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Transforming growth factor-β induces the expression of ANF and hypertrophic growth in cultured cardiomyoblast cells through ZAK

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Abstract

Transforming growth factor-β (TGF-β) has been associated with the onset of cardiac cell hypertrophy, but the mechanisms underlying this dissociation are not completely understood. By a previous study, we investigated the involvement of a MAP3K, ZAK, which in cultured H9c2 cardiac cells is a positive mediator of cell hypertrophy. Our results showed that expression of a dominant-negative form of ZAK inhibited the characteristic TGF-β-induced features of cardiac hypertrophy, including increased cell size, elevated expression of atrial natriuretic factor (ANF), and increased organization of actin fibers. Furthermore, dominant-negative MKK7 effectively blocked both TGF-β-and ZAK-induced ANF expression. In contrast, a JNK/SAPK specific inhibitor, sp600125, had little effect on TGF-β- or ZAK-induced ANF expression. Our findings suggest that a ZAK mediates TGF-β-induced cardiac hypertrophic growth via a novel TGF-β signaling pathway that can be summarized as TGF-β > ZAK > MKK7 > ANF.

Keywords: TGF-β; ANF; Hypertrophic growth; Cardiomyoblasts; MKK7; ZAK

Transforming growth factor-β (TGF-β) is an evolutionarily conserved superfamily of polypeptide growth factors that regulate proliferation, embryonic development, differentiation, and apoptosis in many cell types [1–3]. Virtually every cell in the body produces TGF-β and has receptors for it. TGF-β signals through a heteromeric receptor complex consisting of both type I (TGF-βRI) and type II (TGF-βRII) serine/threonine kinase receptors [4,5]. After TGF-β binds, the type II receptor kinase phosphorylates and subsequently activates the type I receptor kinase. Activated receptor kinase subsequently propagates the signal to downstream effectors and regulatory proteins. Smad proteins act as intermediates that are activated and translocated to the nucleus, where they are able to effect the transcriptional responses. The Smad proteins thus function as the principal molecules for mediating signal transduction from the TGF-β receptors [5–7]. However, although the roles that TGF-β plays as an endocrine and morphogenic factor in many cell types are well established, our understanding of its intracellular signaling mechanisms is limited to this Smad transduction expressway.

This report identifies intracellular TGF-β effectors and provides new mechanistic insight into TGF-β-stimulated hypertrophic growth of cultured cardiomyoblast cells. Gary et al. [8] had suggested that TGF-β is upregulated in myocardium by increased work load and suffices to provoke the hypertrophic program of cardiac gene expression. Characteristic features of cardiac hypertrophy include increased cell size, induction of sacromere organization, and elevated expression of cardiac genes, including atrial natriuretic factor

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(ANF) [9–11]. In humans, sustained cardiac hypertrophy is a key factor in the development of heart failure [12–15]. In a previous study [16], 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 most abundantly expressed in human heart tissue[16]. ZAK expression in mammalian cells leads specifically to JNK/SAPK pathway activation and the activation of NF-κB transcription factor. ZAK can also activate MKK7, the activator of JNK/SAPK [17]. In neonatal cardiac myocytes, MKK7 induces the characteristic features of hypertrophy [9], which suggests that ZAK itself may be involved in signal transduction for the regulation of cultured cardiac cell hypertrophy.

Here, we identify ZAK as a downstream effector for TGF-β-stimulated hypertrophic growth in H9c2 cells. In cultured cardiomyoblast cells, H9c2, we show that ZAK reproduces the effect of TGF-β by inducing the characteristic features of hypertrophic growth. Conversely, dominant-negative ZAK abolishes the effect of TGF-β. Furthermore, ZAK signals to MKK7 for the induction of ANF transcripts, and dominant-negative MKK7 effectively blocks both TGF-β-induced and ZAK-induced ANF expression.

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 (pTRE-ZAK), dominant-negative ZAK (pTRE- ZAKdn), or constitutively active ZAK (pTRE-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 the ZAK genes.

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 of 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).

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 confocal microscope. The cell size was analyzed using Image-Pro Plus software.

Northern blot analysis. Trizol reagents (Life Technologies) were used to isolate 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

TGF-β stimulates hypertrophic growth of cardiac cells through ZAK

Numerous putative extracellular factors have been implicated in the mediation of cellular hypertrophy in vitro, including ligands for G protein coupled receptors (GPCRs), peptide growth factors that signal through receptor protein tyrosine kinases (RPTKs), cell stress, and cytokines [18–23]. To determine the particular ligand that stimulates the ZAK-mediated hypertrophic growth of H9c2 cells, we performed experiments using potential hypertrophic agonists on H9c2 cells that were expressing dominant-negative ZAK (H9c2-ZAKdn). In control H9c2 cells treated with the GPCR agonists angiotensin II and phenylephrine, cell size increased by 1.2 and 1.3 times, respectively (Fig. 1A). The ability to stimulate hypertrophic growth was slightly reduced, but not abolished, in the H9c2-ZAKdn cells, which suggests that ZAK induces hypertrophic growth independently of the signal transduction pathways by which angiotensin II and phenylephrine induce hypertrophic growth in cardiac cells. ZAK and ZAK E/E also stimulated hypertrophic growth, but in the presence of the GPCR agonists, this growth was either reduced or unaffected (data not shown). Of the other agonists tested, the cytokines interleukin-1 (IL-1) and interleukin-6 (IL-6) failed to induce any increase in cell size, while both tumor necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β) increased the cell size by approximately 1.5 and 1.4 times, respectively, compared to the control cells (Fig. 1A). Treatment with TNF-α, however, was still able to increase the cell size even in H9c2-ZAKdn cells; by contrast, in these cells, TGF-β-induced size increase was abolished. Similarly, while both TGF-β and TNF-α markedly increased actin fiber organization, in the H9c2-ZAKdn cells, this TGF-β-induced effect was abolished (Fig. 1B) while the TNF-α-induced effect was unchanged (data not shown). As further evidence that the hypertrophic growth of H9c2 cardiac cells

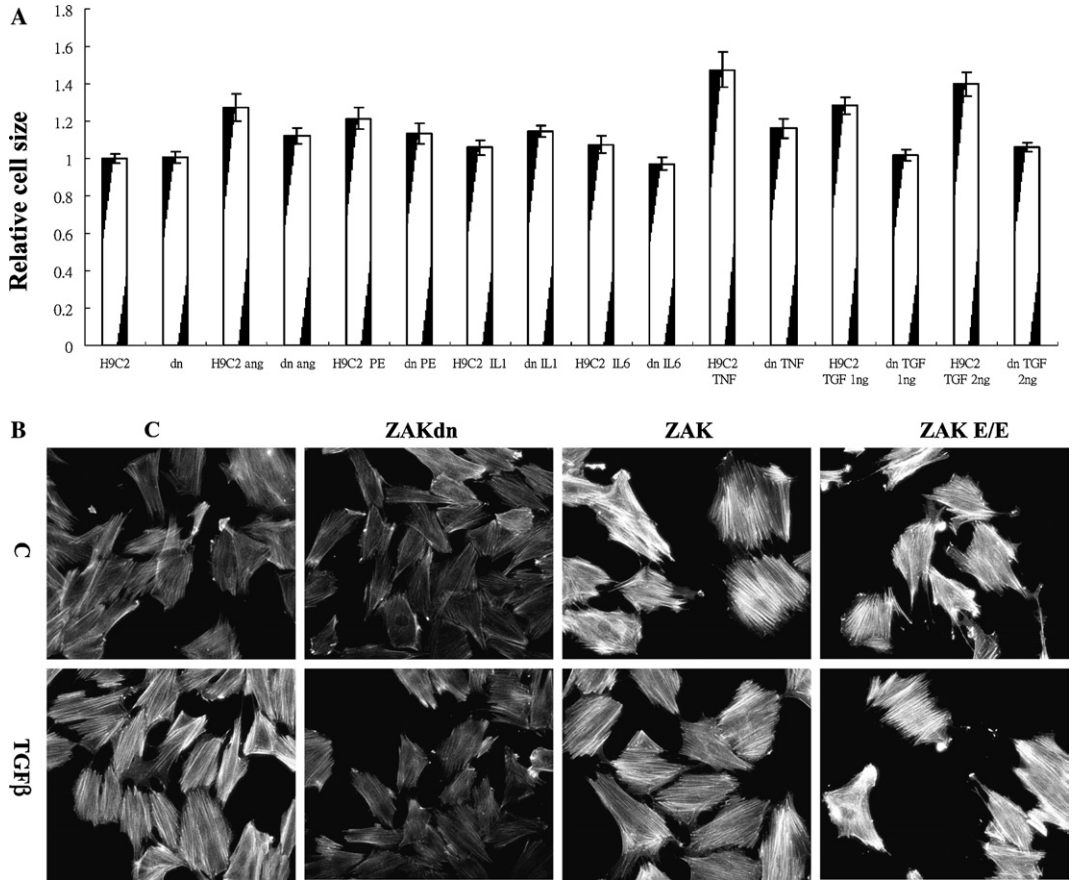

Fig. 1. Modification of the effects of various potential hypertrophic agonists by the dominant-negative form of ZAK in cardiac cells. (A) H9c2 cells and H9c2-ZAKdn cells were cultured in the presence of 100 nM angiotensin II (ang), 10 μM phenylephrine (PE), 100 ng/ml interleukin 1 (IL1), 100 ng/ml interleukin 6 (IL6), 100 ng/ml tumor necrosis factor-α (TNFα) or 1 or 2 ng/ml transforming growth factor-β (TGFβ) with 1 μg/ml doxycycline for 48 h. Cell sizes were analyzed using Image-Pro PLUS software, and the values represent the relative area ±SE from 150 measurements in each group. (B) The indicated tetracyclin-inducible stable H9c2 cell lines were cultured in the presence of 2 ng/ml TGF-β and 1 μg/ml doxycycline for 48 h. The cells were then fixed, permeabilized, and stained with rhodamine-conjugated phalloidin to detect actin filaments.

stimulated by TGF-β is ligand dependent, we further observed that the TGF-β-induced increase in the cell size was concentration dependent (1 ng/ml vs 2 ng/ml). Neither concentration of TGF-β had any effect on size in the H9c2-ZAKdn cells (Fig. 1A). These results suggested that in cardiac cells, signal transduction for TGF-β-induced hypertrophic growth and increased actin organization might be mediated through ZAK.

ZAK autophosphorylation is required for interaction with MKK7

A previous study [17] has demonstrated that the ZAK-activated JNK/SAPK pathway is mediated through MKK7, but not MKK4. ZAK might therefore be expected to physically interact with MKK7, and so to determine whether overexpressed ZAK interacts in vivo with overexpressed MKK7, GFP-tagged ZAK was co-expressed with FLAG-tagged MKK7 in 293T cells. Fig. 2A shows that FLAG-tagged MKK7 was co-immunoprecipitated with GFP-tagged ZAK, which suggests that MKK7 may serve as a necessary intermediate in a pathway between ZAK and JNK/SAPK activation in vivo.

To further explore whether the LZ motif or SAM might be necessary for this interaction, plasmids encoding the GFP-tagged ZAK mutants ΔLZ, ΔSAM, and ΔLS were co-expressed by transient transfection with FLAG-tagged MKK7 in 293T cells. We found that only the GFP-ΔSAM was co-immunoprecipitated with FLAG-MKK7 (Fig. 2B), and this suggested two possibilities: either the ZAK leucine zipper domain interacts directly with MKK7, or else it is the dimerization and autophosphorylation of ZAK that generates a binding site for MKK7. Fig. 2C shows that both GFP-tagged ZAK and ZAK E/E were co-immunoprecipitated with FLAG-MKK7, while GFP-tagged ZAKdn was not. We have already shown that ZAKdn is able to form homodimers via its intrinsic leucine zipper domain (data not shown) but that it lacks the kinase activity to activate the JNK/SAPK pathway. Fig. 2C further shows that ZAKdn fails to autophosphorylate, and taken

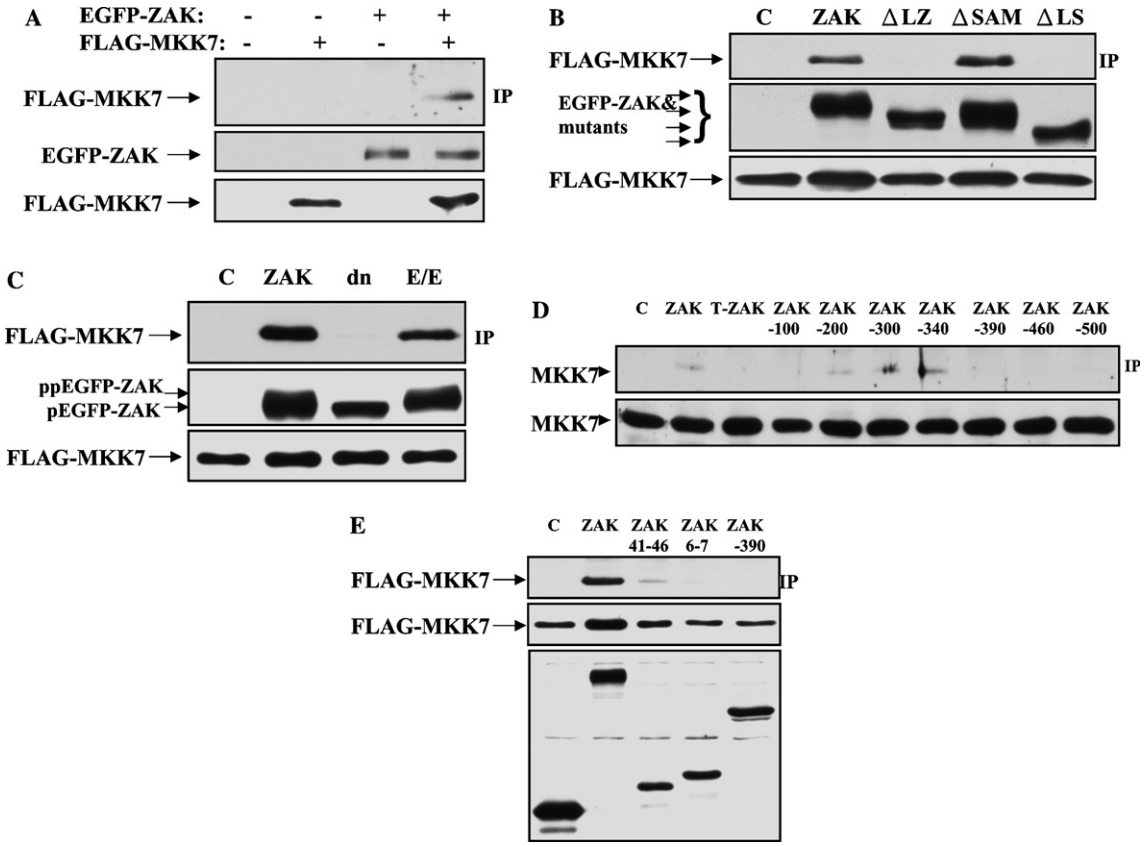

Fig. 2. Role of autophosphorylation and location of the MKK7 binding site in ZAK–MKK7 interaction. Plasmids encoding EGFP-tagged ZAK constructs were transiently co-transfected into 293T cells with plasmid encoding FLAG–MKK7. After 48 h, immunoprecipitation from the cell lysates was performed using anti-GFP antibody (Clontech) followed by immunoblotting using anti-FLAG antibody. (A) Association of ZAK with MKK7. The second and third panels demonstrate expression of the EGFP–ZAK and FLAG–MKK7 constructs. (B) Importance of the leucine zipper region for ZAK–MKK7 interaction. ZAK and ΔSAM include the LZ; in ΔLZ and ΔLS it is deleted. (C) Importance of kinase activity and autophosphorylation in ZAK–MKK7 binding. The ZAK dn mutant, which lacks kinase activity (Fig. 1B), did not autophosphorylate and did not co-immunoprecipitate. (D) Mapping the region of ZAK that binds to MKK7. (E) Confirmation that MKK7 can bind directly to the amino acid 410–460 region of ZAK.

together these results suggest that autophosphorylation of the ZAK protein is required to generate a binding site for MKK7. To determine the region of ZAK that mediates the interaction with MKK7, a series of ZAK deletion mutants was constructed and transiently co-expressed with FLAG-tagged MKK7. We found that only GFP-ZAK, GFP-ZAK-200, GFP-ZAK-300, and GFP-ZAK-340 were co-immunoprecipitated with FLAG-MKK7 (Fig. 2D), which is consistent with our conclusion that the ZAK leucine zipper domain does not mediate the interaction with MKK7, and further suggests that the interaction may be mediated by amino acids 410–460. The lack of binding by GFP-ZAK-100 and the resumption of binding by GFP-ZAK-200 could be explained if there were a positive regulatory region between amino acids 700 and 800 and a negative control region between amino acids 600 and 700. However, results for the GFP-tagged constructs ZAK 41–46 and ZAK 6–7 showed that only ZAK 41–46 was able to co-immunoprecipitate with FLAG-MKK7 (Fig. 2E). This provides more evidence that a binding site between amino acids 410 and 460 of ZAK is necessary for ZAK to physically interact with MKK7, but suggests that there is no binding site in the amino acid 600–700 region. In sum, we interpret these results to mean that ZAK dimerization mediated by the leucine zipper domain is a prerequisite for the activation of the JNK/SAPK pathway and autophosphorylation, and that autophosphorylation exposes an MKK7 binding site between amino acids 410 and 460 of ZAK.

ZAK induces ANF expression through MKK7 in H9c2 cells

To investigate whether the ZAK-induced expression of ANF is mediated through MKK7, H9c2 cells were co-transfected with the ZAK expression construct and different dosages of an expression vector encoding dominant-negative MKK7 (MKK7dn). Northern blot analysis showed that expression of MKK7dn significantly inhibited ANF expression in a dose-dependent manner (Fig. 3A), suggesting that MKK7 does indeed mediate

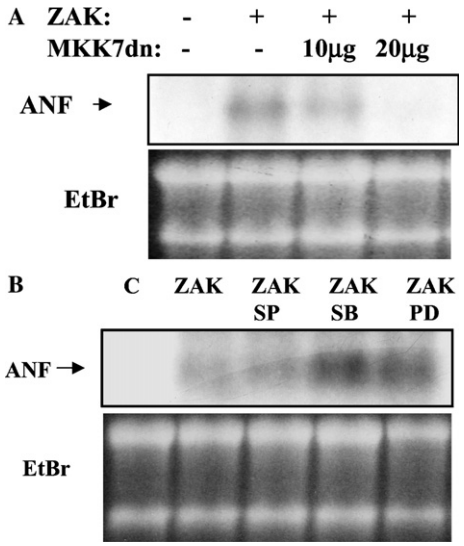

Fig. 3. The effect of dominant-negative MKK7 and MAPK inhibitors on ZAK-induced ANF expression. (A) H9c2 cardiac cells were co-transfected with the ZAK expression construct and either 20 μg of vector or the indicated amount of an expression vector encoding the dominant-negative mutant construct of MKK7, MKK7dn. The upper panel shows Northern blots for 20 μg total RNA isolated from the cell lysates. The blot was probed with full-length rat ANF cDNA. (B) Northern blot analysis of 20 μg total RNA isolated from H9c2 cardiac cells transfected with 10 μg of the ZAK expression construct in the presence of 20 μM JNK/SAPK inhibitor SP600125, 20 μM p38MAPK inhibitor SB203580, or 20 μM MEK 1 inhibitor PD98059. The blot was probed with full-length rat ANF cDNA.

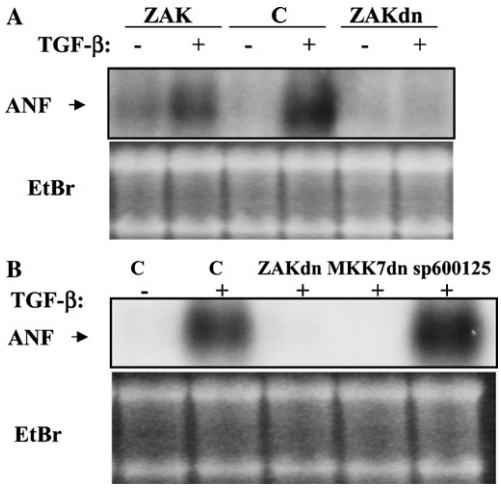

Fig. 4. Effect of the dominant-negative forms of ZAK and MKK7 on the TGF-β-induced expression of ANF transcripts in cardiac cells. (A) Northern blot analysis of 20 μg total RNA isolated from H9c2 cardiac cells transfected with 10 μg of the indicated ZAK expression constructs in the presence of 2 ng/ml TGF-β. The blot was probed with full-length rat ANF cDNA. (B) Northern blot analysis of 20 μg total RNA isolated from 2 ng/ml TGF-β-treated H9c2 cardiac cells that were either transfected with 20 μg of the expression constructs for the dominant-negative forms of ZAK and MKK7, or else cultured in the presence of the JNK/SAPK inhibitor, SP600125.

this pathway. (Wild-type MKK7 alone was not sufficient to induce the expression of ANF; data not shown.) Since these results suggest that MKK7 functions as a mediator for the ZAK-induced expression of ANF, and since Western blot and in vitro kinase assay results (data not shown) have already shown that ZAK activates the JNK/SAPK pathway, we further tested whether JNK activation might be involved in the ZAK-induced expression of ANF transcripts. Treatment with the specific JNK inhibitor SP6000125, however, had no effect on the expression of ANF (Fig. 3B). The p38MAPK inhibitor SB203580 and the MEK1 inhibitor PD98059 also failed to suppress ANF expression. We conclude that although ZAK-induced expression of ANF is mediated through MKK7, the further downstream activation of JNK is evidently not necessary.

TGF-β induces ANF expression through ZAK and MKK7 in H9c2 cells

Northern blot analysis further showed that although ANF transcripts were expressed in H9c2 cells transfected with the ZAK expression construct and treated with TGF-β (2 ng/ml) for 48 h, there were no ANF transcripts detected when the dominant-negative form of ZAK was used instead (Fig. 4A). These results are consistent with the cell size measurement results (Fig. 1A), and taken together, these experiments suggest that TGF-β stimulates the characteristic features of hypertrophic growth, including increase in cell size, induction of actin organization, and elevated expression of atrial natriuretic factor through ZAK.

Since there is now evidence to suggest that MKK7 functions as a key downstream mediator both for ZAK signaling to the JNK/SAPK pathway [17] and for the induction of ANF expression (Fig. 3A), we next tested whether MKK7 also functions as a signaling mediator for TGF-β to induce the expression of ANF in cardiac cells. Results of a Northern blot analysis (Fig. 4B) showed that expression of the dominant-negative form of MKK7 (MKK7dn) significantly inhibited the TGF-β-induced expression of ANF, which suggests that TGF-β-induced expression of ANF is indeed mediated through a linear cascade of ZAK and MKK7. On the other hand, when H9c2 cells were cultured in the presence of both TGF-β and the JNK inhibitor SP600125, ANF expression was not suppressed (Fig. 4B). We therefore conclude that the activation of JNK is evidently not necessary for the TGF-β-induced expression of ANF.

TGF-β induces increased ZAK autophosphorylation and facilitates the assembly of TGF-βRI–ZAK complexes

To further investigate the mechanism whereby ZAK mediates TGF-β stimulation, we examined the phosphorylation status of GFP epitope-tagged ZAK in transfected 293T cells treated with TGF-β. Immunoblotting

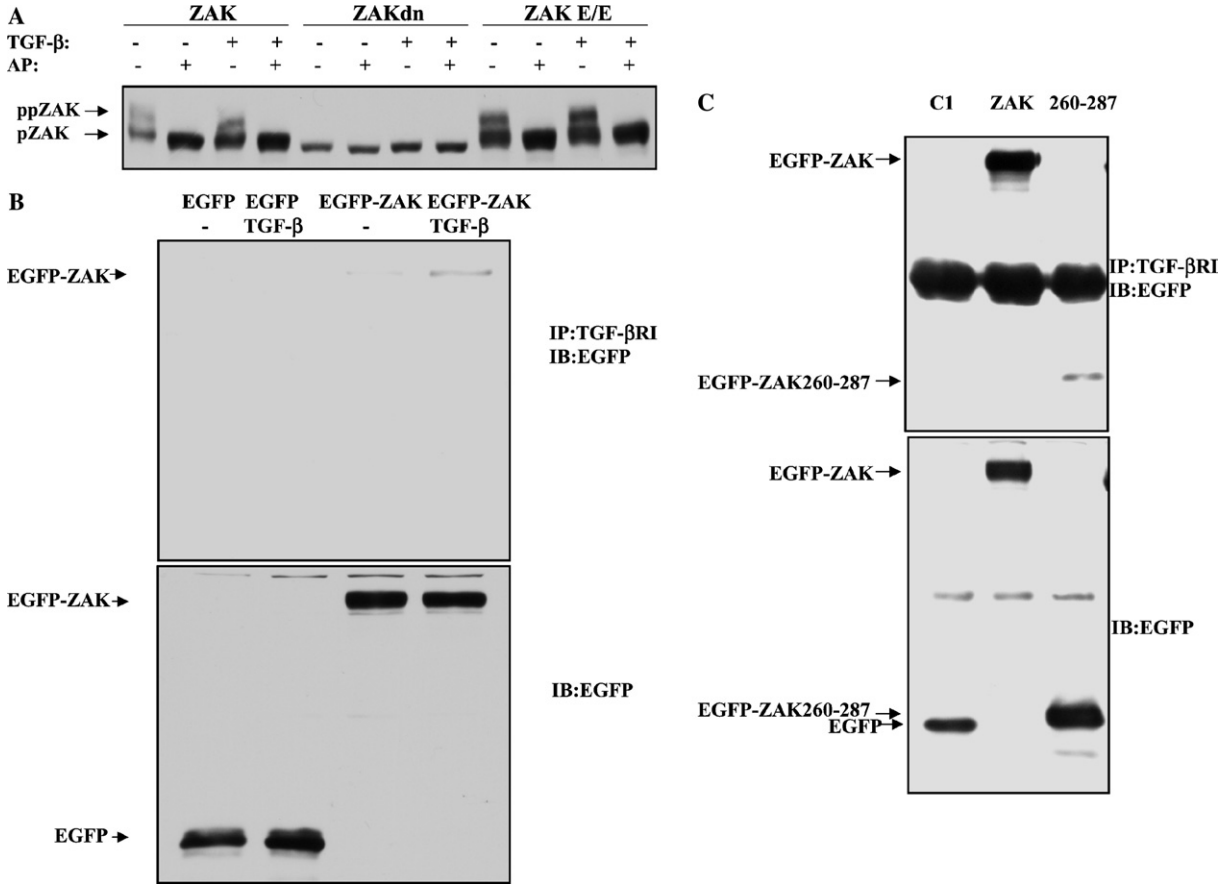

Fig. 5. Effect of TGF-β on ZAK hyperphosphorylation and the formation of TGF-βRI-ZAK complexes. (A) 293T cells were transiently transfected with pEGFP-ZAK, pEGFP-ZAKdn, or pEGFP-ZAK E/E, and then treated with TGF-β for 48 h. The cell lysates were incubated with calf intestine alkaline phosphatase (AP) as indicated and subjected to immunoblotting with anti-GFP antibody. (B) 293T cells were transiently transfected with pEGFP vector or pEGFP-ZAK and then treated with TGF-β for 15 min as indicated. Cell lysates were immunoprecipitated using anti-TGF-βRI antibody (Santa Cruz) and immunoblotted using anti-GFP antibody. The lower panel demonstrates the expression of EGFP and EGFP-ZAK. (C) Confirmation that TGF-βRI can bind directly to the amino acid 260–287 region of ZAK.

results showed that TGF-β induced autophosphorylation in GFP- ZAK and GFP-ZAK E/E, but not in GFP-ZAKdn 293T cells (Fig. 5A). Elimination of the autophosphorylation bands by in vitro treatment with calf intestine alkaline phosphatase further confirmed that the lower mobility SDS–PAGE bands were indeed phosphorylated forms of the ZAK and ZAK E/E proteins. We therefore conclude that TGF-β enhances the autophosphorylation of ZAK.

To investigate whether TGF-β induces a physical interaction between ZAK and TGF-β receptor I (TGF-βRI), GFP or GFP epitope-tagged wild-type ZAK constructs were transfected into 293T cells and the cell lysates were subjected to co-immunoprecipitation analysis. In TGF-β treated cells, GFP-ZAK was co-immunoprecipitated with TGF-βRI (Fig. 5B, upper panel). After TGF-β binds to the TGF-β type II receptor and thereby activates TGF-βRI [5–7], then the formation of a ZAK complex with the type I receptor suggests that the propagation of TGF-β signaling might be mediated through ZAK.

To determine the region of ZAK that mediates the interaction with TGF-βRI, a series of ZAK deletion mutants was expressed in 293T cells and the cell lysates were subject to co-immunoprecipitation analysis. We found that all of the series and T-ZAK were co-immunoprecipitated with TGF-βRI (data not shown), which suggests that the interaction may be mediated by the region between kinase domain and LZ. Fig. 5C shows that ZAK 260–287 was co-immunoprecipitated with TGF-βRI, which provide evidence that a binding site between amino acids 260 and 287 of ZAK is sufficient for ZAK to physically interact with TGF-βRI.

Discussion

The data presented here show that ZAK is a critical component of the signal transduction pathway whereby TGF-β stimulates hypertrophic growth in cardiac cells. Specific evidence is as follows: First, expression of the kinase-dead (dominant negative; dn) mutant ZAK

decreased TGF-β-induced hypertrophic growth (Figs. 1A and B). Second, the TGF-β-induced expression of ANF was dramatically inhibited by ZAKdn (Fig. 4A). Third, having shown that the downstream ZAK effector MKK7 physically interacts with ZAK only when the ZAK proteins are autophosphorylated (Figs. 2A–E), we further showed that the expression of the dominant-negative form of MKK7 inhibited both ZAK-induced ANF expression (Figs. 3A and B) and TGF-β-stimulated ANF expression in cardiac cells (Fig. 4A). Finally, the ectopic expression of ZAK was alone sufficient to induce ANF expression in cardiac cells, even in the absence of TGF-β stimulation (Figs. 3A and B; 4A). Taken together, these data indicate that TGF-β depends on ZAK and its downstream effector, MKK7, to induce hypertrophic growth in cardiac cells. However, the present findings do not rule out the possibility that other factors might also be involved in the ZAK-mediated, TGF-β-induced hypertrophic growth. For example, TGF-β can activate Smad signaling [5,24], which suggests that the possibility of interactions between Smad signaling and ZAK signaling should be investigated. It is also probable that ZAK might induce ANF expression not only through MKK7 (Figs. 4A and B) but also through other downstream effectors such as NF-κB. A previous study [16] 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. At the very least, it seems clear that hypertrophic growth involves an intricate web of interconnected signaling pathways, and interestingly, even the slight influence that ZAKdn has on the hypertrophic growth stimulated by angiotensin II, phenylephrine, or TNF-α (Fig. 1A) suggests that all these stimuli might share common signaling cascades that involve ZAK.

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. This leucine zipper dimerization is required for ZAK autophosphorylation and subsequent activation of the SAPK/JNK pathway. ZAK autophosphorylation is also required for ZAK to bind with MKK7, and we speculate that the intrinsic autophosphorylation of ZAK might expose a binding site between amino acids 410 and 460. We note, however, that since MKK7 co-immunoprecipitated with ZAK 41–46 peptide (Fig. 2E) phosphorylation of this region itself may not be necessary. Other results (Fig. 2D) suggest that there may be two regulatory regions located within ZAK, a positive region between the amino acids 700 and 800, and a negative region between amino acids 600 and 700. These possibilities, as well as the search for the minimum amino acid sequence for MKK7 binding are all currently under investigation.

Acknowledgment

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