Cooperative Interaction between the Basic Helix-loop-helix Transcription Factor dHAND and Myocyte Enhancer Factor 2C Regulates Myocardial Gene Expression*

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Ming-Xi Zang‡, Yong Li‡§, Hao Wang‡, Jun-Bo Wang‡, and Hong-Ti Jia**

From the ‡Laboratory of Development Molecular Biology, Department of Nutrition and Food Hygiene, School of Public Health, Peking University Health Science Center, Beijing 100083, China, the Laboratory of Cardiac Growth and Differentiation, Institut de Recherches Cliniques de Montreal, Quebec H2W 1R7, Canada, and the **Department of Biochemistry and Molecular Biology, Peking University Health Science Center, Beijing 100083, China

Cardiac-restricted transcription factors dHAND and myocyte enhancer factor 2C are expressed in the developing heart and activate several cardiac promoters. However, their regulatory mechanisms are still to be understood. To elucidate their exact regulatory functions, we have developed an RNA interference strategy to specifically inhibit dHAND and myocyte enhancer factor 2C protein production in H9c2 cells, which are derived from rat embryonic heart. Expression of endogenous cardiac genes atrial natriuretic peptide and α-myosin heavy chain was down-regulated in H9c2 cells lacking both dHAND and myocyte enhancer factor 2C, indicating that these factors are required for the maintenance of the cardiac genetic program. Consistent with these, expression of atrial natriuretic peptide and α-myosin heavy chain was up-regulated in H9c2 cells, which overexpressed dHAND and myocyte enhancer factor 2C. In addition, dHAND and myocyte enhancer factor 2C interact to synergistically activate atrial natriuretic peptide and α-myosin heavy chain transcription. Furthermore, chromatin immunoprecipitation analysis in H9c2 cells treated with phenylephrine showed that dHAND and myocyte enhancer factor 2C protein complex bind to the AT sequence on atrial natriuretic peptide promoter. Taken together, these results not only suggest that the complex cis-trans interaction of dHAND, myocyte enhancer factor 2C, and the target gene may fine-tune gene expression in cardiac myocytes but also provide a molecular paradigm to elucidate the mechanisms of action of dHAND and myocyte enhancer factor 2C in the developing heart.

The basic helix-loop-helix (bHLH)1 transcription factors are a large family of genes that play important roles in determination and differentiation of diverse cell types including skeletal cells, hematopoietic cells, some neuronal cells, and cardiomyocytes. The bHLH proteins contain the basic region and the HLH domain. The basic region can bind to the E-box consensus sequence (CANNTG), and the HLH domain can mediate homodimerization or heterodimerization. This family can be divided into several subgroups based either on their structure, DNA binding activity, or expression pattern during development. Tissue-specific bHLH proteins compose the largest subgroup and bind the E-box element (CANNTG) of target gene by dimerization with a ubiquitously expressed bHLH factors, the E protein. In addition, transcriptional activity of the tissue-specific bHLH proteins requires association with other bHLH proteins or non-bHLH proteins (1). For example, in skeletal muscle cells the muscle bHLH protein MyoD regulates skeletal muscle genes through direct interaction with MEF2A, a member of myocyte enhancer factor 2 (MEF2) family (2).

dHAND is a bHLH protein expressed in deciduum, heart, autonomic nervous system and neural crest derivatives. Several laboratories cloned it in 1995 and gave it different names such as HAND2, Th2, and exd. The phenotype of dHAND null mice demonstrates the essential role of dHAND in the formation of the right ventricle, the trabeculae, and the neural crest- derived aortic arches. dHAND is also implicated in the regulation of chamber specification, cardiac looping, and cardiac neural crest (3).

Atria1 natriuretic peptide (ANP) is an important cardiac hormone expressed during heart development, and it regulates the blood volume and pressure. Reduced ANP expression in dHAND −/− mice suggests that dHAND is potentially important for the regulation of ANP. The ANP promoter harbors four functional E-box elements that might bind dHAND (4).

Another transcription factor that is required for cardiogenesis is myocyte enhancer binding factor 2C (MEF2C), which belongs to the MADS (MCM1, agamous, deficiens, and serum response factor) box family. In mammals, the MADS-box family is composed of four members, MEF2A, MEF2B, MEF2C, and MEF2D. These transcription factors contain an almost identical N-terminal region necessary to mediate DNA binding, and they are divergent in their C-terminal region, which is implicated in transcription activation. The MEF2 proteins can form homo- and heterodimers to bind the consensus DNA sequence (T/C)TA(AGT)TA(G/A) to regulate gene expression. Inactivation of MEF2C in mouse leads to cardiac morphogenetic defects, vascular abnormalities, and embryonic lethality, which demonstrates an essential role of MEF2C in heart development (5).

Transcriptional regulation of cardiac development requires...
the coordinated expression of several factors in a temporally and spatially defined manner. These cardiac-restricted transcription factors form multiprotein complexes around cardiac-restricted genes through interaction to regulate target genes. But the mechanism by which these genes regulate has yet to be determined. Here we demonstrate that transcriptional factors dHAND and MEF2C interact to synergistically activate expression of cardiac gene promoters in H9c2 cells. Inhibiting expression of dHAND and MEF2C in H9c2 cells result in down-regulation of ANP and α-myosin heavy chain (α-MHC). Moreover, overexpression of dHAND and MEF2C in H9c2 cells result in up-regulation of ANP and α-MHC. In addition, the functional interaction on ANP promoter is mediated through the MEF2C-binding site but not dHAND-binding sites. This interaction suggests that dHAND protein was recruited by MEF2C to AT sequence on ANP promoter to activate transcription.

MATERIALS AND METHODS

Plasmids—The pcDNA3-MEF2C expression vector was a gift of Dr. Coralie Poizat and Larry Kedes (University of Southern California). The pcDNA3-His2B-dHAND expression vector, the ANP reporter plasmid (~638 base pairs upstream from the transcriptional start site), and the α-MHC-luciferase (~330 base pairs upstream from the transcriptional start site) were gifts from Jeffery D. Molkenk and Dr. Yan-Shan Dai (Cincinnati, OH).

RNAi Plasmids Constructs—The U6iHAND and U6iMEF2C RNAi constructs were designed following pSilencer neo™ instruction manual (Ambion). Briefly, 21-nucleotide-long inverted repeats separated by a 9-nucleotide linker were inserted downstream of the U6 promoter. Six thymidines were inserted downstream of the antisense strand to provide a stop signal for the RNA polymerase III. The sense strand of the hairpin was homologous to a 21-nucleotide region in the target mRNA (MEF2C or dHAND) and were analyzed by BLAST research to ensure that they did not have significant sequence homology with other genes. The sense strand of the MEF2C small interfering RNA (siRNA) was designed to be homologous to a region covering the MEF2C-coding sequence (nucleotides 538–558 bp) from the start codon of the mouse MEF2C or RNAi plasmid of dHAND and MEF2C or empty vector were expressed in U2OS cells and subjected to luciferase assay. The luciferase activity was normalized to β-galactosidase activity to correct for differences in transfection efficiency. The data were statistically analyzed with Student’s t test.

Northern Blot Analysis—After transfection with dHAND and MEF2C or RNAi plasmid of dHAND and MEF2C or empty vector pcDNA3 and pSilencer2.1-U6, total cellular RNA was isolated from H9c2 cells with Trizol reagent (Invitrogen). Twenty micrograms of total RNA were size-fractionated on formaldehyde-agarose gel (1%), transferred to a nylon membrane (Hybond N+; Amersham Biosciences) by capillary blotting in the presence of 20× SSC (300 mM sodium chloride and 300 mM sodium citrate), and then cross-linked by irradiation with a germicidal ultraviolet lamp at 2 °C for 30 s at 30°C. Blots were hybridized with random prime-labeled rat cDNA probes for ANP, α-MHC, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The hybridization signal obtained with the GAPDH probe was used to correct differences in loading and/or transfer efficiencies. The probes were labeled with [32P]dCTP by a Prime-a-Gene labeling system (Promega). The hybridization and washing were performed according to standard methods. The membranes were then exposed to x-ray film for 24 h at −70 °C. The ANP probe was obtained by using the specific oligonucleotide primers 5′-AACCAGAGAGTGAGCCGGAGACAGCAAGAGAGTAATTTCTTGC-3′ and 5′-TGCTCTCAGAGTATTTTGCTGTATTCTT-3′, which are based on the nucleotide sequence of rat ANP cDNA. The primers for α-MHC probe were 5′-TGCCGCCAAGCAGAAATGCA-3′ and 5′-ACAGCAGAAGTCAGCATGCTTATCAT-3′. Immunoprecipitation and Western Blot Analysis—H9c2 cells were lysed at 4 °C with a gentle rotation in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Triton-100) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml aprotinin). Lysates were cleared by centrifugation at 4 °C. Protein lysates were incubated with 1% SDS, 0.1MNaHCO3, the nuclei were separated from the supernatant by 12,000 × g for 10 min. Protein concentration was measured using a bicinchoninic acid protein assay. To perform the ChIP assay, H9c2 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Cells were collected and cross-linked with 1% formaldehyde for 10 min at 37 °C. Cells were kept in ice-cold phosphate-buffered saline twice and centrifuged for 5 min at 2000 × g. Cells were then resuspended in 0.2 ml of lysis buffer (1% SDS, 100 mM EDTA, 50 mM Tris–Cl, pH 8.1, protease inhibitor) and sonicated 12 times for 10 s each followed by centrifugation for 10 min. Supernatants were collected and diluted in buffer (1% Triton X-100, 0.01% SDS, 2.0 mM EDTA, 167 μM NaCl, 16.7 μM Tris–Cl, pH 8.1) followed by immunocrosslinking with 80 μl of salmon sperm DNA/protein A-agarose for 2 h at 4 °C. Immunoprecipitation was performed overnight at 4 °C with dHAND antibody or MEF2C antibody (Santa Cruz). After immunoprecipitation, 60 μl of salmon sperm DNA/protein A-agarose was added, and the incubation was continued for another hour. Precipitates were washed sequentially in the following three different washing buffers for 5 min each: a low salt immune complex washing buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.1, 150 mM NaCl), a high salt immune complex washing buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.1, 150 mM NaCl), and 1% deoxycholate, 1 mM EDTA, 0.1% Tween-20. Elutions were pooled and heated at 65 °C for at least 6 h to reverse the formaldehyde cross-linking. DNA fragments were purified; 1 μl from 50 μl of DNA extract was reserved for 25–30 cycles of PCR amplification. Chromatin immunoprecipitation assay kit was purchased from Upstate Biotechnology. The primers for the E-box were 5′-TCCACCCACGAGCCAATGATG-3′ (sense primer) and 5′-CCGGCCTGGTTGCTGGCTTTCAAG-3′ (antisense primer). The primers for the A/T site were 5′-GACCTGGAAAGAGGCCTTGG-3′ (sense primer) and 5′-ATGGCCCTGCTGGTGGAGCT-3′ (antisense primer).
the function of dHAND and MEF2C in the activation ANP and suggest that the ANP and endogenously expressed in H9c2 cells.

Endogenous ANP and cardiac-specific gene expression by dHAND and MEF2C, we investigated whether these two transcription factors could functionally interact to induce the activation of the ANP and α-MHC promoters. We employed H9c2 cells to address this issue. The H9c2 cell line is derived from 13.5-embryonic day BDIX rat heart tissue and was cloned by Kimes and Brandt in 1976 (6). ANP, α-MHC, dHAND, and MEF2C are all endogenously expressed in H9c2 cells.

Cotransfection of both dHAND and MEF2C in H9c2 cells leads to a 7- and 5-fold increase in synergistic activation of the ANP and α-MHC promoter respectively (Fig. 1). These data suggest that the ANP and α-MHC genes are synergistically activated by co-expression of dHAND and MEF2C. Collectively, these results indicate that the transcription factors dHAND and MEF2C can functionally synergize to activate the promoters of the ANP and α-MHC gene.

RESULTS

Synergistic Activation of the ANP and α-MHC Promoter by dHAND and MEF2C—Both dHAND and MEF2C regulate cardiac promoters and are expressed in cardiomyocytes. To gain great insight into the transcriptional mechanism regulation of cardiac-specific gene expression by dHAND and MEF2C, we investigated whether these two transcription factors could functionally interact to induce the activation of the ANP and α-MHC promoters. We employed H9c2 cells to address this issue. The H9c2 cell line is derived from 13.5-embryonic day BDIX rat heart tissue and was cloned by Kimes and Brandt in 1976 (6). ANP, α-MHC, dHAND, and MEF2C are all endogenously expressed in H9c2 cells.

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dHAND and MEF2C Synergistically Activate Expression of Endogenous ANP and α-MHC in H9c2 Cells—To further assess the function of dHAND and MEF2C in the activation ANP and α-MHC, we examined the effects of overexpression of dHAND and MEF2C on the expression of the endogenous ANP and α-MHC gene in H9c2 cells. As shown in Fig. 2, expression of the ANP and α-MHC gene was enhanced in H9c2 cells transfected with both dHAND and MEF2C compared with the empty vector pcDNA3.1. Furthermore, when repressing dHAND and MEF2C expression by transfecting the H9c2 cells with RNAi plasmid of dHAND and MEF2C, the expression of the ANP gene was reduced compared with the transfection of H9c2 cells with pSilencer2.1-U6 (Fig. 2A). Similar results were obtained for the α-MHC gene (Fig. 2A). Importantly, endogenous expression of dHAND and MEF2C was dramatically reduced in H9c2 cells transfected with the RNAi plasmid of dHAND and MEF2C, respectively (Fig. 2B). Moreover, both expression of dHAND and MEF2C was dramatically reduced in H9c2 cells transfected with RNAi plasmid of dHAND and MEF2C, respectively (Fig. 2B).
and MEF2C were increased in transfected H9c2 cells with dHAND and MEF2C. These findings suggest that dHAND and MEF2C maintain transcription of ANP and α-MHC gene.

**dHAND and MEF2C Physically Interact In Vivo**—Our previous studies indicate that dHAND directly interacts with full-length MEF2C *in vitro* (7). To examine whether dHAND and MEF2C interact *in vivo*, co-immunoprecipitation experiments were performed in H9c2 cells. Protein lysates from H9c2 cells were first incubated with dHAND antibody, then protein A was used to precipitate dHAND from protein lysates, which was resolved in a SDS-PAGE and subjected to Western blot with anti-MEF2C antibody. The data demonstrated that MEF2C interacted with dHAND *in vivo* but not with preimmune goat serum (Fig. 3). These data suggest that MEF2C and dHAND physically interact *in vivo* to form a protein complex.

**dHAND-MEF2C Protein Complex Bind to the A/T Sequence of ANP Promoter in H9c2 Cells Treated with Phenylephrine (PE)**—Our previous studies show that the dHAND-MEF2C protein complex binds to the A/T sequence of ANP promoter in unstimulated H9c2 cells (7), which also suggests that in the H9c2 cell line the interaction of dHAND and MEF2C takes place in the A/T sequence of ANP promoter. To further address whether the dHAND and MEF2C complex change their binding sites on ANP promoter under PE stimulation, a serial of ChIP experiments was performed in H9c2 cells treated with PE. PE is a well known stimulator of cell enlargement (hypertrophy) in rat ventricular myocytes. This hypertrophic response induces the expression of ANP (8). To further examine whether dHAND-MEF2C protein complexes are assembled on one or both sites of ANP promoter, we divided the soluble chromatin into two aliquots; one was immunoprecipitated with dHAND antibodies and the other with MEF2C antibodies. Both of the eluted immunoprecipitates were amplified by the two site sequences of the ANP promoter (Fig. 4). The data demonstrate that both dHAND and MEF2C can occupy the DNA fragment from −628 to −494 bp, which harbors the A/T sequence (Fig. 4A), whereas they do not assemble on the DNA fragment from −511 to −62 bp, which harbors the E-box sequence (Fig. 4B). All these results demonstrate that the dHAND-MEF2C protein complex still binds to the A/T sequence of ANP promoter in H9c2 cells treated with PE.

**DISCUSSION**

In this study we have obtained the following results. 1) dHAND and MEF2C interact to synergistically activate transcription of ANP and α-MHC in H9c2 cells, 2) ANP and α-MHC mRNA levels are reduced when inhibiting the expression of dHAND and MEF2C, and 3) dHAND-MEF2C synergy is dependent on MEF2C DNA binding, and dHAND protein is recruited by MEF2C to the A/T sequence on the ANP promoter to activate ANP transcription.

Roles of MEF2C and dHAND in Regulating Heart Development—MEF2C is a member of the MEF2 transcriptional factors family that contains MADS and MEF2 domains to mediate DNA binding and dimerization. These MEF2 transcriptional factors have been shown to be expressed at high level in all muscle cells, but they are also found in brain and lymphoid tissue (9). They are important regulators of muscle-specific gene expression and differentiation of all three muscle lineages. For example, during myogenesis, MEF2A and muscle bHLH proteins cooperatively activate skeletal muscle genes. Thus, skeletal myogenesis is mediated by MEF2A, which can initiate the developmental cascade. In fact, the MEF2 family directly associates with cell-specific transcriptional factors to regulate the determination, proliferation, and differentiation of cardiac muscle, smooth muscle, and neural cells. They control the development of multiple tissues including heart, vasculature, neural tubes, and skeletal muscle (2). MEF2C is expressed in heart, skeletal muscle, spleen, and brain. In the developing mouse heart, MEF2C is initially expressed in the precardigenetic mesoderm beginning at embryonic day 7.75 (E 7.75). Consistent with its expression pattern, inactivation of the MEF2C gene in mice leads to cardiac morphogenetic defects, vascular abnormalities, and lethality by E 9.5, suggesting that MEF2C functions as a critical regulator of cardiac development. Indeed, MEF2C plays a crucial role in the looping of the cardiac tube, development of the right ventricle, and expression of a subset of cardiac muscle genes (10).

The direct transcriptional targets of MEF2C in regulation of diverse cell fates are largely unknown. Four members of the MEF2 transcriptional factor family bind to a DNA sequence element, referred to as an A/T-rich sequence, which consists of the consensus DNA sequence (T/C/TAA/TG/A). MEF2C forms homodimers and heterodimers that interact with the A/T-rich sequence element to directly promote transcriptional activation of the target gene. Here we demonstrated that MEF2C functions in concert with dHAND as a mechanism of enhancing cardiac-specific ANP and α-MHC gene transcription. This synergy between MEF2C and dHAND was independent of the ability of dHAND to bind the E-box on ANP promoter, and it required the dHAND-MEF2C protein complex to bind the A/T-rich sequence element on the ANP promoter. These observations suggest that MEF2C can participate in programming the cardiac gene expression through combinatorial interactions with other transcription factors such as dHAND. These results also indicate that dHAND can function in regulating ANP gene expression independently of the E-box sequence elements.

Like MEF2C, dHAND is also thought to function in regulating cell type-specific gene expression through combinatorial interactions with other transcriptional factors. dHAND is a member of a large transcription factors family that contains the bHLH domain which mediates DNA binding and dimerization. These transcription factors are expressed in a wide array of eukaryotic organisms from yeast to humans. For example, dHAND is expressed in numerous tissues including the heart, limbs, and multiple neural crest-derivatized tissues during development. Most notably, dHAND forms both homodimers with itself and heterodimers with the ubiquitously expressed E12/E47 bHLH proteins or with non-bHLH proteins to enhance transcriptional activation (4, 11). Here we demonstrated that dHAND was recruited by MEF2C to potentiate cardiac-specific ANP gene expression. dHAND has also been reported to inter-
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Mechanism for MEF2C and dHAND Transcriptional Regulation of ANP—ANP and MHC play important roles during heart development. For example, ANP is a cardiac peptide hormone that possesses significant diuretic, natriuretic, and vasodilatory activities and plays a critical role in the maintenance of blood pressure and sodium balance. During embryonic development, the ANP gene is expressed in both the atrium and the ventricle, but after birth, the expression of ANP is mainly in the cardiac atria. However, if the ventricle is subjected to diverse stimuli causing ventricular hypertrophy, including growth factors, cytokines such as phenylephrine or angiotensin II, mechanical stretch, and activators of protein kinase C, the ventricular ANP gene is stimulated and starts to be expressed. ANP, once released into the circulation, causes natriuresis, diuresis, and vasodilation. Thus, ANP plays a potentially important role in hemodynamic regulation during hypertension. Because expression of ANP is abundant in ventricular cells during embryogenesis and subsequently is extinguished during normal adult development, the reexpression of ANP within hypertrophied ventricular myocytes has been considered to be representative of the induction of an embryonic gene program (14).

α-MHC, another cardiac-specific gene, encodes a gene for a cardiac contractile protein and plays an important role in the speed of heart contraction because it has a characteristic adenine triphosphatase (ATPase) activity, which is correlated with the energy transduction responsible for the generation of force in muscle. The myosin heavy chains are encoded by a highly conserved multigene family, and their expression is tissue- and developmental stage-specific. In cardiac muscle, two distinct MHC genes, α- and β-MHC isoforms, have been characterized. Both cardiac MHC genes are coexpressed in the early heart tube when septation and the cardiac compartments are distinguished during normal adult development, the reexpression of MHC promoters occurs during the development (15). Although ANP and α-MHC play important roles during heart development, little is understood in the mechanisms of regulation in cardiomyocytes. Delineation of the ANP and α-MHC regulatory elements has further yielded insight into the transcriptional regulation of a cardiac-specific gene. ANP promoter harbors four copies of the E-box sites and an A/T-rich sequence. α-MHC promoter harbors two copies of the E-box sites and an A/T-rich sequence. Interactions between these cis-elements and cardiac-specific transcriptional factors fine-tune transcription of ANP and α-MHC genes. Here we demonstrated that dHAND interacts with MEF2C to potentiate cardiac-specific expression of ANP and α-MHC. Most notably, dHAND protein was recruited by MEF2C to an A/T sequence on the ANP promoter to activate ANP transcription in H9c2 cells.
et al. (4) reported that dHAND functions as a transcriptional activator partially independent on DNA binding. Indeed, interaction between transcription factors may take place at one of their binding sites. For example, among these cardiac-specific transcription factors, GATA4 recruits MEF2C (5), NKX2.5 recruits dHAND (4), and NKX2.5 recruits GATA4 (15). All these recruitments lead to an increase of ANP promoter activity. On the other hand, interaction between transcription factors may take place at both of their binding sites. For example, the synergistic activation of cardiac promoters by NKX2.5-TBX5 interaction has been shown to be based on stable ternary complexes composed of NKX2.5-TBX5 interaction as well as the simultaneous binding of each protein to its binding site (17, 18). Accordingly, our results provide a molecular paradigm for elucidating the regulation mechanisms of dHAND and MEF2C on heart development.

During cardiogenesis and cardiac differentiation, many transcription factors play important roles on ANP transcription. For example, dHAND interacts with MEF2C, GATA4, or NKX2.5 (4, 11), and GATA4 interacts with MEF2C or NKX2.5 (5, 16). All these interactions contribute to enhance the activity of ANP promoter. These transcription factors and their target genes generate a unique network of protein-protein and protein-DNA interactions to form a cardiac-specific enhanceosome, which coordinates expression of target gene. The enhanceosome is made of stable multiprotein complexes that promote the cooperative recruitment of coactivators and RNA polymerase II complex to activate transcription. Assembly of the enhanceosome is regulated by specific extracellular events and intracellular signals (19–22).

Although we have shown that MEF2C forms a complex with dHAND, it is not clear how complex formation is regulated. A challenge for future investigations is to discover the temporal and spatial regulation of these interactions during heart development. It is reported that p38 MAPK (mitogen-activated protein kinase) activates MEF2 in cardiac muscle (23–25) and dHAND has been implicated in epithelial 1-induced transcription (12), so the analysis of interaction between dHAND and MEF2C in response to different stimuli in cardiomyocytes at various development stages will be important to understand the mechanisms of enhanceosome formation on ANP and α-MHC transcription regulation.

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