abundantly expressed in human heart tissue [12]. ZAK expression in mammalian cells leads specifically to JNK/SAPK pathway activation and the activation of the NF-κB transcription factor. ZAK can also activate MKK7, the activator of JNK/SAPK [13]. In neonatal cardiac myocytes, MKK7 induces the characteristic features of hypertrophy [6], which suggests that ZAK itself may be involved in signal transduction for the regulation of cardiac hypertrophy.

Here we identify ZAK stimulated hypertrophic growth in cardiomyoblast cells. In cultured H9c2 cardiomyoblast

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cells, we show that ZAK induces the characteristic features of cardiac hypertrophy.

Materials and methods

Cell culture and transfection. H9c2 cells and 293T 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 by the expression vectors was performed using the Ca-PO_4 method. The parental H9c2 Tet-on clone, which expresses the tetracycline-repressible transactivator, was used to generate stable lines expressing wild-type ZAK, ZAKdn, or ZAKE/E, and pTK-Hyg (Clontech) which carries the hygromycin resistance gene. Clone selection was carried out in the presence of 100 μg/ml hygromycin B (Calbiochem). Drug-resistant clones were further tested for expression of the transgenes after adding doxycycline to induce expression of ZAK genes.

Expression constructs. DNA fragments corresponding to various ZAK domains were amplified by PCR. Sequences were verified by sequence analysis. These fragments were cloned into the pEGFPC1 vector. Kinase-negative (K45M), constitutive (S230E, S234E), or domain deleted (ΔLZ, ΔSAM, or ΔLS) ZAK forms were produced using a QuickChange site-directed mutagenesis kit (Stratagene) by PCR and cloned into the pEGFPC1 expression vector. See Fig. 1A for schematics of all the constructs used in this study.

Immunoprecipitation and Western blot analysis. Cell lysates were prepared in IP buffer (40 mM Tris–HCl [pH 7.5], 1% NP40, 150 mM NaCl, 5 mM EGTA, 1 mM DTT, 1 mM PMSF, 20 mM NaF, proteinase inhibitors, and 1 mM sodium vanadate). Cell extracts (600 μg) were incubated with 5 μg anti-GFP mAb (Clontech) for 6 h at 4 °C, mixed with 20 μl protein A–Sepharose suspension, and incubated for an additional hour. Immunoprecipitates were collected by centrifugation, washed three times with IP buffer plus 0.5% deoxycholate and five times with IP buffer alone, and then subjected to SDS–PAGE. Immunoblot analysis was performed with anti-FLAG (Sigma). Cells expressing ZAK or the empty vector 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 specific protein bands were visualized with an ECL chemiluminescent detection system (Amersham).

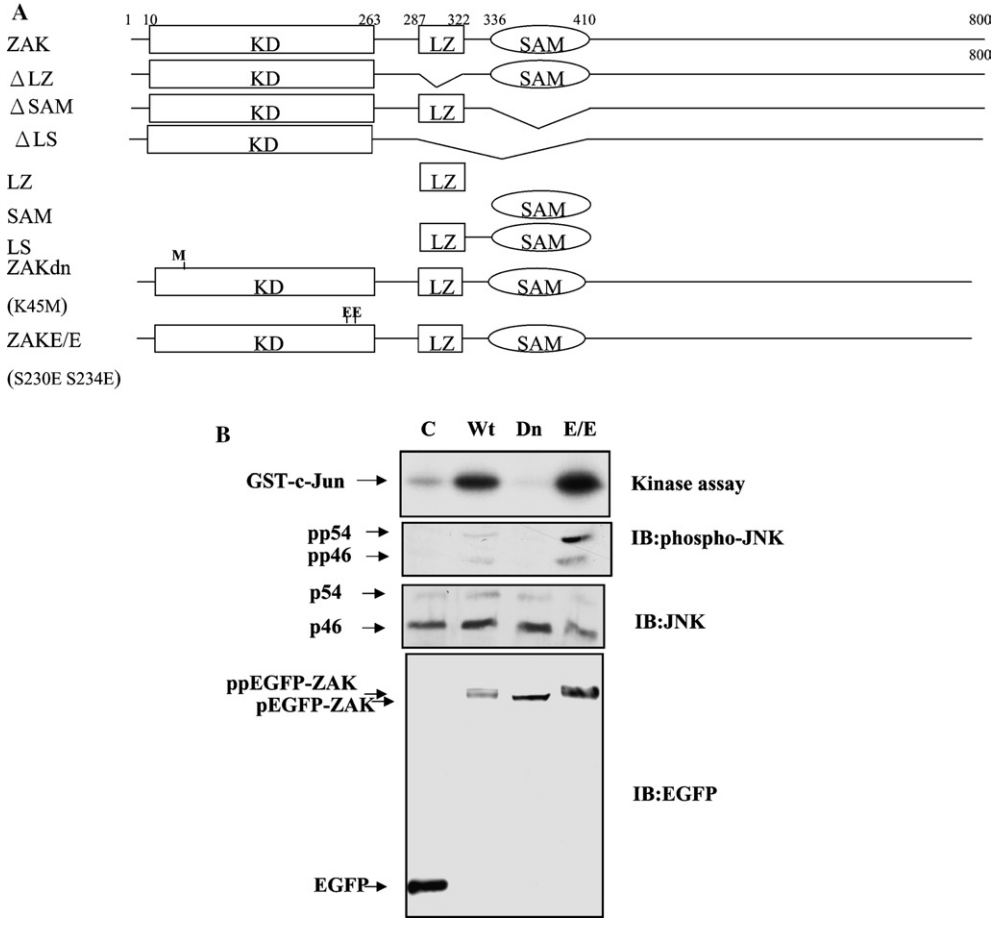

Fig. 1. Schematic representations of various expression constructs used in this study and activation of the JNK/SAPK pathway by dominant-negative and constitutively active ZAK proteins. (A) ZAK is characterized by the presence of a kinase domain (KD), a leucine zipper motif (LZ), and a sterile α motif (SAM). (B) 293T cells were transfected with pEGFPC1 (C), pEGFPC1-ZAK (Wt), pEGFPC1-ZAKdn (dn), and pEGFPC1-ZAK E/E (E/E) and incubated for 48 h. The lysates from the transfected 293T cells were then subjected to an in vitro kinase assay using GST-c-Jun (1–79) proteins as the substrate (upper panel). After resolution using 10% SDS–PAGE, the middle two panels show the immunoblotting with antibodies specifically against phosphorylated JNK/SAPK and JNK/SAPK. To determine the expression levels of EGFP, cell lysates were separated on 10% SDS–PAGE and immunoblotted using anti-GFP monoclonal antibody (lower panel).

Detection of the JNK/SAPK activities. Protein kinase assays were carried out using a fusion protein of glutathione *S*-transferase (GST) and c-Jun (amino acids 1–79) as the substrate. The GST-c-Jun fusion proteins were bound to glutathione Sepharose beads and incubated for 15 min on ice with 20 μg of cellular extract that contains JNK, in the presence of 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 collected 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) for 15 min at 25 °C 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-c-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 c-Jun substrate was determined by autoradiography.

Cell staining and measurement. H9c2 cells were fixed and permeabilized. Actin filaments were visualized using rhodamine-labeled phalloidin. Cells were examined and photographed using a Zesis Axioskop and a confocal microscope. The cell size was analyzed using Image-Pro Plus software.

Cell cycle analysis. Cells were grown for 48 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 Cell Quest (BD PharMingen) and ModFit (Verity) analysis software.

Northern blot analysis. Trizol reagents (Life Technologies) were used to isolate the total RNA from H9c2 cells transfected with the indicated recombinant plasmids. RNAs were separated on a formaldehyde agarose gel, transferred to a nylon filter, and then hybridized with a probe corresponding to the full length of rat ANF cDNA. The blot was washed with SSC/SDS solutions before autoradiography. Ethidium bromide (EtBr) staining was used to check the integrity of all samples.

Results

Generation of kinase-negative and constitutively activate mutants of ZAK

Previous studies [12] have shown that ZAK acts as a MAPK-kinase kinase (MKKK) to activate c-Jun amino-terminal kinase (JNK) pathway. To determine if ZAK activation of the JNK pathway depends on the integrity of the ZAK kinase domain, we produced and expressed kinase-negative ZAKdn and constitutively active ZAK E/E. The ZAKdn mutant was generated by a substitution of Lys^{45} with Met and the ZAK E/E mutant was generated by substitution of both Ser^{230} and Ser^{234} with Glu (see Fig. 1A for a full list of the various constructs used in this study). When 293T cells were transfected with GFP-tagged forms of wild-type ZAK and the two mutants, an in vitro kinase assay and immunoblot assay showed that JNK activities were induced in the wild-type transfected cells (Fig. 1B), a result which is consistent with the previous studies. No JNK activity was detected in the ZAKdn transfected cells but the ZAK E/E transfected cells showed increased induction of JNK. Autophosphorylation was clearly seen in wild-type ZAK, but was even more marked in the ZAK E/E cells, where most of the ZAK E/E proteins were in their autophosphorylated form. No autophosphorylation took place in the case of ZAKdn. Taken together, we conclude that ZAK activates the JNK pathway and its autophosphorylation is dependent on the intrinsic activity of the kinase domain.

The LZ region is critical for the formation of activated disulfide-bridged ZAK homodimers

It has been shown previously that ZAK forms oligomers (homodimers) when over-expressed in 293T cells [12]. This kinase contains a leucine-zipper region (LZ) and a sterile-α motif (SAM), and because both LZ and SAM are known to mediate oligomerization in a wide array of proteins, here we investigate whether ZAK oligomerization is mediated through LZ or SAM. Using a co-immunoprecipitation approach, a FLAG-tagged ZAK expression construct was transfected either alone or together with GFP epitope-tagged wild-type or mutated ZAK expression constructs (ΔLZ, ΔSAM, and ΔL, which, respectively, lacked the LZ region, the SAM motif, or both) into 293T cells. After anti-GFP antibody was used to immunoprecipitate the putative complexes, immunoblotting with an anti-FLAG antibody was used to detect any FLAG-ZAK that was pulled down by GFP-ZAK. As expected, FLAG-ZAK bound to GFP-ZAK (Fig. 2A). The SAM-deleted mutant was also able to bind FLAG-ZAK. However, a GFP-ZAK protein without the LZ motif was unable to co-precipitate FLAG-ZAK, which suggests that the formation of ZAK oligomers is mediated through the LZ and not the SAM region. The second panel of Fig. 2A shows that the LZ domain is also required for autophosphorylation. Further evidence of the importance of the LZ domain in oligomerization was provided by subcloning three ZAK cDNA fragments consisting only of the LZ, SAM, or LZ + SAM (LS) regions into the pEGFPC1 vector and again co-transfecting the 293T cells. Co-immunoprecipitation results showed that both LZ and LS readily formed complexes with FLAG-ZAK, but SAM did not (Fig. 2B). Since both the second panel of Fig. 2A and the kinase assay (Fig. 2C) further showed that deletion of the zipper domain but not SAM compromised the ZAK autophosphorylation activity and abrogated its ability to activate JNK. Taken together, these results indicate that ZAK uses the LZ motif for self-association and for autophosphorylation initiation and JNK activation.

To establish whether ZAK in its oligomeric form is stabilized by disulfide bonds, we expressed GFP-tagged ZAK in 293T cells, and two days after transfection, the GFP-ZAK protein was separated via SDS–PAGE in

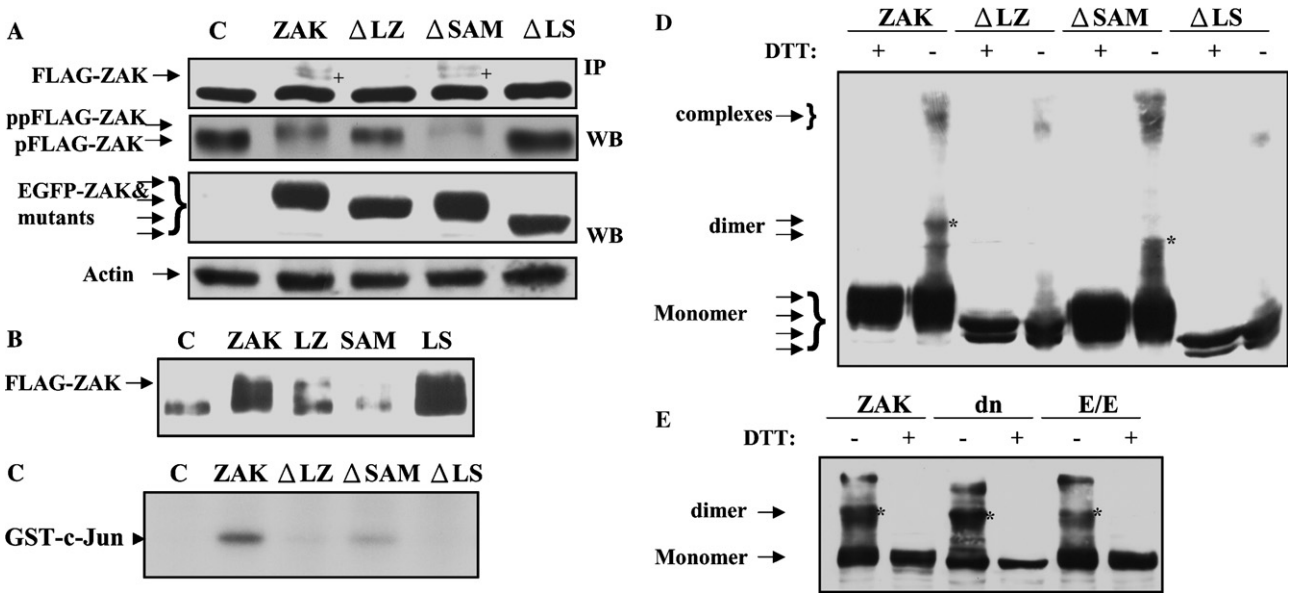

Fig. 2. Role of the leucine zipper domain in ZAK oligomerization, autophosphorylation, and JNK/SAPK activation. (A) The domain deletion effect on ZAK's oligomerization potential. The plasmid encoding FLAG-ZAK was transiently co-transfected into 293T cells with plasmids encoding the indicated ZAK constructs pEGFPC1, pEGFPC1-ZAKΔLZ, pEGFPC1-ZAKΔSAM, and pEGFPC1-ZAK ΔLS and incubated for 48 h. Cell lysates were immunoprecipitated using anti-GFP antibody (Clontech) and immunoblotted using anti-FLAG antibody. Bands marked with a plus (+) indicate co-immunoprecipitated protein. The second and third panels demonstrate FLAG-ZAK expression and the indicated ZAK constructs. The last panel demonstrates actin expression for quantity control loading. IP, immunoprecipitation; WB, Western blot. These experiments were performed four times; representative data are shown. (B) Interaction of domain-only fragments with ZAK. FLAG-ZAK and the indicated ZAK constructs were co-transfected into 293T cells. After 48 h the indicated EGFP-tagged ZAK domains were immunoprecipitated using anti-GFP antibody and immunoblotted using anti-FLAG antibody. (C) In vitro kinase assay. Cell lysates from 293T cells transfected with the indicated constructs were subjected to the kinase assay using GST-c-Jun (1–79) protein as the substrate. (D) Effect of DTT on dimerization. Wild-type and indicated domain-deleted ZAK (ΔLZ, ΔSAM, and ΔLS) expression constructs were transfected into 293T cells, and after 48 h the EGFP-tagged ZAK or ZAK mutants in the cell lysates were separated by SDS–PAGE in the presence or absence of the DTT reducing agent. Bands marked with an asterisk (*) indicate the presence of the dimeric form. (E) Effect of intrinsic kinase activity on ZAK dimerization. Wild-type ZAK and its dn and E/E expression constructs were transfected into 293T cells, and after 48 h the EGFP-tagged ZAK or ZAK mutants were separated by SDS–PAGE in the presence or absence of the DTT reducing agent in the cell lysates. Asterisks indicate the existence of the dimeric form.

either the presence or absence of the DTT reducing agent. In the presence of DTT, ZAK migrated as a monomer, however under non-reducing conditions we detected protein complexes approximately twice the size of the ZAK monomeric form (Fig. 2D). This result suggests that when the ZAK protein is over-expressed in cells, it occurs as a homodimer that is stabilized by disulfide bonds. Fig. 2D also shows that while the ΔLZ and ΔLS mutants were unable to form the homodimers, the ΔSAM mutant was able to oligomerize under non-reducing conditions. Because the LZ region is evidently essential for dimerization, we next determined whether the kinase function of this region was also critical. Fig. 2E suggests that dimerization is not dependent on ZAK's intrinsic kinase activity because under non-reducing conditions, the disulfide-bridged homodimers were detected in cells transiently expressing not only ZAK and E/E, but also in cells expressing the dominant-negative (dn) mutant. The picture that emerges is that while the formation of ZAK homodimers is mediated by its intrinsic zipper region, its autophosphorylation occurs only subsequently. Autophosphorylation is presumably also essential for JNK activation.

Expression of ZAK induces hypertrophic growth in cultured cardiomyoblast cells

To characterize the functional effects of ZAK on cardiac cells, we generated stable H9c2 cardiomyoblast cells that expressed wild-type, dominant-negative (dn), or constitutively active (E/E) cells under tetracycline-responsive transactivator control. In this system, the addition of the tetracycline analog doxycycline induces the expression of recombinant proteins. Fig. 3A shows that the expression of ZAK and ZAK E/E resulted in a marked increase in the organization of the actin fibers. Cell size also increased significantly (by approximately 1.9- and 2.4-fold, respectively) compared to the control cells (Fig. 3B). The dominant-negative mutant failed to induce either of these changes. Moreover, the hypertrophic growth of cardiac cells correlated with ZAK activity was further provided by the domain deletion mutants: expression of ΔLZ and ΔLS, neither of which is able to form homodimers or autophosphorylation (Fig. 2), did not induce in the organization of the actin fibers, and cell size (Figs. 3A and B), whereas ΔSAM, which does form homodimers and autophosphorylation

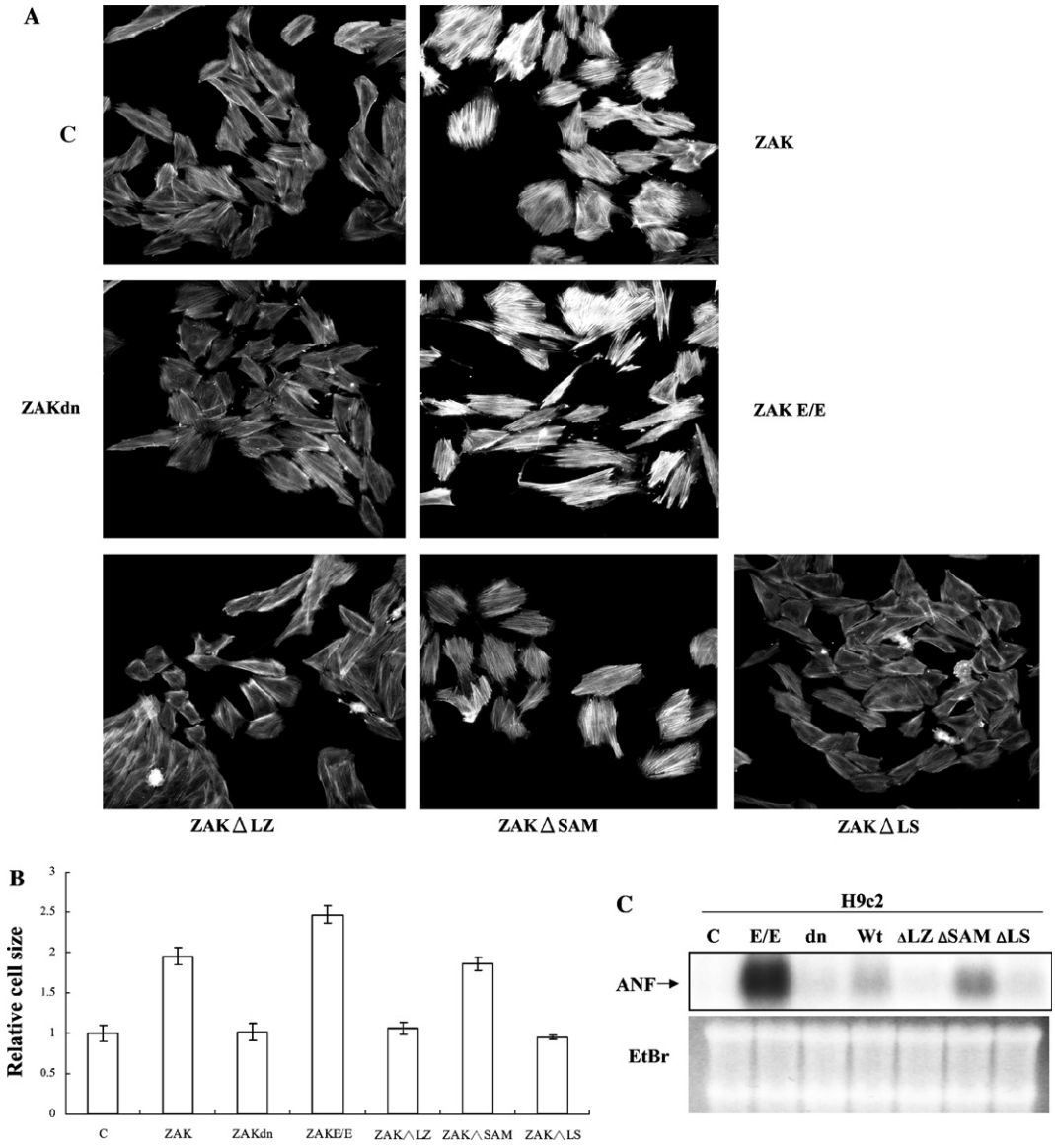

Fig. 3. Effect of ZAK and mutants (dn, E/E, ΔLZ, ΔSAM, and ΔLS) on hypertrophy and actin organization in H9c2 cardiac cells and effect of ZAK on ANF gene expression in H9c2 cardiac cells. (A) Hypertrophic growth and actin organization. The indicated tetracycline-inducible stable H9c2 cell lines were cultured in the presence of 1 μg/ml doxycycline for 48 h. The cells were then fixed, permeabilized, and stained with rhodamine-conjugated phalloidin to detect the actin filaments. (B) Relative cell size. Cell size was analyzed using Image-Pro PLUS software. The values represent the relative area ± SE from 150 measurements in each group. The increased cell sizes induced by ZAK and ZAK E/E are highly significant ($P < 0.05$). (C) Northern blot analysis of 20 μg total RNA isolated from H9c2 cardiac cells transfected with 20 μg of the indicated ZAK expression constructs. The blot was probed with full-length rat ANF cDNA.

(Fig. 2), also induced in actin fiber organization, and cell size (Figs. 3A and B). These results suggest a linear relationship between ZAK activity and the hypertrophic growth of cardiac cells, and further suggest that this growth is effected by pathway(s) that are downstream of ZAK.

ZAK regulates atrial natriuretic factor expression in cardiac cells

Since cardiac hypertrophy is associated with the reprogramming of fetal gene expression such as atrial natriuretic factor (ANF) and β-myosin heavy chain (β-MHC) [14,15], here we investigate whether ZAK expression induces the expression of one of these hypertrophy molecular marker genes. Northern blot analysis of the total RNA isolated from H9c2 cells transiently transfected with the indicated ZAK constructs (Fig. 3C) indicated that over-expression of ZAK and ZAK E/E but not ZAKdn specifically increased ANF mRNA levels. Further evidence that induction of the ANF transcripts is correlated with ZAK activity was provided by the large deletion mutants: expression of ΔLZ and ΔLS,

neither of which is able to form homodimers or autophosphorylate (Fig. 2), did not affect the expression of the ANF transcripts (Fig. 3C), whereas ΔSAM, which does form homodimers and autophosphorylation (Fig. 2), also induced ANF mRNA expression. We therefore conclude that ZAK must be activated before it can have any effect on the ANF transcripts.

ZAK induction of hypertrophic growth is correlated with the expression of p21$^{Waf1/Cip1}$ but is independent of cell cycle regulation

Although the expression of ZAK in H9c2 cardiomyoblast cells led to hypertrophic growth (Figs. 3A and B), surprisingly we found that the proliferation of cells expressing the dn and E/E mutants was reduced with respect to the parental control cells, whereas proliferation was unaffected in the ZAK-expressing cells (Fig. 4A). To further investigate this phenomenon, asynchronous cultures were trypsinized and their cell cycle distributions were analyzed using flow cytometry. Fig. 4B shows that the dn and E/E-expressing cells had a significantly higher percentage of cells in the G_0/G_1 phase of the cell cycle (83.32% and 87.93%, respectively) compared to the control (67.42%) and ZAK-expressing cells (68.38%). Moreover, there were fewer dn and E/E-expressing cells in the S phase (11.31% and 8.60%, respectively) compared to the control (21.64%) and ZAK-expressing cells (21.59%). These findings suggest that the dn and E/E-expressing cells were arrested in the G_0/G_1 phase.

Because cell cycle progression commences in G_1 with cyclin D-CDK4/6 assembly and activation, and is followed in the G_1/S transition by subsequent cyclin E-CDK2 [16] activation, we next studied the effect of

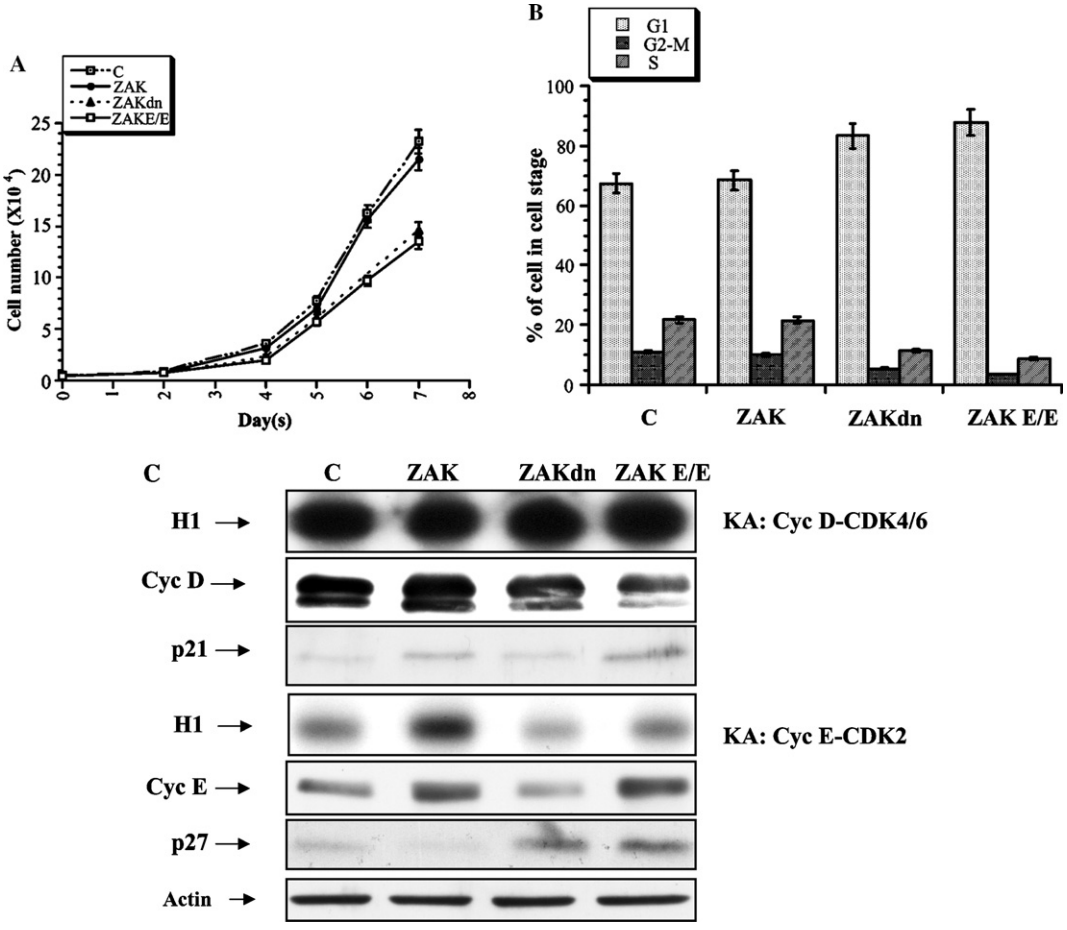

Fig. 4. Effects of ZAK on H9c2 cardiac cell proliferation and cell cycle machinery. (A) Cell proliferation growth curves of H9c2 cells expressing ZAK, ZAKdn, and ZAK E/E. Stable H9c2 cell lines under the control of a tetracycline-responsive transactivator were seeded at an original density of 5000 cells per six-well dish in the presence of 1 μg/ml doxycycline, and cell counts were taken at the indicated times. (B) The ZAK and 2 mutant effects on cell cycle progression. Stable H9c2 cell lines were induced to express the indicated genes for 48 h and then analyzed by FACS. The percentage of cells in each cell cycle phase was calculated using ModFit software. Experiments were performed three times and gave similar results. (C) ZAK effect on the cyclin D, cyclin E, p21$^{Waf1/Cip1}$, and p27^{Kip1} expression levels, and on cyclin D-CDK4/6 and cyclin E-CDK2 activity. Stable H9c2 cell lines were induced to express the indicated genes for 48 h and cell lysates were then analyzed by Western blot and in vitro kinase assay. Cell-cycle protein expression was determined by immunoblots of whole-cell lysates. The histone H1 kinase assay was performed after immunoprecipitation with either anti-cyclin D or anti-cyclin E. The last panel demonstrates expression of actin for quantity control loading. These experiments were performed four times; representative data are shown.

ZAK, ZAKdn, and ZAK E/E on the G_1 cell cycle machinery. In H9c2 cells ZAK up-regulated cyclin D expression levels, ZAKdn had no effect, and ZAK E/E decreased cyclin D expression (Fig. 4C, panel 2). However, the cyclin D-CDK4/6 complex activity, which is controlled by cyclin D in early G_1, was not affected (Fig. 4C, panel 1). Because the activity of this complex is also regulated by the cyclin-dependent kinase inhibitor (CKI) $p21^{Waf1/Cip1}$ [17], we also measured the CKI levels and found that it was up-regulated in the ZAK and E/E cells, but unchanged in the dn cells (Fig. 4C, panel 3). Moreover, the E/E cells, had higher levels of $p21^{Waf1/Cip1}$ expression than the ZAK cells. This expression level pattern for $p21^{Waf1/Cip1}$ parallels the hypertrophic response pattern for the corresponding ZAK constructs (compare Figs. 4C, panel 3 with 3B). We note too that other investigators have reported that elevated CKI expression levels are similarly associated with vascular smooth muscle and mesangial cell hypertrophy [18–22]. These observations suggest that $p21^{Waf1/Cip1}$ levels might play a role in the response to hypertrophic stimuli. This role, however, appears to be independent of p21's regulation of cyclin D-CDK4/6 activity.

A similar series of tests were run for the G_1/S transition machinery (Fig. 4C, last 3 panels). The expression levels of cyclin E were elevated in the ZAK and E/E cells, but decreased in the dn cells (Fig. 4C, panel 5). Further, the E/E cells had higher cyclin E expression levels than the ZAK cells. Kinase activity of the cyclin E-CDK2 complexes was unchanged in the E/E cells, elevated in the ZAK cells and reduced in the dn cells (Fig. 4C, panel 4). Since the kinase activities were not correlated with the expression levels of cyclin E, we next determined the expression levels of the cyclin E-CDK2 associated CKI, $p27^{Kip1}$. Levels of $p27^{Kip1}$ were down-regulated in ZAK cells, but elevated in dn and E/E cells (Fig. 4C, panel 6). These results suggest that while the expression of $p27^{Kip1}$ is not correlated with hypertrophic growth, it might be associated with cell proliferation.

Discussion

The data presented here showed that ZAK plays a role in the signal transduction pathway thereby stimulating hypertrophic growth in cardiomyoblast cells. The specific evidence is as follows: first, expression of wild-type ZAK induces hypertrophic growth (Figs. 3A and B). Second, the ectopic expression of ZAK was alone sufficient to induce ANF expression in cardiac cells (Fig. 3C). Taken together, these data indicate that ZAK induces hypertrophic growth in cardiomyoblast cells. However, these findings do not rule out the possibility that other downstream effectors might also be involved in the ZAK-mediated hypertrophic growth. For example, ZAK can activate JNK and NF-κB signaling, which suggests that the possibility of interactions between the JNK and the NF-κB signaling should be investigated. It, therefore, suggests that ZAK might induce ANF expression not only through MKK7 but also through other downstream effectors such as NF-κB. A previous study [12] has already indicated that ZAK signaling leads to the activation of NF-κB, and we are now investigating whether NF-κB cooperates with MKK7 for ANF expression in cardiac cells.

We have also shown that ZAK dimerization is necessary for the activation of ZAK, and that the covalently linked ZAK homodimers are formed by a direct interaction of the leucine zipper domain (Fig. 2). This leucine zipper dimerization is required for ZAK autophosphorylation and subsequent activation of the SAPK/JNK pathway.

Al-Awqati and Preisig proposed that cell hypertrophy results from both cell cycle-independent and cell cycle-dependent mechanisms, while a study [18–23] on mesangial hypertrophy caused by high glucose suggested that cell hypertrophy is associated with increased levels of the CKIs $p21^{Waf1/Cip1}$ and $p27^{Kip1}$. Here, however, we found that although ZAK expression in cardiac cells increased the $p21^{Waf1/Cip1}$ protein levels (Fig. 4C), cell proliferation was unaffected in these cells (Fig. 4A) and cell cycle distribution was the same as in the parental control cells (Fig. 4B). Assuming that $p21^{Waf1/Cip1}$ does in fact play a role in the hypertrophic growth of ZAK-expressing H9c2 cells, it therefore remains to be determined how this CKI might exert this function independently of its cell cycle regulatory role. Although $p21^{Waf1/Cip1}$ and cyclin E protein levels are increased in ZAK and E/E expression cells, the $p27^{Kip1}$ levels are decreased in E/E cells. Thus, one interpretation could be that the increase in the expression levels of $p27^{Kip1}$ by E/E cells might be secondary to cell-cycle arrest. Another interpretation could be that ZAK has biphase effects on regulating the expression of $p27^{Kip1}$. ZAK-activated pathway(s) are necessary for inhibiting the expressing of $p27^{Kip1}$ and overstimulation (by expression of the more active E/E mutant) might result in inducing the expressing of $p27^{Kip1}$. We purpose that E/E mutant-activated pathway(s) likely mimic the extracellular stimuli that overactivates the ZAK signaling pathway(s). To identify the extracellular factor(s) would be valuable in addressing this question. Most examples of hypertrophy are associated with growth arrest. Our results show that $p27^{Kip1}$ might be involved in growth arrest whereas $p21^{Waf1/Cip1}$ could play a role in the induction of hypertrophic growth. We propose that cell hypertrophy is independent of cell-cycle arrest and depends on the $p21^{Waf1/Cip1}$protein levels. It remains to be elucidated how $p21^{Waf1/Cip1}$ exerts the hypertrophic function independently of cell-cycle regulation. Although our results implicate cell cycle regulatory molecules in mediating the hypertrophic effects of ZAK in

cultured cardiomyoblast cells, we are unable to determine whether the expression of these molecules, such as $p21^{Waf1/Cip1}$ and $p27^{Kip1}$, is the cause of hypertrophic growth. To examine the possible involvement of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ in the hypertrophic growth of cardiomyoblasts, we will use the SiRNA technique to knock down expression of these proteins in ZAK expressing cells. This should offer an approach to determine the role of these molecules in cardiomyoblast hypertrophy.

Another curious finding was that both ZAKdn and ZAK E/E expression reduced cell proliferation (Fig. 4A), evidently by arresting the cell cycle in the G_1 phase (Fig. 4B). It may be that some ZAK-activated pathway(s) are necessary for cell cycle entry, and further, that both blocking this pathway (by expression of the dominant negative ZAK) and overstimulation (by expression of the more active E/E mutant) might result in blocking the cell cycle progression. This explanation would account for the observed results, but it is a hypothesis that still remains to be tested.

Acknowledgments

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