

Hypertrophic stimuli augment expression of cMG1/ERF-1, a putative zinc-finger motif transcription factor, in rat cardiomyocytes

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Abstract We isolated the gene for cMG1/ERF-1, a known putative zinc-finger transcription factor, by differential display of mRNA extracted from cardiomyocytes with and without leukemia inhibitory factor (LIF) stimulation. LIF induced cMG1/ERF-1 mRNA at 15 min, and levels peaked at 10-fold initial levels at 30 min. cMG1/ERF-1 expression was inhibited by AG490 (JAK2 inhibitor) and genistein, but was unaffected by PD98059 or wortmannin. Phenylephrine, angiotensin II and endothelin-1 also induced cMG1/ERF-1 expression. Mechanical stretch in vitro and acute pressure overload in vivo increased cMG1/ERF-1 expression. To our knowledge, this is the first report showing that the cMG1/ERF-1 gene can be induced by various hypertrophic stimuli, and that Janus kinase 2 is involved in this process.

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Key words: Leukemia inhibitory factor; cMG1/ERF-1; Cardiac hypertrophy; Rat cardiomyocyte

1. Introduction

Cardiac hypertrophy, ventricular volume overload and reduced contractility are observed in all cases of heart failure, regardless of cause. Cardiac hypertrophy is a compensatory response that allows the heart to cope with the pathogenic stimuli associated with many cardiovascular diseases. Cardiac hypertrophy is induced by mechanical load [1,2] and neuro-humoral factors such as angiotensin II (AngII) [3,4], endothelin-1 (ET-1) [5,6] and norepinephrine [7,8]. Therefore, clarification of the molecular mechanisms of the development of cardiac hypertrophy is a very important step in elucidating the mechanisms of heart failure. We have described the intracellular signaling pathway in cardiac hypertrophy that occurs via gp130 or AngII receptor [9,10]. The current study aimed to clarify the molecular mechanisms of cardiac hypertrophy by cloning genes that are expressed downstream of gp130 or AngII.

RNA differential display (DD) is a powerful means of detecting genes whose expression is augmented or decreased in

response to various stimuli [11–13]. Using this method, we cloned a gene that was differentially expressed between treated or untreated with leukemia inhibitory factor (LIF). This clone encoded a known putative transcription factor with a zinc-finger motif that is regulated downstream of LIF. In the present study, we report that expression of this gene is changed in response to various hypertrophic stimuli.

2. Materials and methods

2.1. Cell culture

Primary cultures of cardiomyocytes were prepared from the ventricles of 1-day-old neonatal Wistar rats by enzymatic dissociation in 0.03% trypsin, 0.03% collagenase, and 20 µg/ml DNase I as described [9,10]. They were incubated in the same medium for 24 h and then stimulated with various agents.

2.2. RNA DD analysis

Cardiomyocytes were stimulated with LIF (1000 U/ml) for 1, 6 and 24 h. Total RNA was isolated by the acid guanidinium/thiocyanate phenol/chloroform (AGPC) extraction method. DD was conducted as described previously [13] to identify genes differentially expressed between cardiomyocytes stimulated or unstimulated with LIF. DD-polymerase chain reactions (PCR) were performed using a combination of three 21-bp anchored LH11N primers and 21-bp extended OPA or 18-bp LH-AP arbitrary primers [12,13]. The PCR products were fractionated by electrophoresis through a 6% acrylamide sequencing gel, eluted, and reamplified as described [11]. The reamplified cDNA band was subcloned into the pCR2.1 TOPO vector (Invitrogen) and sequenced on both strands with T7 and M13 reverse primers using an ABI PRISM 310 Genetic Analyzer or an ABI 373A Sequencer (Applied Biosystems).

2.3. Northern blot analysis

Total RNA was separated on 1% agarose/formaldehyde gels and transferred to nylon membranes. The membranes were hybridized overnight to a cDNA probe labeled with [α -³²P]dCTP using the Rediprime DNA labeling system (Amersham Life Science) at 42°C. Full-length cDNA plasmid was a generous gift from Dr. Kenneth D. Brown [14]. Prior to stimulation with LIF, PD98059, wortmannin, AG490 or genistein was added to the culture medium.

2.4. Mechanical stretch

Cells were seeded on a fibronectin-coated deformable, silicone rubber dish at a density of 1×10^5 cells/cm². The culture medium was changed 24 h after seeding to a medium containing 0.1% fetal bovine serum (FBS). After 8 h in the 0.1% FBS medium, the culture dishes were stretched by 20%. Stretching of cardiomyocytes was accomplished as described previously [15]. A 20% stretch was shown to be sufficient to activate various signal transduction pathways and not to damage the cells.

2.5. Animal experiment protocol

Wistar rats (8 weeks) were anesthetized with ether, and the abdominal aorta was ligated between the right and left renal arteries. The left

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Abbreviations: LIF, leukemia inhibitory factor; JAK, Janus kinase; ET-1, endothelin-1; AngII, angiotensin II; DD, differential display; ERF-1, EGF-response factor 1; STAT, signal transducers and activators of transcription

ventricular myocardium was excised at 0 and 120 min, homogenized with a Polytron homogenizer in solution D, and RNA was extracted by the AGPC method.

3. Results

3.1. Expression of *cMG1/ERF-1* is increased by the stimulation with LIF

To isolate genes that were involved in gp130-mediated cardiac hypertrophy, we initially performed DD-PCR analysis of cardiomyocytes in the absence or presence of LIF. We found several cDNA fragments that were upregulated in cardiomyocytes stimulated with LIF (Fig. 1). Some of them were reamplified by PCR with the same primers and subcloned. Sequence analysis revealed that one of these cDNAs was 520 bp in length and had 100% homology with rat *cMG1* and 94.2% homology with human *ERF-1*. *cMG1/ERF-1* is known as a putative immediate-early gene, and is considered to be a zinc-finger transcription factor based on its protein structure. Northern blot analysis using the cDNA fragment was performed to confirm the DD result (Fig. 1B), and a full-length *cMG1* probe yielded the same result (data not shown). LIF

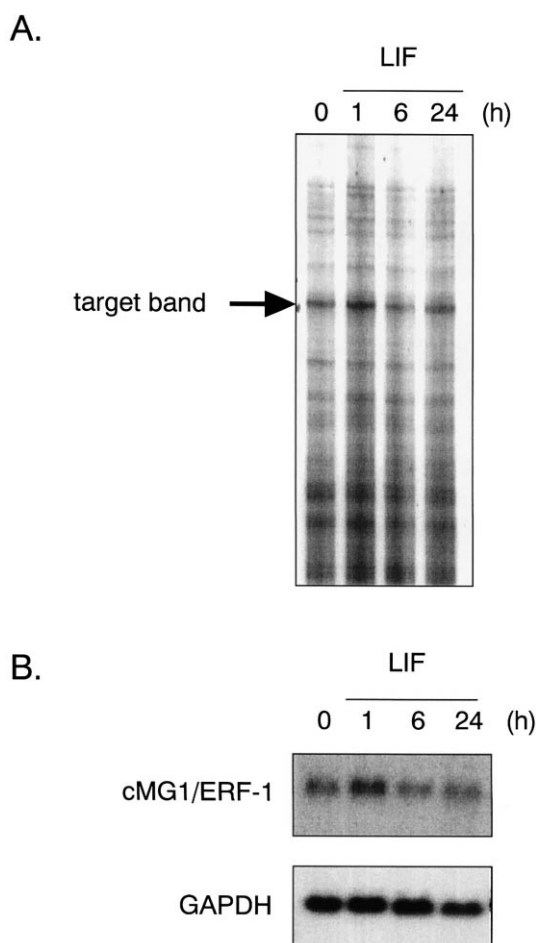


Fig. 1. Identification of differentially expressed genes from rat cardiomyocytes stimulated with LIF. A: Autoradiograms of differential display as described in Section 2. The arrow indicates a cDNA isolated in this study. Expression of this cDNA increased at 1 h after LIF stimulation. B: Confirmation of the DD pattern by Northern blot analysis using the reamplified PCR product as a probe. A GAPDH probe was used as control.

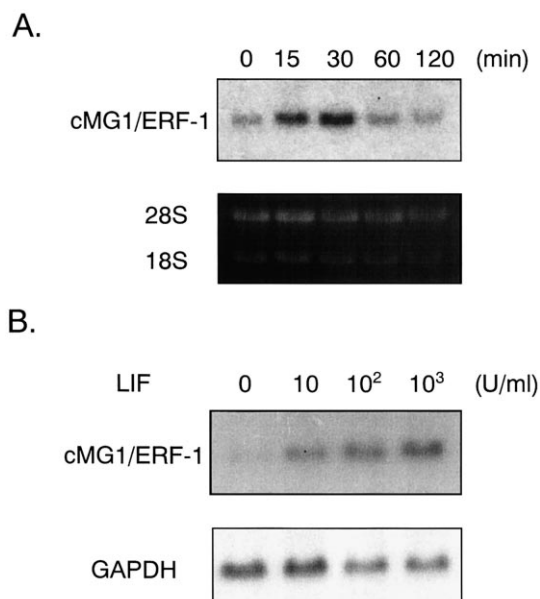


Fig. 2. Time course and dose dependence of *cMG1/ERF-1* expression in LIF-stimulated cardiomyocytes. A: Time course of *cMG1/ERF-1* expression. LIF induced *cMG1/ERF-1* mRNA expression in rat cardiomyocytes as early as 15 min, which peaked at a 10-fold increase at 30 min, and returned to the control levels at 120 min (RNA 10 μ g). B: Dose dependence of *cMG1/ERF-1* expression by LIF stimulation.

induced *cMG1/ERF-1* mRNA expression in rat cardiomyocytes as early as 15 min. It peaked by approximately 10-fold at 30 min, and returned to the control level at 120 min (Fig. 2A). *cMG1/ERF-1* expression was increased with LIF in a dose-dependent manner (Fig. 2B).

3.2. LIF-induced *cMG1/ERF-1* expression is mediated by the JAK/STAT pathway

We and others have previously reported that LIF activated the JAK/STAT, raf-1/MEK/ERK and PI3-K/p70S6 kinase pathways in rat cardiomyocytes [9,16,17]. To determine which pathway mediates *cMG1/ERF-1* expression, we preincubated the cells with PD98059 (MEK inhibitor), wortmannin (PI3-K inhibitor), AG490 (JAK2 inhibitor) or genistein (tyrosine kinase inhibitor), followed by stimulation with LIF. LIF-induced *cMG1/ERF-1* expression is almost completely inhibited by AG490, partially inhibited by genistein, but only slightly attenuated by PD98059 or wortmannin. These results indicated that *cMG1/ERF-1* expression was mediated by the JAK/STAT pathway. We also compared the role of these signaling pathways in LIF-induced expression of other hypertrophic marker genes such as *c-fos* and BNP (Fig. 3B). In contrast to *cMG1/ERF-1*, *c-fos* was strongly inhibited by PD98059, and moderately inhibited by AG490. BNP was attenuated by AG490, but only minimally inhibited by PD98059. These findings indicate that *c-fos* and *cMG1/ERF-1* are distinctly regulated by LIF-mediated signaling events.

3.3. *cMG1/ERF-1* is induced by other hypertrophic ligands

LIF relays its signal via gp130, a co-receptor of the interleukin-6 (IL-6) family of cytokines. However, other ligands such as AngII, ET-1 and phenylephrine induce cardiac hypertrophy via a seven-transmembrane G protein-coupled receptor. To determine other hypertrophic stimuli which may in-

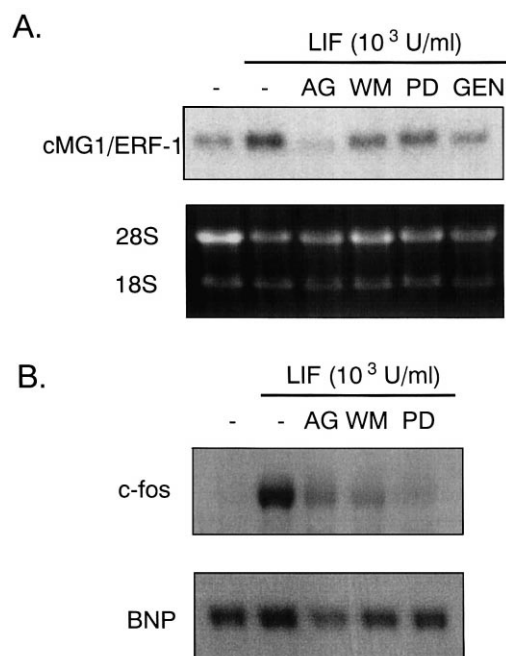


Fig. 3. Upstream pathway of cMG1/ERF-1 induction. Cells were preincubated with AG490 (JAK2 inhibitor, 20 μ M), PD98059 (MEK inhibitor, 50 μ M), wortmannin (PI3-K inhibitor, 10 nM), or genistein (tyrosine kinase inhibitor, 25 μ M) for 30 min, and stimulated with LIF for 30 min. Induction of cMG1/ERF-1 by LIF is almost completely inhibited by AG490, is partially inhibited by genistein, but is unaffected by PD98059 or wortmannin.

fluence cMG1/ERF-1 expression, we stimulated the cardiomyocytes with AngII, ET-1 or phenylephrine (Fig. 4). AngII, ET-1 and phenylephrine all augmented cMG1/ERF-1 expression compared with the controls. ET-1-induced cMG1/ERF-1 expression levels were equivalent to those induced by LIF, while induction by AngII or phenylephrine was slightly lower than that by LIF. These results indicate that cMG1/ERF-1 is not only specifically induced by LIF but is also a hypertrophic marker gene.

3.4. Mechanical stretch in vitro and acute aorta banding in vivo increased cMG1/ERF-1 expression

Previous reports revealed that acute pressure overload in vivo [18–20] or mechanical stretch in vitro induced immediate-early genes such as *c-fos*, *c-myc*, *egr-1* and *c-jun* [2,21]. To confirm whether acute pressure overload in vivo or mechanical stretch in vitro induces cMG1/ERF-1 expression in car-

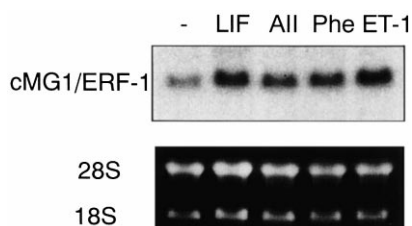


Fig. 4. Effect of AngII (AII), phenylephrine and ET-1 on cMG1/ERF-1 expression in cardiomyocytes. Cells were stimulated with either AngII, phenylephrine or ET-1 for 30 min. Phenylephrine and ET-1 induced cMG1/ERF-1 expression to almost the same levels as LIF. AngII also induced cMG1/ERF-1 expression, but to lower levels than LIF.

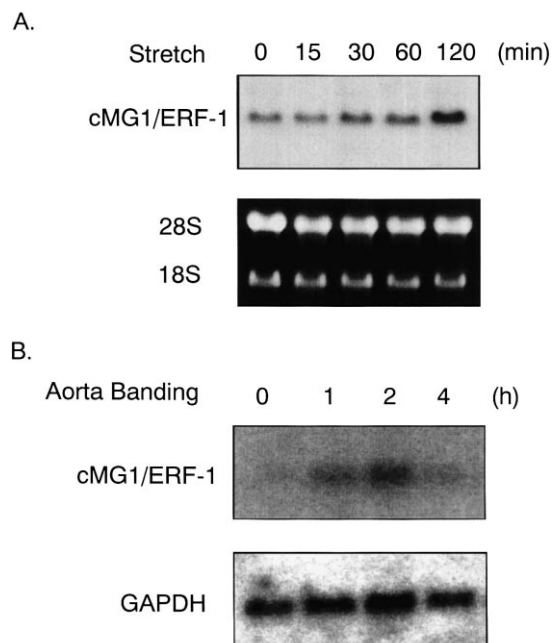


Fig. 5. Effect of mechanical stretch and pressure overload on cMG1/ERF-1 expression in cardiomyocytes. **A:** Mechanical stretch induced cMG1/ERF-1 expression. Cardiomyocytes were seeded on deformable silicone rubber dishes, and were stretched by 20% for the indicated times. Mechanical stretch induced cMG1/ERF-1 expression at 30 min, and expression reached a maximum at 120 min. **B:** cMG1/ERF-1 is augmented by acute aortic banding in vivo. Rat abdominal aortas were ligated between the right and left renal artery for the indicated times, and total RNA was isolated from the left ventricle. Acute pressure overload induced cMG1/ERF-1 by eight-fold at 2 h.

diomyocytes, we performed the following experiment. We prepared cardiomyocytes on deformable silicone rubber dishes, and stretched them by 20%. Mechanical stretch increased cMG1/ERF-1 expression after 30 min, and its expression peaked at three-fold at 120 min (Fig. 5A).

Next we ligated the abdominal aorta of rats for 1, 2 and 4 h, and also prepared sham-operated rats. Then, we examined cMG1/ERF-1 expression (Fig. 5B). Aortic banding induced cMG1/ERF-1 expression more than eight-fold. This induction was reproducible in three separate experiments. These findings indicate that hypertrophic stimuli such as acute pressure overload in vivo and mechanical stretch in vitro induce cMG1/ERF-1 expression in cardiomyocytes.

4. Discussion

We have cloned some genes that are specifically induced by stimulation with LIF, which causes cardiac hypertrophy in vitro. In the present study, we characterized one of these genes, cMG1/ERF-1 [14,22–27]. This gene contains a zinc-finger motif and is a putative transcription factor, although its action as a transcription factor, its binding sequence and target genes are largely unknown. Gomperts et al. isolated the cMG1 gene, and showed that growth factors such as EGF, PDGF, IGF-1 and AngII augmented cMG1 expression in a rat intestinal cell line (RIE-1), and they suggested that cMG1 might be a primary response gene [14]. Bustin et al. isolated ERF-1, a human homologue of cMG1, and reported that the promoter/enhancer region and intron sequences contain multi-

ple putative transcription factor binding motifs characteristic of early-response genes [22]. We found that the expression of this gene was increased not only by LIF but also by other cardiac hypertrophic ligands such as AngII, ET-1 and phenylephrine in rat cardiomyocytes. cMG1/ERF-1 expression peaked at 30 min after the stimulation, similar to the time course of *c-fos*. Moreover, cMG1/ERF-1 expression was induced by pressure overload in vivo and mechanical stretch in vitro. These findings are compatible with cMG1/ERF-1 being an immediate-early gene.

We showed that cMG1/ERF-1 expression was significantly inhibited by the JAK2 inhibitor AG490 and the tyrosine kinase inhibitor genistein, and that it was only slightly attenuated by blockers of the raf-1/MEK/ERK or PI3-K/p70S6k pathways. This was in contrast to the findings that *c-fos* expression is reduced by MEK inhibitors or PI3-K inhibitors. We previously showed that AngII activates JAK2, Tyk2, STAT1 and STAT2 in cultured rat cardiomyocytes [10]. If cMG1/ERF-1 is induced through the JAK2 pathway, it is reasonable to assume that AngII can induce cMG1/ERF-1 expression. However, it is not yet clear whether ET-1 or phenylephrine operates via this pathway. Since this gene was strongly induced by ET-1 and phenylephrine, another signaling pathway might also be involved.

The present study revealed that cMG1/ERF-1 expression was induced by mechanical stretch, peaking at 120 min. Previous reports showed that mechanical stretch could induce immediate-early gene expression, but could also induce secretion of other growth factors such as AngII and ET-1 [28–30]. We also reported that mechanical stretch activated the JAK/STAT pathway, and that autocrine/paracrine secreted IL-6 family cytokines might largely be involved in this activation [14]. The fact that cMG1/ERF-1 induction by mechanical stretch peaked at 120 min, which was later than ligand stimulation, may suggest that cMG1/ERF-1 was induced not only directly by mechanical stretch, but also by secondary humoral factors secreted by mechanical stretch.

Several reports have shown that acute pressure overload can induce cardiac hypertrophy, via activation of various signal transduction pathways, and that autocrine/paracrine secreted AngII and ET-1 might be critically involved in this activation [31–33]. We also showed that acute pressure overload produced by aortic banding activates the JAK/STAT pathway, and that angiotensin II and IL-6 family cytokines were involved in this activation [34]. The present study has shown that LIF, AngII and ET-1 strongly induced cMG1/ERF-1 expression in cultured cardiomyocytes. These findings suggest that acute pressure overload can induce cMG1/ERF-1 expression.

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