Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 1142–1146, February 1995
Cell Biology

Expression cloning of cardiotrophin 1, a cytokine that induces cardiac myocyte hypertrophy

(embryonic stem cell/gp130 ligand family)

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Communicated by David V. Goeddel, Tularik, Inc., South San Francisco, CA, October 24, 1994

ABSTRACT **Heart failure continues to be a leading cause of mortality worldwide. A hallmark of this disease is dilated cardiac hypertrophy, which is accompanied by a reactivation of genes expressed in fetal heart development. Reasoning that fetal or embryonic growth factors may mediate the onset of cardiac hypertrophy, we have coupled expression cloning with an embryonic stem cell-based model of cardiogenesis to isolate a 21.5-kDa protein, cardiotrophin 1, that potently induces cardiac myocyte hypertrophy *in vitro*. Amino acid similarity data indicate that cardiotrophin 1 is a member of the leukemia inhibitory factor/ciliary neurotrophic factor/oncostatin M/interleukin 6/interleukin 11 family of cytokines. Several members of this family that are known to signal through the transmembrane protein gp130 stimulate cardiac myocyte hypertrophy, like cardiotrophin 1, suggesting that the gp130 signaling pathway may play a role in cardiac hypertrophy. A 1.4-kb cardiotrophin 1 mRNA is expressed in the heart and several other mouse tissues.**

Cardiac muscle cell hypertrophy is one of the most important adaptive responses of the heart and is a central feature of many cardiac diseases in man (1). Following long-standing hypertension, myocardial injury, or other demands for increased cardiac output, the heart adapts through the activation of a hypertrophic response, which is characterized by an enlargement of myocardial cells and an accumulation of sarcomeric proteins in the absence of cell division (2–7). Although this process is initially compensatory, there can be a pathological transition in which the myocardium becomes irreversibly enlarged and dilated, with the accompanying onset of overt cardiac muscle failure. The identification of the factors which mediate the onset of these various phases of cardiac hypertrophy and failure remains a major pursuit in cardiac biology and medicine (3).

The development of an *in vitro* assay system for myocardial cell hypertrophy has offered the possibility of isolating and characterizing novel activities which might mediate this important physiologic response (2, 8–11). After stimulation with α-adrenergic agonists (such as phenylephrine) (8, 12–15) or with endothelin (16), neonatal rat ventricular myocytes display a number of features indicative of a hypertrophic response (17), including the induction of a set of immediate early genes (c-*fos*, c-*jun*, c-*myc*, and *Egr-1*) (14, 18), an increase in cell size without concomitant proliferation (8–10), and an organization of contractile proteins into sarcomeric units (13, 14). In addition, ventricular hypertrophy *in vivo* as well as *in vitro* is associated with the induction of several embryonic genes, including the reexpression of a fetal heart marker protein, atrial natriuretic peptide (ANP) (12, 16, 19). The induction of ANP is one of the most conserved and well-characterized markers of the hypertrophic response; it is found in all forms of hypertrophy and in all species examined thus far (2, 11).

Totipotent mouse embryonic stem cells differentiate into multicellular, cystic embryoid bodies when cultured in the absence of a fibroblast feeder layer or with the removal of leukemia inhibitory factor (LIF) (20). Since these embryoid bodies spontaneously beat and display cardiac specific markers (20–22), it has been suggested that they might serve as a valuable source of novel factors that can induce a hypertrophic response *in vitro* (3, 22). In this work we show that embryoid bodies do indeed elaborate a factor(s) that induces a hypertrophic response. An expression cloning approach to the characterization of the protein(s) responsible for this activity has led to the isolation of a cDNA clone encoding a 21.5-kDa protein, designated cardiotrophin 1 (CT-1), that induces cardiac myocyte hypertrophy.¶

MATERIALS AND METHODS

Cytokines were purchased from Genzyme with the exception of rat ciliary neurotrophic factor (CNTF), which was produced at Genentech. Collagenase was from Worthington.

Hypertrophy Assay. The assay described previously (24) was adapted for 96-well plates. In brief, ventricular cardiac myocytes were isolated from neonatal rats by collagenase digestion and Percoll gradient purification (14). These cells were suspended at 75 cells per μl in Dulbecco's modified Eagle's medium/Ham's nutrient mixture F-12 (DMEM/F-12, 1:1, vol/vol) supplemented with transferrin (0.01 mg/ml), insulin (0.001 mg/ml), aprotinin (0.001 mg/ml), L-glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml) and were plated in aliquots of 200 μl in a 96-well plate that had been previously coated with supplemented DMEM/F-12 containing 4% fetal bovine serum for 8 hr at 37°C. After culture for 24 hr at 37°C in 5% CO_2/95% air, test substances were added, and the cells were cultured for an additional 48 hr. The cells were then stained with crystal violet, and the hypertrophy was scored visually. For historical reasons, a score of 3 is given to cells incubated without a hypertrophy factor; a score of 7 is for maximal hypertrophy, such as that induced by 0.1 mM phenylephrine. The cells shown in Fig. 1 are representative of a hypertrophy score of 3 (unconditioned) and 7 (conditioned).

For staining of myosin light chain 2 (MLC2), myocytes were fixed and stained on microscope slides by indirect immuno-

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Abbreviations: ANP, atrial natriuretic peptide; LIF, leukemia inhibitory factor; CNTF, ciliary neurotrophic factor; OSM, oncostatin M; IL, interleukin; MLC, myosin light chain; CT, cardiotrophin.

¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. U18366).

fluorescence as described (13), with the exception that the cells were blocked with 5% normal donkey serum and indirectly labeled with fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG $F(ab')_2$ fragments (Jackson ImmunoResearch). Rat ANP concentrations were determined by competition for the binding of ^{125}I-labeled rat ANP for a rat ANP receptor A–IgG fusion protein (25) in an eight-well dilution series.

Embryoid Bodies. Mouse embryonic stem cells (American Type Culture Collection CRL 1934) were differentiated into embryoid bodies by growth for 6 days in DMEM (high glucose) containing 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol, 20% heat-inactivated fetal bovine serum, and penicillin/streptomycin. These cells were then changed to serum-free DMEM/F-12 for 24 hr. This conditioned medium was concentrated 10-fold (Amicon YM10) and assayed for cardiac hypertrophy at a 2- to 4-fold dilution (in parallel with concentrated unconditioned medium).

Expression Cloning. Poly(A)$^+$ RNA (26, 27) was used to prepare a cDNA library (EBL3) in the plasmid expression vector pRK5B (28) by a vector priming strategy (29). In brief, pRK5B was linearized at the *Not* I site, treated with alkaline phosphatase, and ligated to the single-stranded oligonucleotide ocdl.1.3 (5′-GCGGCCGCGAGCTCGAATTCT$_{30}$-3′). The ligated product was then cut with *Bst*XI, and the 4700-bp vector fragment was isolated by agarose gel electrophoresis. The vector was further purified by oligo(dA) chromatography. The expression library was constructed with 1 μg of poly(A)$^+$ RNA, 5 μg of vector primer, and reagents from Amersham. After first- and second-strand synthesis and T4 DNA polymerase fill-in reactions, the material was sized for inserts of >500 bp by gel electrophoresis and circularized by blunt-end ligation without the addition of linkers. The ligation products were used to transform *Escherichia coli* DH5α cells by electroporation. From 1 μg of poly(A)$^+$ RNA, 499 ng of double-stranded cDNA was generated, 17 ng of cDNA was ligated, and 3.3 ng was transformed to yield 780,000 clones, 83% of which had inserts with an average size of 1470 bp.

DNA was isolated from pools of 75–15,000 clones (primary pools) and transfected into human embryonic kidney 293 cells by Lipofectamine transfection (GIBCO/BRL). Two micrograms of DNA was used to transfect ≈200,000 cells in six-well dishes; the cells were incubated in 2 ml of serum-free medium for 4 days. Transfection and expression efficiency were monitored by the inclusion of 0.2 μg of DNA for a plasmid expressing a secreted form of alkaline phosphatase (30). One hundred microliters of conditioned culture medium from each transfected pool was assayed for hypertrophy in a final volume of 200 μl. For some pools the conditioned medium was concentrated (Amicon, Centricon 3) 4- to 5-fold before assay. For sib selection, the primary positive pool of 190 clones was divided into subpools of 80, 20, and finally individual clones.

The DNA sequence of the cDNA clones was determined by dideoxy DNA sequencing (31). Clone pRK5B.chf.781 has a cDNA insert of 1345 bp followed by a poly(A·T) stretch of ≈100 bp. The cDNA insert of clone pRK5B.chf.437.48 matches pRK5B.chf.781 beginning at base 27 and extending through the 3′ poly(A) sequence.

Expression and Purification of the CT-1 Fusion Protein. The reading frame encoding CT-1 (beginning at aa 2) was cloned C-terminal to the sequence encoding the first 53 aa of the herpes simplex virus glycoprotein D followed by a factor Xa cleavage site. Following expression in 293 cells and cleavage of the herpes secretion signal sequence, this construct is expected to give a 34-aa N-terminal extension to CT-1 of KYALADASLKMADPNRFRGKDLPVLDQLLEIEGR followed by the CT-1 sequence SQREGSL .... This fusion protein was purified from conditioned medium with a monoclonal antibody (5B6) affinity column. This antibody is specific for this portion of the herpes glycoprotein D sequence. The fusion protein was quantified by colorimetric assay (Bio-Rad), and the N-terminal sequence was confirmed by amino acid sequencing.

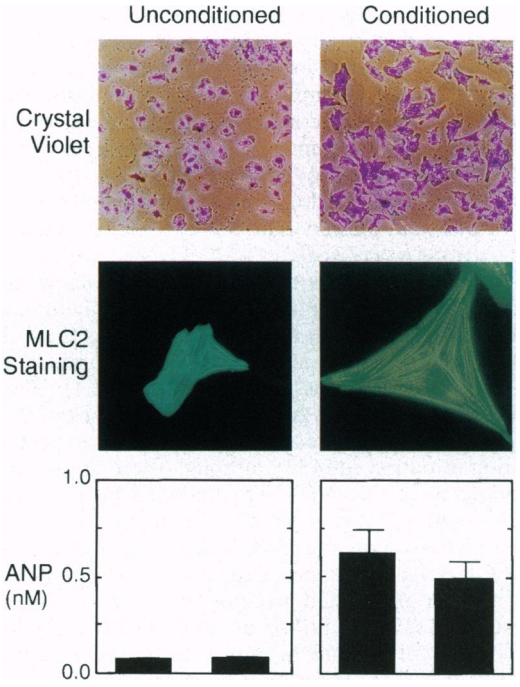

FIG. 1. Hypertrophy induced by embryoid-body conditioned medium. Hypertrophy was assessed by visual examination of the cells following crystal violet staining (*Top*) (×90), by staining with antibody to MLC2 (*Middle*) (×90), and by determination of the ANP concentration of duplicate hypertrophy supernatants (*Bottom*). Error bars show the estimated standard error of the ANP determinations.

RESULTS

Conditioned medium from differentiated embryoid bodies induced a clear hypertrophic response in neonatal cardiac myocytes as judged by three independent criteria: an increase in cell size, organization of MLC2 into sarcomeric units, and ANP secretion (Fig. 1). Significantly greater hypertrophy was induced than that found for maximally effective doses of endothelin or angiotensin II (Table 1), peptides that have been reported to induce hypertrophy *in vitro* (16, 32). The hypertrophic activity produced by embryoid bodies peaked at 6–7 days of differentiation (data not shown), and the activity was sensitive to proteases (Pronase) and heat (90°C, 60 min).

As the basis for expression cloning of the hypertrophic factor, a miniaturized assay was developed in which hypertro-

Table 1. Hypertrophy assay

Test cytokine(s)	Conc., nM	Hypertrophy score*
EB conditioned medium†	3‡	7
Unconditioned medium	3‡	3
None	0	3
Endothelin 1	1	5
	10	5
	100	5
Angiotensin II	10	3
	100	3
	1000	3

*A score of 3 is no hypertrophy; 7 is maximal hypertrophy (see *Materials and Methods*).
†Conditioned medium of 6- to 7-day embryoid bodies.
‡Fold concentration of the medium.

phy is scored visually following crystal violet staining of neonatal rat cardiac myocytes (*Materials and Methods*). Pools of clones from an expression cDNA library prepared from 6- to 7-day differentiated embryoid bodies were transfected into human 293 cells, and the conditioned medium was assayed for hypertrophic activity. From 1172 pools screened, two weakly positive pools containing 190 and 700 clones were found. Isolation of the positive clone from each pool by sib selection and hybridization showed that the two clones were closely related.

DNA sequencing of the two positive clones shows that the cDNA inserts are about 1400 bp and differ only in length. A G+C-rich region of about 650 bp encompasses the 5′ half of the inserts and includes an open reading frame of 203 aa (translated molecular mass, 21.5 kDa) (Fig. 2*A*). The DNA sequence surrounding the ATG codon at the start of this open reading frame matches the consensus sequence expected for a translation initiation site (35), although there are no in-frame stop codons 5′ of this ATG. The encoded protein sequence (Fig. 2*B*) contains one cysteine residue and one potential N-linked glycosylation site (33); it does not contain a hydrophobic N-terminal sequence indicative of a secretion signal (36). A number of secreted proteins lack a cleaved N-terminal signal sequence; some utilize an internal hydrophobic segment, whereas others are proposed to be secreted via nonclassical routes (37). The clones contain a mouse B1 repeat (38) of about 150 bp in the 3′ untranslated region and a poly(A) sequence at the 3′ end (preceded by two AATAAAA polyadenylylation signals) (Fig. 2*A*). No match of the DNA sequence was found in searches of GenBank, December 1994.

Transfection into human 293 cells of the two initially identified clones or of an expression plasmid containing only the 203-aa open reading frame yielded conditioned media that induced cardiac hypertrophy (Fig. 3). The induced response was positive for all three measures of hypertrophy—cell enlargement, MLC organization, and ANP induction. Coupled *in vitro* transcription/translation of the isolated clone gave one major band (≈22 kDa) that was active for hypertrophy as well (data not shown). Based on its cardiac hypertrophy-inducing activity, we designate this protein CT-1.

The amino acid sequence of CT-1 has some similarity with that of LIF (24% identity) and CNTF (19% identity) (Fig. 2*B*). These proteins are members of a family including oncostatin M (OSM), interleukin 6 (IL-6), and IL-11 (34, 39, 40). While members of this family are only distantly related in primary sequence (15–20% amino acid identity), they are predicted to have similar tertiary structures containing four amphipathic helices (34). Analysis of the helices predicted for CT-1 based on the sequence alignment (Fig. 2*B*) indicates that they are amphipathic, as would be expected for a member of this family. CNTF, like CT-1, lacks a hydrophobic N-terminal secretion signal sequence.

CT-1 is a potent inducer of myocyte hypertrophy; activity can be detected with 0.1 nM or lower concentrations of an N-terminal fusion protein (Fig. 3; Table 2). Proteins related to CT-1 also induce hypertrophy (Table 2) with a ranking of potency of CT-1 ≥ mouse LIF > human IL-11 > human OSM >> rat CNTF or mouse IL-6. The shape of the hypertrophied cells induced by these proteins and by CT-1 is more elongated than that induced by phenylephrine or by embryoid-body conditioned medium (compare Figs. 1 and 3). Thus, additional factors that influence cardiac hypertrophy may be produced by embryoid bodies.

Seven-day embryoid bodies express a single 1.4-kb mRNA species encoding CT-1 (Fig. 4*A*), approximately the size of the isolated cDNA clones. A 1.4-kb band was also expressed in adult mouse mRNA from heart, skeletal muscle, liver, lung, and kidney; a weaker band was found in testis and brain; no

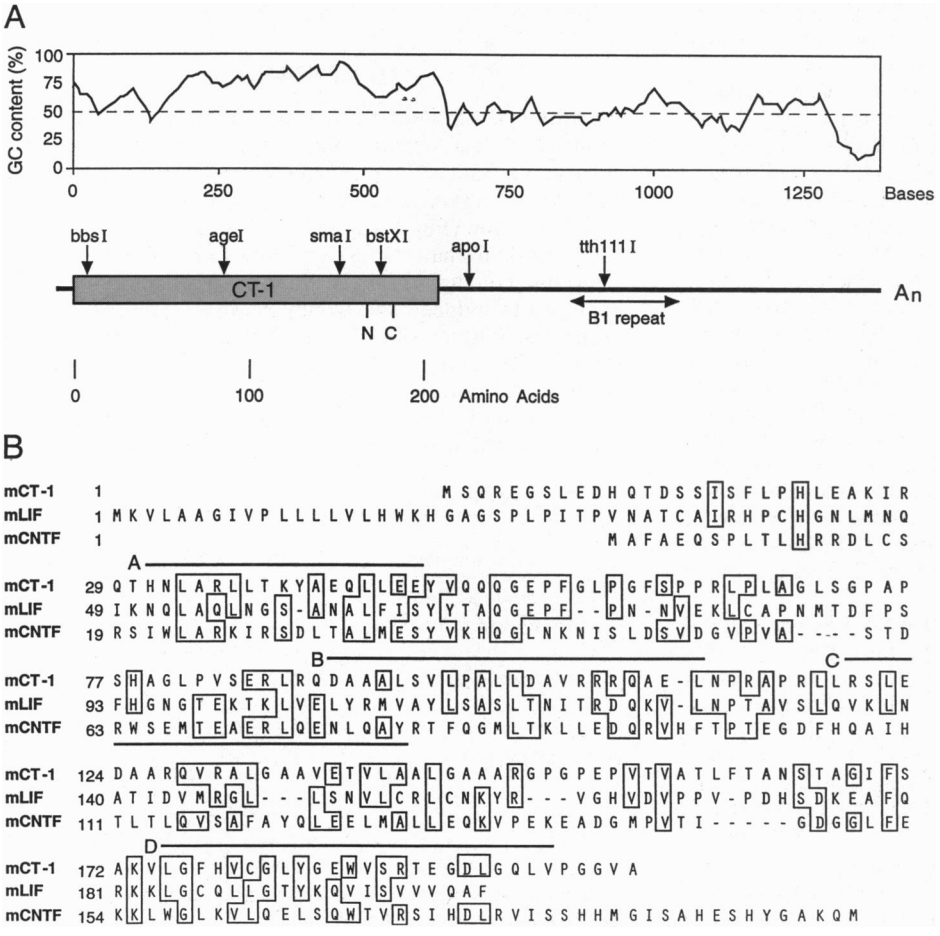

FIG. 2. Map of cDNA clones encoding CT-1 and an alignment of the protein sequence. (*A*) Map of the DNA sequence. Boxed region is the open reading frame encoding CT-1. Unique restriction enzyme sites are shown with arrows; N indicates the potential N-linked glycosylation site (33); C, the cysteine residue; An, poly(A). (*B*) Encoded amino acid sequence of mouse CT-1 (mCT-1) aligned with that of mouse LIF (mLIF) and mouse CNTF (mCNTF). Overlining indicates the location of four amphipathic helices based on their proposed locations in CNTF (34). As a quantitative measure of their amphipathic character (34), the mean helical hydrophobic moments ($\langle\mu_H\rangle$) for the four CT-1 segments (maximum of 18 residues) are 0.59, 0.34, 0.59, and 0.34 for helices A–D, respectively.

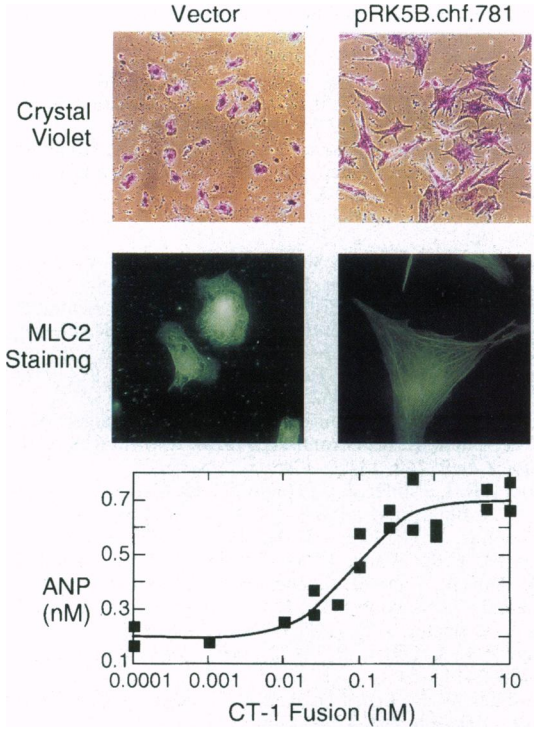

FIG. 3. Hypertrophy induced by recombinantly expressed CT-1. Human 293 cells were transfected by lipofection with pRK5B.chf.781, one of the two initially identified cDNA clones expressing CT-1, or with the vector, pRK5B. After 4 days of incubation without serum, the conditioned medium (without concentration) was assayed for hypertrophy. Hypertrophy was assessed by visual examination of the cells following crystal violet staining (*Top*) and by staining with MLC2 antibody (*Middle*). For the determination of potency, the ANP response was quantified for CT-1 that was expressed and purified as a fusion protein (*Materials and Methods*) (*Bottom*).

expression was observed in spleen (Fig. 4*B*). Thus, CT-1 is expressed in a number of mouse tissues and might be expected

Table 2. Hypertrophy assay of CT-1-related cytokines

Cytokine	Conc., nM	Hypertrophy score*
None	0	3
CT-1 fusion	0.05	6
	0.1	5
	0.25	6
	0.5	6.5
	1.0	7
Mouse LIF	0.05	4
	0.25	5.5
	2.5	6
Human IL-11	0.1	3.5
	0.2	4.5
	0.5	4.5
	1.0	4.5
	2.0	5.5
Human OSM	6.25	4.5
	12.5	4.5
	25	5
	50	6
Mouse IL-6	50	3.5
	100	3.5
Rat CNTF	25	4
	100	4

*A score of 3 is no hypertrophy; 7 is maximal hypertrophy (see *Materials and Methods*).

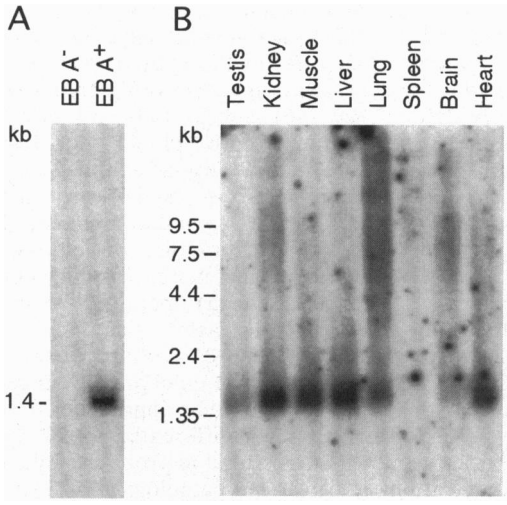

FIG. 4. Expression of RNA encoding CT-1. (*A*) Samples (4 μg per lane) of poly(A)$^-$ and poly(A)$^+$ RNA from seven-day embryoid bodies (EB A$^-$ and EB A$^+$) were electrophoresed in a formaldehyde/1.2% agarose gel and transferred to a nylon membrane (41). (*B*) Blot of mouse tissue poly(A)$^+$ RNA (Clontech). For both blots the labeled CT-1 probe was a 282-bp PCR fragment centered near the stop codon. Hybridization was in 50% formamide/0.75 M NaCl/0.075 M trisodium citrate at 42°C, with washes in 30 mM NaCl/3 mM trisodium citrate at 55°C.

to have functions outside the heart. A clear band of 1.4 kb was also found in mRNA isolated from cardiac non-myocytes (mostly fibroblasts) (data not shown) suggesting that CT-1 may be responsible for at least a portion of the hypertrophic activity produced by these cells (42).

DISCUSSION

Embryonic stem cells have proven to be an extraordinarily valuable resource in the study of biological systems. Here, we show that upon differentiation into embryoid bodies, these cells produce an activity that induces cardiomyocyte hypertrophy *in vitro*. An initial characterization of this activity suggested that it might be due to a novel protein or proteins, and we have utilized an expression cloning approach to identify one such protein, CT-1. When expressed as the native material, CT-1 induces cardiac hypertrophy as judged by cell enlargement, MLC organization, and ANP induction. Determination of the potency of CT-1, based on the expression and purification of an N-terminal fusion protein, shows that CT-1 is active at 0.1 nM or lower concentrations. The precise phenotype induced by CT-1 differs somewhat from that induced by embryoid-body conditioned medium, suggesting that additional factors that influence or induce cardiomyocyte hypertrophy remain to be characterized from this source. Determination of the quantitative contribution CT-1 makes to the activity found in embryoid-body condition medium will await the availability of suitable antibodies.

Amino acid sequence similarity data as well as structural considerations indicate that CT-1 is a member of the LIF/CNTF/OSM/IL-6/IL-11 family of cytokines. It is about as similar in sequence to members of the family as the other members are to one another. These cytokines mediate an overlapping set of pleiotropic actions on a variety of cell types including hepatocytes, megakaryocytes, osteoclasts, and neuronal cells (43, 44). Members of this family—in particular LIF, IL-11, and OSM—are active, like CT-1, in inducing cardiac hypertrophy *in vitro*. The members of this family are ligands for receptors that use the transmembrane signaling protein gp130 (43–45). Perhaps these cytokines and the gp130 signaling pathway have a greater role in cardiac hypertrophy or devel-

opment than previously recognized. It will be of interest to determine whether CT-1 uses similar signaling pathways to activate the distinct features of the hypertrophic phenotype and whether these pathways intersect downstream with the previously defined Ras (46) and G_q (23) pathways for the activation of this important adaptive physiological response.

These studies document the utility of coupling expression coupling to an embryonic stem cell model of cardiogenesis to isolate embryonic-derived growth and developmental factors. The recent development of an embryonic stem cell-based assay system for the specification and maturation of ventricular muscle lineages (unpublished data) opens the possibility of using this approach to isolate factors that mediate other important steps during cardiogenesis. Because other cell types also arise during differentiation of embryonic stem cells, our studies show the feasibility of similar approaches to identify factors which influence other differentiated cell lineages. While further work will be required to probe the role of CT-1 in the onset of physiological and pathological hypertrophy *in vivo* and in the complex process of vertebrate cardiogenesis, the identification of cytokines such as CT-1 represents an important step in the understanding and potential treatment of heart failure.

We thank Kirk Knowlton, Wanda Miller-Hance, Cathy Newton, and Monique LaCorbiere, who participated in early stages of this work, and Audrey Goddard for proofreading the DNA sequence.

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