Activation of the Cytochrome c Gene by Electrical Stimulation in Neonatal Rat Cardiac Myocytes

ROLE OF NRF-1 AND c-Jun

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Activation of cytochrome c (cmt c) transcription in electrically stimulated neonatal rat cardiac myocytes is preceded by transient expression of the activating protein-1 family of transcription factors, c-Fos, c-Jun, and JunB, as well as nuclear respiratory factor-1 (NRF-1). Mutations in either the NRF-1 or in the two cyclic AMP response elements on the cyt c promoter significantly reduce cyt c promoter activation produced either by electrical stimulation (Xia, Y., Buja, L. M., Scarpulla, R. C., and McMillin, J. B. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11399–11404) or by transfection of c-jun into nonpaced cardiac myocytes. Electrical stimulation of cardiac myocytes activates the c-Jun N-terminal kinase (McDonough, P. M., Hanford, D. S., Sprenkle, A. B., Mellon, N. R., and Glembozski, C. C. (1997) J. Biol. Chem. 272, 24046–24053) so that the fold-activation of the cyt c promoter is increased by pacing when either c-jun or c-fos/c-jun were cotransfected. Physical association of NRF-1 protein with the NRF-1 enhancer element and of c-Jun with the cyclic AMP response element binding sites on the cyt c promoter was determined by gel shift competition assays and by antibody super shifts. This is the first demonstration that induction of NRF-1 and c-Jun by pacing of cardiac myocytes directly mediates cyt c gene expression and mitochondrial proliferation in response to hypertrophic stimuli in the heart.

Hypertrophic growth is a basic mechanism for increasing cell size in post-mitotic cardiac muscle. Hearts that have been subjected to hemodynamic stress increase intermediate early gene and contractile protein expression as part of the hypertrophic response. Likewise, in cardiac myocytes in culture, application of mechanical stimuli produces increases in the intermediate early genes c-fos, c-jun and junB (1–4), and re-expression of β-myosin heavy chain, all characteristic of hypertrophic growth (for review, see Ref. 5). When neonatal rat cardiac myocytes in culture are paced, both the content and organization of cellular contractile proteins increase (6). The increased energy demand resulting from electrical stimulation of cardiac myocytes is associated with increased mitochondrial number and cytochrome oxidase activity (7). The proliferation of mitochondria observed with electrical stimulation is coincidental with increased mRNA content for cytochrome oxidase subunit Va and cytochrome c (1, 7). Pacing also increases c-fos, β-myosin heavy chain, and cytochrome c promoter activities which coincide with the rise in their respective gene transcripts. These results are consistent with the interpretation that electrical stimulation of cardiac myocytes produces transcriptional activation of nuclear-encoded mitochondrial proteins. Therefore, electrical stimulation provides a model whereby physiologically meaningful expression of transcription factors specific to nuclear-encoded mitochondrial target genes in cardiac myocytes can be identified.

Pacing of cardiac myocytes induces a rise in cytoplasmic calcium, an event which has been demonstrated to activate multiple signaling pathways (8, 9). Atrial natriuretic factor (ANF) gene induction by electrical stimulation in neonatal cardiac myocytes is dependent on Ca2+ influx and calmodulin activity (6). Whereas the cAMP response element binding protein (CREB) functions as a Ca2+-regulated transcription factor and as a substrate for depolarization-activated Ca2+-calmodulin dependent protein kinases I and II in neuronal cell lines (10), McDonough and Glembozski (6) observed no change in cAMP formation after electrical stimulation of neonatal cardiac myocyte cultures. Ramirez et al. (11) demonstrated that activation of c-Jun N-terminal kinase (JNK) in neonatal rat ventricular myocytes and in adult myosin light chain-2v-Val-12 Ras mice is associated with cardiac hypertrophy. These authors concluded that maintenance of elevated JNK activity is important for development of the hypertrophic state. The latter data, together with observations that JNK is activated by Ca2+ (12, 13) and that transcriptional activation of the cardiac-specific anf promoter occurs by collaborative interactions between JNK, c-Jun, serum response factor and Sp-1 (2), suggest that there is participation of c-Jun in pathways involving Ca2+-activated gene expression and the hypertrophic response.

Recent studies from our laboratory also support a potential regulatory role for activating protein-1 (AP-1) as well as nuclear respiratory factor-1 (NRF-1) in the initiation of mitochondrial proliferation in response to electrical stimulation. The promoters of many nuclear genes encoding respiratory subunits are at least partially dependent on the nuclear respiratory factors, NRF-1 and NRF-2 (reviewed in Ref. 14). These nuclear transcription factors also act on genes that encode key components of mitochondrial transcription and replication suggesting that they may act to coordinate nuclear-mitochondrial interactions (15). Consistent with an important role of NRF-1

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1 CRE, cAMP response element; CREB, CRE binding protein; mCRE, murine CRE; JNK, c-Jun N-terminal kinase; AP-1, activating protein-1; NRF-1, nuclear respiratory factor-1; mNRF-1, murine NRF-1; cyt, cytochrome; Luc, luciferase; β-gal, β-galactosidase; Sp-1, GC-rich binding protein; TRE, phorbol 12-O-tetradecanoate 13-acetate-responsive element.
in mitochondrial gene activation, mutations in the NRF-1 site of the cyt c promoter inhibited the induction by electrical stimulation by 5-fold (1). Likewise, mutations in the CRE (ATF) sites of the cyt c promoter also significantly reduced the induction of this gene by electrical stimulation (1). Recent findings suggest that the Fos/Jun and ATF/CREB families of transcription factors interact and should be grouped into a superfamily of transcription factors (16). Since both NRF-1 and Fos/Jun mRNAs appear in time just prior to activation of cyt c gene expression, and since AP-1 factors play a central role in mitogenesis (17), we asked whether AP-1 is involved in cyt c gene expression, and whether this involvement is a direct interaction. The results herein describe the transcriptional response of the cyt c promoter to a hypertrophic stimulus in the neonatal cardiac myocyte.

**EXPERIMENTAL PROCEDURES**

**Cardiac Cell Culture and Electrical Stimulation—**Neonatal (1-3 day old) cardiac myocytes were isolated and plated in 12-well dishes in Dulbecco's modified Eagle's medium supplemented with 10% Hyclone calf serum at a plating density of 4 x 10^3 cell/cm^2 as described previously by this laboratory (7). After 24 h, the serum-containing medium was removed, and the cells were washed and subsequently maintained for 72 h in Dulbecco's modified Eagle's medium, in the absence of serum, containing 1% bovine serum albumin (fraction V). The medium was exchanged for fresh serum-free medium at 2 days. After 4 days in culture (24 h + serum, 72 h – serum), three wells containing myocytes were stimulated for 72 h using the method of Brevet et al. (18) as modified by McDonough and Glembocksi (6). Three additional wells in the dish were maintained in the same medium as the stimulated cells, but in the absence of contractile stimulation. During the experimental period, the medium bathing the control and stimulated cells was changed after 48 h to fresh Dulbecco's modified Eagle's medium + 1% bovine serum albumin.

**Preparation of cDNA Probes for Electrophoretic Mobility Shift Assays—**DNA sequences of oligonucleotides used to generate the cDNA probes for electrophoretic mobility shift (EMSA) assays were as follows. CRE-1-cyt (5'-CGTAAGCCGTCTGCGGCGTTT-3'); CRE-2-cyt (5'-GGAGATCGGGCGTACGCGCGCG-3'); CRE-3-cyt (5'-GTTATCGGATCCCCGACCTG-3'); NRF-1-cyt (5'-CCGTACGCGCGCCGGCC-3'); mFos-1 (5'-G TAGTGGCGACGAGGATTAATTTG-3'); mFos-2 (5'-G TGGCGACGAGGATTAATTTG-3'); mJun (5'-GAATATTTGATTT-3'); mNRF-1 (5'-ATTTTGGGTTTGGTTTTT-3'); EXP-1 (5'-GGGATTGCCTTTCGCTTG-3'); EXP-2 (5'-GGGATTGCCTTTCGCTTG-3').

**Preparation of RNA probes for Northern blot analysis—**After washing, the blots were visualized by autoradiography following storage at -70 °C for 24 h. The gel containing the 487-base pair CRE-Sp1 fragment includes coding exon 2 of the mouse mrf-1 gene in pGEM7zf. Radiolabeled RNA transcripts were synthesized from template DNA linearized with EcoRI as described by the Promega technical manual using Sp-6 RNA polymerase, ATP, GTP, UTP and [α-32P]CTP (0.5 μM each) in the presence of RNasin and spermidine. After DNase digestion, the cDNA probes were hybridized to 20 μg of total RNA and then digested with a double RNase A (Boehringer Mannheim), and the RNA/RNA hybrid was analyzed using 3.5% PAGE in the presence of 8 M urea. Autoradiography was performed as described above for Northern blots.

**Preparation of Plasmids for Transfection—**The plasmids pCB4Luc/172 and pCB4Luc/236 containing 1.1 kilobase pairs of the first intron and the 5' flanking region of rat cyt c were constructed by cloning the BglII and EcoRI sites of the cyt c promoter between pGEM-2 vector and EcoRI sites. The plasmids pJunB was constructed by cloning 1.2-kilobase pair fragment of JunB cDNA into pGEM-2 vector between EcoRI and Smal sites. The canonical AP-1 response element, TRE-Luc, and the neutral core promoter, Δ55 P, Luc, were generous gifts of Dr. Michael Schneidman-Duhme (Baylor College of Medicine).

**DNA Transfection—**Cardiac myocytes were grown in serum-containing medium for 24 h; the myocytes were transfected by the (Ca3P)2 precipitation method (19). To detect the interrelationship of the early gene products with subsequent expression of later genes, e.g. cyt c, 0.1 μg of pc-Fos, 1 μg of pc-Jun, and 1 μg of pJunB were cotransfected singly or in combination with 2 μg of wild type or the mutated cyt c promoter constructs (18). In each case, 2 μg of Rosa sarcoma virus β-galactosidase (β-gal) expression plasmid was cotransfected to monitor transfection efficiency. Six hours after transfection, the cells were washed twice and maintained in serum-free medium for another 12 h. Electrical stimulation was initiated and cell extracts were isolated either at the point when electrical stimulation began (zero time) or after 6, 12, 24, 48, and 72 h. The control cells were maintained under the same conditions as the stimulated cells, only in the absence of electrical stimulation. Luciferase and β-gal assays were performed (20) and normalized to protein content (21).

**Preparation of cDNA Probes for Electrophoretic Mobility Shift Assays—**DNA sequences of oligonucleotides used to generate the cDNA probes for electrophoretic mobility shift (EMSA) assays were as follows. CRE-1-cyt (5'-CGTAAGCCGTCTGCGGCGTTT-3'); CRE-2-cyt (5'-GGAGATCGGGCGTACGCGCGCG-3'); CRE-3-cyt (5'-GTTATCGGATCCCCGACCTG-3'); NRF-1-cyt (5'-CCGTACGCGCGCCGGCC-3'); mFos-1 (5'-G TGGCGACGAGGATTAATTTG-3'); mFos-2 (5'-G TGGCGACGAGGATTAATTTG-3'); mJun (5'-GAATATTTGATTT-3'); mNRF-1 (5'-ATTTTGGGTTTGGTTTTT-3'); EXP-1 (5'-GGGATTGCCTTTCGCTTG-3'); EXP-2 (5'-GGGATTGCCTTTCGCTTG-3').**
were cotransfected with the c-Fos-Luc and c-Jun promoter expression constructs and the TRE-Luc vector (controls). The results are representative of RNA isolated from three different cultures.

The NRF-1 site in the cyt c promoter resulted in 57% decrease from reporter gene activity when myocytes were transected with cyt c-jun (p < 0.05, Fig. 2). The dependence of the cyt c promoter on intact NRF-1 sites in the absence of electrical stimulation is consistent with up-regulation of NRF-1 mRNA observed with transfection of cyt c-jun (Fig. 1). Together, these results suggest that induction of cyt c expression is, either by transfection or by electrical stimulation (1), is important for subsequent expression of NRF-1. These experiments also raise the possibility that, in addition to NRF-1, intermediate early genes may also play a direct role in cyt c gene transcription in the heart.

**Transactivation of the Cyt c Promoter by Jun Requires both CRE and NRF-1 Sites**—Mutational analysis has revealed the essential role of CRE and NRF-1 sites in the cyt c promoter in the response of cardiac myocytes to electrical stimulation (1). To test the hypothesis that AP-1 proteins might map to one or both electrical stimulation response elements of the cyt c promoter (i.e. CRE and NRF-1), unstimulated cardiac myocytes were cotransfected with the cyt c-jun expression vector and a luciferase reporter gene driven by the wild-type or by series mutants of the cyt c promoter. Negative control experiments were carried out by transfection of empty vector or β-gal alone or with cyt c-jun, as well as transfection of the neutral core promoter, Δ56 Fos-Luc/β-gal alone or with cyt c-jun. There was no inductive response of the reporter gene in either of the negative control transfections (Fig. 2). The TRE-Luc/β-gal transfection served as a positive control. The unstimulated cardiac myocytes demonstrated a slight inductive response (p < 0.05) with the TRE expression construct alone, and this response was significantly increased (p < 0.01) with cotransfection with cyt c-jun (Fig. 2). Similarly, transfection of cyt c-β-gal alone produced a small but significant (p < 0.05) augmentation of reporter gene expression, and this increase was significantly enhanced with cotransfection with cyt c-jun (Fig. 2). Mutation of Sp-1 sites in the cyt c promoter did not alter transactivation by cyt c-jun. In contrast, mutation of both CRE sites reduced cyt c-jun-dependent promoter transcription by 80% (p < 0.05, Fig. 2).
petition assays were carried out using unlabeled CRE-1, CRE-2, mCRE-1, mCRE-2, and NRF-1, as well as authentic AP-1 oligonucleotide (Fig. 4B). Unlabeled probes specific for each element were effective competitors, whereas the mutated probes were not (Fig. 4B). Unlabeled AP-1 was tested for its ability to compete for extract protein binding to labeled CRE-1 and CRE-2. In each case, the observed shifts of both labeled enhancer oligonucleotides were abolished (Fig. 4B). These results demonstrate that protein factors binding to the CRE sites also bind the AP-1 probe with high affinity, consistent with the suggestion that there is a Jun-dependent transactivation of cyt c.

**Electrical Stimulation Activates Cyt c Transcription by c-Jun Binding to the CRE Sites**—The ability of the AP-1 oligonucleotide to compete with protein associated with the CRE sites on the cyt c promoter suggests that c-Jun might activate CRE by physical association with these sites. To identify the protein associated with CRE, supershifts were performed by using anti-c-Jun (Fig. 5). Anti-c-Jun completely blocked both CRE shifts (Fig. 5, CRE-1 and -2, lanes 3), suggesting that the protein associated with the CRE sites is c-Jun. Conversely, anti-NRF-1 did not interact with CRE-bound protein (Fig. 5, CRE-1 and -2, lanes 4). Parallel experiments were carried out using AP-1 as the consensus probe. Anti-c-Jun, but not anti-NRF-1, produced a supershift with protein bound to the AP-1 oligonucleotide (Fig. 5, AP-1, lanes 3 and 4), confirming that c-Jun was present in the nuclear extracts from the electrically stimulated myocytes. Other control experiments were carried out to confirm the specificity of the anti-c-Jun results. Anti-NRF-1 resulted in a supershift of protein bound to the NRF-1 site but not to the CRE sites (Fig. 5, NRF-1, lanes 3 and 4). In the negative control experiment, neither anti-c-Jun (Fig. 5, Sp-1, lane 3) nor anti-NRF-1 (Fig. 5, Sp-1, lane 4) was able to produce supershifts of protein bound to the Sp-1 probe.

CREB Is Not a Major Component of the CRE Site—Depolarization-induced calcium entry in pheochromocytoma cells leads to phosphorylation of CREB, contributing to Ca^{2+} activated gene transcription (22). Association of c-Jun protein with the CRE site on the cyt c promoter may involve formation of a tertiary complex with CREB, where activation of CREB is mediated through a calcium-dependent pathway (22). Gel shifts were carried out using anti-CREB to detect possible CREB association with the CRE sites on the cyt c promoter (Fig. 6A). While anti-Jun reduced the shift observed with labeled CRE probe, anti-CREB had no effect on the interaction between CRE and nuclear proteins extracted from paced myocytes (Fig. 6, lane 3 versus 4). Anti-NRF-1 had no effect on the shift with labeled CRE (Fig. 6, lane 5). To ensure that the absence of both supershifts and blocking by anti-CREB-1 was not due to an inadequate amount of antibody, larger amounts of anti-CREB (1.5, 2.0, and 3.0 μg) were used to interact with DNA-protein complex. The increased amounts of anti-CREB had no effect on the shifts (Fig. 6B, lanes 2–5).

**DISCUSSION**

Electrical stimulation of neonatal cardiac myocytes in culture is associated with expression of the immediate early genes, c-fos and c-jun, followed by the appearance of mRNA for the transcription factor, NRF-1. Up-regulation of these transcription factors precede the transcriptional activation of cyt c in these cells (1). NRF-1 plays a pivotal role in activation of nuclear-encoded mitochondrial gene expression as well as in the coordination of this process with increased mitochondrial DNA synthesis (14, 15). In electrically stimulated cardiac myocytes, both NRF-1 and CRE elements in the cyt c promoter are required for enhanced transcription of the cyt c gene (1). There is ample evidence that AP-1 components, and in particular
CRE-1, CRE-2, NRF-1, Sp-1, and AP-1 were end-labeled with $^{32}$P and incubated with nuclear extracts from control and stimulated cardiac myocytes as described under "Experimental Procedures." B, CRE-1 and CRE-2 were end-labeled with $^{32}$P and incubated with nuclear extracts from stimulated myocytes in the absence or presence of a 100-fold excess of the indicated, unlabeled competitors. The first two lanes in both panels (−) represent the absence of competitor where lane 1 is probe alone and lane 2 represents probe incubated with nuclear extracts from stimulated myocytes. The results are representative of three different cultures of cardiac myocytes.

**FIG. 4.** Increased binding of transcription factors to the CRE and NRF-1 elements with electrical stimulation. A, CRE-1, CRE-2, and NRF-1 were end-labeled with $^{32}$P and incubated with nuclear extracts from control and stimulated cardiac myocytes as described under "Experimental Procedures." B, CRE-1 and CRE-2 were end-labeled with $^{32}$P and incubated with nuclear extracts from stimulated myocytes in the absence or presence of a 100-fold excess of the indicated, unlabeled competitors. The results are representative of three different cultures of cardiac myocytes.

**FIG. 5.** The binding of c-Jun from nuclear extracts of stimulated cardiac myocytes to CRE sites in the cyt $c$ promoter. CRE-1, CRE-2, NRF-1, Sp-1, and AP-1 were end-labeled with $^{32}$P and incubated with nuclear extracts from electrically stimulated cells for 20 min before the addition of either 1 μg of anti-c-Jun or 1 μg of anti-NRF-1. In the lanes represented by A–E, lane 1 represents probe alone, lane 2 contains probe plus nuclear extract alone. In A, the nuclear extracts in lane 3 were incubated with anti-NRF-1, while extracts in lane 4 were incubated with anti-c-Jun. In panels B–E, nuclear extracts in lane 3 were incubated with anti-c-Jun and in lane 4 with anti-NRF-1. The results are representative of three different cultures.

c-Jun, are essential for hypertrophy and expression of fetal cardiac genes, as well as being involved in the proliferative and differentiation responses of many cell types (5, 23). Mitochondrial proliferation is a necessary component of cell growth, suggesting a potential role for c-Jun in the activation of cyt $c$ transcription. The temporal relationship between c-Jun, JunB, and NRF-1 expression relative to transcriptional activation of cyt $c$ was the first indication that there is a physiologically relevant role for these transcription factors in cyt $c$ expression in heart (1).

The present results provide evidence that there is physical association of NRF-1 with the cyt $c$ promoter. Our data also provide the first demonstration that the protooncogene product, c-Jun, mediates the transcriptional response of cyt $c$ to pacing in neonatal cardiac myocytes. The initial piece of evidence supporting this contention is the dependence of the c-Jun-de-
tion of this kinase by electrical stimulation does not enhance the JunB effect on cyt c. The combination of c-Jun/junB transfection was also unaltered by electrical stimulation, reflecting the antagonistic or neutral effects of JunB under conditions where the formation of c-Jun/JunB dimers is favored (25–27). Finally, evidence of a physical association of c-Jun with the CRE elements of the cyt c promoter was the first direct demonstration that induction of this transcription factor by pacing plays an essential role, along with NRF-1, in the proliferative response of mitochondria to cardiac myocyte hypertrophy. The induction of NRF-1 mRNA by transfection of cells with c-jun has not been previously reported. The time course of this induction relative to activation of c-jun transcription (1) suggests that transcription of nrf-1 may be activated by c-Jun in electrically stimulated cardiac myocytes.

Recent findings suggest that the Fos/Jun and ATF/CREB families of transcription factors interact and should be grouped into a “superfamily” of transcription factors (16). The CRE (ATF) elements (TGACGTA) present in the cyt c promoter are defined as the activating transcription factor binding site or the site for binding of the CARD response element binding protein (CREB). In neuronal cell lines, activation of L-type voltage-sensitive Ca2+ channels stimulates phosphorylation of CREB contributing to calcium-activated gene transcription through a Ca2+-calmodulin-dependent protein kinase pathway (22). However, neither phosphoinositide hydrolysis or CaMP formation are contributory to gene expression in electrically stimulated neonatal cardiac myocytes (6). The latter observation is consistent with our findings using the cardiac myocyte model developed by McDonough and Glembotski (6), i.e. there is no detectable contribution of CREB to the protein complex associated with the CRE sites in the cyt c promoter. The strong blockage of the CRE shift by anti-Jun suggests that the binding affinity of c-Jun to the CRE binding sites is low compared with binding of the cardiac nuclear protein extracts to authentic AP-1. In the latter case, supershifts are observed with anti-c-Jun. This supershift pattern was also characteristic of anti-NRF-1 when the antibody was used against cardiac nuclear proteins associated with the NRF-1 binding element. This differential effect of antibodies on the binding patterns reflects the finding that Fos/Jun proteins bind to the ATF/CRE sites with a lower affinity than to the AP-1/LATRE site (16).

Our data represent the first physiological evidence that the activity of AP-1 proteins, particularly, c-Jun, is essential for cyt c gene expression, contributing to the proliferation of mitochondria during cardiac myocyte cell growth and differentiation (1, 7). Cyt c represents a target gene which plays an important role not only in assuring adequate energy supplies to an actively contracting cardiac myocyte, but is also important to the initiation of apoptosis (28). This situation is particular relevant in heart where compensatory hypertrophy progresses to failure, the latter characterized by programmed cell death (29). It is intriguing that c-Jun and c-Jun N-terminal kinase have been implicated in induction of programmed cell death (30). Although AP-1 is not an integral part of the basic elements involved in cell death, it may be required to initiate expression of limiting components that are required for apoptosis to occur. One of these components which is essential to programmed cell death is cyt c, which activates the cytochrome c protease, CPP32 (28). The cellular pathway that leads directly to cyt c accumulation in the cytosol has been the subject of recent interest with release of cyt c from the mitochondrial inner membrane proposed as an initiating event (31). Other investigators have measured no change in the difference spectra for cyt c of mitochondria isolated from control and apoptotic cells (32). The latter results suggest that cyt c remains on the mitochondrial inner membrane but is instead inactivated during programmed cell death (32). One could also speculate that, under certain conditions, activation of cyt c gene transcription could enhance myocyte concentrations of cyt c for initiation of apoptosis. Finally, a function for c-Jun N-terminal kinase activation in the apoptotic effect of ceramides has been demonstrated (30). Furthermore, Fas- or ceramide-mediated cell death is inhibited by transfection of Jurkat cells with an inhibitory Jun construct (30). AP-1 may play a role in directing the dual functions of the c-Fos, c-Jun family members to mediate cell proliferation or cell death by activating target genes, e.g. cyt c, which respond to the intracellular milieu by amplification of the appropriate signaling pathways.

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