c-Src Activation Plays a Role in Endothelin-dependent Hypertrophy of the Cardiac Myocyte*

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Atrial natriuretic peptide (ANP) is a cardiac hormone involved in the regulation of intravascular volume and blood pressure (1). Under normal conditions, the ANP gene is expressed almost exclusively in the cardiac atria, although modest levels of expression are also detected in the cardiac ventricle, hypothalamus, aortic arch, and lung (2, 3). Ventricular ANP gene expression is elevated during embryonic and early neonatal life but remains low throughout adulthood unless the ventricle is subjected to hemodynamic stress (e.g. volume or pressure overload). In this context, the ventricular myocyte undergoes characteristic biochemical and morphological changes that signal the initiation of hypertrophy. At the level of gene expression, this includes activation of the immediate early genes (proto-oncogenes like c-fos, c-jun, c-myc, and egr-1) followed by reactivation of a fetal gene program (e.g. ANP, β-myosin heavy chain, and α-skeletal actin) and subsequent up-regulation of sarcomeric contractile proteins (e.g. myosin light chain-2 and cardiac actin) (4). The high degree of fidelity with which ANP gene expression is activated in this process has led to its identification as one of the earliest and most reliable markers of hypertrophy. While no in vitro system has been shown to mimic hypertrophy in vivo with absolute fidelity, the neonatal rat cardiac myocyte responds to a number of mechanical (e.g. passive stretch) (5, 6) and biochemical (e.g. endothelin (ET) (7, 8), angiotensin II (AII) (9), phenylephrine (PHE) (10), or growth factors (11, 12)) stimuli with phenotypic changes that closely parallel those seen with hypertrophy in the whole animal.

Endothelin-1, a 21-amino acid vasoconstrictor, is one of the most potent hypertrophic stimuli in the neonatal myocardial system (7, 13). ET-1 binds to a specific heterotrimeric G protein-coupled receptor that is linked to a number of well-defined intracellular signaling pathways. Activation of phospholipase C, mobilization of intracellular calcium, activation of protein kinase C, and stimulation of MAP kinase activity each have been linked to ET-1 in the neonatal cardiac myocyte (4, 7, 14). The latter is of particular interest in that MAP kinase has been classically associated with activation of tyrosine kinase activity. Several recent studies indicate that G protein-coupled receptors employ a very unique mechanism to effect this stimulation. This involves a Gβγ subunit-mediated increase in Src-tyrosine kinase activity, which in turn leads to phosphorylation of the Shc adapter protein, activation of the Shc-Grb2-Sos-Ras pathway, and increased MAP kinase activity (15–18).

ET has been shown to stimulate tyrosine kinase activity in a number of systems (19–21) and, more recently, has been linked directly to activation of c-Src in rat mesangial cells (22, 23). In cardiac myocytes, the picture is incomplete. Thorburn et al. (24) have shown that inhibitors of tyrosine kinase activity (e.g. genistein) reduce phenylephrine-dependent activation of c-Fos, ANP, and myosin light chain-2 (MLC-2) promoter activity in cultured myocytes. Sadoshima and Izumo (25) have recently demonstrated that AII activates Fyn, a tyrosine kinase closely related to Src, in these cells. They hypothesize that this activation, through Shc/Grb2/Sos intermediates, leads to stimulation of Ras and MAP kinase, two biochemical markers that have been linked to the hypertrophic process (6, 26, 27). Note-worthy, however, are the findings of Zou et al. (28), which

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§ The abbreviations used are: ANP, atrial natriuretic peptide; hANP, human ANP; ET, endothelin; AII, angiotensin II; PHE, phenylephrine; MAP, mitogen-activated protein; SRE, serum response element; TK, thymidine kinase; PBS, phosphate-buffered saline; PIPES, 1,4-piperazine-ethanesulfonic acid; SRF, serum response factor; Ets, E26 transformation-specific; CArG: CA-rich G; AP-1, activator protein-1.
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question the dependence of the hypertrophic response on tyrosine kinase activation, suggesting instead that stimulation of MAP kinase by AII is strongly linked to activation of protein kinase C. Thus, at present, there is no consensus as to the role of the tyrosine kinases in the signaling cascades traditionally associated with the development of hypertrophy.

Given the controversy surrounding this issue, we have investigated the role of c-Src and Src-related tyrosine kinases in initiating the signaling cascade linking the liganded ET receptor to activation of the ANP gene promoter and, by inference, to the development of hypertrophy in the neonatal cardiac myocyte. We have determined that ET does activate c-Src in the myocyte and that this activation is closely tied to the subsequent activation of ANP gene transcription.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The hANPCAT constructs contain the 5' flanking sequence of the human ANP gene linked to coding sequence for bacterial chloramphenical acetyl transferase (CAT); the accompanying numerical designation defines the 5' limit of the included genomic sequence relative to transcriptional initiation. A 5.1-kb hANP promoter fragment was cloned into pSVβCAT vector, as described previously (29, 30). —466 hANPCAT was constructed from —1150 hANPCAT by polymerase chain reaction (Perkin-Elmer) using an upstream sense oligonucleotide (5'-GGTCTAGAATTTCATTCGGCTGGT-3') , which incorporated an XbaI site at its 5' terminus and downstream antisense oligonucleotide (5'-CGCGTTGCAGGCACTGCTAATGTA- GTCTGCT-3') derived from CAT coding sequence. Mutants in the response element sequence (SER) element-like element of the hANP gene were generated using the Transformer™ site-directed mutagenesis kit from CLONTECH (Palo Alto, CA). Mutagenic primers for the Ets motif, CarG box, and AP-1 sites were GGTGCGCTGCAATTCTGTGTCGTCATCACCATTA TCTCTCCACCATTAAGGTGACCTGACAGCTGA, and GGCCCGTACTGAATTTAAAACATGAGGG (reading 5' to 3') , and those for the type II site were GCCACTGCTTTTGCGTTTCTT, respectively. The TKCAT hybrid gene, containing 109 base pairs of the herpes simplex virus thymidine kinase (TK) promoter (positions —109 to —47) fused to CAT, has been described previously (31). The following restriction enzyme fragments of the hANP gene were subcloned into pTKCAT and tested for regulatory activity: BamHI/PvuII (positions —472 to —400), PvuII/PvuI (positions —400 to —322), and PvuII/SalI (positions —208 to —16). Wild type —472 to —372 TKCAT and its CarG mutant were generated by polymerase chain reaction using wild type —466 hANPCAT and its homologous CarG mutant as templates; an upstream sense oligonucleotide (5'-CTCCTGAACCTGATGATTCCACGTTG- GCTCGGTGCTGCTGCT-3') , which incorporates PstI and HindIII sites at the 5' terminus and downstream antisense oligonucleotide (5'- ACAAGCTGCCATCCGGTTGTGTTG-3') , which incorporates SalI and HindIII sites at the 3' end, were used to amplify the relevant fragments, which were then cloned into the HindIII site of pTKCAT. All mutants and polymerase chain reaction-generated fragments were sequenced. Dominant negative Ras (p12Nras) was provided by W. J. Fanti. pGR Csk was obtained from Dr. Shigeyuki Nada. c-Src and v-Src came from Dr. J. A. Cooper, and the v-SRC and v-SRC mutants of c-Src were from Dr. Tony Hunter. pMiK Neo Yes was from Dr. Marius Sudol, and SRa Fyn and SRa Lck were provided by Dr. Tadashi Yamamoto.

**Antibodies**—Rabbit polyclonal antibodies directed against c-Src (SRC2), c-Yes (c-Yes3) and Fyn (FYN3-G) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). A monoclonal antibody (clone 28) that specifically recognizes the region adjacent to Tyr253 in the C-terminal regulatory domain of c-Src was generously provided by Dr. Hisashi Kowakatsu. This region is recognized by the antibody only when Tyr253 is in the unphosphorylated state, i.e. when the kinase is in an activated form (32). Clone 28 antibody was raised against the peptide sequence LEDYFTSTPQYQPGENIL, which occurs in the C termini of Src, Fyn, and Yes; consequently, the antibody recognizes all three proteins. Src-specific monoclonal antibody (Hy9d3/H9) was a gift from Dr. Joan Brugge, and monoclonal anti-phosphotyrosine antibody (clone 4G10) was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Monoclonal antibodies that specifically recognize c-Yes (c-Yes3H9) and Fyn (Fyn301) were obtained from WAKO Chemicals USA (Richmond, VA). Anti-α-actinin (sarcomeric) monoclonal antibody (EA-53) was purchased from Sigma. All secondary antibodies used for immunofluorescence were from Jackson Laboratories (West Grove, PA); those used for Western blots were from Amersham Pharmacia Biotech. Streptavidin conjugates were from Vector Laboratories, Inc. (Burlingame, CA). Dynabeads M-280 (sheep anti-rabbit IgG) were from Dynal A.S. (Oslo, Norway).

**Cell Culture, Transfection and CAT Assay**—Primary cultures of rat ventricular myocytes from 1-day-old rats were prepared as described (33) and plated on 0.1% gelatin-coated plates in DME-H21/10% enriched calf serum (Gemini Bioproducts Inc., Calabasas, CA). After cell attachment (~18 h), the culture medium was changed to a defined DME-H21/10% serum substrate medium (34). All experiments were performed after 24–48 h incubation in serum substitute medium. For transfection, 10 million cells/group were resuspended in 400 μl of phosphate-buffered saline (PBS), 0.1% glucose containing 25 μg of reporter plasmid together with varying concentrations of different expression vectors, as described in the figure legends. DNA concentration for all samples was adjusted to 40 μg with inert DNA (pUC 18). Cells were electroporated using the Bio-Rad Gene Pulsar at 280 V 2500 microfarads. Independent measurements of transfection efficiency, using pBSV β-galactosidase, typically show less than 15% variation within a given experiment. After transfection, each group was plated onto 6-cm dishes (15 × 10⁴ cells/cm²) in DME-H21/10% EC. After the initial 24 h, medium was changed to DME-H21/10% serum substitute medium. Endothelin (10⁻⁷ M) was added at different intervals thereafter. The cells were harvested at 66–72 h post-transfection, and the cell lysates were assayed for CAT activity and β-galactosidase activity as described (33).

**Isolation of RNA and Northern Blot Analysis**—Total RNA was isolated from ventricular cardiomyocytes using the RNeasy kit (QIAGEN, Inc.). 5 μg of ventricular RNA was size-fractionated on 1% agarose containing 2.2 M formaldehyde, transferred by capillary action to GeneScreen Plus hybridization Transfer Membrane (NEN Life Science Products) in 10⁻³ standard saline citrate (SSC, 1.5 M sodium chloride, and 0.15 M sodium citrate) for 8–16 h and fixed to the membrane by UV irradiation (DNA transfer lamp, Fotodyne Inc., New Berlin, WI). Membranes were then baked at 80 °C for 1 h and probed with a full-length rat ANP cDNA labeled with [γ-³²P]dCTP using the random primer technique. Hybridizations were performed in Rapid-Hyb buffer (Amersham Pharmacia Biotech) according to instructions of the manufacturer. Blots were later stripped and reprobed with radiolabeled glyceraldehyde dehydrogenase cDNA to control for differences in loading and transfer of RNA among samples. To quantitate mRNA levels, autoradiograms were scanned by laser densitometry.

**Immunoprecipitation and Western Blot Analysis**—Incubations with ET were terminated by aspiration of medium and two rapid washes with PBS. All subsequent steps were performed in ice-cold buffers. Cells were lysed in modified radioimmune precipitation lysis buffer (50 mm HEPES, pH 7.4, 150 mm NaCl, 10 mm glycerol, 1.5 mm MgCl₂, 1 mm EDTA, 0.25 mm sodium orthovanadate, 10 mm sodium pyrophosphate, 100 mm NaF, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 10 μg/ml each of leupeptin and aprotinin, and 1 mM phenylmethylsulfonyl fluoride) (35). After brief sonication, lysates were centrifuged for 10 min at 15,000 × g and the supernatant was used for centrifugation (15 min at 15000 × g). Lysates were preclayed by 30-min incubation with 5 μl of agarose conjugated to mouse, goat, and rabbit IgG, followed by 5-min centrifugation at 7000 × g. Equal concentrations of total protein (250 μg) were then incubated with specific antibodies at concentrations of 1–2 μg/250 μg of protein for 60 min at 4 °C. The immune complexes were collected after the addition of 25 μl of Dynabeads and incubation for 30 min at 4 °C. The pellets were washed once with lysis buffer and two times with buffer A (20 mM Tris, pH 7.4, 1 mM EDTA, 0.1% Nonidet P-40, 10% glycerol, and 5 μg β-mercaptoethanol) and subjected to immunoblotting or the in vitro kinase reaction. Lysates, normalized for protein content, or immunoprecipitates were boiled in Laemmli sample buffer, electrophoretically separated on 12% polyacrylamide gel containing 1% SDS, and transferred to a nitrocellulose membrane. Filters were blocked in TBST (10 mM Tris, pH 8.0, 150 mm NaCl, 0.05% Tween 20/0.5% skim milk buffer, except on those occasions when anti-phosphotyrosine antibodies were used, when 5% bovine serum albumin was exchanged for skim milk. Following treatment with a blocking kit (Vector Laboratories), as recommended by the manufacturer, the filters were incubated with the appropriate primary antibody (1:500) for 1 h. Membranes were washed 0.5–1 × TBST buffer for 60 min. Filters were then incubated with biotinylated donkey anti-mouse (1:1000) and a 15-min incubation with streptavidin-horseradish peroxidase conjugate (1:1000), immunoreactive bands were visualized by enhanced chemiluminescence using the ECL system (Amersham Pharmacia Biotech). When necessary, membranes were stripped with 2% SDS in 62.5 mM Tris- HCl, pH 6.7, in the presence of 200 μl β-mercaptoethanol (freshly

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Endothelin-1-dependent accumulation of tyrosine-phosphorylated proteins in ventricular myocytes. Freshly isolated ventricular myocytes (5 x 10^6) were plated in serum substitute media on 6-cm plates coated with gelatin. 24 h post-plating, cells were treated with 100 nM ET for the indicated times. Whole cell lysates were prepared as described under "Experimental Procedures." Samples were gel-fractionated, transferred to nitrocellulose membranes, and immunoblotted with anti-phosphotyrosine antibody (4G10). To verify equal loading, the membrane was stained with Amido Black (data not shown). Molecular mass markers are presented on the right.

added) for 60 min at 50–60 °C. Following overnight blocking in TBST/1% skim milk and treatment with a commercial blocking kit (Vector Laboratories), membranes were reblotted with appropriate antibody, and the protocol described above was repeated.

Src Kinase Assay—To measure c-Src kinase activity, endogenous c-Src was isolated by immunoprecipitation with monoclonal antibody mAB327. The immunoprecipitates from ~500 μg of total ventricular cell protein were washed three times with Src kinase reaction buffer (100 mM Tris-HCl, pH 7.2, 125 mM Mg(C2H3O2)2, 25 mM MnCl2, 2 mM EGTA, 0.25 mM sodium orthovanadate, 2 mM dithiothreitol), and reactions were carried out using components of a commercially available Src kinase assay kit (Upstate Biotechnology, Inc., Lake Placid, NY). The assay is based on Src-dependent phosphorylation of a substrate peptide (KVEKIGETGYGGVYK) derived from p34^CDC2 (36). For the autophosphorylation assays, immunoprecipitates were washed twice with kinase buffer (10 mM MnCl2, 10 mM HEPES, pH 7.4) and incubated with kinase buffer plus 1 μg of [γ-32P]ATP and 1 μl unlabeled ATP for 15 min. Reactions were terminated by boiling in sample buffer. Samples were resuspended in buffer and electrophoresed on 12% polyacrylamide gels containing 1% SDS.

Immunofluorescence—Cells were plated on round 12-mm coverslips (Esco, Germany) coated with fibronectin (10 μg/ml) at a density of 10^4 cells/cm^2, treated with ET as described above, and washed with sterile PBS three times. For staining of Src, Yes, and Fyn, cells were fixed either in acetone at 20 °C for 5 min or in 3.7% paraformaldehyde at room temperature for 20 min and washed three times with PBS. When fixation was performed in paraformaldehyde, cells were permeabilized for 2 min in 0.2% Triton X-100/PBS. Fixed/permeabilized cells were then washed in PBS, blocked with blocking kit (Vector Laboratories), and incubated in wet chambers with primary antibody (1:100 dilution in PBS) overnight at 4 °C. Following three successive 10-min washes in PBS, cells were incubated for 30 min at room temperature with biotinylated donkey anti-rabbit or rhodamine-conjugated goat anti-mouse secondary antibody diluted 1:100 in PBS. Cells were then washed three times in PBS for 10 min and incubated for 15 min at room temperature with a 1:100 dilution of fluorescein isothiocyanate-streptavidin. After sequential PBS washes (three times, 10 min each), the cells were mounted with Vectashield mounting medium (Vector Laboratories). Controls performed with only secondary antibodies resulted in negligible signals. Conventional epifluorescence microscopy was carried out using a Zeiss Axiophot microscope. Photography was performed with Kodak TMAX 400 black and white film.

RESULTS

Endothelin Activates Src in Cultured Neonatal Cardiac Myocytes—Treatment of neonatal rat ventricular myocytes with 100 nM ET resulted in a time-dependent increment in whole lysate tyrosine kinase activity, assessed by immunoblotting with the anti-phosphotyrosine antibody (4G10) (Fig. 1). Accumulation of phosphotyrosine-containing proteins in the lysates was maximal at 1 min and began to fall after 5 min of exposure to the peptide. Phosphotyrosine content of at least 10 cellular proteins, ranging from ~20 to ~110 kDa was increased by treatment with ET. Prolonged exposure of the film revealed fainter signals corresponding to higher molecular mass, phosphotyrosine-containing proteins (~120–200 kDa) (data not shown).

Western blot analysis of immunoprecipitates of Src in these lysates revealed no change in the levels of Src protein following treatment with ET (Fig. 2A). However, when the same blot was probed with an antibody that selectively recognizes the activated form of Src (clone 28), an ET-dependent increase in signal intensity was detected. Pooled results from three independent experiments indicated a 2.95 ± 1.32-fold induction of activated Src following ET treatment (data not shown).

The ET-dependent activation of Src implies stimulation of the intrinsic tyrosine kinase activity associated with this protein. To confirm this, we carried out an autophosphorylation assay (Fig. 2B) as well as a conventional Src kinase assay using a synthetic peptide sequence derived from p34^CDC2 (36) as the target substrate (Fig. 2C). In both cases, ET provoked an almost 7-fold increase in Src kinase activity, which peaked around 1 min and returned to near basal levels after 15 min of incubation. This induction was largely blocked by inclusion of the nonselective tyrosine kinase inhibitor genistein during ET stimulation (Fig. 2C).

Endothelin Activates Fyn and Yes in Cardiac Myocytes—To explore the possibility that other members of the Src family of tyrosine kinases might be stimulated by ET, we examined extracts from ET-treated cells for the presence of activated Fyn and Yes. As shown in Fig. 3, Western blot analysis of immunoprecipitates generated with anti-Fyn or anti-Yes antibodies showed no change in the absolute levels of these two proteins following ET treatment. However, when the same blots were reprobed with antibody that selectively recognizes the activated forms of Yes or Fyn, a time-dependent increase in levels of the individual activated kinases was observed. These increases were present at 30 s and peaked at 2–15 min of ET exposure.

Endothelin Causes a Redistribution of c-Src, Fyn, and c-Yes in the Cardiac Myocyte—Activation of the Src family tyrosine kinases was accompanied by changes in their topographical distribution within the cell. Primary cultures of neonatal rat ventricle myocytes were analyzed by indirect immunofluorescence with polyclonal antibodies directed against c-Src. In quiescent myocytes, two patterns of c-Src distribution were apparent: a fine punctate staining throughout the cytoplasm and a concentrated staining adjacent to the membrane (Fig. 4A). Treatment of cells with ET for 120 s produced a striking redistribution of c-Src into large nonuniform aggregates in a perinuclear distribution. The ET-induced redistribution was reversible. After exposure to ET for 15 min, the original diffuse pattern of immunostaining returned (data not shown). Staining with a monoclonal antibody (mAB 327) directed against c-Src revealed a similar distribution pattern (data not shown). The levels of Src immunoreactivity detected in mesenchymal cells (i.e. primarily cardiac fibroblasts) present in these cultures were considerably lower than those seen in cardiac myocytes.

A similar approach using antibody directed against Fyn (Fig. 4C) demonstrated an ET-dependent redistribution of the kinase from a diffusely punctate pattern to spherical cytoplasmic vesicles clustered in a perinuclear distribution. In the case of Yes (Fig. 4B), ET promoted a redistribution from the diffusely pattern seen in the quiescent myocyte to a pattern suggesting a linear array of Yes molecules along the myofilaments of the
c-Src and Yes was detected as a 85-kDa polypeptide by mAB 327, which recognizes both inactive and active c-Src. Following visualization of immune complexes by ECL, the membrane was washed and reblotted with clone 28, a c-Src antibody that specifically recognizes the active form of Src (anti-active Src). Results were quantified by densitometric analysis, normalized for differences in loading, and expressed as -fold activation compared with untreated control. Each analysis was repeated three times with similar results.

**Fig. 2.** ET-1 stimulates Src kinase activity in ventricular cardiocytes. Freshly prepared ventricular myocytes were plated as described in the legend to Fig. 1. 24 h post-plating, cells were stimulated for the indicated times with 100 nM ET. Cells were washed and lysed, and used for a quantitative immunoblot assay. Immunoprecipitates were separated on 12% SDS-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membranes. These were probed with mAB 327, which recognizes both inactive and active c-Src. Following visualization of immune complexes by ECL, the membrane was washed and reblotted with clone 28, a c-Src antibody that specifically recognizes the active form of Src (anti-active Src). Results were quantified by densitometric analysis, normalized for differences in loading, and expressed as -fold activation compared with untreated control. Each analysis was repeated three times with similar results.

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cyte hypertrophy (7), we sought to determine if Src could be linked mechanistically to downstream markers of hypertrophy in this in vitro system. To address this issue, we employed a transiently transfected ANP promoter-driven reporter, a marker that has been shown to respond to a number of different hypertrophic stimuli in this in vitro model (7–10, 12). As shown in Fig. 5A, ET treatment effected approximately a 3-fold increase in reporter activity, which was suppressed by a dominant negative Ras mutant (N17S), by a dominant negative Src mutant (K297M) or by Csk, a kinase that negatively regulates Src activity through phosphorylation of Tyr527 near the carboxyl terminus of the molecule (37).

**Fig. 3.** Activation of Fyn and Yes in ET-treated ventricular cardiocytes. Fyn and Yes were immunoprecipitated (ip) from cell lysates (250 μg) using rabbit polyclonal anti-Yes (c-Yes3) and anti-Fyn (FY3N) antibodies. Immunoprecipitates were separated on 12% SDS-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membranes. These were probed with monoclonal antibodies Fyn301 and Yes3H9, which recognize both inactive and active forms of Fyn and Yes, respectively (top panels). Following visualization of immune complexes by ECL, the membranes were washed and reblotted with antibody (clone 28) that specifically recognizes only active forms of Fyn and Yes (bottom panels). Results were quantified by densitometric analysis, normalized for differences in loading, and expressed as -fold activation compared with untreated control. Each analysis was repeated three times with similar results.
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Next, we explored the functional link between tyrosine kinase activity and ANP gene expression. Pretreatment of the cultures with a potent and Src family-selective tyrosine kinase inhibitor PP1 (38) resulted in a dose-dependent reduction in the ET-dependent increase in ANP mRNA levels (Fig. 6A), as well as c-Src-induced hANP promoter activity (Fig. 6B). A similar inhibition was obtained in the presence of the less selective protein tyrosine kinase inhibitor, genistein (data not shown). By inference, the inductive properties of each (i.e. ET and Src) appear to be linked to their tyrosine kinase activating properties in the cardiac myocyte.

Src Response Localizes to a CArG Motif on hANP Promoter—We next attempted to identify the Src-sensitive locus on the hANP promoter. As shown in Fig. 7, activation of the promoter was observed with 5'-deletion mutants containing 1150 and 466 base pairs of 5'-flanking sequence; however, the induction was largely, although not completely, lost as the deletion was moved to −222, implying the presence of a Src-sensitive element or elements between −466 and −222 in this promoter. We generated a number of restriction fragments spanning this region and introduced them upstream from the core viral thymidine kinase gene promoter linked to CAT. Of the group of reporters analyzed, only constructs harboring sequences spanning the region between −472 and −400 proved to be activable by v-Src (Fig. 8) (v-Src was selected to provide maximal levels of promoter activity). Examining the sequence of this fragment, we identified a region structurally homologous to the serum response element (SRE) of the c-fos promoter, a locus that has previously been identified as a target of Src in cultured glomerular mesangial cells (22). Like the c-fos SRE (39), the SRE-like structure of the hANP gene promoter features a CArG element at its core with a potential Ets binding site.
Ventricular cardiocytes were transfected with 25 \( \mu \)g of hANP CAT vector. Total RNA was isolated, size-fractionated on formaldehyde/agarose gels, transferred to nitrocellulose, and hybridized to a radiolabeled rat ANP cDNA. The top part of A depicts a representative experiment. The bottom part presents results of quantitative densitometric analyses of three separate experiments. Values (mean ± S.E.) are normalized for glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA. The top part of A shows the induction of ANP mRNA by ET. The bottom part shows the induction of ANP protein by ET. The results were normalized to control (CTL) or in the presence of 1 \( \mu \)g of c-Src. Twenty-four hours later, the indicated concentration of PPI was added to half the cultures, and extracts were generated after an additional 24-h incubation. Results represent the means ± S.E. of three individual experiments performed in duplicate. +, \( p < 0.01 \); **, \( p < 0.001 \) compared with control; + +, \( p < 0.05 \) compared with control. *, \( p < 0.01 \); ***, \( p < 0.005 \) compared with ET-stimulated hANP RNA (A) or Src-stimulated hANP CAT reporter (B).

**FIG. 6.** Inhibition of tyrosine kinase activity by PP1 prevents ET or Src-induced transactivation of ANP gene expression. A. Northern blot analysis of ET-dependent ANP gene expression. Cells were treated for 24 h either with vehicle or ET (10\(^{-7}\)M), in the presence or absence of the Src family-selective kinase inhibitor PP1. Total RNA was isolated, size-fractionated on formaldehyde/agarose gels, transferred to nitrocellulose, and hybridized to a radiolabeled rat ANP cDNA. Following autoradiography, the blot was stripped and reprobed with radiolabeled glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA. The top part of A depicts a representative experiment. The bottom part presents results of quantitative densitometric analyses of three separate experiments. Values (mean ± S.E.) are normalized for glyceraldehyde phosphate dehydrogenase signal in each sample and expressed relative to the control. B, cells were transfected with −1150 hANPCAT (25 \( \mu \)g) alone (CTL) or in the presence of 1 \( \mu \)g of c-Src. Twenty-four hours later, the indicated concentration of PPI was added to half the cultures, and extracts were generated after an additional 24-h incubation. Results represent the means ± S.E. of three individual experiments performed in duplicate. +, \( p < 0.01 \); **, \( p < 0.001 \) compared with control; + +, \( p < 0.05 \) compared with control. *, \( p < 0.01 \); ***, \( p < 0.005 \) compared with ET-stimulated hANP RNA (A) or Src-stimulated hANP CAT reporter (B).

**FIG. 7.** Mapping the region of hANP promoter that confers Src sensitivity. Ventricular cardiocytes were transfected with 25 \( \mu \)g of SV\(_{40}\)CAT or the individual hANP-CAT 5'-deletion mutant in the absence (basal) or presence of 1 \( \mu \)g of v-Src expression vector. The hANP promoter constructs are identified by the 5'-border of the included hANP gene sequence. Cells were cultured and assayed for CAT activity as described in the legend of Fig. 5. CAT activity is expressed in cpm of [\( ^{3} \)H]acetylchloramphenicol produced per 100 \( \mu \)g of protein and represents the average of 3–5 separate experiments done in triplicate. *, \( p < 0.01 \) versus reporter alone (without v-Src).

There is, in addition, a structure similar to an AP-1 binding element 3′ from CArG that also has a homologue in the c-fos promoter (Fig. 9B). We examined the role of each of these three structural elements, alone and in combination, as potential cis-acting mediators of the Src effect. Site-directed mutations were introduced within the context of −466 hANP-CAT and examined for functional response to a cotransfected v-Src expression vector. As shown in Fig. 9A, mutation of the CArG element or the AP-1 binding site resulted in a decrease in basal promoter activity to approximately 50% of the control level, while mutation of the Ets binding site, the site responsible for ternary complex assembly on the c-fos promoter (40), was without effect. Furthermore, while mutation of the CArG motif resulted in a significant decrease in the level of induction by v-Src, mutation of the AP-1 or Ets binding site was almost completely ineffective. Introduction of the same mutation in the CArG binding site into −100 TKCAT (haboring the region between −472 and −372 in the hANP promoter) significantly decreased v-Src sensitivity (10.2–versus 2.9-fold induction) (Fig. 8). These findings suggest that the CArG motif plays a dominant role in mediating Src-dependent activation of the hANP promoter.

Finally, to confirm the importance of this locus to the ET-mediated induction of hANP gene promoter activity, we tested the same constructs in transiently transfected myocytes after different intervals of exposure to the peptide. As shown in Fig. 10, ET effected a time-dependent increase in the activity of wild type −1150 and −466 hANPCAT reporters. The induction was largely suppressed by introduction of a single mutation into the CArG element or by a triple mutation (i.e. at the CArG, Ets, and AP-1 motifs) into the −466 hANPCAT reporter.

**DISCUSSION**

The findings presented here demonstrate that an agonist of hypertrophy in the cardiac myocyte (i.e. endothelin) signals at least a portion of its activity through Src-like nonreceptor tyrosine kinases. We show that 1) ET stimulates the activity of c-Src, Fyn, and Yes in primary cultures of neonatal rat cardiac myocytes, 2) ET-dependent induction of the hANP gene promoter requires participation of c-Src, and 3) activation of the hANP promoter by either c-Src or ET requires the presence of a CArG motif positioned between −422 and −413 in that pro-
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A schematic representation of hANP promoter fragments in a heterologous promoter context is shown. The inclusive sequence for each construct is identified at the termini of the individual fragments. -472/-372 TKCAT MUT contains the CArG box (positions -423/-413) mutation described under “Experimental Procedures.” Ventricular myocytes were transfected with 15 μg of the indicated constructs in the absence or presence of 1 μg of the v-Src expression vector. Cells were cultured and processed for CAT activity as described under “Experimental Procedures.” Data presented are representative of three separate experiments done in duplicate.

moter. This demonstrates, for the first time, that the hANP gene represents a downstream target for c-Src and suggests a role for c-Src in promoting cardiac hypertrophy.

Until recently, G protein-coupled receptors were thought to signal selectively through a well defined group of enzymatic effectors (e.g. adenyl cyclase and phospholipase C) and ion channels, while activation of tyrosine kinases was largely seen as a function of the cytokine and growth factor receptors. Recent investigation has provided convincing support for G protein-dependent activation of tyrosine kinase activity (specifically Src family kinase activity) within target cells. Tyrosine kinase activation by lysophosphatidic acid has been linked to the βγ subunits of the associated G protein complex (15), implying that the activation involves a novel mechanistic pathway. Liganded α- and β-adrenergic, AII, and ET receptors have been shown to stimulate tyrosine kinase activity (19, 23, 41, 42), and in some cases, tyrosine kinase inhibitors have been shown to block their effects (6, 24).

By analogy to the growth factor receptors, activation of cellular tyrosine kinases by norepinephrine, AII, and ET could account for a significant portion of the growth-promoting properties that these ligands display in cardiovascular tissues.

Each of the Src family kinases (i.e. c-Src, Fyn, and c-Yes) that we examined was activated by ET. It is difficult at this stage to determine the relative contribution of each in promoting the growth response associated with hypertrophy. AII has been shown to promote activation of the tyrosine kinase Fyn in ventricular myocytes (25) as well as assembly of downstream signaling complexes (i.e. Shc-Grb2-Sos) thought to be involved in hypertrophy. However, as seen in Fig. 3, Src is considerably more effective than either Yes or Fyn in stimulating at least one marker of hypertrophy (i.e. hANP promoter activity), and dominant negative Src (43), which should selectively antagonize Src activity, partially inhibited ET-dependent stimulation of the hANP gene promoter. Furthermore, a recent study by Kuppuswamy et al. (44) demonstrated cytoskeletal association of c-Src and focal adhesion kinase, but not Fyn, in pressure-overloaded right ventricular myocardium, lending further support to a specialized role for Src in the hypertrophic process.

Previous studies carried out with cultured mesangial cells showed that ET induction of the c-fos promoter requires activation of c-Src (22, 29), a finding similar to that reported here. In the case of the c-fos promoter, the activation depends upon the presence of an intact CArG motif in the SRE, while the upstream Ets motif, which is required for assembly of the ternary complex (specifically, association of serum response factor (SRF) accessory protein (SAP) or Elk-1 with SRF), proved to be dispensable. Subsequent analyses revealed an additional requirement for a calcium/cAMP response element located more proximally (23) in the c-fos promoter. In the present study, the CArG motif in the hANP promoter also proved to be critical for the response to Src and ET. The level of promoter induction by v-Src was reduced from 7.2- to 2-fold when point mutations were introduced into the CArG motif. As seen with the c-fos promoter, mutation of the Ets binding site
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or an AP-1 binding site located downstream from the CArG element had little effect on induction by Src. The residual 2-fold induction of the CArG mutant by Src could reflect the involvement of a second element (e.g. a calcium/cAMP response element or a second, as yet unidentified, CArG motif) in mediating the Src effect.

The lack of dependence of the ET induction on the Ets motif in the promoter suggests that formation of a ternary complex is not required. Although a number of studies have shown that growth factor activation of the fos promoter (presumably through the MAP kinase pathway) involves ternary complex formation (36, 45), other studies have demonstrated that certain trophic stimuli (e.g. serum, lysophosphatidic acid, and intracellular activators of heterotrimeric G proteins) traffic through the SRF itself (46). This is believed to result from activation of the Rho family of small G proteins (i.e. Rho, Rac, and CDC42hs) and does not correlate with activation of the MAP kinases (i.e. extracellular signal-regulated kinase, stress-activated protein kinase/c-Jun N-terminal kinase, or MPK2/p38) (47). Other studies have suggested that some growth factors may signal the c-fos SRE through selective phosphorylation of Ser103 in the SRF molecule (48), a modification that could alter SRF’s signaling capabilities and/or binding kinetics on the SRE. ET-dependent activation of the hANP promoter promoter appears to conform to the ternary complex-independent paradigm, relying almost exclusively on the CArG motif for the inductive effect. Similar conclusions have been drawn with regard to the regulation of the rat ANP promoter by PHE (49).

The latter induction appears to signal through a CArG motif in the proximal promoter of the gene without a requirement for ternary complex formation.

A unifying mechanism linking hypertrophic stimuli to enhanced gene transcription in the cardiac myocyte is lacking. Activation of the c-fos gene by passive mechanical stretch or AII depends upon an intact serum response element (including Ets and CArw box binding sites) in the promoter of that gene (42, 50). PHE- and protein kinase C-dependent induction of the β-myosin heavy chain gene requires the presence of an M-CAT site in the proximal promoter (51). Activation of the skeletal α-actin gene by PHE (52), as well as tumor growth factor-β (12), requires intact M-CAT, CArwG, and Sp-1 binding sites. A CArwG motif in the same promoter has been linked to activation by basic fibroblast growth factor (53). The activation of the rat ANP gene by PHE (49) or electrical depolarization (54) requires an intact CArwG motif and a GC-rich phenylephrine response element, a structure resembling an Sp-1 binding site (49, 55), while PHE induction of the rat brain natriuretic peptide gene is heavily dependent upon an M-CAT site between −109 and −102 in that promoter (56). Thus, there is considerable heterogeneity in the molecular circuitry used by ventricular myocytes to link hypertrophic stimuli to the activation of gene expression. A second CArwG motif positioned further upstream in the rat ANP promoter has been shown to interact with its proximal homologue in regulating transcriptional activity (49). It will be of interest to determine whether a similar motif is present in the proximal human promoter and, more importantly, whether it subserves the same type of regulatory function.

In summary, we have shown that the hypertrophic properties of ET in cardiac myocytes traffic, at least in part, through c-Src. We have also demonstrated that ET and Src activate the hANP gene promoter through a discrete CArwG motif present in the promoter without a requirement for ternary complex formation. Collectively, these findings suggest a potentially important role for c-Src as a mediator of cardiac hypertrophy.

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c-Src Activation Plays a Role in Endothelin-dependent Hypertrophy of the Cardiac Myocyte

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