Functional Role of c-Src in Gap Junctions of the Cardiomyopathic Heart

Toshihiko Toyofuku, Masanori Yabuki, Kinya Otsu, Tsunehiko Kuzuya, Michihiko Tada, Masatsugu Hori

Abstract—Given the essential role played by gap junctions in the coordination of cardiac muscle contraction, it is plausible that down-regulation of gap junctional conduction is in part responsible for the contractile dysfunction observed in hypertrophied and failing hearts. In the present study, we analyzed the expression and function of the gap junction protein, connexin43, in the ventricular myocardium of hereditary cardiomyopathic, Syrian BIO 14.6 hamsters. Immunoprecipitation and immunoblot analyses revealed that levels of tyrosine phosphorylated connexin43 were increased in BIO 14.6 hamsters at the late stage of congestive heart failure. Furthermore, the increased tyrosine phosphorylation was correlated with increased c-Src activity. The functional consequences of tyrosine phosphorylation of connexin43 in gap junction were assessed using transfected cells expressing constitutively active c-Src. It was found that constitutively active c-Src diminished propagation of Ca\(^{2+}\) waves in HEK293 cells and reduced gap junctional conductance between pairs of cardiac myocytes. We, therefore, conclude that during the progression of cardiac dysfunction in the cardiomyopathic heart, gap junctional communication is reduced via c-Src–mediated tyrosine phosphorylation of connexin43. (Circ Res. 1999;85:672-681.)

Key Words: cardiomyopathic hamster ■ connexin43 ■ connexin45 ■ c-Src ■ gap junction

Low-output congestive heart failure is a syndrome characterized by both systolic and diastolic dysfunction; the velocity and magnitude of ventricular contractions and the rate of pressure development are all decreased.\(^1\) The cause of the decline in contractility may be quantitative in nature, with decreased numbers of cardiac myocytes with accompanying fibrosis, and/or it may be qualitative, resulting from changes in the structure and function of subcellular elements, including contractile and cytoskeletal proteins, excitation-contraction coupling, cardiac metabolism, and/or signal transduction. Impaired electrical conduction is another feature of the hypertrophied and failing ventricular myocardium. Indeed, the electrical uncoupling between myocytes that has been observed in hypertrophied hearts\(^2\) may underlie the reentry mechanism responsible for ventricular tachycardia and may degrade contractile performance, even before the onset of heart failure.

Gap junctions, composed of connexins, allow propagation of electrical activity between cardiac myocytes, thereby coordinating the contractile behavior of the cardiac muscle.\(^3\)\(^-\)\(^5\) Connexin43 is the major connexin present in the cardiac ventricular myocardium, although connexin45 is also expressed. Regulation of gap junction–mediated coupling can occur at several levels, that is, acutely by changing gating properties and on a long-term basis by changing the overall expression of connexins and/or switching among different connexin isoforms.\(^3\) Physiological studies have shown that the gating properties of cardiac gap junctions can be affected by activation of various second-messenger pathways, including cAMP-dependent protein kinase, protein kinase C (PKC), mitogen-activating protein (MAP) kinase, and Src family tyrosine kinases.\(^6\)\(^-\)\(^10\) When terminally differentiated cardiac myocytes are subjected to hemodynamic overloads, tyrosine phosphorylation of intracellular substrates has been found to play a major role in the resultant development of cardiac hypertrophy.\(^11\)\(^-\)\(^13\) Because Src family tyrosine kinases are enriched at intercalated discs,\(^14\) we hypothesized that connexin43 may be phosphorylated by tyrosine kinase in hypertrophied and failing hearts, thereby reducing electrical coupling between cardiac myocytes.

A number of animal models have been used to study contractile performance in the failing heart. The most widely used model is the hereditary cardiomyopathic Syrian hamster. At an early stage in the cardiomyopathy, strain BIO 14.6 hamsters exhibit a thickened ventricular wall resembling hypertrophic cardiomyopathy; congestive heart failure then develops in the late stage.\(^15\) In the present study, we analyzed expression and function of connexin43 in the ventricular myocardium of cardiomyopathic BIO 14.6 Syrian hamsters. We used a biochemical approach involving immunoprecipi-
tation and immunoblot analyses to selectively detect tyrosine phosphorylated connexin43 at the late stage of the disease. To confirm the extent to which electrical coupling is diminished at the late stage of the failing process, we had originally planned to measure gap junctional conductance between pairs of myopathic myocytes. Unfortunately, we were unable to isolate intact cardiac myocytes without affecting the phosphorylation state of connexin43, perhaps in part because of the high level of tyrosine phosphatase activity in adult heart tissues.\textsuperscript{16} We therefore applied another functional approach entailing overexpression of constitutively active c-Src in connexin43-expressing HEK293 cells and cardiac myocytes. Our findings indicate that c-Src-mediated tyrosine phosphorylation of connexin43 is correlated with reduced gap junctional communication between transfected cells.

**Materials and Methods**

**Reagents**

Mammalian expression vectors, pcDNA3 and pZeoSV, and Zeocin were obtained from Invitrogen Corp. Plasmid pGreen Lantern-1 and lipofectin reagent were from Gibco-BRL. G418, genistein, random copolymer poly(Glu, Tyr) 4:1, and sodium orthovanadate were from Sigma-Aldrich. Molecular biology–grade calf intestinal alkaline phosphatase and protein A-Sepharose were from Pharmacia Biotech. Mouse monoclonal anti-connexin43 IgG antibody was from Transduction Laboratories. Rabbit polyclonal anti-connexin45 antibody was from Chemicon International Inc. Because this antibody cross-reacts with connexin43,\textsuperscript{17} we used it only after excluding the anti-connexin43 fraction by incubating the whole antibody with purified connexin43 blotted on the nitrocellulose. Mouse monoclonal anti-phosphoserine and anti-phospho-Thr antibodies and peroxidase-labeled, affinity-purified, anti-mouse IgG and anti-rabbit IgG antibodies were from Zymed Laboratories, Inc. Mouse monoclonal anti-FLAG antibody was from Eastman Kodak. Rabbit polyclonal anti-c-Src antibody and anti-C-terminal Src kinase (Csk) antibodies were from Santa Cruz Biotechnology, Inc. Biotinylated anti-mouse IgG antibody, FITC-conjugated streptavidin and mounting medium Mowiol were from Vector Laboratories, Inc. Rhodamine-conjugated anti-mouse IgG antibody was from Bio-Science. An enhanced chemiluminescence (ECL) assay kit and \( [γ^32\text{P}]\)ATP were from Amersham Pharmacia Biotech Ltd. A Src kinase assay kit was from Upstate Biotechnology Inc. Fura-2-acetoxyethyl ester (Fura 2-AM) and 0.06% pluronic F127 were from Molecular Probes Inc.

**Experimental Models**

**BIO14.6 Cardiomyopathic Hamster**

Animal experiments were conducted in accordance with the guidelines issued from the Animal Research Committee of the Osaka University Graduate School of Medicine. Breeding male and female BIO 14.6 strain cardiomyopathic hamsters and BIO F1B strain control hamsters at 2 (early stage) and 10 (late stage) months of age were obtained from KAC Inc (Kyoto, Japan). At 10 months of age, BIO 14.6 hamsters consistently showed cardiac dysfunction, but BIO F1B control hamsters exhibited little change in cardiac function.\textsuperscript{15,18} Ventricles were minced, and the cells were dispersed by digestion with 0.1% collagenase at 37°C. The dispersed cells were resuspended in high-glucose DMEM supplemented with 10% FCS and 10 μg/mL bromodeoxyuridine and preplated onto culture dishes for 30 minutes to remove fibroblasts. Cells were then plated on glass coverslips to an initial density of 10⁶ cells/mL and maintained at 37°C under an atmosphere of 5% CO\textsubscript{2}/95% air. HEK293 cells were grown in high-glucose DMEM supplemented with 10% FCS and penicillin at 37°C under an atmosphere containing 5% CO\textsubscript{2}/95% air.

**Biochemical Analyses**

**Immunoblot Analysis**

Ventricles were excised, homogenized with a Polytron homogenizer, and then lysed in lysis buffer containing (in mmol/L) HEPES (pH 7.5) 50, NaCl 150, MgCl\textsubscript{2} 1.5, EGTA 1, sodium orthovanadate 100, and PMSF 1; 10% glyceral; 1% Triton-X-100; 10 μg/mL aprotinin; and 10 μg/mL leupeptin. The lysates were then centrifuged for 15 minutes at 2000g (Beckman TL-100 centrifuge) to remove fibrous debris probably derived from pericardium, and the resultant supernatants were solubilized in SDS loading buffer. HEK293 cells were lysed in the lysis buffer without low-speed centrifugation and solubilized in SDS loading buffer. Samples were subjected to SDS-PAGE and transferred to nitrocellulose. The nitrocellulose blots were then incubated with primary antibody against the respective target proteins. The blots were then washed 3 times with Tris-buffered saline containing 0.1% Tween 20, incubated with peroxidase-labeled affinity-purified antibody against the primary antibodies, washed again, and then developed using an ECL system.

**Alkaline Phosphatase Treatment**

By repeated concentration and redilution in a Centriprep 30 microconcentrator, lysates prepared from cardiac ventricles were equilibrated against phosphate reaction buffer containing (in mmol/L) Tris-HCl (pH 8.0) 50, MgCl\textsubscript{2}, 10, and NaCl 150. The protein was then incubated for 4 hours at 30°C in the presence of 2 units of molecular biology-grade calf intestinal alkaline phosphatase. Control reactions were run after addition of phosphate inhibitors (in mmol/L) Na\textsubscript{2}VO\textsubscript{4}, 100, EDTA 10, and PO\textsubscript{4}, 10 to the reaction mixture.

**Immunoprecipitation Analysis**

By repeated concentration and redilution in a Centriprep 30 microconcentrator, the lysates prepared from cardiac ventricle or HEK293 cells were equilibrated against immunoprecipitation buffer containing (in mmol/L) Tris-HCl (pH 8.0) 50, MgCl\textsubscript{2}, and NaCl 150. The lysates were then incubated with 0.1% albumin-coated protein A-Sepharose for 2 hours at 4°C and clarified by centrifugation for 15 minutes at 15 000g. In a rotating vessel, the supernatants were then incubated for 2 hours at 4°C with mouse monoclonal anti-phosphoserine, anti-phosphotyrosine, or anti-connexin43 antibodies bound to protein A–Sepharose. After incubation, the immune-complexes were extensively washed with the immunoprecipitation buffer. Samples were then subjected to immunoblot analysis with primary antibody raised against respective target proteins. Primary antibody-antigen complexes were incubated with peroxidase-conjugated secondary antibody against mouse IgG or rabbit IgG antibodies and then visualized using ECL.

**Immunofluorescence Analysis**

HEK293 cells coexpressing connexin43 and either FLAG-tagged c-Src (WT) or c-Src (Y527F) were grown on glass cover slides for 3 days, fixed with 3% paraformaldehyde for 10 minutes, and then permeabilized with 0.1% Triton X-100 for 10 minutes. After blocking with 5% BSA in PBS for 30 minutes, the cells were incubated for 2 hours with mouse monoclonal anti-connexin43 or anti-FLAG antibodies. Primary antibody-connexin43 complexes were visualized by incubation 1 hour with biotinylated anti-mouse IgG antibody, followed by incubation for 1 hour with FITC-conjugated streptavidin. Primary antibody–FLAG-tagged c-Src complexes were visualized by incubation with rhodamine-labeled anti-mouse IgG antibodies. Coverslides were then mounted in Mowiol 4-88, and cells were photographed on an Olympus Provis AX80 microscope equipped with the appropriate filters.
C-src Immune Complex Kinase Assay
The tyrosine kinase activity of c-Src was determined in immune complexes using an Src kinase assay kit that utilized a synthetic peptide (KVEKIGEGTYGVVYK) corresponding to amino acids 6 to 20 of p34 as a substrate. Immunoprecipitates prepared with rabbit polyclonal anti-c-Src antibody bound to protein A-Sepharose beads were incubated for 10 minutes at 30°C with an assay buffer containing (in mmol/L) Tris-HCl (pH 7.2) 100, MgCl₂ 125, MnCl₂ 25, EGTA 2, Na₃VO₄ 0.25, and DTT 2, and 125 μmol/L cold ATP and 10 μCi of [γ³²P]ATP. The phosphorylated substrates were separated from residual [γ³²P]ATP with P81 phosphocellulose paper and quantified in a scintillation counter. Autophosphorylation of c-Src was determined under the same assay conditions except that no synthetic peptide was used.

Csk Immune Complex Kinase Assay
The tyrosine kinase activity of Csk was determined in immune complexes using the random copolymer, polycl(Glu, Tyr) 4:1. Immunoprecipitates prepared with rabbit polyclonal anti-Csk antibody bound to protein A-Sepharose beads were incubated for 10 minutes at 30°C with an assay buffer containing (in mmol/L) Tris-HCl (pH 7.4) 60, MgCl₂ 2, Na₃VO₄ 0.25, DTT 10; 200 μg/mL BSA; and 125 μmol/L cold ATP and 10 μCi of [γ³²P]ATP. The phosphorylated substrates were separated from the residual [γ³²P]ATP with P81 phosphocellulose paper and quantified in a scintillation counter.

Functional Analysis

Construction of Epitope-Tagged c-Src and Mutant c-Src cDNAs
Full-length c-Src cDNA was amplified by reverse transcription–polymerase chain reaction (PCR) using mRNA isolated from chicken heart. Initially, 2 domains of the c-Src cDNA (nucleotides 1 to 774 and nucleotides 775 to 1599) were amplified and cloned into Bluescript KS(+) vector, after which the cloned sequences were verified by nucleotide sequencing. To construct the N-terminal domain, each sense and antisense PCR primer contained an EcoRI site at the 5′end, whereas to construct the C-terminal domain, only the sense PCR primer contained an EcoRI site at the 5′end. The antisense PCR primer was designed to delete the endogenous stop codon and replace it with a FLAG sequence containing an MluI site at nucleotide position 774, thereby producing the full-length, wild-type c-Src cDNA (c-Src(WT)). To construct the constitutively active c-Src,23 a point mutation substituting tyrosine527 with phenylalanine was introduced in the C-terminal domain using PCR-based mutagenesis.24 After verifying the nucleotide sequences, the 2 domains were excised and ligated using the MluI site at nucleotide position 774, thereby producing the full-length cDNA encoding c-Src(WT) or c-Src(Y527F). For transfection, the full-length cDNAs encoding c-Src(WT) or c-Src(Y527F) were ligated into the EcoRI sites of either the pcDNA3 mammalian expression vector or the pZeoSV expression vector.

Stable Coexpression of Connexin43 and c-Src(WT) or c-Src(Y527F) in HEK293 Cells
HEK293 cells were transfected with the pcDNA vector containing connexin43 cDNA using the calcium phosphate precipitation technique, and a stable cell line overexpressing connexin43 was established by subsequent selection with 800 μg/mL G418.24 Clones expressing connexin43 were then transfected with pZeoSV vectors containing c-Src(WT) or c-Src(Y527F) cDNAs using the calcium phosphate precipitation technique, and the transfecants were grown for selection in DMEM containing 250 μg/mL Zeocin and 400 μg/mL G418. Each clone selected was analyzed by Northern blot and immunoblot analyses.

Transient Coexpression of c-Src(WT) or c-Src(Y527F) With Plasmid pGreen Lantern-1 in Cardiac Myocytes
Cardiac myocytes plated on coverslips were cotransfected with plasmid pGreen Lantern-1 and pcDNA3 vectors containing the cDNA for either c-Src(WT) or c-Src(Y527F) using lipofect agent. Expression of green fluorescent protein (GFP) and c-Src by the transfectants was assessed 24 hours later using immunofluorescence microscopy and alternating between the appropriate filter sets for GFP (absorption, blue; emission, green) and for c-Src labeled with anti-FLAG and rhodamine-conjugated anti-mouse IgG antibodies (absorption, blue; emission, red). The molar ratio of pGreen Lantern-1 and c-Src cDNAs for transfection was optimized to be 1:5. At this ratio, GFP-positive cells always expressed c-Src. Once cells expressing c-Src were identified, they were subjected to electrophysiological analysis to assess the effect of c-Src expression on the gap junctional conductance between cardiac myocytes.

Measurement of Intercellular Ca²⁺ Propagation
Intercellular Ca²⁺ propagation was measured as described previously.25 HEK293 cells were used in this experiment, because they contain neither endogenous connexin 43 nor functional connexin 40,43. When HEK293 cells were transiently transfected with ryanodine receptor cDNAs, only a few percent of total cells expressed exogenous ryanodine receptor.25 If caffeine, an activator of ryanodine receptor, was applied to these cells, ryanodine receptor–expressing cells could become the primary cells that initially increase intracellular Ca²⁺ in response to caffeine, thereby triggering intercellular Ca²⁺ propagation. To determine whether c-Src mediates the gap junctional property of connexin43, we created 2 stable cell lines expressing connexin43 with or without constitutively active c-Src, namely c-Src(W527F).

In the experiment, cells plated on glass coverslips were transfected with the PMT2 expression vector containing the cDNA for the ryanodine receptor. Forty-eight hours after transfection, the cells were incubated in DMEM containing 5 μmol/L Fura 2-AM and 0.06% pluronic F127. Coverslips with dye-loaded cells were mounted in a laminar flow perfusion chamber on the stage of an inverted microscope, where the cells were continuously superfused with HEPES buffer containing (in mmol/L) NaCl 140, KCl 5, MgCl₂ 0.3, HEPES (pH 7.4) 15, glucose 10, and CaCl₂ 1.8. An Argus-50Ca image processor (Hamamatsu Photonics) was used to acquire video frames containing fluorescent images of Fura 2-AM–loaded cells at 2-second intervals and to calculate maps of the distribution of intracellular free Ca²⁺ ([Ca²⁺]) within the cells present in each frame; [Ca²⁺²], was calculated from the ratios of the fluorescence intensities excited by illumination at 340 and 380 nm. Caffeine (15 μmol/L), an activator of the ryanodine receptor, was added to HEPES buffer. The effect of the tyrosine kinase inhibitor genistein on cell-cell coupling among cells overexpressing connexin43 and c-Src(Y527F) was examined by superfusing Fura 2-AM–loaded cells for 10 minutes with HEPES buffer containing 30 μmol/L genistein and then exposing the cells to caffeine.

Electrophysiology
Gap junctional currents (Iₓ) were measured using a Geneclamp 500 amplifier (Axon Instruments, Inc) and a double whole-cell patchclamp procedure.26–27 Pairs of GFP-positive cardiac myocytes overexpressing c-Src(WT) or c-Src(Y527F) were selected by fluorescence microscopy. Coverslips were then transferred to the stage of a Nikon Diaphot microscope, where experiments were performed at room temperature while exchanging the bath solution containing (in mmol/L) NaCl 133, KCl 3.6, CaCl₂ 1.0, MgCl₂ 0.3, glucose 16, and HEPES (pH 7.2) 3.0.

For each cell in the pair, access to the cell interior was achieved by applying gentle suction to the tip of a fire-polished glass pipette (3 to 5 MΩ) filled with solution containing 10 mmol/L free Ca²⁺ and (in mmol/L) CsCl 135, CsCl 0.5, MgCl₂ 2, EGTA 5.5, and HEPES 5.0 (pH 7.2) and sealed to the cell membrane (seal resistance, >1 GΩ). Cells were voltage clamped at a holding potential of −40 mV, and voltage pulses (10 mV, 200 to 500 ms in duration) were alternately applied to each cell. Within each cell pair, Iₓ was calculated from the ratios of the fluorescence intensities excited by illumination at 340 and 380 nm. Caffeine (15 μmol/L), an activator of the ryanodine receptor, was added to HEPES buffer. The effect of the tyrosine kinase inhibitor genistein on cell-cell coupling among cells overexpressing connexin43 and c-Src(Y527F) was examined by superfusing Fura 2-AM–loaded cells for 10 minutes with HEPES buffer containing 30 μmol/L genistein and then exposing the cells to caffeine.
measured keeping constant the membrane potential in one cell and applying the voltage steps ($V_j$) to the other cell. Junctional conductances ($G_j$) were calculated from the equation:

$$G_j = I_j / V_j.$$  

The theory behind measuring gap junctional conductance has been considered in detail elsewhere. 26 The nonjunctional membrane resistance was usually on the order of 0.2 to 0.5 GΩ, which was more than 100 times greater than the series resistance (3 to 5 MΩ). Consequently, the current flowing through the junction was approximately equal to the recorded current change ($I_j$). The series resistance of the electrode, which was repeatedly checked during the experiments and corrected by occasional suction if the electrode started to clog, was always <1% of the parallel sum of the seal resistance (>1 GΩ) and the nonjunctional membrane resistance (0.2 to 0.5 GΩ). Thus, because changes in series resistance had minimal impact on the recording, we rarely used series resistance compensation in this study.

Statistics

Data are presented as mean±SD. Statistical analysis was performed using ANOVA and unpaired Student t tests as appropriate. Values of $P<0.05$ were considered significant.

Results

General Characteristics of BIO 14.6

Cardiomyopathic and BIO F1B Control Hamsters

The experimental animals used in this study were routinely assessed for penetrance of the cardiomyopathic phenotype. Significant pathological alterations are usually undetectable during the early stages of the cardiomyopathy (up to 2 to 3 months), although the characteristic gross appearance caused by muscular weakness and heart failure develops progressively and becomes prominent during the late stage (7 to 8 months after birth). 15 To assess cardiac hypertrophy in our experimental animals, cardiac ventricle/body weight ratios were measured at various stages during the development of the cardiomyopathy. When compared with values from age-matched, BIO F1B controls, the cardiac ventricle/body weight ratio of BIO 14.6 hamsters was comparable with control at 2 months of age (early stage) but then increased substantially, so that by 10 months of age (late stage; Figure 1), cardiac hypertrophy was evident.

Phosphorylation of Connexin43 and Connexin45 in Cardiomyopathic Heart

To determine whether during the development of cardiomyopathy there were changes in the relative expression levels of connexin43 or connexin45 and/or whether isoform switching took place, connexin43 and connexin45 were quantified by densitometric analysis of immunoblots (Figure 2A). These measurements showed that among the groups tested, levels of connexin43 and connexin45 were not significantly different.

Figure 1. Cardiac ventricle/body weight ratios of experimental animals. Cardiac ventricles were obtained from F1B (control) and BIO14.6 hamsters at early (E) and late (L) stages of cardiomyopathy. Each sample was weighed and then divided by the body weight. Data are mean±SD obtained from 5 samples in each group. *$P<0.05$ vs control at early stage.

Figure 2. Immunoblot analysis for detection of connexin43 and connexin45 in cardiomyopathic heart. A, Cardiac cell lysates were prepared from F1B (control) and BIO14.6 hamsters at early and late stages of cardiomyopathy. Proteins were resolved by SDS-PAGE and immunoblotted with anti-connexin43 and anti-connexin45 antibodies. B, Cardiac cell lysates from control and BIO14.6 hamsters were treated with (+) or without (−) alkaline phosphatase (AP) and then resolved by SDS-PAGE and immunoblotted with specific antibodies. Note that the slower mobility band of connexin43 was selectively sensitive to alkaline phosphatase. P and NP indicate phosphorylated and unphosphorylated forms of connexin43, respectively. Mobility of molecular mass markers is shown on the right.
Figure 3. Immunoprecipitation and immunoblot analyses for detection of serine- and tyrosine-phosphorylated connexin43 and connexin45 in cardiomyopathic hearts. A, Cardiac cell lysates were immunoprecipitated with either anti-phosphoserine or anti-phosphotyrosine antibody. The immunoprecipitates were resolved by SDS-PAGE and immunoblotted with anti-connexin43 or anti-connexin45 antibodies. B, Cardiac cell lysates were immunoprecipitated with anti-connexin43 antibody, and then the resultant immunoprecipitated connexin43s were immunoblotted with either anti-phosphoserine or anti-phosphotyrosine antibody. A and B, Band at 55 kDa (arrow) represents mouse immunoglobulin heavy chains. IP indicates immunoprecipitation; anti-P-Ser, anti-phosphoserine; and anti-P-Tyr, anti-phosphotyrosine antibody. The mobility of molecular mass markers is shown on the right.

doubledt with a major band of 43 to 45 kDa (indicated by P) and a minor band of 41 kDa (indicated by NP). Consistent with previous reports, connexin45 migrated as a major band of 48 kDa and a very faint band of 46 kDa; levels of the latter varied substantially among experiments and were thought to correspond to a proteolytic degradation product of the 48-kDa polypeptide. No differences were found among experimental groups in the relative quantities of connexin43 and connexin45.

To determine whether connexin43 and connexin45 were modified by phosphorylation, protein samples were treated with alkaline phosphatase and immunoblotted with mouse anti-connexin43 or rabbit anti-connexin45 antibody (Figure 2B). Alkaline phosphatase treatment collapsed the 43- to 45-kDa bands into the 41-kDa form of connexin43, indicating that the lower electrophoretic mobilities could be attributed to phosphorylated products. In contrast, alkaline phosphatase treatment did not alter the mobility of connexin45; this was despite the fact that we (in this study) and others have shown it to be phosphorylated.

To obtain an indication as to whether the connexins were phosphorylated on serine or tyrosine, cardiac cell lysates were immunoprecipitated with mouse monoclonal anti-

phosphoserine and anti-phosphotyrosine antibodies linked to protein A–Sepharose beads. The immunoprecipitated proteins were then immunoblotted with mouse anti-connexin43 or rabbit anti-connexin45 antibody (Figure 3A). Anti-phosphoserine antibody immunoprecipitated both connexin43 and connexin45 in all cardiac cell lysates, indicating that in all of the experimental groups, connexin43 and connexin45 were serine phosphorylated to a similar degree. In contrast, anti-phosphotyrosine antibody selectively immunoprecipitated connexin43 in cardiac cell lysates from late-stage BIO 14.6 hamsters.

To eliminate the possibility that other phosphorylated proteins pulled down by the immunoprecipitation procedure cross-react with anti-connexin43 antibody, the experiments were carried out in reverse order: cardiac lysates were first immunoprecipitated with anti-connexin43 antibody, and then the resultant immunoprecipitated connexin43s were immunoblotted with anti-phosphoserine or anti-phosphotyrosine antibodies (Figure 3B). Phosphorylation of both tyrosine and serine residues was detected in the connexin43s of late-stage BIO 14.6 hamsters. Thus, in the late stage of the cardiomyopathic heart, connexin43s, which were usually phosphorylated only at serine residues, were further phosphorylated at tyrosine residues.

**Specific Activity of c-Src in the Cardiomyopathic Heart**

To determine whether c-Src, a principal tyrosine kinase in cardiac myocytes, is involved in the tyrosine phosphorylation of connexin43, the specific activity of c-Src was measured by immune complex kinase assay (Figure 4). c-Src activity was 3-fold greater in late-stage BIO 14.6 hamsters than in control hamsters (Figure 4A, left), despite the fact that expression of c-Src protein was similar in both hamster types (Figure 4B, left). In addition, immunoprecipitation-immunoblot assay showed that c-Src was tyrosine phosphorylated in late-stage BIO 14.6 hamsters (Figure 4B, middle), confirming the correlation between tyrosine phosphorylation of c-Src and its activation; c-Src is activated by autophosphorylation at Tyr419 and inactivated by phosphorylation at Tyr527 by Csk. Autophosphorylation of c-Src was measured by assaying the c-Src immune complex in the absence of substrates and was found to be increased in late-stage BIO 14.6 hamsters (Figure 4A, middle). In contrast, Csk activity and protein levels were similar in both hamster types (Figure 4A, right and 4B, right). Apparently then, c-Src autophosphorylation was responsible for its activation in the late-stage cardiomyopathic heart.

**Tyrosine Phosphorylation of Connexin43 by Activated c-Src**

Our biochemical approach clearly showed that tyrosine phosphorylation of connexin43 was correlated with activation of c-Src in the failing cardiomyopathic heart. But it left open the question of the in vivo, functional significance of this finding. To address this question, the phosphorylation state of connexin43 was examined in HEK293 cells coexpressing connexin43 with either wild-type c-Src(WT) or constitutively active c-Src(Y527F) (Figure 5A). Immunoblot analysis...
We previously showed that propagation of Ca$^{2+}$ waves between cardiac myocytes overexpressing c-Src(Y527F) propagated Ca$^{2+}$ waves through gap junctions, and the effect was reversed by the tyrosine kinase inhibitor genistein. We therefore hypothesized that during intercellular Ca$^{2+}$ wave propagation, inositol triphosphate and/or Ca$^{2+}$ permeate gap junctions to trigger Ca$^{2+}$ release from intracellular Ca$^{2+}$ stores in adjacent cells. On the other hand, Lamont et al$^{34}$ showed that Ca$^{2+}$ does not pass between cardiac myocytes, suggesting that the transport properties of connexin43 are tissue-specific.

To gain further insight into the selectivity of gap junction permeability, we examined the role of c-Src in gap junctional communication by studying intercellular propagation of Ca$^{2+}$ waves between HEK293 cells expressing connexin43 (Figure 6) as well as the electrical coupling between cardiac myocytes (Figure 7). We first measured the intercellular propagation of Ca$^{2+}$ between HEK293 cells transiently transfected with ryanodine receptor cDNA. Application of caffeine to HEK293 cells, which lack endogenous connexin43, increased [Ca$^{2+}$]i in one member of a cell cluster but did not induce intercellular propagation of Ca$^{2+}$ (Figure 6A). Application of caffeine to cells coexpressing connexin43 with c-Src(WT), by contrast, typically increased [Ca$^{2+}$], in one member of a cell cluster first, but then a wave of increased [Ca$^{2+}$], was propagated from one cell to another (Figure 6B). Cells coexpressing connexin43 with c-Src(Y527F) propagated Ca$^{2+}$ waves only minimally, although in those cells, treatment with genistein significantly increases the propagation (Figure 6C). This means that tyrosine phosphorylation of connexin43 by constitutively active c-Src decreased propagation of Ca$^{2+}$ waves through gap junctions, and the effect was reversed by the tyrosine kinase inhibitor genistein.

**Effect of c-Src on Gap Junctional Conductance Between Cardiac Myocytes**

We next examined the effect of activated c-Src on gap junction formation in cardiac myocytes (Figure 7). Because we were unable to generate stable cultures of cardiac myocytes overexpressing c-Src, a transient expression strategy showed that both cell lines expressed similar amounts of c-Src(WT) and c-Src(Y527F) (left panel), and neither altered coexpression of connexin43 (right panel). Anti-phosphoserine antibody immunoprecipitated connexin43 in cells coexpressing c-Src(WT) or c-Src(Y527F), indicating that in this cell line, expressed connexin43 is endogenously serine phosphorylated as is observed in gap junction–competent cells (eg, cardiac myocytes). Anti-phosphotyrosine antibody immunoprecipitated connexin43 in cells coexpressing c-Src(Y527F) but not in cells expressing c-Src(WT). Furthermore, pretreatment for 10 minutes with 30 mmol/L of genistein diminished the tyrosine phosphorylated connexin43 band, indicating that activated c-Src directly tyrosine phosphorylated connexin43 in vivo.

The immunofluorescent analysis shown in Figure 5B was carried out to further investigate the effect of overexpressed c-Src on the distribution of connexin43 in the transfected cells, because connexin43 signal intensity appeared to vary among experiments, and reduced labeling might have resulted from increased phosphorylation of connexin43 in the c-Src(Y527F) transfectants. Indeed, using the same monoclonal mouse anti-connexin43 antibody used in the present study, fibroblast growth factor-2–evoked decreases in connexin43 labeling were previously shown to coincide with increased phosphorylation of connexin43 in the absence of substrate (middle panel). Autophosphorylation of c-Src was measured by immune complex kinase assay using poly(Glu) as a substrate (right panel). The kinase reaction was carried out as described in Materials and Methods. Data are normalized to the mean of control at the early stage, which was set to a value of 1. Data are mean ± SD obtained from 15 samples in each group. *P < 0.05 vs control at the early stage.
was used. Cardiac myocytes were cotransfected with plasmid pGreen Lantern-1 and either c-Src(WT) or c-Src(Y527F) at a molar ratio of 1:5. At that ratio, 0.5% to 2% of the total number of cardiac cells expressed GFP, and cells expressing GFP always coexpressed c-Src; 1% to 5% of the cells expressed only c-Src (Figure 7A). Consequently, cells expressing c-Src(WT) or c-Src(Y527F) were readily identified as GFP-positive cells using fluorescence microscopy without immunostaining. Once identified, pairs of the GFP-positive cells were subjected to electrophysiological analysis.35 Repetitive 10-mV pulses were applied to pairs of voltage-clamped cells, and $G_j$ was measured as described in Materials and Methods. In control cells and cells transfected with c-Src(WT), $G_j$ was measured to be 126.6 ± 55.8 nS ($n=9$) and 122.4 ± 60.3 nS ($n=13$), respectively, conductances that were similar in amplitude to those previously recorded in rat cardiac myocyte pairs.36 In contrast, $G_j$ measured in cardiac myocytes transfected with c-Src(Y527F) was only 28.5 ± 16.3 nS ($n=16$). However, when cells overexpressing c-Src(Y527F) were treated with 20 mmol/L genistein, which is sufficient to block tyrosine phosphorylation (Figure 5A), $G_j$ increased to 101.5 ± 30.6 nS ($n=16$).

To eliminate potential effects of changes in nonjunctional membrane resistance on the value of $G_j$, nonjunctional membrane resistance was measured before and after each experiment. It was found to range from 0.2 to 0.5 GΩ and it did not vary among experimental groups.

Discussion

Connexin43 is a major connexin in cardiac myocytes and is required for proper contractile performance of cardiac muscle; when connexin43 expression is disrupted by gene targeting, conduction velocity is slowed in the ventricular muscle of mice heterozygous for the connexin43 null mutation.5 In the cardiomyopathic hamster, the striking feature of the end stage of heart failure is the presence of substantial scarring.37,38 Myolytic foci are also found at this stage, although infiltration by inflammatory cells and fibroblasts is usually minimal. Distribution of connexin43 becomes diffuse, coinciding with the irregular structure of the intercalated discs,39 and gap junctional conductance between isolated cardiac myocytes is decreased to 10% to 20% of control.40 Given that the expression level of connexin43 does not change in the late-stage, cardiomyopathic heart,49 decreased conductance is most likely caused by functional changes in the gap junction. This idea is supported by our findings that connexin43 is tyrosine phosphorylated in the late-stage, cardiomyopathic hamster in parallel with activated c-Src activity, and that the gap junctional conductance between cardiac myocytes is reduced by constitutively active c-Src.

At its C-terminal tail, connexin43 contains potential phosphorylation sites for a variety of serine/threonine protein kinases, including cAMP-dependent protein kinase,6 PKC,41 MAP kinase,42 and Src family tyrosine kinases.9,43 Consistent with earlier studies,28,44 we observed that in gap junction–competent cells (eg, normal cardiac myocytes), most connexin43 is serine phosphorylated. Despite the fact that it is thought to be required for gap junction formation,29 serine phosphorylation decreases the conductance through the channels formed by the connexin43 molecules.41 Furthermore, additional serine phosphorylation induced by α-adrenergic stimulation in cardiac myocytes,45,46 and in mitotic vascular cells at the M phase of the cell cycle47 further decreases gap junctional communication. Thus, the functional properties of connexin43 appear to be determined by the relative levels of serine and tyrosine phosphorylation. Therefore, we suggest that the phosphorylation state of connexin43 is an important regulator of the function of gap junctions in the cardiomyopathic heart, which is supported by our finding that tyrosine phosphorylation of connexin43 occurs during the progression of cardiac dysfunction in cardiomyopathic hamster. This is in contrast to other cases, such as hypertrophied and hibernating myocardium, in which electrical uncoupling between cardiac myocytes is accompanied by decreased levels of connexin43.48,49
The gene encoding δ-sarcoglycan, one of the dystrophin-associated glycoproteins, was recently identified as a candidate gene for the etiology of BIO14.6 cardiomyopathic hamster, suggesting that disruption of the membrane architecture caused by mutated δ-sarcoglycan is a primary cause of the observed cardiomyopathic changes. Moreover, the resultant humoral and hemodynamic changes induced by the cardiac dysfunction likely trigger signaling to increase tyrosine phosphorylation of connexin43, as shown by our functional assay using constitutively active c-Src. This notion is consistent with evidence that cell-cell interfaces are enriched in c-Src, especially in the intercalated discs of cardiac myocytes, and when fibroblasts are transfected with v-Src, gap junctional conductance is reduced on tyrosine phosphorylation of connexin43. Although increased c-Src activity could potentially be caused in part by inflammatory cells infiltrating myolytic lesions in the cardiomyopathic heart, the small number of these cells present within the lesions suggest that their contribution is minimal.

c-Src activity is itself regulated by its phosphorylation state: autophosphorylation on Tyr419 activates c-Src, whereas phosphorylation on Tyr527 by Csk inhibits it. In the late-stage, cardiomyopathic heart, activation of c-Src was correlated with autophosphorylation, but not with Csk activity. Several lines of evidence suggest a role for c-Src in signaling via G-protein–coupled receptors. For example, c-Src is activated after stimulation of thrombin, endothelin I, or angiotensin II receptors. There is also evidence that humoral factors, including an α-adrenergic agonist, endothelin I, and angiotensin II, are released during cardiac dysfunction, and inhibitors of angiotensin-converting enzyme increase conduction velocity in cardiomyopathic ventricles by as much as 30%. It is therefore plausible that such humoral factors may affect the contractile performance of the failing heart through c-Src–mediated tyrosine phosphorylation of connexin43.

The major features of heart failure are contractile dysfunction and arrhythmia. Based on the results of the present study, it is plausible that gap junctional incompetence caused by tyrosine phosphorylation of connexin43 contributes to these abnormalities by disrupting the coordinated excitation of cardiac myocytes.

Figure 6. Effect of c-Src on intercellular propagation of Ca$^{2+}$ waves between connexin43-expressing cells. Distributions of [Ca$^{2+}$]i in ryanodine receptor–transfected HEK293 cells are shown at 0, 10, and 20 seconds after onset of the peak increase in [Ca$^{2+}$]i elicited by caffeine; [Ca$^{2+}$]i is calibrated to the pseudocolor scale bar. A, Caffeine increased [Ca$^{2+}$]i in one cell but did not induce Ca$^{2+}$ propagation. B, Caffeine induced propagation of Ca$^{2+}$ between cells coexpressing connexin43 and c-Src(WT). C, Caffeine induced propagation of Ca$^{2+}$ between cells coexpressing connexin43 and c-Src(Y527F). Amplitudes of propagated Ca$^{2+}$ waves were markedly smaller than those shown in panel B. C2, Same cells seen in panel C1 were then exposed to genistein for 10 minutes, after which they were stimulated with caffeine. Amplitudes of propagated Ca$^{2+}$ were significantly increased by genistein.
cardiac myocytes. Although previous studies have assigned a central role to PKC and MAP kinase in the regulation of connexin43-mediated communication, c-Src appears to be a key element during the progression of heart failure. Interestingly, activation of Src family tyrosine kinases has until now been thought to be a favorable consequence of the hypertrophic response of cardiac myocytes to pressure overload. However, it appears that once cardiac muscle dysfunction reaches an uncompensated state, activation of c-Src, instead leads to further deterioration of cardiac performance. Accordingly, pharmacological intervention designed to restore gap junctional function may prove valuable in the clinical management of arrhythmia and decreased contractility in heart failure.

Acknowledgment

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

References


Functional Role of c-Src in Gap Junctions of the Cardiomyopathic Heart
Toshihiko Toyofuku, Masanori Yabuki, Kinya Otsu, Tsunehiko Kuzuya, Michihiko Tada and Masatsugu Hori

Circ Res. 1999;85:672-681
doi: 10.1161/01.RES.85.8.672

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1999 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/85/8/672

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/