KLF4 is a novel regulator of the constitutively expressed HSP90

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Abstract Krüppel-like factor 4 (KLF4) is a zinc finger-containing transcription factor with diverse regulatory functions in cell growth, proliferation, and differentiation. But little is known about the regulation of KLF4 on the expression of HSP90 (HSP84 and HSP86). In the current study, overexpression of KLF4 was firstly identified to promote the basal expression of HSP90 (HSP84 and HSP86) but not the inducible expression in the C2C12 cells and RAW264.7 cells. Conversely, KLF4 inhibition by antisense oligonucleotides markedly decreased the constitutive expression of HSP90 (HSP84 and HSP86). Here, we also presented data that overexpression of KLF4 resulted in enhanced promoter activities of HSP84. Consistently, KLF4 bind to the KLF4 binding sites in the promoter regions of HSP84 directly. Together, these findings support a role for KLF4 as a novel regulator of the constitutive expression of HSP90.

Keywords KLF4 · HSP90 · HSP84 · HSP86 ·
Gene regulation

Introduction

Heat shock proteins (HSPs) are a large family of highly conserved proteins which are ubiquitous, occurring in all

organisms from bacteria and yeast to humans. Among the HSPs, the HSP90 family is one of most abundant cytoplasm proteins, which represents about 2% to 5% of the total cellular proteins even in the absence of stress stimulation. HSP90 is a molecular chaperone and has been demonstrated to be involved in the folding of signal transduction molecules such as protein kinases (Src, Raf1, cdk4) and steroid receptors and involved in many important aspects of cellular processes (Pratt et al. 1996; Richter and Buchner 2001). In vertebrates, there are two cytoplasmic versions of HSP90 encoded by two distinct genes giving rise to products of 84 and 86 kDa. In mice, the expression of these two genes, *hsp84* and *hsp86*, vary with respect to each other in responses to stress and also in response to signals for growth and development. HSP86 is more sensitive to heat shock induction, whereas the HSP84 is the major cellular counterpart responding to mitogenic stimulation (Moore et al. 1989). It is demonstrated that the inducible HSP90 expression is mainly regulated by heat shock transcription factor 1 (HSF1). In response to various inducers such as elevated temperatures, oxidants, heavy metals, and bacterial and viral infections, HSF1 acquires DNA binding activity to the heat shock element (HSE), thereby mediating transcription of the *hsp90* genes, which results in the accumulation of HSP90 (Pirkkala et al. 2001). But it is not well known whether the expression of HSP90 is regulated by other factors.

Krüppel-like factor 4 (KLF4), also known as Gut-enriched Krüppel-like factor (GKLF) or epithelial zinc finger (EZF), is a member of Sp1-like/KLF transcription regulator family, which has a highly conserved DNA binding domain at the carboxyl terminus that has three tandem Cys₂His₂ zinc finger motifs. It has been demonstrated that KLF4 both activates and represses gene transcription (Bieker 2001; Ghaleb et al. 2005). Analysis

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of KLF4 target genes reveals the function of KLF4 in the regulation of cell proliferation and differentiation, which includes roles in downregulation of ornithine decarboxylase promoter activity (Chen et al. 2002) and upregulation of several keratin genes (Brembeck and Rustgi 2000; Jaubert et al. 2003; Okano et al. 2000). In addition, KLF4 was shown to be necessary and sufficient in mediating the checkpoint function of p53 at both the G1/S and G2/M transition points (Yoon et al. 2003). KLF4 accomplishes this task both through its transcriptional activation of p21^{WAF1/Cip1} (Zhang et al. 2000) and through direct suppression of cyclin D1 (Shie et al. 2000) and cyclin B1 (Yoon and Yang 2004) expression, which are required for the G1/S and G2/M transitions, respectively. However, a role for KLF4 in regulating the expression of HSP90 remains unknown.

Previously, we used cDNA microarray to identify genes altered by KLF4 overexpression in murine C2C12 myogenic cells and significant increase in mRNA level for HSP84 were observed. The result indicated that HSP84 may be a potential target gene of KLF4. In this study, coexpression of KLF4 and HSP90 (HSP84 and HSP86) was demonstrated in murine C2C12 cells and RAW264.7 cells. And then the effects of KLF4 overexpression or inhibition on HSP90 (HSP84 and HSP86) expression were detected. As a result, we identified that KLF4 was required for the constitutive expression of HSP90 (HSP84 and HSP86) but not the inducible expression. In addition, the ability of KLF4 to activate the HSP84 promoter was determined and the region responsible for the binding of KLF4 in the HSP84 promoter was demonstrated. The result indicated that KLF4 upregulated the expression of HSP90 by its transcription factor manner.

Materials and methods

Antibodies The following antibodies were used: mouse anti-HSP90 polyclonal antibody (recognizing both HSP84 and HSP86 proteins; Stressgen); rabbit anti-KLF4 polyclonal antibody (Santa Cruz); mouse antiglyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (Sigma); peroxidase-conjugated antimouse and antirabbit IgG (Boster Biological Technology).

Cell culture and heat shock treatment Murine C2C12 myoblast cells and murine RAW264.7 macrophages were routinely grown at 37°C in Dulbecco's modified Eagle's medium, 10% fetal calf serum in a humidified atmosphere with 5% CO₂. For heat shock experiments, actively growing cells were fed with the medium preincubated at 42°C and transferred to a 42°C preset incubator for 1 h. Control cells were maintained at 37°C.

Western blot analysis Cells were lysed in B-buffer containing 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0, and 1% sodium dodecyl sulfate. Proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred onto Protran nitrocellulose membranes (Schleicher & Schuell). The membranes were blocked overnight in phosphate-buffered saline (PBS) containing 10% nonfat dry milk and 0.5% Tween-20 and incubated with primary antibodies for 2 h. Horseradish peroxidase-conjugated antirabbit or antimouse IgG was used as the secondary antibody. The immunoreactive bands were visualized using 3,3'-diaminobenzidine (Boster Biological Technology). Anti-GAPDH antibody was used to normalize for equal amounts of proteins and calculate the relative induction ratio.

RNA extraction and RT-PCR analyses Total RNA was extracted by using the TRIzol reagent (Invitrogen) according to the instructions of the manufacturer. Dilutions corresponding to 1 µg of total RNA were reverse transcribed (Invitrogen) and reverse transcription polymerase chain reaction (RT-PCR) was performed using the iCycler Apparatus (Biometra). For polymerase chain reaction amplification, the following primers were used. GAPDH-forward: 5'-AAG CCC ATC ACC ATC TTC CA-3', GAPDH-reverse: 5'-CCT GCT TCA CCA CCT TCT TG-3'; KLF4-forward: 5'-GCG GGA AGG GAG AAG ACA C-3', KLF4-reverse: 5'-GGG GAA GAC GAG GAT GAA GC-3'; HSP84-forward: 5'-CTG CTC TGC TCT CCT CTG GT-3', HSP84-reverse: 5'-CCC AAC CCT GCT ATT CTG TG-3'; HSP86-forward: 5'-CAT CAA TCT CAT TCC CAG CA-3', HSP86-reverse: 5'-TCA GCA ACC AAA TAG GCA GA-3'.

Plasmids Expression plasmids for KLF4 were generated by RT-PCR and cloned into pcDNA3.1 vector (Rezzani et al. 2003). Generation of the HSP84 promoter constructs (−1,229 to +12 and −729 to +12) was performed by RT-PCR and cloned into the pGL3 vector. All the constructs were authenticity verified by sequencing (data not shown).

Loss-of-function assay using antisense oligos A KLF4 antisense oligonucleotide (Liu et al. 2006) was designed to target the initiation site for KLF4 translation (KLF4-AS, agactcgccaggtggctgcctcatt) and was synthesized commercially (Invitrogen). Antisense oligonucleotides were transfected into cells with lipofectamine according to the manufacturer's instructions (Invitrogen) 24 h after plating. The specificity of the antisense oligonucleotide was validated by employing a control oligonucleotide (KLF4-Ctrl, ttactcgtcgggtggaccgctcaga) and a group treated only with lipofectamine (Lipo). Cell samples were collected 48 h

after transfection with Trizol reagent (Invitrogen) according to the manufacturer's instructions.

Electrophoretic mobility shift assay After the treatment, cells were harvested and washed twice with cold PBS. Briefly, the cell pellet was resuspended in 400 μ l cold buffer A (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM ethylene glycol tetraacetic acid [EGTA], 1 mM dithiothreitol [DTT], and 0.5 mM phenylmethanesulfonylfluoride [PMSF]). The cells were allowed to swell on ice for 15 min, then 25 μ l of a 10% solution of Nonidet P-40 (NP-40) was added, and the tube was vortexed vigorously for 10 s. The homogenate was centrifuged at 10,000 $\times$ g for 30 s, and the nuclear pellet was resuspended in 50 μ l ice-cold buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF). After vigorously rocking at 4°C for 15 min on a shaking platform, the nuclear extract was centrifuged at 10,000 $\times$ g for 5 min in a microfuge at 4°C, and the supernatant was frozen in aliquots at -80°C. The protein content of the different fractions was determined by a Bradford method.

Electrophoretic mobility shift assay (EMSA) were performed according the manufacturer's instructions (LightShift Chemiluminescent EMSA Kit, PIERCE). DNA probes were generated according to the KLF sites at positions -780 to -751 bp of the mouse HSP84 promoter as double-stranded, HRP-labeled oligonucleotides corresponding to the wild-type sequences (5'-ggggaaaaaaaaaaaaaaaaagccaccc-3') and mutant sequence (5'-ggggaaCCaaaTTaaaaaaaaagccaccc-3').

Luciferase reporter assay For luciferase reporter assay, exponentially growing C2C12 cells and RAW264.7 cells were seeded in 24-well culture dishes. Transfection of murine C2C12 cells and murine RAW264.7 cells was carried out according the manufacturer's instructions (LIP-OFECTAMINE 2000™, Invitrogen). Each transfection experiment contained 0.5 μ g of reporter plasmid with 500 ng of transcription factor expression vector and with 20 ng of pRL-null vector (Promega) as an internal transfection control. And luciferase activities were measured with the dual luciferase system according to the manufacturer's instructions (Promega). Transfections were performed in triplicate. A LUMIstar Luminometer (BMG, Germany) was used to quantify light signal.

Statistical analysis Data in the figures and text were expressed as the means $\pm$ standard errors of the mean. Each experiment was performed at least three times, and statistical analysis was performed using two-tailed Student's *t* test or Fisher's least significant difference test; otherwise, representative data were shown. $P < 0.05$ was considered significant.

Results

Coexpression of KLF4 and HSP90 in C2C12 cells and RAW264.7 cells

KLF4 and HSP90 are expressed in various tissues and cells. In the current study, we detected the expression of KLF4 and HSP90 in C2C12 cells and RAW264.7 cells by Western blot. As shown in Fig. 1, the expression of KLF4 and HSP90 was detectable under normal conditions in both cell lines; following sublethal hyperthermia, the expression of both proteins was significantly induced. The expression patterns of KLF4 and HSP90 were parallel. These results indicated that the coexpression of KLF4 and HSP90 was present in C2C12 cells and RAW264.7 cells.

KLF4 promotes the basal expression of HSP90

To test the effect of KLF4 overexpression (Liu et al. 2006, 2007) on the regulation of HSP90 expression, we used RT-PCR and Western blot to detect the amount of HSP90 (HSP84 and HSP86) mRNA and protein in the C2C12 cells and the RAW264.7 cells overexpressed with KLF4. As shown in Fig. 2, the basal expression of HSP84 and HSP86 mRNA and protein was significantly upregulated by KLF4 overexpression under normal conditions. But when exposed to heat stress, no greater accumulation of HSP84 and HSP86 mRNA and protein was measured in KLF4-overexpressed cells compared with the vector control. These results suggested that KLF4 was not a major regulator of HSP90 expression in response to heat shock but it was a novel activator of the constitutive expression of HSP90.

To further test whether KLF4 is an endogenous regulator of HSP90 basal expression, antisense oligonucleotides were used to knockdown KLF4 in the C2C12 cells and RAW264.7 cells (Liu et al. 2006, 2007). When the cells were transfected with antisense oligonucleotides for 48 h, expression of KLF4 was detected by Western blot for identification of endogenous KLF4 inhibition (Fig.3a, c).

Fig. 1 Coexpression of KLF4 and HSP90. Cultured cells were harvested under the normal conditions or with 12 h recovery following a sublethal heat stress, and total proteins were assessed by Western blot analysis in the C2C12 cells (a) and RAW264.7 cells (b). GAPDH was used as an internal control. *Normal* group under the normal conditions, *HS* group treated with heat stress

Fig. 2 Effect of KLF4 overexpression on the expression of HSP90 (HSP84 and HSP86). C2C12 cells (a) and RAW264.7 cells (b) were stably transfected with pcDNA3.1-KLF4 plasmid and the expression of KLF4 protein was detected by Western blot compared with the vector control. And then the levels of HSP90 protein were detected in the KLF4-overexpressing C2C12 cells (c) and RAW264.7 cells (e) compared with the vector control by Western blot; the levels of HSP84 and HSP86 mRNA were detected in the KLF4-overexpressing C2C12 cells (d) and RAW264.7 cells (f) compared with the vector control by RT-PCR. GAPDH was used as an internal control

After the expression of KLF4 was inhibited, the expression of HSP90 was determined by RT-PCR and Western blot. As shown in Fig. 3a, c, the level of HSP90 protein in the KLF4-deficient cells was significantly decreased and less than one third of the level in the group transfected with control oligonucleotides (Ctrl) and the group treated only with lipofectamine (Lipo) under the normal conditions. As expected, the HSP84 and HSP86 mRNA in KLF4-deficient cells was also dramatically down to less than one fourth of the two control groups under normal conditions (Fig. 3b, d). These results further suggested that KLF4 was a regulator of HSP90 constitutive expression.

KLF4 activates the HSP84 promoter

To determine whether the potential KLF4 binding element found at -770 to -757 bp of the promoter region of HSP84 by bioinformatics methods are capable of binding to KLF4, EMSA was used to detect KLF4-specific DNA binding activity to the HSP84 promoter in both KLF4-overexpressed cells and the vector controls. As shown in Fig. 4a, b, KLF4 bound to the element with high affinity and specificity was verified by mutant radiolabeled oligonucleotides to which KLF4 did not bind and by supershift studies. KLF4 overexpression significantly increased the

Fig. 3 Effect of KLF4 deficiency on the expression of HSP90 (HSP84 and HSP86). C2C12 cells (a) and RAW264.7 cells (c) were transiently transfected with KLF4 antisense oligonucleotide and the expression of HSP90 protein was detected compared with the control oligonucleotide (Ctrl) and the group treated only with lipofectamine (Lipo) by Western blot. The expression of HSP84 and HSP86 mRNA in C2C12 cells (b) and RAW264.7 cells (d) was detected by RT-PCR

Fig. 4 Transcription activity of KLF4 to HSP84 promoter. The potential KLF4 binding sites of HSP84 promoter were assessed for the ability to bind KLF4 by EMSA. KLF4 bound to the KLF4 binding elements at -780 to -751 bp of the HSP84 promoter in the C2C12 cells and KLF4 overexpression promoted the binding of KLF4 to the corresponding probes containing KLF4 binding elements (**a**). KLF4 also bound to the KLF4 binding elements at -780 to -751 bp of HSP84 promoter in the RAW264.7 cells (**b**). *pcDNA3.1* the vector control group, *pcDNA3.1-KLF4* KLF4 overexpression group, *Cold probe* competition with cold probe (200-fold excess concentration), *Mutant probe* competition with mutant cold probe (200-fold excess

concentration), *KLF4 Ab* supershift group by KLF4 antibody. The indicated HSP84 promoter deletion constructs were used and the ability of KLF4 to regulate the promoter activity of HSP84 was assessed by transient transfection studies in the C2C12 cells (**c**) as well as the RAW264.7 cells (**d**). The luciferase activity was detected using the dual luciferase reporter system. All transfections were performed at least three times in triplicate. *KLF4* the cell group transfected with full-length KLF4, *Neo* the vector control group. # $P < 0.05$, statistically significant versus the vector control group (Neo); * $P < 0.05$, statistically significant versus the group HSP84 (-729 to +12)

binding activity. These data provide direct evidences that KLF4 is able to bind to the promoter region of HSP84.

To further understand how KLF4 induce HSP84 transcription, we assessed its effect on HSP84 promoter activity. For these studies, we used a fragment of the mouse HSP84 promoter containing -1,229 to +12 bp linked to the promoterless coding domain of the luciferase gene. The reporter construct was then cotransfected with either the control pcDNA3.1 or the pcDNA3.1-KLF4 expression vector into C2C12 cells and RAW264.7 cells. Figure 4c, d showed that KLF4 was able to activate the

promoter activity of HSP84 at levels (above twofold) comparable with the control vector. However, this transactivation was almost completely lost upon further deletion up to the -729 to +12 bp construct. These data suggest that the critical regulatory elements necessary for mediating KLF4 effects on the HSP84 promoter lies within the -1,229 to -729 bp region. Together with the results of bioinformatics analysis and EMSA, it is suggested that the KLF4 DNA binding sites at -770 to -757 bp play a major role in the regulation of HSP84 promoter activity by KLF4.

Discussion

Our present study provides the first evidence for the upregulation of HSP90 basal expression by KLF4, a member of the Krüppel-like family of transcription factors. To confirm whether the expression of HSP90 was influenced by KLF4, murine C2C12 cells and RAW264.7 macrophages were overexpressed with KLF4 and expression of HSP90 (HSP84 and HSP86) was examined at the mRNA level as well as the protein level in this study. As expected, the basal expression of HSP90 (HSP84 and HSP86) was significantly induced by KLF4 overexpression. But when treated with heat stimuli, little increase in the HSP90 (HSP84 and HSP86) levels occurred in the cells overexpressed with KLF4 compared with the vector controls. It seemed that KLF4 regulated the basal expressions of HSP90 (HSP84 and HSP86), but had little effect on their induction in response to heat stress. Because constitutive expression of HSP90 is associated with various human diseases, it seems important to illustrate the regulation of HSP90 basal expression. Therefore, we further focused on the effect of antisense oligonucleotides against KLF4 on the basal expression of HSP90 (HSP84 and HSP86). In contrast to KLF4 overexpression, KLF4 inhibition dramatically downregulated the basal expressions of HSP90 (HSP84 and HSP86). Evidences from us suggest that KLF4 is a novel regulator of HSP90 basal expression.

To further disclose the correlation between KLF4 and HSP90, we checked the promoter regions of the HSP84 gene and found a KLF4 binding element at −770 to −757 bp by bioinformatics analysis. EMSA studies indicated that KLF4 could bind to the KLF4 binding site at −770 to −757 bp of the HSP84 gene with high affinity. And luciferase reporter assay showed that KLF4 activated the transcription of the HSP84 gene significantly. Furthermore, we demonstrated that KLF4 and its binding element were indispensable in the regulation of KLF4 on the HSP84 gene. Firstly, deletion of the KLF4 binding site at −770 to −757 bp altered the promoter activity of the HSP84 gene induced by KLF4 in C2C12 cells as well as RAW264.7 cells. Secondly, the specific bands in EMSA only showed up in the presence of both KLF4 and the binding site at −770 to −757 bp of the HSP84 gene. In summary, we demonstrated that KLF4 was capable of binding to the promoter region of the HSP84 gene and promoted the transcription of the HSP84 gene.

In this study, we revealed the strong induction of HSP90 by KLF4 in C2C12 myoblast cells. Previous studies have addressed that the function of HSP90 is essential for the regulation of myoblast differentiation. The multimeric complexes of nascent myosin filaments associated with HSP90 are intermediates in the folding and assembly pathway of muscle myosin. Inhibition of HSP90 by geldanamycin caused C2C12 cells to become depleted of

multiple signal transduction proteins whose functions are essential for myoblast differentiation (Yun and Matts 2005a, b). Furthermore, HSP90 functions to balance the phosphorylation state of Akt by modulating the ability of Akt to be dephosphorylated by PP2Ac during C2C12 myoblast differentiation (Yun and Matts 2005a, b). As a transcription factor with various functions, the roles of KLF4 in skeletal muscles are not well known. In our previous work, the basal expression of KLF4 was detected in skeletal muscles both in vivo and in vitro (Rezzani et al. 2003). Together with our current results, it is suggested that KLF4 may be a novel regulator of skeletal muscles by induction of the expression of HSP90.

In addition, induction of HSP90 by KLF4 may have important implications in the activation of macrophages. HSP90 function has been demonstrated to be necessary for the activation of macrophages (Byrd et al. 1999). Geldanamycin blocked the nuclear translocation of NF- κ B and expression of tumor necrosis factor in macrophages treated with lipopolysaccharide. It was shown that NO synthesis from iNOS could be profoundly modulated by HSP90. HSP90 is an allosteric enhancer of iNOS. In cells, HSP90 is coupled with iNOS, and thus facilitates NO synthesis (Rezzani et al. 2003). KLF4 was also identified as a critical regulator in macrophage activation. KLF4 was found to regulate the macrophage differentiation marker CD11d (Noti et al. 2005). KLF4 interacts with the NF- κ B family member p65 to cooperatively induce the iNOS promoter, but inhibits the TGF- β 1/Smad3 induction of the PAI-1 promoter independent of KLF4 DNA binding through a novel antagonistic competition with Smad3 for the C terminus of the coactivator p300/CBP (Feinberg et al. 2005). In light of the critical role of KLF4 and HSP90 in the activation of macrophages, together with the regulation of HSP90 by KLF4 in RAW264.7 macrophages that was first identified in the present study, we suggested that KLF4 could orchestrate its effects by upregulating the expression of HSP90 in the activation of macrophages.

In brief, we demonstrate that KLF4 is required for the basal expression of HSP90 in C2C12 cells and RAW264.7 cells. It is conceivable that KLF4 and HSP90 may participate in modulating several biological states such as myoblast differentiation and macrophage activation.

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