Signals Through gp130 Upregulate *bcl-x* Gene Expression Via STAT1-binding *cis*-Element in Cardiac Myocytes

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Abstract

We described recently the activation of the Janus kinase-signal transducer and activator of transcription (Jak-STAT) and mitogen-activated protein (MAP) kinase pathways by leukemia inhibitory factor (LIF) through gp130, a signal transducer of IL-6–related cytokines, that transduces hypertrophic signals in cardiac myocytes. In addition, stimulation of gp130 by IL-6–related cytokines is known to exert a cytoprotective effect.

In the present study, we investigated the possibility that activation of gp130 initiates activation of the cytoprotective genes in cardiac myocytes. Incubation of cardiac myocytes with LIF induced the expression of bcl-x, and the isoform that was induced by LIF was identified as bcl-xL. Induction of bcl-xL protein was also identified by Western blotting. Antisense oligonucleotide against bcl-x mRNA inhibited protective effect of LIF accompanied with the reduction in bcl-xL protein.

We constructed *bcl-x* promoter-luciferase reporter gene plasmids (−639/+10- or −161/+10-luciferase), and transfected them to cardiac myocytes. LIF stimulation increased the luciferase activity of −639/+10-luciferase plasmids. Although −161/+10-luciferase plasmids presented comparable responsiveness to LIF, the basal transcription level was impaired. The LIF-responsive *cis*-element was localized to a DNA fragment (positions −161 to +10) that contains an interferon-γ activation site (GAS) motif (TTCGGAGAA) at position −41 of the *bcl-x* gene promoter. This motif bound to STAT1, not to STAT3, and site-directed mutagenesis revealed that this motif was essential for LIF-responsive promoter activity.

These data suggest that LIF induces bcl-x mRNA via STAT1 binding *cis*-element in cardiac myocytes, presenting cytoprotective effect. (*J. Clin. Invest.* 1997. 99:2898–2905.)
Key words: IL-6–related cytokine • leukemia inhibitory factor • signal transduction • *bcl-2* family • Jak-STAT pathway

Introduction

gp130 is a signal transducer of interleukin 6 (IL-6)–related cytokines (1), and is widely expressed in various organs, including the heart (2). Binding of the IL-6–related cytokine to its receptor induces the receptor–gp130 complex to transduce the signal. Transgenic mice overexpressing IL-6 and IL-6 receptor (that constitutionally activate gp130) demonstrate myocardial hypertrophy (3). In contrast, disruption of the gene for gp130 was found to be lethal in mice, and these animals demonstrated immature hypoplastic ventricular myocardium (4).

Recently, a novel IL-6–related cytokine, cardiotrophin-1 (CT-1),[1] was cloned and characterized as a cardiac myocyte hypertrophy-inducing factor (5). CT-1 binds to leukemia inhibitory factor receptor, and exhibits biological activity in cardiac myocytes, such as induction of immediate early genes through the activation of gp130 (6, 7).

Cytoprotective function is one of the most remarkable activities of the IL-6 family of cytokines. Ciliary neurotrophic factor, which also signals through the activation of gp130, protects neural cells from the degeneration induced by axotomy (8). Similar results were observed in the IL-6 and IL-6 receptor double-transgenic mice (9).

CT-1 is reported to prevent cell death induced by serum depletion in neonatal rat cardiac myocytes (10). As a molecular basis of the cytoprotective effects of IL-6–related cytokines, IL-6 prevented the apoptosis that was induced by IL-6 depletion with the upregulation of bcl-xL in an IL-6–dependent myeloma cell line (11). *Bcl-x* was identified as a *bcl-2*–related gene that functions as a regulator of apoptotic cell death (12). Bcl-x has two isoforms; one is bcl-xL, a preventive isoform against apoptosis, and the other is bcl-xS, a promotive isoform.

To elucidate the molecular mechanisms of the cytoprotective effects of the IL-6–related cytokines, we examined the effects of leukemia inhibitory factor (LIF)—one of the IL-6–related cytokines that exerts biological effects on cardiac myocytes (7, 13)—on the expression of bcl-xL mRNA in cardiac myocytes. In addition, the localization of the LIF-responsive *cis*-element in the promoter region of the *bcl-x* gene was determined using *bcl-x* promoter–luciferase reporter plasmids. The electrophoretic mobility shift assay indicated that signal transducer and activator of transcription 1 (STAT1), but not STAT3, binds to the LIF-responsive *cis*-element of the *bcl-x* gene.

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1. *Abbreviations used in this paper:* CT-1, cardiotrophin 1; EMSA, electrophoretic mobility shift assay; GAS, interferon-γ activation site; Jak-STAT, Janus kinase-signal transducer and activator of transcription; LIF, leukemia inhibitory factor; M-199, medium-199; MAP, mitogen-activated protein; NCS, newborn calf serum; NMC, nonmyocardial cells; RT, reverse transcription; SIE, *sis*-inducible element.

Methods

Materials. Recombinant murine LIF (10^6 U/ml) from Amrad Biotech (Boronia, Victoria, Australia) and norepinephrine (1 mg/ml) from Sankyo Co. Ltd. (Tokyo, Japan) were used in the present study. Anti-STAT1 and anti-STAT3 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti–*bcl-x* monoclonal antibody was obtained from Transduction Laboratories (Lexington, KY). Human recombinant IL-6 and soluble IL-6 receptor were kindly donated by Dr. T. Taga (Department of Molecular Cell Biology, Tokyo Medical and Dental University).

Cell culture. Primary cultures of neonatal cardiac myocytes were prepared by collagenase and trypsin digestion from the ventricles of 1-d-old Sprague Dawley rats, or 1–2-d-old DDY mice, obtained from Nippon Dobutsu (Osaka, Japan) as described previously (14). In brief, cultures were enriched with cardiac myocytes by preplating for 60 min to deplete the population of nonmyocardial cells (NMC). Nonattached cells were then suspended in medium-199 (M-199) supplemented with 10% newborn calf serum (NCS), plated onto plastic tissue culture dishes, and cultured for 24 h at 37°C in 95% air/5% CO_2. 15 h after plating, the culture medium was changed to M-199 with 1% NCS and 0.1 mM bromodeoxyuridine for 24 h. Over 90% of the cells displayed spontaneous contractile activity in the case of rat cardiac myocyte culture, compared with 80–90% in murine cardiac myocyte culture. The culture medium was changed to M-199 48 h before the experiments.

Attached cells during preplating were cultured as NMC, and were maintained for 4 d with the medium changed every 2 d. NMC were passaged twice by treatment with trypsin/EDTA. NMC cultures were composed of > 95% fibroblasts.

Northern blotting. Murine cardiac myocytes were cultured at a cell density of 10^6/8 ml in 100-mm dishes. Total RNA was prepared by the acid guanidinium thiocyanate-phenol-chloroform (AGPC) method (15). 10 μg of total RNA was size-fractionated by formaldehyde–agarose gel electrophoresis, and blotted onto a nylon membrane (Hybond N^+; Amersham International PLC, Little Chalfont, United Kingdom). Hybridization and washing were performed as previously described (12). Mouse bcl-x cDNA, kindly donated by Dr. Y. Tsujimoto (Osaka University Medical School), was used as a probe.

Reverse transcription (RT)-PCR assay. RNAs from murine cardiac myocytes or NMC were prepared as described above. Total RNA (0.1 μg) was subjected to first-strand synthesis using oligo-dT (Pharmacia Biotech AB, Uppsala, Sweden) and moloney murine leukemia virus reverse transcriptase (GIBCO BRL, Gaithersburg, MD) at 37°C for 2 h, and the reaction was stopped by incubation at 70°C for 10 min. The primers used for bcl-x amplification by PCR were synthesized according to the sequence reported by Fang et al. (16).

For semiquantitative PCR for bcl-x, the 5′ primer and the 3′ primer were 5′-AGTCAGTTTAGTGATGTC-3′ and 5′-GGATGTTAGATCACTGAA-3′, respectively. PCR (1 min at 96°C, 1 min at 55°C, 2 min at 72°C) was performed at 35 cycles. In this condition, relative quantitative analysis of PCR products was achieved (see Fig. 1 *A*). PCR products were subcloned and sequenced to identify the sequence of mouse bcl-x cDNA (16) (data not shown). For determining the isoform of bcl-x, another 3′ primer (5′-GTTCCCGTAGAGATCCAC-3′) was synthesized. PCR was performed at 40 cycles to attain maximal sensitivity of the reaction, leading to the loss of quantitative accuracy.

PCR for *bcl-2* was performed at 33 cycles (1 min at 96°C, 1 min at 58°C, 2 min at 72°C) using the primers derived from the mouse bcl-2 cDNA sequence (17) (5′-GCGCAAGCCGGGAGAACA-3′ as 5′ primer, and 5′-AGACGTCCTGGCAGCCAT-3′ as 3′ primer). In this condition, relative quantitative analysis of PCR products was achieved (data not shown). PCR for β-tubulin was performed according to Miller-Hance et al. (18) with a slightly modified protocol (5 cycles with 45 s at 94°C, 45 s at 72°C, and 2 min at 72°C; 23 cycles with 45 s at 94°C, 1 min at 55°C, and 2 min at 72°C).

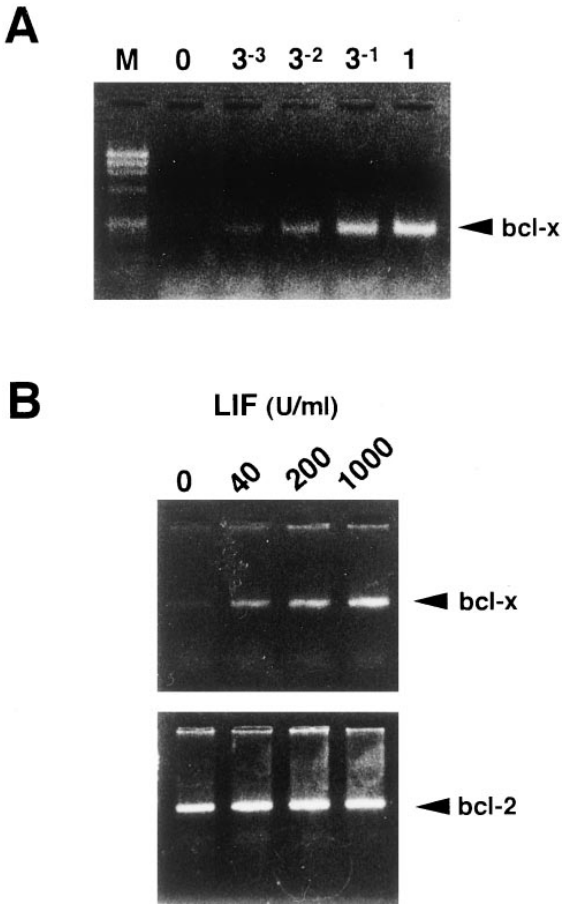

Figure 1. Semiquantitative RT-PCR analysis of bcl-x mRNA expression. (*A*) RNA was prepared from murine cardiac myocytes treated with LIF (10^3 U/ml) for 24 h. RT reaction was performed, and the product was diluted by threefold in order. Thereafter, PCR was performed as described in Methods. Molecular weight markers from HaeIII-digested ϕX 174 are indicated (*M*). (*B*) Cardiac myocytes were treated with various concentrations of LIF for 24 h, and RT-PCR was performed using bcl-x primers (*top*) or bcl-2 primers (*bottom*) as described in Methods.

Cell viability assay. Neonatal rat cardiac myocytes were cultured at a density of 2×10^4/100 μl in 96-well dishes. After culturing with M-199 with 10% NCS for 15 h, the medium was changed to M-199 with 1% NCS, and was incubated for 24 h. Thereafter, cardiac myocytes were cultured without serum for another 24 h, and were stimulated with LIF. 72 h later, myocytes were washed with PBS twice and treated with 25 μl of collagenase trypsin for harvest. The protease reactions were terminated with the addition of medium containing trypan blue. Thereafter, trypan blue–excluded cells were counted. Each assay was performed in quadruplicates.

Western blotting. Neonatal rat cardiac myocytes were cultured at a cell density of 3×10^5/3 ml in 60-mm dishes in duplicates. After being cultured with M-199 with 10% NCS for 15 h, the medium was changed to M-199 with 1% NCS, and was incubated for 24 h. Thereafter, cardiac myocytes were cultured without serum for another 24 h. The cells were was then stimulated with LIF for 24 h. One of the duplicated dishes was harvested for counting the cell number. The other dish was lysed directly with SDS-PAGE solution, and was immediately boiled for 5 min. Cell lysates containing 5×10^3 cells were SDS-gel electrophoresed, transferred onto polyvinylidene difluoride membrane, and immunoblotted with anti–bcl-x antibody according to Kunisada et al. (14).

Antisense assay. The antisense and sense phosphorothioate oligonucleotides were designed according to Wang et al. (19); antisense: CTGAGACATTTTTAT, sense: ATAAAAATGTCTCAG. This region of murine *bcl-x* is completely identical to that of rat *bcl-x* (20). Antisense oligonucleotide assays were performed according to previously described methods (21). Cardiac myocytes were preincubated with antisense or sense oligonucleotides for 24 h, and were stimulated with LIF.

Luciferase assay. Neonatal rat cardiac myocytes were cultured on 60-mm dishes at a cell density of 5×10^5 cells/dish. 3 μg of DNA (2 μg of luciferase reporter DNA and 1 μg of RSVβ-gal DNA) was transfected after 18 h by the calcium phosphate method (22) with some modification. In brief, cardiac myocytes were treated with DNA aggregated by calcium phosphate for 4 h, followed by 15% glycerol treatment for 3 min. Cells were cultured in M-199 containing 1% NCS for 24 h; thereafter, NCS was depleted for 24 h. Cells were stimulated for 24 h and harvested. Luciferase and β-gal assays were performed as described previously (23).

Plasmid construction. The promoter region of the human *bcl-x* gene was obtained by hot-start PCR using anti-Taq antibody (Clontech Laboratories, Inc., Palo Alto, CA). PCR was performed using human genomic DNA (100 ng) as a template. Oligonucleotide primers were designed according to the sequence reported in GenBank (accession No. D30746); 5′-CAGATTGCAGATCTGAGGCA-3′ (forward) and 5′-CAGTGGACTCTGAATCTC-3′ (reverse). We numbered the first nucleotide in the first exon as +1. Construct −639/+10 luciferase, which contains 639 bp upstream from the transcription start site and 10 bp of the 5′-untranslated region, was constructed as described below. The PCR product was subcloned into a pGEM-T vector and constructed into the NcoI and SacI sites of the luciferase basic vector (Nippon Gene, Toyama, Japan) in which the NcoI site was newly made by NcoI linker (TaKaRa Shuzou, Otsu, Japan) at the SmaI site. The deletion construct −161/+10 region was obtained by cutting with NcoI and bglII in the −639/+10 region in the pGEM-T vector.

Site-directed mutagenesis. Site-directed mutagenesis was carried out according to instructions (LA PCR in vitro mutagenesis kit; TaKaRa Shuzou). In brief, the promoter region of the *bcl-x* gene was inserted into the multicloning sites between the SacI and SphI sites of pUC18. The mutagenesis primer was synthesized as follows: 5′-CGTCCTCTCCTAGATGCCTT-3′. Two PCRs were performed separately with two combinations of the primers; with the mutagenesis primer and M13 primer M4, or with MUT4 and M13 primer RV, respectively. These two PCR products were combined and amplified by subsequent PCR with M13 primers M4 and RV. The fragments digested with SacI and SphI were subcloned into the pGEM-T vector, and were sequenced to verify the mutant DNA.

Electrophoretic mobility shift assays (EMSAs). EMSAs were carried out according to the method previously described (24), with modifications. Whole-cell lysates used for EMSAs were prepared as follows: rat myocytes (10^7 cells/100-mm dish) were stimulated with LIF (10^3 U/ml) for 10 min, washed with phosphate-buffered saline containing 1 mM sodium orthovanadate twice, and harvested using cell scrapers. The cells were collected by centrifugation (5,000 rpm for 1 min), and lysed with 100 μl of the lysis buffer (50 mM Hepes, pH 7.8, 420 mM KCl, 0.1 mM EDTA, 0.1 mM DTT, 5 mM $MgCl_2$, 1 mM sodium orthovanadate, 20% glycerol, 0.1 mM PMSF). The samples were frozen at −80°C and were homogenized by pipetting with a 26.5G syringe. The probes were labeled with polynucleotide kinase (TOYOBO, Osaka, Japan) and added to the mixture (5×10^4 cpm/assay). The binding reactions were carried out for 30 min at room temperature in a total volume of 20 μl, containing 10 mM Hepes, pH 7.8, 105 mM KCl, 1 mM EDTA, 5 mM DTT, 5 mM $MgCl_2$, 0.2 mM sodium orthovanadate, 10% glycerol, 4 μg of poly(dI-dC)poly(dI-dC) (Pharmacia Biotech AB, Uppsala, Sweden), and 3 μg of whole cell lysate.

In some assays, competitive oligonucleotides or 2 μg of antibody was added to the mixture. The sample mixture was resolved by 4% polyacrylamide gel electrophoresis in TGE buffer (50 mM Tris, 380 mM glycine, 2 mM EDTA). The gel was dried and autoradiographed. *sis*-inducible element (SIE) oligonucleotide (24) was kindly donated by Dr. A. Yokota (Osaka University Medical School).

Statistics. Results were given as mean±SE. Statistical analysis was performed by unpaired *t* test; $P < 0.05$ was considered significant.

Results

LIF induces bcl-x mRNA in murine cardiac myocytes. As shown in Fig. 2, the expression of bcl-x mRNA was examined by Northern blotting analysis. Neonatal murine cardiac myocytes were stimulated with 10^3 U/ml of LIF, or with vehicle for 24 h. Compared with untreated cells, LIF-treated cardiac myocytes exhibited a markedly enhanced 2.7-kb band (Fig. 2, lanes *1* and *2*). The upexpression of bcl-x mRNA was also detected by RT-PCR methods (Fig. 1 *B*, *top*). LIF induced bcl-x mRNA in a dose-dependent manner. Increased expression of bcl-x mRNA was detected 6 h after the addition of LIF to the culture medium (data not shown). The expression of bcl-2 mRNA in cardiac myocytes was not induced by LIF (Fig. 1 *B*, *bottom*). In NMC, bcl-x mRNA was constantly expressed, however, the expression was not influenced by LIF (data not shown).

To identify the isoform of bcl-x in murine cardiac myocytes, we arranged the oligonucleotide primers for RT-PCR spanning the alternative splicing sites (as described in Methods), and performed PCR at 40 cycles to detect the minor com-

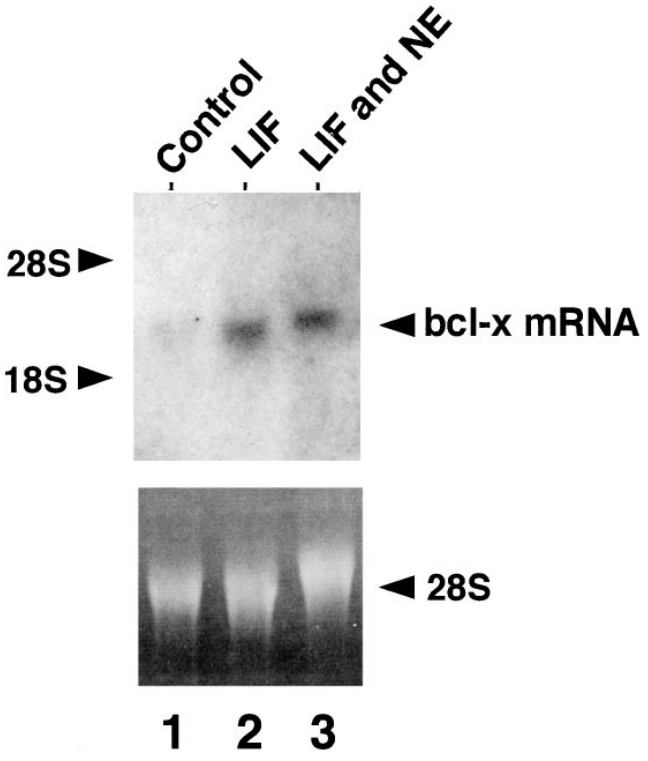

Figure 2. LIF induced bcl-x mRNA expression in cardiac myocytes. Cultured murine cardiac myocytes were treated with LIF (10^3 U/ml) (lane *2*) or with the combination of LIF (10^3 U/ml) and norepinephrine (2 μM) (lane *3*) for 24 h. 10 μg of total RNA was separated by 1.2% formaldehyde agarose gel electrophoresis. BamHI fragment of psk-mouse bcl-xL cDNA was used as a probe (*top*). The bottom panel indicates the 28S ribosomal RNA.

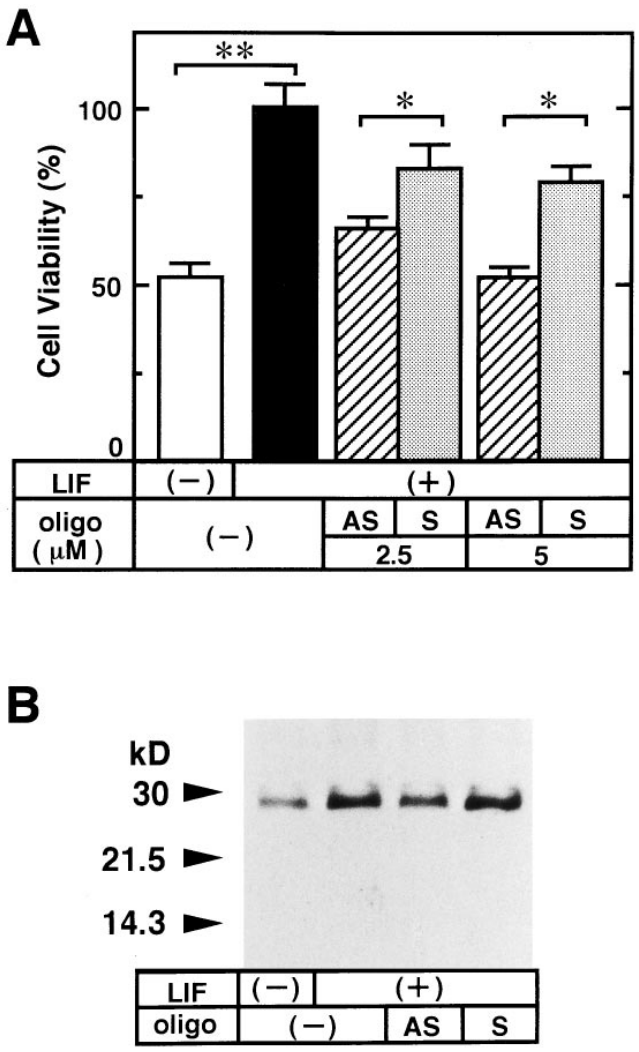

Figure 3. LIF induced protective effect on cardiac myocyte survival. (*A*) Neonatal rat cardiac myocytes were treated with LIF (10^3 U/ml) in the presence of antisense (*hatched bars*) or sense (*gray bars*) oligonucleotides (2.5, 5 μM) for 72 h. Cells were harvested with collagenase/trypsin and counted. Values are given as mean±SE of four samples. Results are given as a percentage of basal cell number under LIF-stimulated condition without oligonucleotides (7.2×10^3 cells per dish). Similar results were obtained in two independent experiments. $^{**}P < 0.01$. $^{*}P < 0.05$. (*B*) Cardiac myocytes were treated with LIF (10^3 U/ml) in the presence of 5 μM antisense or sense oligonucleotides for 24 h. Cells were directly lysed with SDS-PAGE solution. The cell lysates, containing 5×10^3 cells, were Western blotted with anti–bcl-x antibody. Similar results were obtained in two independent experiments.

ponent of the isoforms. Only a fragment 519 bp in size, corresponding to the bcl-xL isoform, was obtained by PCR in the presence or absence of LIF stimulation. The fragment 330 bp in size, corresponding to bcl-xS, was not detected (data not shown).

LIF induces protective effect on cardiac myocyte survival. The cytoprotective effects of LIF upon myocardial cell death induced by serum depletion were analyzed by trypan blue exclusion assays. Neonatal rat cardiac myocytes were cultured in the presence or absence of LIF for 72 h. As shown in Fig. 3 *A*, LIF promoted myocardial cell survival, and the effect was observed as early as 24 h after LIF addition. Antisense oligonucleotides against bcl-x mRNA canceled LIF-mediated protection on cell survival in a dose-dependent manner. Although the sense oligonucleotides also inhibited LIF-mediated cell protection, the inhibitory effects of sense oligonucleotides were significantly less than those observed by antisense.

The protein level of bcl-xL was analyzed by Western blotting. As shown in Fig. 3 *B*, LIF stimulation induced threefold upexpression of 29-kD band, corresponding to bcl-xL. In the presence of antisense oligonucleotides, the increase of bcl-xL was inhibited by 80%, but not in the presence of sense oligonucleotides.

Bcl-x gene promoter region contains both basal and LIF-responsive elements. To investigate the transcriptional activation of bcl-x mRNA, promoter-luciferase plasmids were constructed and transfected into rat cardiac myocytes transiently. As shown in Fig. 4, luciferase activity was measured in the presence or absence of LIF stimulation. The −639/+10-luciferase plasmid presented significantly high basal activity in the absence of cytokine stimulation, and LIF stimulation enhanced activity (2.6±0.3-fold). The deletion construct −161/+10-luciferase plasmid also presented equally augmented activity (3.8±0.5-fold) with LIF stimulation, while basal transcription activity was markedly reduced. These data suggested that the −639/−162 region is involved in basal transcription.

Bcl-x mRNA is not activated by norepinephrine. The effect of norepinephrine on the expression of bcl-x mRNA in murine cardiac myocytes was compared with that of LIF by RT-PCR (Fig. 5). Norepinephrine was added to the culture medium at a concentration of 2 μM, which activated mitogen-activated protein (MAP) kinase to a level similar to that obtained with 10^3 U/ml of LIF stimulation (data not shown). Although norepinephrine showed little effect on the induction of bcl-x mRNA, stimulation with the combination of IL-6 and soluble IL-6 receptor induced the upregulation of bcl-x mRNA. To confirm the results described above, the construct −161/+10-luciferase

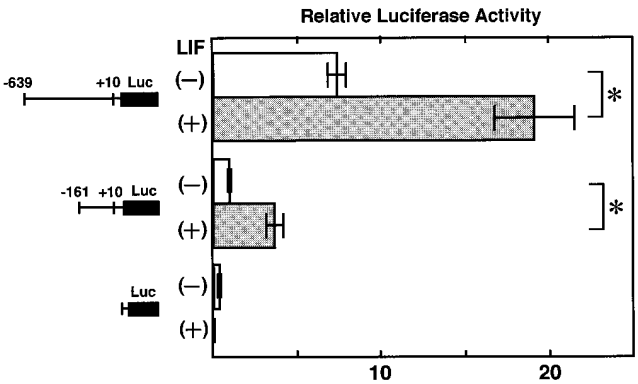

Figure 4. Transcriptional activation of bcl-x promoter-luciferase constructs in response to LIF. bcl-x promoter (positions −639 to +10 or positions −161 to +10) was ligated to luciferase reporter gene (*Luc*) and transiently transfected into cardiac myocytes. The transfected cells were cultured in the presence (*gray bars*) or absence (*open bars*) of LIF (10^3 U/ml) for 24 h, and harvested. The luciferase activity was normalized by β-galactosidase activity. Values are given as mean±SE of three samples. Similar results were obtained in three independent experiments. $^{*}P < 0.05$.

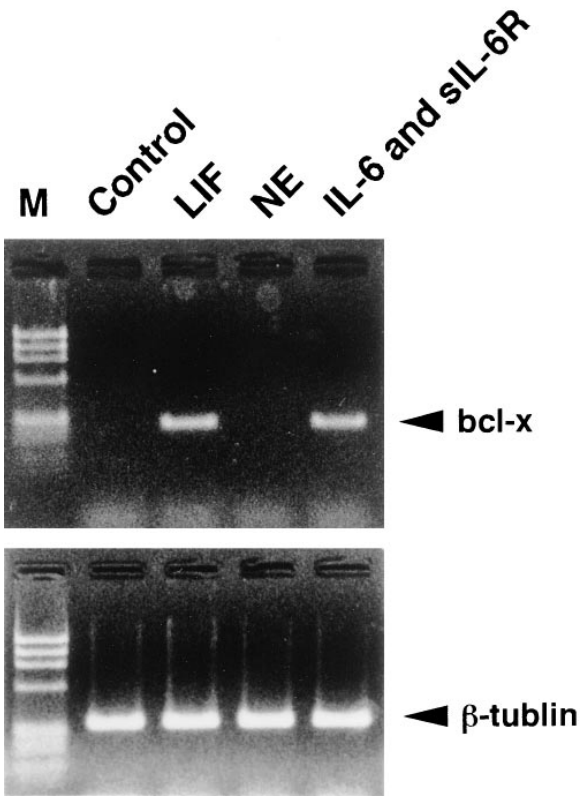

Figure 5. Expression of bcl-x mRNA in cardiac myocytes stimulated with LIF, norepinephrine (*NE*), or the combination of IL-6 and soluble IL-6 receptor (*sIL-6R*). Cardiac myocytes were treated with LIF (10^3 U/ml), norepinephrine (2 μM), or the combination of IL-6 (10^3 U/ml) and soluble IL-6 receptor (0.5 μg/ml) for 24 h. RT-PCRs for bcl-x (*top*) and for β-tubulin (*bottom*) were performed as described in Methods. Molecular weight markers from HaeIII-digested ϕX 174 are indicated (*M*).

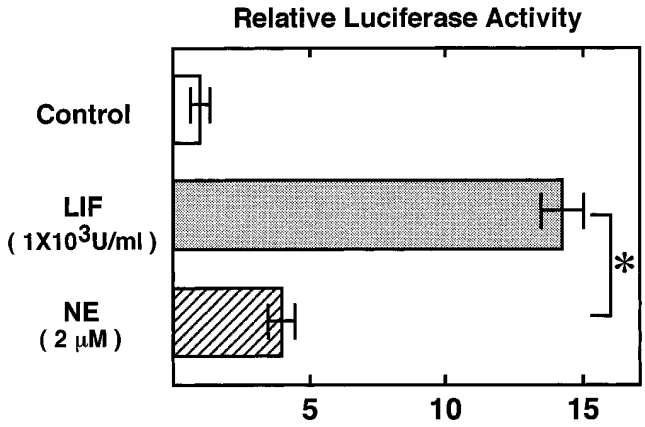

Figure 6. Transcriptional activation of bcl-x promoter-luciferase construct in response to LIF or norepinephrine (*NE*). The construct −161/+10-luciferase was transfected into cardiac myocytes, and luciferase assays were performed as described in Fig. 4. The cells were treated with LIF (10^3 U/ml) or norepinephrine (2 μM) for 24 h. *Open bar*, control; *gray bar*, LIF; *hatched bar*, NE. The luciferase activity was normalized by β-galactosidase activity. Values are given as mean±SE of three samples. Similar results were obtained in three independent experiments. *$P < 0.05$.

was transfected into rat cardiac myocytes and stimulated by norepinephrine. As shown in Fig. 6, the luciferase activity was enhanced by norepinephrine significantly, but to a lesser extent than the enhancement obtained with LIF. Moreover, norepinephrine did not present additional effects on the induction of the endogenous bcl-x mRNA that is upexpressed by LIF (Fig. 2, lanes *2* and *3*).

LIF responsive element in the promoter region of the bcl-x gene contains the interferon-γ activation site (GAS) motif. The promoter region from −161 to +10 contains the GAS motif, TTCGGAGAA, at position −29. EMSAs using SIE as a probe indicated that the oligonucleotide containing the GAS motif inhibited the formation of SIE-STAT1 complex induced by LIF stimulation in rat cardiac myocytes (data not shown). To clarify the functional significance of this motif, a site-directed mutagenesis analysis was performed. As shown in Fig. 7, the mutation led to a significant reduction in the promoter activity in the presence of LIF.

STAT1 is the transcriptional factor bound to the bcl-x gene LIF-responsive element. EMSAs were carried out using the oligonucleotide from positions −44 to −29 (which contains the GAS motif) as a probe (Fig. 8). The labeled probe was incubated with the cellular extracts from LIF-stimulated or -un-

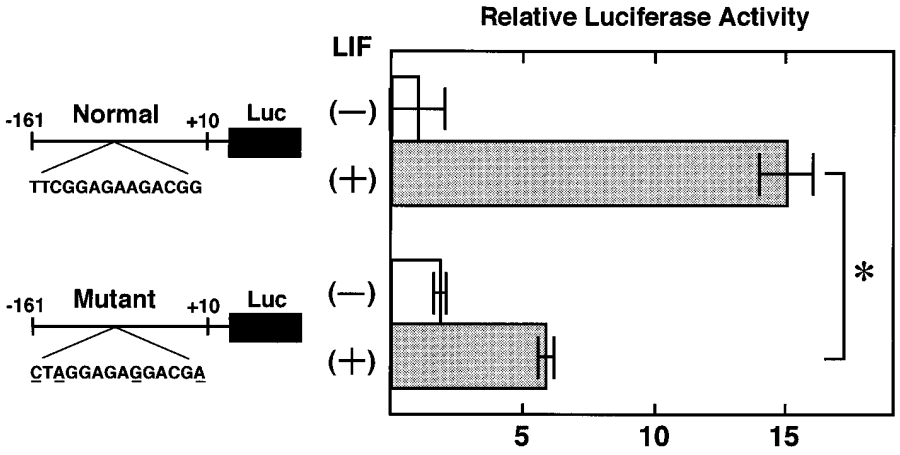

Figure 7. Effect of disruption of the GAS motif in the promoter activity of the *bcl-x* gene. Normal (TTCGGAGAAGACGG) or mutant (CTAGGAGAGGACGA) promoter-luciferase construct was transfected into cardiac myocytes, and luciferase assay was performed as described in Fig. 4. The transfected cells were treated with (*gray bars*) or without (*open bars*) LIF (10^3 U/ml) for 24 h. The luciferase activity was normalized by β-galactosidase activity. Values are given as mean±SE of three samples. Similar results were obtained in three independent experiments. *$P < 0.05$.

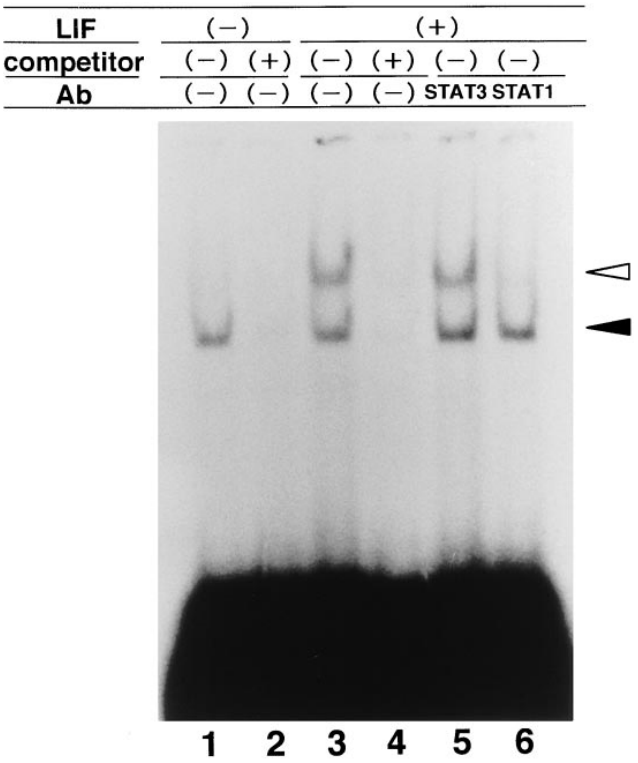

Figure 8. STAT1 bound to the *cis*-element in the promoter region of the *bcl-x* gene in response to LIF. By using the oligonucleotide −44/−29 as a probe, EMSAs were performed. Labeled oligonucleotide (0.15 ng) was incubated with 2 μg of cell lysates untreated (lanes *1* and *2*) or treated (lanes *3–6*) with LIF (10^3 U/ml) for 10 min, in the presence of a 250-fold molar excess of unlabeled oligonucleotide (lanes *2* and *4*), 2 μg of anti-STAT3 antibody (lane *5*), or 2 μg of anti-STAT1 antibody (lane *6*). DNA–protein complexes were resolved by 4% polyacrylamide gels. *Closed arrowhead*, unknown band detected either in the presence or absence of LIF. *Open arrowhead*, LIF-induced DNA–protein complex.

stimulated rat cardiac myocytes. The probe formed an unknown complex, which was diminished by an excess amount of cold probe, even in the absence of LIF (Fig. 8, *closed arrowhead*). The probe led to the appearance of an additional complex with LIF stimulation (Fig. 8, *open arrowhead*). This LIF-inducible complex was canceled in the presence of 250-fold unlabeled cold probe or by anti-STAT1 antibody, but not by anti-STAT3 antibody. The LIF-inducible complex was also diminished by the addition of the DNA fragment from positions −161 to +10, but not by the fragment with a mutation in the GAS motif (data not shown).

Discussion

One of the major findings in the present study was induction of the *bcl-xL* gene by LIF stimulation in cardiac myocytes. Though *bcl-x* is a member of *bcl-2*–related genes (12), the expression of *bcl-2* was not influenced by LIF stimulation in cardiac myocytes. The transcriptional regulation of the *bcl-x* gene is known to be different from that of the *bcl-2* gene (25). IL-6 is reported to induce bcl-xL mRNA without upregulating bcl-2 in an IL-6–dependent myeloma cell line (11). We also confirmed in an IL-6–dependent myeloma cell line (MH-60 cells) that IL-6 upregulated bcl-xL mRNA, and exerted an inhibitory effect on the progression of apoptosis induced by cytokine depletion (data not shown). Since LIF and IL-6 share gp130 in their receptor complexes as a signal transducer, it is reasonable that LIF exerts biological functions similar to those observed by IL-6. In addition, we have reported that LIF transduces signals by activating gp130 in cardiac myocytes (14).

LIF promoted cardiac myocyte survival (Fig. 3 *A*), associated with the increased level of bcl-xL (Fig. 3 *B*). Antisense oligonucleotides against bcl-x mRNA reduced the LIF-mediated cytoprotection with the reduced bcl-xL protein level. The induced expression of the *bcl-x* gene by LIF stimulation in cardiac myocytes may underline a valuable function of the gp130 signaling pathway in myocardial protection. Considering that sense oligonucleotides' slight impairment of the cytoprotection induced by LIF might partially be because of the cytotoxicity of the oligonucleotides, antisense oligonucleotides may not be sufficient for the complete inhibition of cytoprotective effects of LIF. It is suggested that other cytoprotective proteins might be induced with LIF stimulation.

We next investigated the molecular mechanisms by which *bcl-x* gene expression is regulated under LIF stimulation in cardiac myocytes. Promoter-reporter gene transfection analysis revealed that the *bcl-x* gene promoter (positions −639 to +10) contains two transcriptional regulatory elements, one basal, and the other LIF-inducible. As shown in Fig. 5, norepinephrine did not induce endogenous *bcl-x* gene expression. This was consistent with our additional finding that the LIF-responsive *cis*-element (positions −161 to +10) responded to norepinephrine to a lesser extent (Fig. 6).

gp130 transduces signals through both the Janus kinase-signal transducer and activator of transcription (Jak-STAT) and MAP kinase pathways, while norepinephrine signals through the MAP kinase pathway (14), but not the Jak-STAT pathway. The difference in *bcl-x* gene induction between LIF and norepinephrine may result from the difference in their signaling pathway. We identified a GAS motif (TTCGGAGAA) within an LIF-responsive element (positions −161 to +10), and disruption of the sequence by site-directed mutagenesis resulted in an impaired response to LIF stimulation (Fig. 7). The oligonucleotide DNA fragment that contains the GAS motif is bound to STAT1, which was activated by LIF stimulation (Fig. 8).

IL-6 activates STAT1 and STAT3 differentially and independently via the cytoplasmic domain of gp130 (26). It is reported that LIF also activates both STAT1 and STAT3 by activating gp130 (27). The specificity of STAT-directed transcription in response to the stimulation of gp130 may depend in part on the 5-bp sequence between TT and AA in the GAS-like motif: $TT(N)_5AA$. Although STAT1 and STAT3 are known to bind the same GAS-like element (28), this is not the case for the GAS-like motif in the promoter region of *bcl-x* gene. From the present study, this element bound to STAT1, but not to STAT3 (Fig. 8).

Kinoshita et al. (29) and Miyazaki et al. (25) provided contradictory results regarding *bcl-x* gene induction by IL-2 and IL-3 in hematopoietic cells. Kinoshita et al. found that upregulation of *bcl-x* gene expression was induced by transfection of the ras protein (29), while Miyazaki et al. stated that activation of the EGF receptor, which activates the ras pathway, did not induce bcl-xL mRNA expression (25). The latter result is consistent with our findings, demonstrating that the *bcl-x* gene was

significantly induced by LIF stimulation (which activates both the STAT and MAP kinase pathways) but not by norepinephrine stimulation (which activates the MAP kinase pathway). Our data, however, do not mean that the MAP kinase pathway is not related to the expression of bcl-x, because the MAP kinase pathway cross-talks with other signaling pathways, including the STAT pathway. It is reported that MAP kinase regulates tyrosine-phosphorylated STAT by serine phosphorylation (30). Moreover, it is well known that some acute phase protein genes contain both STAT- and MAP kinase–responsive elements in the promoter region, and that the expression of these genes is regulated coordinately by both pathways (1). Indeed, norepinephrine activated the promoter activity of the *bcl-x* gene significantly, while norepinephrine did not exhibit the additional effects on the upexpression of the endogenous bcl-x mRNA by LIF. Therefore, it is possible to hypothesize that activation of the STAT pathway may trigger the induction of the *bcl-x* gene in a rate-limiting step, while the MAP kinase pathway may be responsible for enhancement of the expression of the *bcl-x* gene. It is reported that erythropoietin-dependent inhibition of cell death is blocked by transfecting dominant-negative form of Jak 2 (31). Thus, it is at least likely that the Jak kinase plays a critical role in cell protection under various kinds of stimulation that induce apoptosis.

Although no abnormality was reported in the heart in either bcl-x–deficient mice (32) or STAT1-disrupted mice (33, 34), *gp130* knockout mice demonstrated hypoplastic ventricles (4). Therefore, the signaling pathway observed in the present study may be involved during stress, but not during heart development. Recently, cytokines were shown to be involved in many cardiovascular diseases. In particular, IL-6–related cytokines were reported to be produced by cardiac myocytes upon stimulation with hypoxia, IL-1, or epinephrine (35). Therefore, the induction of the bcl-xL mRNA with the activation of gp130 in cardiac myocytes may provide a cytoprotective effect with these types of stimulation.

In conclusion, a GAS-like *cis*-element is involved in the transcriptional regulation of the *bcl-x* gene in response to LIF, and STAT1 may be a candidate for the *trans*-regulatory factor in the induction of the *bcl-x* gene in cardiac myocytes.

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