Cardiotrophin 1 (CT-1) Inhibition of Cardiac Myocyte Apoptosis via a Mitogen-activated Protein Kinase-dependent Pathway

DIVERGENCE FROM DOWNSTREAM CT-1 SIGNALS FOR MYOCARDIAL CELL HYPERTROPHY*

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Cardiac myocyte survival is of central importance in the maintenance of the function of heart, as well as in the development of a variety of cardiac diseases. To understand the molecular mechanisms that govern this function, we characterized apoptosis in cardiac muscle cells following serum deprivation. Cardiotrophin 1 (CT-1), a potent cardiac survival factor (Sheng, Z., Pennica, D., Wood, W. L., and Chien, K. R. (1996) Development (Camb.) 122, 419–428), is capable of inhibiting apoptosis in cardiac myocytes. To explore the potential downstream pathways that might be responsible for this effect, we documented that CT-1 activated both signal transducer and activator of transcription 3 (STAT3)- and mitogen-activated protein (MAP) kinase-dependent pathways. The transfection of a MAP kinase kinase 1 (MEK1) dominant negative mutant cDNA into myocar
dial cells blocked the antiapoptotic effects of CT-1, indicating a requirement of the MAP kinase pathway for the survival effect of CT-1. A MEK-specific inhibitor (PD98059) (Dudley, D. T., Pang, L., Decker, S.-J., Bridges, A. J., and Saltiel, A. R. (1995) Proc. Natl. Acad. Sci. USA 92, 7686–7689) is capable of blocking the activation of MAP kinase, as well as the survival effect of CT-1. In contrast, this inhibitor did not block the activation of STAT3, nor did it have any effect on the hypertrophy response elicited following stimulation of CT-1. Therefore, CT-1 promotes cardiac myocyte survival via the activation of an antia apoptotic signaling pathway that requires MAP kinases, whereas the hypertrophy induced by CT-1 may be mediated by alternative pathways, e.g. Janus kinase/STAT or MEK kinase/c-Jun NH2-terminal protein kinase.

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CT-1. In contrast, this inhibitor did not block the activation of STAT3, nor did it have any effect on the hypertrophy response elicited following stimulation of CT-1. Therefore, CT-1 promotes cardiac myocyte survival via the activation of an antia apoptotic signaling pathway that requires MAP kinases, whereas the hypertrophy induced by CT-1 may be mediated by alternative pathways, e.g. Janus kinase/STAT or MEK kinase/c-Jun NH2-terminal protein kinase.

Cardiac muscle cell survival plays a critical role in maintaining the normal function of the heart and possibly in cardiac development. Adult cardiac muscle cells are terminally differentiated and therefore have lost their proliferative capacity. In contrast to skeletal muscle, the myocardium does not contain satellite heart muscle cells, and irreversible heart injury results in scarring and an eventual decrease in global cardiac function. In response to mechanical stimuli and hemodynamic stress, the adult myocardium activates an adaptive hypertrophic response that is characterized by an increase in myocardial cell size without a concomitant increase in myocyte number (For review, see Refs. 1 and 2). However, during long-standing exposure to hypertension or other forms of hemodynamic stress, a distinct form of myocardial cell hypertrophy can be activated in which the heart becomes dilated and individual cardiac myocytes exhibit an increase in cell length, reflecting the addition of new sarcomeric units in series (3, 4). This dilatation of the heart is usually accompanied by fibrosis, microscarring, and the loss of viable cardiac myocytes throughout the myocardium. As a result of cardiac dilatation and myocyte dropout, the myocardium ultimately develops an irreversible loss of function and ensuing cardiac muscle failure (4). As such, the identification of the signaling pathways that mediate distinct forms of cardiac muscle cell hypertrophy, dysfunction, and cardiac muscle cell survival are critical to the ultimate elucidation of the molecular basis of cardiac muscle failure.

By coupling expression cloning with an embryonic stem cell-based model of in vitro cardiogenesis (5), recent studies have identified cardiotrophin 1 (CT-1), a novel cardiac cytokine that was isolated in a search for new factors that induce cardiac myocyte hypertrophy (5). CT-1 is a new member of the IL-6 family of cytokines that exert their biological effects through the shared signaling subunit gp130 (3, 5–7) and can activate a distinct form of myocardial cell hypertrophy that is characteristic of volume overload cardiac hypertrophy at the molecular, morphological, and cellular levels (5). Importantly, cardiotrophin 1 has been shown to be capable of promoting survival of both embryonic and neonatal rat ventricular muscle cells (8). Recent studies have demonstrated that CT-1 exerts its effects on cardiac muscle cell hypertrophy through promoting the het
erodimerization of gp130 with the leukemia inhibitory factor

1 The abbreviations used are: CT-1, cardiotrophin 1; MAP, mitogen-activated protein; MEK, MAP kinase kinase; STAT, signal transducer and activator of transcription; PBS, phosphate-buffered saline; IL-6, interleukin 6; LIF, leukemia inhibitory factor; JAK, Janus kinase; CNTP, ciliary neurotrophic factor; MLC-2v, ventricular myosin light chain 2; ANF, atrial natriuretic factor; ERK, extracellular regulated kinase; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling.
programmed cell death of cardiac myocytes, known concerning the mechanisms by which CT-1 promotes the downstream hypertrophic response (3). Relatively less is characterization of an CT-1 on myocyte survival and hypertrophic responses. To address these questions, the current study reports the characterization of an in vitro cardiac muscle assay system in which apoptosis is induced following serum deprivation of myocytes that are plated at a relatively low density. In this assay system, we document the onset of cardiac myocyte cell death via apoptotic pathways by two independent criteria, i.e., scoring for nuclear changes associated with apoptosis and the presence of internucleosomal DNA fragmentation. The addition of CT-1 is capable of promoting cardiac myocyte survival and blocking apoptosis. To explore the potential downstream pathways that might be responsible for this effect, we documented that CT-1 is capable of activating both STAT3- and MAP kinase-dependent pathways. To directly relate the activation of these pathways to the biological functions of CT-1, we transfected a MAP kinase kinase 1 (MEK1) dominant negative mutant cDNA into neonatal ventricular myocardial cells and found that the mutant was capable of blocking the antiapoptotic effects of CT-1 on individual cardiac myocytes, thereby indicating a requirement of MAP kinase activity for the survival effect of CT-1 on cardiac myocytes. In addition, in studies applying the MEK inhibitor (PD098059) (9), we observed that the inhibitor was capable of blocking the activation of MAP kinase, as well as the survival effect of CT-1. The inhibitor displayed specificity for the MAP kinase pathway, as it did not inhibit the activation of STAT3, nor did it have any effect on the hypertrophic response elicited following stimulation of CT-1. Taken together, these studies indicate that CT-1 promotes cardiac myocyte survival by preventing apoptosis through a signaling pathway that requires MAP kinase. In addition, MAP kinase does not appear to be required for the activation of a CT-1-dependent hypertrophic response, indicating that CT-1 uses divergent signaling pathways for the activation of the survival and hypertrophic responses, the latter of which may be mediated by a JAK/STAT or MEK kinase/c-Jun NH2-terminal protein kinase pathway.

**EXPERIMENTAL PROCEDURES**

**Cytokines and Antibodies**—Murine LIF, IL-6, and ciliary neurotrophic factor (CNTF) were obtained from Life Technologies, Inc., Genzyme, and Boehringer Mannheim, respectively. Mouse CT-1 was a kind gift from Diane Pennica (Genentech Inc., South San Francisco, CA); STAT3 monoclonal antibodies were purchased from Upstate Biotechnology Inc.; ERK1 and ERK2 monoclonal antibodies were obtained from Transduction Laboratories and protein A-Sepharose CL-4B (Pharmacia Biotech Inc.) for 1 h at 4 °C. The precipitate samples were then diluted with a kinase buffer containing 20 mM Hapes, pH 7.2, 4 mM MgCl2, 1 mM dithiothreitol, 0.05 mg/ml bovine serum albumin, and 0.25 μg of dNTPs was added to the cells. Following incubation at room temperature for 30 min and boiling for 5 min, equal samples were loaded onto 15% SDS-polyacrylamide gels electrophoresis. Bradford protein assay was used to equilibrate the cells. Terminal deoxynucleotidyl transferase reaction solution containing 0.2 mM potassium carboxylate H7.2, 4 mM MgCl2, 1 mM dNTPs, 0.5 μg/ml bovine serum albumin was used to equilibrate the cells. MAP kinase activity was measured in the presence or absence of cytokines for various periods. The cells were then washed with cold PBS, and the DNA was collected by using a DNA isolation kit (Purogene). 2 μg of DNA was loaded onto a 2% agarose gel. TUNEL assays were performed after the cells were fixed with 4% paraformaldehyde for 30 min at 25 °C and washed with PBS three times. Terminal deoxynucleotidyl transferase reaction solution containing 2 mM biotin-conjugated dUTP (Boehringer Mannheim) and 10 units of terminal deoxynucleotidyl transferase (Life Technologies) was added to the cells for 60 min in a 37 °C humidified incubator. After washing with PBS containing 0.2% Tween 20, the cells were incubated with a fluorescein isothiocyanate-tagged anti-biotin monoclonal antibody (Jackson Laboratory) (1:500) for 1 h at 37 °C. After washing with PBS containing 0.2% Tween 20, the cells were then imaged with fluorescent microscopy.

**Transfection and Immunostaining**—For calcium phosphate transfections, purified cardiac myocytes were exposed to a cDNA-calcium phosphate precipitate 24 h after plating (11). A firefly luciferase cDNA controlled by a Rous sarcoma virus promoter and pcDNA3 containing the MEK1 K97M mutant (12) (a kind gift from Natalie Ahn, University of Colorado, Boulder, CO) were co-transfected. Following transfection, the cells were washed and maintained in serum-free medium in the presence of 2% FBS for 4 days and fixed. Immunostaining was performed as described previously (10). MLC-2v antibody or α-smooth muscle actin monoclonal antibody (Jackson Laboratory) were used to identify cardiac myocytes; an anti-luciferase antibody (Cortex) was used to identify successfully transfected cells. Hoechst dye was used to stain nuclei to identify apoptosis.

**Tyrosine Phosphorylation Assay for STAT-3**—After serum starvation for 24 h, cardiac myocytes (4 × 105/10 cm) were treated with agonists (1 ng/ml IL-6) and then incubated with a solution containing 10 μM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 μM DTT, 0.2 mM sodium vanadate, 0.2 μM phenylmethylsulfonyl fluoride, 1% Triton X-100, and 0.5% Nonident P-40. Cell lysates were centrifuged at 100,000 g for 1 h at 4 °C and with 20 μl of 50% suspension of anti-mouse IgG-agarose (Sigma) for an additional 30 min. The precipitate was extracted and subjected to Western blotting analysis using anti-STAT3.
were determined using one way analysis of variance.

mosomal DNA condensation, and fragmentation (Fig. 2, independent features of apoptosis, such as cell shrinkage, chromatin condensation and cell shrinkage that is characteristic of myocyte apoptosis. LIF and insulin-like growth factor 1 (100 nM), and transforming growth factor β (10 nM) failed to inhibit apoptosis (Fig. 1B; data not shown). Therefore, these results indicate that CT-1 is a potent survival factor for preventing myocyte apoptosis.

CT-1 Pathways of Apoptotic Blockade

RESULTS

Serum Deprivation Induces Apoptosis in Cultured Neonatal Rat Ventricular Myocytes—Previous studies have established a loss of cardiac myocyte survival in short-term culture in the absence of serum (8). To investigate whether cardiac myocyte death after serum deprivation is secondary to apoptosis, we first evaluated cells for the presence of internucleosomal cleavage by monitoring for DNA laddering, a hallmark of apoptosis, on agarose gels. The DNA fragmentation of cultured neonatal ventricular cells was observed after day 2 of serum deprivation and persisted throughout day 6 in the present assay system (Fig. 1A), whereas little DNA laddering was observed in the presence of serum (data not shown). DNA fragmentation cleavage was not present in noncardiac cells even after 10 days in serum-free media (Fig. 1C), documenting that the fragment DNA did not come from the small proportion (5%) of contaminating nonmuscle cells in the culture. Cells undergoing apoptosis were also identified at the single cell level by TUNEL staining and by nuclear staining with the Hoescht 33258 dye (Fig. 2). About 70% of myocytes maintained in serum-free media for 5 days were TUNEL stained positive and displayed small condensed nuclei, cell shrinkage, and nuclear fragmentation consistent with apoptosis (Fig. 2). Thus, it is clear that cardiac myocytes in the absence of serum displayed several independent features of apoptosis, such as cell shrinkage, chromosomal DNA condensation, and fragmentation (Fig. 2, D–F). These results indicate that, similar to other cell types such as lymphocytes and neural cells, cardiac myocytes also undergo apoptosis in the absence of defined growth factors.

CT-1 Inhibits Apoptosis in Serum-deprived Neonatal Rat Myocytes—To determine whether the effects of CT-1 on promoting embryonic and neonatal cardiac survival (8) are associated with inhibition of cardiac myocyte apoptosis, we examined the effects of CT-1 in cultured cardiac myocytes. As shown in Fig. 1B, we found that 1 nM CT-1 inhibited internucleosomal cleavage of genomic DNA in serum-deprived cardiac myocytes. This inhibition occurred in a concentration-dependent manner (data not shown). The antiapoptotic effect of CT-1 was further revealed by TUNEL staining and by staining the nuclei with Hoescht 33258 dye (Fig. 2, A–C). Fewer CT-1-treated cells (less than 10%) were positive for internucleosomal cleavage by TUNEL staining. In addition, CT-1-treated cells did not display chromatin condensation and cell shrinkage that is characteristic of myocyte apoptosis. LIF and insulin-like growth factor 1 were also effective in preventing apoptosis in cardiac myocytes, whereas IL-6, CNTF, basic or acidic fibroblast growth factors (100 nM), and transforming growth factor β (10 nM) failed to inhibit apoptosis (Fig. 1B; data not shown). Therefore, these results indicate that CT-1 is a potent survival factor for preventing myocyte apoptosis.

CT-1 Activates MAP Kinase in Cardiac Myocytes—Previous studies have documented that IL-6 could activate MAP kinase via gp130 (7, 13). To determine whether CT-1 can also activate MAP kinases in cardiac myocytes, neonatal ventricular muscle cells maintained in serum-free media were stimulated with CT-1 and other cytokines for various periods. Cellular lysates were then purified, and two forms of MAP kinase present in cardiac myocytes (ERK1 or ERK2) were immunoprecipitated using specific ERK1 and ERK2 antibodies. MAP kinase activity was subsequently determined by measuring its ability to phosphorylate (myelin basic protein). CT-1 activated both ERK1 and ERK2 by 4–5-fold at 10–15 min after stimulation (Fig. 3). This activity decreased to basal level after 30 min (data not shown), similar to the time course that was seen following phenylephrine stimulation of cardiac myocytes (11). In addition, Western blotting of immunoprecipitated proteins by ERK2 antibody revealed that CT-1 could induce the increase of the phosphorylation form of ERK2 protein (data not shown). In comparison with other cytokines, we found that LIF also activated MAP kinase activity (Fig. 3), but IL-6 had less of an effect in some experiments. Since IL-6 did not display detectable biological effects on cardiac myocyte proliferation, hypertrophy, and survival, or on the phosphorylation of gp130 and STAT3 (3, 8, Fig. 8), the low activation of MAP kinase most likely represents a signal derived from the effects of IL-6 on the 5% noncardiac cells that are present in the cultures. Thus, these results demonstrated that both CT-1 and LIF can activate MAP kinase pathways in cardiac myocytes.

MEK1 Dominant Negative Mutant Blocks the Survival Effects of CT-1—To determine whether the activation of MAP kinase is required for CT-1 inhibition of apoptosis in cardiac myocytes, we transiently expressed the dominant negative mutant of MEK1 (K97M) in cardiac myocytes. This mutant protein can be phosphorylated but does not activate the downstream kinases (12). The MEK1 (K97M) expression vector was cotransfected with Rous sarcoma virus luciferase into neonatal rat cardiac myocytes. The cells were then triple immunostained with an anti-luciferase antibody for identifying the transfected cells, an anti-myosin heavyweight chain monoclonal antibody for identifying cardiac myocytes, and Hoescht 33258 dye for identifying cells that were undergoing apoptosis. 27% of myocytes transfected with MEK1 (K97M) were apoptotic even in the presence of CT-1. However, only 6% of the myocytes transfected with the luciferase vector or empty backbone vector were apoptotic in the presence of CT-1 (Fig. 4). These results were independently confirmed by TUNEL staining, in which
23% of cells transfected with MEK1 (K97M) and stimulated with CT-1 were positive for apoptosis, compared with 5% in control groups (data not shown). Since transfection efficiency in cardiac myocytes is generally low (about 1–2% of cells), it is difficult to confirm using other assays, such as DNA laddering on agarose gels, that the MEK1 dominant negative mutant can inhibit antiapoptotic effects of CT-1. However, the results from the above assays are consistent with the results with a MEK inhibitor (see below). These data suggested that activation of a MAP kinase-dependent pathway is required for the survival effects of CT-1 on cardiac myocytes.

**PD098059 Inhibited the Activation of MAP Kinases and Survival Effects of CT-1**—Although the application of dominant negative mutants provides useful information for dissecting signaling pathways, it has limitations that include the low transfection efficiency in certain cell types and unwanted interaction of the mutants with other components. Small cell-permeable molecules, such as inhibitors for intracellular serine and threonine kinases, provide an alternative approach (9). PD098059 is a specific MEK inhibitor that selectively inhibits MEK1 activity (9, 14). This inhibitor blocks phosphorylation and activation of MAP kinase-induced growth factors (9, 14, 15). For example, nerve growth factor-induced differentiation of PC-12 cells was blocked effectively by this agent. To confirm that activation of MAP kinase is required for CT-1-stimulated inhibition of apoptosis in cardiac myocytes, we used PD098059 in our experimental system to determine whether this agent could inhibit the activation of the ERK1 and ERK2 kinases concomitantly with relieving the CT-1 inhibition of myocyte apoptosis. Similar to results in other cell systems, PD098059 inhibited the activation of both ERK1 and ERK2 in cardiac myocytes in a concentration-dependent fashion (Fig. 5). At 1 μM PD098059 a partial inhibition and at 10 μM a complete inhibition of CT-1-stimulated activation of ERK1 and ERK2 were...
observed. PD098059 had an almost identical inhibitory effect on these kinases in cardiac myocytes stimulated by phenylephrine (11). This inhibitor was then used to determine whether activation of MAP kinase by CT-1 was required for cardiac myocyte survival. As shown in Fig. 6, 10 \( \mu M \) PD098059 was able to completely inhibit the survival effects of 1 \( nM \) CT-1. Increasing the concentration of CT-1 to 100 \( nM \) was not able to prevent apoptosis in the presence of 10 \( \mu M \) PD098059. This result suggested that PD098059 might block the downstream signaling pathway by which CT-1 prevents apoptosis. It is notable that the survival effects of CT-1 in the current study are relatively higher than previously reported (8). The difference between the current and previous study is the cell density that was used in the in vitro assay system. It is clear that a higher cell density provides a more suitable condition for cardiac myocyte survival. Although the conclusions regarding the protective effects of CT-1 on both lower and higher cell density cultures are qualitatively identical, we believe that the currently modified experimental conditions constitute a more optimal assay system for determining the survival effects of growth factors on cardiac myocytes. To exclude the possibility of a nonspecific cytotoxic effect of PD098059, we tested whether it was capable of inducing cell death in the presence of serum. 5% fetal bovine serum was added to the myocytes after the addition of PD098059. Serum-induced survival was not inhibited by 10 or 100 \( \mu M \) PD098059 (Fig. 6B; data not shown). This result is in agreement with previous studies that showed that PD098059 has no obvious nonspecific cytotoxic effects on PC-12 cell lines (15). This result suggests that PD098059 may induce cell death by specifically inhibiting the MAP kinase pathway and not through a nonspecific cytotoxic effect on cardiac myocytes. Taken together, these results provided additional evidence that the activation of the MAP kinase pathway is required for the survival effects of CT-1 on cardiac myocytes.

**PD098059 Does Not Inhibit CT-1-stimulated Hypertrophy or Induction of ANF**—Since CT-1 also promotes cardiac myocyte hypertrophy (5), it is important to determine whether shared or divergent signaling pathways mediate the survival and hypertrophic effects of CT-1 on cardiac myocytes. One of the advantages of the in vitro hypertrophy model is that cultured cardiac myocytes can quickly respond to many stimuli that can induce morphological, biochemical, and molecular changes characteristic of hypertrophy. To determine whether CT-1 could stimulate hypertrophy in the presence of PD098059, we evaluated the changes in myocyte morphology and ANF gene expression. CT-1 stimulated an increase in cell size and an increase in ANF mRNA within 1–3 days after stimulation. This period precedes the onset of significant myocyte cell death in the presence of PD098059. Even though 10 \( \mu M \) PD098059 effectively inhibited the effect of CT-1 on the activation of MAP kinase and cell survival, it had little effect on the approximate 4-fold increase of ANF mRNA stimulated by CT-1 at 48 h after stimulation (Fig. 7A). Thus, PD098059 did not significantly affect ANF expression induced by CT-1 (1 \( nM \)). As reported previously, CT-1 induces a form of hypertrophy characterized by elongation of cardiac myocytes and the assembly of new sarcomeric units in series (3, 5). PD098059 was not able to block the ability of CT-1 to induce these morphological changes, even at day 4, when PD098059 induced a significant amount of cell death (Fig. 7B). These findings suggested that CT-1 activation of myocyte hypertrophy does not require MAP kinase signaling.

**PD098059 Does Not Block the Activation of the STAT3 Pathway by CT-1**—The findings described above suggest that there is a MAP kinase-independent signaling pathway that may mediate the CT-1-stimulated myocyte hypertrophy. Recently, constitutive activation of gp130 by the overexpression of both IL-6

**Fig. 5. Inhibition of the activation of MAP kinases by a MAP kinase inhibitor, PD098059.** PD098059 (PD) was added to cardiac myocytes 30 min prior to the addition of CT-1. The cells were cultured for 15 min, and ERK1 and ERK2 were immunoprecipitated. The precipitates were assayed for the ability to phosphorylate myelin basic protein. A, effects on the activation of ERK1. B, effects on the activation of ERK2. Each experiment was repeated twice.

**Fig. 6. Effects of PD098059 on the survival of cardiac myocytes promoted by CT-1 and serum.** PD098059 (PD) was added to the culture 30 min prior to the addition of CT-1 or 5% fetal bovine serum. The cells were then assessed for viability at various time points with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (see “Experimental Procedures”). A, concentration dependence. The cells were cultured for 6 days in the presence of PD098059 and CT-1. B, time course. Each point is the average from three wells, and each experiment was repeated three times. In a control, 80% cardiac myocytes cultured in 5% fetal bovine serum remained alive up to 7 days (data not shown).
and IL-6 receptor in the hearts of transgenic mice was shown to be associated with ventricular hypertrophy and increased JAK/STAT3 activity (16). Thus, we sought to determine whether CT-1 is also able to activate STAT3 kinase. Stimulation of ventricular myocytes with both CT-1 and LIF increased phosphorylation of STAT3, whereas stimulation with IL-6 had no effect on STAT3 phosphorylation. To determine whether the activation of STAT3 by CT-1 required activation of MAP kinase, we incubated ventricular myocytes in the presence of CT-1 and 10 μM PD098059. Inhibition of MAP kinase activity was not able to prevent phosphorylation of STAT3, suggesting that CT-1 can activate both MAP kinase and MAP kinase-independent signaling cascades (Fig. 8B). These data also indicate that STAT3 activation is not sufficient to maintain cardiac myocyte survival when MAP kinase is inhibited by PD098059. It remains possible that JAK/STAT3 may be needed for CT-1-induced cardiac hypertrophy. In addition, these studies suggest the simultaneous activation of both JAK/STAT and MAP kinase pathways by CT-1 in cardiac muscle cells and may provide a useful system to study the differential regulation of signaling pathways for survival versus hypertrophy in cardiac myocytes.

**DISCUSSION**

*CT-1-dependent Pathways Promote Myocardial Cell Survival via Inhibition of Myocyte Apoptosis—* Adult cardiac muscle cells are terminally differentiated and have lost their proliferative capacity. As a result, the maintenance of cardiac muscle cell survival is critical for the maintenance of normal cardiac function. Although a wide variety of survival factors have been found for neuronal cells and several other terminally differentiated cell types (17–19), and several growth factors may play important roles in cardiac hypertrophy and cardiac development (20), relatively little is known about the specific combination of growth factors and/or cytokines that are required to maintain long-term survival of cardiac myocytes. In neuronal cell types, a number of peripherally derived neurotrophic factors have been shown to play a vital role in the regulation of the survival of spinal motoneurons at a variety of stages of development (21). In this regard, two members of the IL-6 family of cytokines, LIF and CNTF, have been shown to play an important role in maintaining the viability of motoneurons in long-
term culture and also in the in vivo context (21–25). Interestingly, mice that harbor a targeted disruption of the α subunit of the CNTF receptor or the β receptor of the LIF receptor die shortly after birth and display a severe loss of more than 48% of the motoneurons in the spinal cord and brain stem (26, 27). These results suggest that ligands that activate gp130 may play a critical physiological role in regulating survival of terminally differentiated cell types in vivo. However, the function of the growth factors or cytokines in cardiac myocyte survival and its signaling pathways by which this survival is conferred remain unclear.

Recently, CT-1 has been identified and cloned based on its ability to induce hypertrophy in cultured neonatal rat ventricular muscle cells (5). CT-1 is expressed relatively early during murine cardiogenesis (E8.5), at which time it displays preferential expression in cardiac muscle, with little expression found in the mesenchymally derived atrioventricular cushions and conotruncal ridges, which contribute to septation of the chambers and outflow tract, respectively (8). In later stages, cardiotrophin 1 is found in a wide variety of other tissues, including neuronal cells, dorsal root ganglion, and skeletal muscle (8). Functionally, CT-1 promotes in vitro cultured cardiac myocyte survival (8). Recently, cardiotrophin 1 has also been shown to display both in vitro and in vivo effects on survival of neonatal motoneurons following sciatic nerve axotomy (28). However, the mechanism underlying the survival effects of CT-1 remains unclear.

In this regard, the present study provides direct evidence that one of the mechanisms by which cardiotrophin 1 can promote the survival of terminally differentiated cell types is via the activation of pathways that ultimately lead to inhibition of the apoptotic signaling pathway. The present study characterizes an in vitro assay system whereby cardiac myocytes enter the apoptotic signaling pathway after the deprivation of serum. Using three independent criteria, TUNEL staining, internucleosomal DNA fragmentation, and nuclear condensation, we have documented that CT-1 can block the onset of apoptosis in individual cardiac muscle cells. This effect appears to be specific and does not simply represent an indirect trophic effect on the cells, as agents such as transforming growth factor β, which has been shown to have trophic effects on cultured neonatal rat ventricular muscle cells (20), are without significant effect. Finally, the addition of IL-6 or CNTF has relatively little effect, whereas the addition of another member of the IL-6 family (LIF), which uses an identical heterodimer pathway for activating downstream cardiac muscle cell responses via gp130 and LIF receptor β (3), has a similar effect on the blockade of the apoptotic signaling pathway. Insulin-like growth factor 1, which has been shown to be cardioprotective in a murine model of myocardial ischemia reperfusion (29), could also inhibit cardiac myocyte apoptosis. Taken together, these studies provide the first evidence that cardiotrophin 1 promotes cardiac myocyte survival through the activation of pathways that can interrupt the apoptotic signaling cascade. Recently, we also found that CT-1 could block the apoptosis in neonatal cardiac myocytes infected with Coxsackie virus. The effects of CT-1 on adult heart cells are currently under study.

MAP Kinase Is Required for CT-1-dependent Inhibition of Apoptosis—All members of the IL-6-LIF cytokine family trigger downstream signaling pathways in multiple cell types through the homodimerization of gp130 or the heterodimerization of gp130 and LIF receptor β (7). Until now, intracellular signaling pathways that couple the gp130 activation with the downstream cardiac cell responses have remained unclear. In other cell types, members of this family have been shown to activate the JAK/STAT pathway (7, 30–32). STAT3 is phosphorylated in response to IL-6-related cytokines and plays a critical role in gp130-mediated terminal differentiation and growth arrest of a myeloid cell line (32–34). It is also becoming clear that this family of cytokines can activate Ras and MAP kinase cascades, as well (7, 13). However, the distinctive role of this Ras/MAP kinase pathway versus the JAK/STAT pathway in the activation of downstream cellular responses of the IL-6 family is not completely clear.

Using two independent approaches, the current study provides direct evidence that MAP kinase-dependent pathways are required for the inhibition of cardiac myocyte apoptosis. Transfection studies with the MEK1 dominant negative mutant protein vector result in blockade of the CT-1 inhibition of myocyte apoptosis. To confirm the essential role of the MAP kinase pathway in the survival function of CT-1, we applied a MEK-specific inhibitor, PD098059, in our assay system. In addition, PD098059 prevents MEK1 activation by Raf and has been shown to have little effect on other kinases, including cAMP-dependent kinase, protein kinase C, and other serine and threonine kinases (9, 14, 15). This inhibitor does not inhibit c-Jun NH2-terminal protein kinase activation either. In the current study, PD098059 effectively inhibited the activation of ERK1 and ERK2 following CT-1 stimulation in a concentration-dependent manner but had little effect on activation of the JAK/STAT pathway, as assessed by the phosphorylation of STAT3, thereby providing further evidence for selectivity of these inhibitory effects on MAP kinase. In addition, treatment with this inhibitor significantly blocked the survival effects of CT-1 and led to an increase in the loss of cell viability. The time course of the onset of cell death induced by the inhibitor was similar to the time course found during serum deprivation, thereby suggesting that cell death may result from similar pathways. The fact that the effects of PD098059 on the cells cannot be rescued by higher concentrations of CT-1 suggested that PD098059 is selectively targeting downstream CT-1-dependent signaling pathways.

Recently, MAP kinase pathways have also been found to be necessary for nerve growth factor effects on promoting the survival of neuronal cell types (PC-12), whereas c-Jun NH2-terminal protein kinase activation and inhibition of MAP kinase has been shown to be critical for induction of apoptosis in these cells (35). The current study provides further evidence that MAP kinase-dependent pathways might play a particularly important role in promoting the survival of terminally differentiated cell types. However, it should be noted that MAP kinase-independent pathways for the inhibition of cardiac myocyte apoptosis also exist, since the current studies document that serum can block the apoptosis of cardiac myocytes in this cultured assay system even in the presence of the selective MAP kinase inhibitor. It will become of interest to determine whether similar MAP kinase-dependent signaling pathways operate in the in vivo context and to identify the downstream cellular effectors of this inhibition. In this regard, recent studies have suggested that the induction of B-cell lymphoma/leukemia X and 2 can result in the inhibition of apoptosis in a wide variety of cell types (36, 37). It will certainly become of interest to determine whether this induction is dependent on MAP kinase and whether this is sufficient to confer a protective effect on myocardial cells.

CT-1-dependent Hypertrophy Is Independent of MAP Kinase—In addition to its effects on promoting cardiac myocyte survival, CT-1 is also capable of activating a distinct form of

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2 E. Jeon, Z. Sheng, and K. Knowlton et al., unpublished observations.

3 J. H. Brown, unpublished data.
myocardial cell hypertrophy in a well characterized in vitro assay system (3). The overexpression of both IL-6 and IL-6 receptors in the heart of transgenic mice results in cardiac hypertrophy, which is associated with constitutive activation of STAT3 activity (16). In addition to gp130-dependent pathways for cardiac myocyte hypertrophy, it has been well documented that G protein-coupled receptors, including α-adrenergic and endothelin 1 receptor subtypes, can activate features of myocardial cell hypertrophy in an in vitro assay system (38–41). Moreover, Ras-dependent pathways appear to be both necessary and sufficient to activate hypertrophy in vitro and in vivo, as recently revealed by transgenic mice that express constitutively active Ras in the ventricular chamber under the control of the MLC-2v promoter (42). A sub strain of these mice display a distinct form of obstructive cardiac hypertrophy4 indistinguishable at a morphological, physiological, and pathological level from hypertrophic cardiomyopathy in the clinical setting. Interestingly, the phenotype seen in CT-1-stimulated cells correlates mostly with the volume overload hypertrophy phenotype, resulting in the addition of sarcomeric units in series as opposed to in parallel (3). In addition, divergent signaling pathways appear to mediate the activation of these distinct forms of hypertrophy in this neonatal assay system. Thus, although Ras-dependent pathways appear to be sufficient for activating hypertrophy in the system, it is not clear that MAP kinase is the main downstream effector for this response. In fact, recent studies have shown conflicting results regarding the requirement of MAP kinase pathways in hypertrophy induced by α-adrenergic agonists (11, 43, 44).

The latter activates both STAT3- and MAP kinase-dependent pathways. MAP kinase-dependent pathways appear to be responsible for the survival effects of CT-1; whereas STAT3 may mediate the effects of CT-1 on other cellular activities, such as morphological or gene expression changes.

CT-1 on cardiac muscle cells (Fig. 9). CT-1 activates downstream responses through promoting the heterodimerization of the LIF receptor and gp130 and subsequently activates JAK. The activated JAK may activate both STAT3 and the Ras/Raf/MAP kinase pathway. The activated MAP kinase pathway is responsible for the survival effects of CT-1; whereas STAT3 may mediate the effects of CT-1 on other cellular activities, such as morphological or gene expression changes.

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FIG. 9. A model of the potential mechanism for the biological effect of CT-1 and other cytokines. After CT-1 forms a heterodimer with its receptor and gp130, the latter may phosphorylate JAK. The activated JAK may activate both STAT3 and the Ras/Raf/MAP kinase pathway. The activated MAP kinase pathway is responsible for the survival effects of CT-1; whereas STAT3 may mediate the effects of CT-1 on other cellular activities, such as morphological or gene expression changes.

CT-1 or LIF gp130 and/or LIFRβ JAKs Ras/Ref STAT3 MAPK HYPERTROPHY SURVIVAL

Based on the results of the present and previous studies (7, 16, 30–33), we have proposed a working model of the downstream signaling pathways that may mediate the actions of

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