Activation of gp130 Transduces Hypertrophic Signals via STAT3 in Cardiac Myocytes

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Background—gp130, a signal transducer of the IL-6–related cytokines, is expressed ubiquitously, including in the heart. The activation of gp130 in cardiac myocytes was reported to induce myocardial hypertrophy. The downstream side of gp130 consists of two distinct pathways in cardiac myocytes, one a Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, the other a mitogen-activated protein kinase (MAPK) pathway. In the present study, we examined whether the JAK/STAT pathway, especially the STAT3-mediated pathway, plays a critical role in gp130-dependent myocardial hypertrophy by transfecting wild-type and mutated-type STAT3 cDNA to cardiac myocytes.

Methods and Results—We constructed three kinds of replication-defective adenovirus vectors carrying wild-type (AD/WT) or mutated-type (AD/DN) STAT3 cDNA or adenovirus vector itself (AD). Cultured murine cardiac myocytes infected with adenovirus were stimulated with leukemia inhibitory factor (LIF), and the expression of c-fos and atrial natriuretic factor (ANF) mRNAs and [3H]leucine incorporation were examined. There were no significant differences in MAPK activity among the three groups. Compared with AD-transfected cardiac myocytes, induction of c-fos and ANF mRNAs and protein synthesis after LIF stimulation were significantly augmented in AD/WT-transfected cells. In contrast, induction of c-fos and ANF mRNA expression and protein synthesis were attenuated after LIF stimulation in cardiac myocytes transfected with AD/DN.

Conclusions—These results suggest that the STAT3-dependent signaling pathway downstream of gp130 promotes cardiac myocyte hypertrophy under stimulation with LIF. (Circulation. 1998;98:346-352.)

Key Words: interleukins ■ hypertrophy ■ signal transduction ■ STAT3 ■ myocytes

Myocardial hypertrophy is induced by various stimuli in vivo, such as pressure or volume overload. This hypertrophic response is assumed to be an important compensation to maintain mechanical function under these conditions. Regarding cardiac myocyte hypertrophy, mechanical stretch and various growth factors utilizing G protein–coupled receptors, including PE, endothelin-1, and angiotensin II, are well-known stimuli. Various molecules are reported to exist downstream of G protein–coupled receptors, for example, ras, protein kinase C, and MAPK. Hunter et al reported that transgenic mice carrying constitutive active ras, which is an upstream molecule of MAPK, showed ventricular hypertrophy. In contrast, the inhibition of MAPK by use of antisense oligonucleotides was reported to downregulate PE-induced hypertrophic responses in cardiac myocytes.

Although it has long been accepted that MAPK is critical in myocardial hypertrophy, some recent evidence suggested that activation of MAPK was not sufficient for the induction of hypertrophy following a G protein–coupled receptor-dependent pathway. Neither carbachol nor ATP, which activates MAPK, can induce cardiac myocyte hypertrophy. In addition, PE retains the ability to induce hypertrophy despite the inhibition of MAPK activation in cardiac myocytes.

Both epidermal growth factor and nerve growth factor are known to activate MAPK; however, only nerve growth factor can induce neural cell differentiation that is associated with prolonged activation of MAPK. Therefore, the kinetics of MAPK activation are important for various subsequent physiological functions.

We and others have reported that IL-6–related cytokines, such as CT-1 or LIF, induced hypertrophy in cardiac myocytes not through G protein–coupled receptors but through gp130, which is a common β-receptor of the IL-6–related cytokine family. The signaling pathway downstream of gp130 is reported to consist of two distinct pathways, one a JAK/STAT pathway, the other a MAPK pathway. gp130-dependent MAPK activation induced by LIF in cardiac myocytes is characteristic, that is, the magnitude is half of and the duration is shorter than that of PE-induced MAPK activation. The distinct kinetic pattern of MAPK activation

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observed after gp130 activation might be associated with different physiological functions in the gp130-dependent signaling pathway. Recently, Sheng et al.\(^{19}\) reported that MAPK activation induced by CT-1 was important for the prevention of apoptosis and was not required for cardiac myocyte hypertrophy through gp130. However, the precise physiological function of the JAK/STAT signaling pathway has not yet been elucidated in cardiac myocytes.

In the present study, we examined whether the JAK/STAT pathway, especially the STAT3-dependent pathway, is important in inducing cardiac myocyte hypertrophy through gp130, using cultured cardiac myocytes transfected with a replication-defective recombinant adenovirus carrying STAT3 or mutated STAT3 cDNA.

### Methods

#### Materials

Murine LIF, medium 199, NCS, and M-MLV reverse transcriptase were purchased from Gibco BRL. Oligo-dT [d(T)12-18, 5'-OH, Na\(^+\)] and protein A sepharose were obtained from Pharmacia Biotech. Ty peel polymerase and the human c-fos cDNA (0.48-kb fragment) were from Takara. PRIME IT for labeling cDNA was from Stratagene. Polyvinylidene difluoride membrane (Immobilon-P) was from Millipore Co. \([\text{H}]\)leucine, [α-\(^32\)P]dCTP, the BIOTRAK p42/p44 MAPK assay kit, and the enhanced chemiluminescence (ECL) detection system were from Amersham. PD98059, a specific MAPK kinase inhibitor, and a phosphospecific STAT1 antibody that recognizes tyrosine phosphorylated STAT1 were from Cell Signal. The samples were separated in a 7.5% or a 5.0% SDS–polyacrylamide gel, and the resolved proteins were electrophoretically transferred onto an Immobilon-P membrane with a transfer buffer.

#### Immunoprecipitation

Cardiac myocytes were washed with TBS buffer (50 mmol/L Tris HCl, pH 7.4; 150 mmol/L NaCl; and 1 mmol/L sodium orthovanadate) and homogenized with RIPA buffer (20 mmol/L Tris HCl, pH 7.4; 1% NP-40; 0.1% SDS; 150 mmol/L NaCl; 1 mmol/L EDTA; 10 μg/ml aprotinin; 1 mmol/L sodium orthovanadate; and 0.5 mmol/L PMSF) with 15 strokes in a Teflon-glass homogenizer at 4°C. Aprotinin, PMSF, and sodium orthovanadate were added just before homogenization. The homogenates were centrifuged at 100 000 ×g for 30 minutes at 4°C, Supernatants were incubated with anti-STAT3 antibody and protein A sepharose for 4 hours at 4°C, then washed three times with TBS buffer, eluted with 25 μL of sample buffer (62.5 mmol/L Tris, pH 6.8; 2% SDS; 5% 2-mercaptoethanol; and 10% glycerol), and boiled for 10 minutes. Thereafter, the samples were centrifuged at 20 000 g for 1 minute, and the supernatants were collected and stored at −80°C until assay.

#### Preparation of Recombinant Adenovirus

Cardiac myocytes were washed with TBS buffer and collected with 100 μL of sample buffer (62.5 mmol/L Tris HCl, pH 6.8; 2% wt/vol SDS; 10% glycerol; 50 mmol/L DTT; 0.1% bromophenol blue). They were lysed with a sonicator for 10 seconds at 4°C, boiled for 5 minutes, and then centrifuged for 10 minutes at 100 000g. Supernatants were collected and stored at −80°C until assay.

#### Western Blot Analysis

The samples were separated in a 7.5% or a 5.0% SDS–polyacrylamide gel, and the resolved proteins were electrophoretically transferred onto an Immobilon-P membrane with a transfer buffer.
(25 mmol/L Tris, 190 mmol/L glycine, and 20% methanol). Membranes were blocked with 5% skim milk and probed either with anti-phosphotyrosine antibody at a 1:1000 dilution for 1 hour to detect phosphorylated STAT3 or with phosphospecific STAT1 antibody to detect phosphorylated STAT1. The immune complexes were visualized with Kodak X-OMAT-AR film with the enhanced chemiluminescence system used according to the manufacturer’s instructions. The filters were incubated in stripping buffer (62.5 mmol/L Tris-HCl, pH 6.8; 100 mmol/L 2-mercaptoethanol; and 2% SDS) for 30 minutes at 50°C and reprobed with anti-STAT3 or anti-STAT1 antibody.

MAPK Assay

Protein kinase activity was measured by the P42/P44 MAPK assay system as previously described, with modification.18 The stimulated cardiac myocytes were lysed at 4°C with a RIPA buffer and centrifuged at 100 000g for 30 minutes at 4°C. Supernatants were collected and incubated with anti-ERK1 and anti-ERK2 antibodies and protein A sepharose for 4 hours at 4°C, then washed three times with MAPK reaction buffer containing 20 mmol/L Tris HCl, pH 7.4; 20 mmol/L β-glycerophosphate; 1 mmol/L sodium orthovanadate; 2 mmol/L EDTA; 0.5 mmol/L PMSF; 10 mg/mL aprotinin; and 20 mmol/L NaF. Thereafter, 15 µL of MAPK reaction buffer, 10 µL of synthetic peptide, and 5 µL of [γ-32P]ATP solution were added to protein A sepharose. The synthetic peptide used in this assay contains the phosphorylation sequence PLS/TP as MAPK substrates. This peptide is phosphorylated more specifically by MAPK than myelin basic protein, which is commonly used to detect MAPK activity.24 The mixture was incubated for 30 minutes at 30°C. The reaction was terminated by the addition of a stop buffer. The phosphorylated synthetic peptide was isolated by application of the reaction mixture onto a phosphocellulose paper. The papers were then washed twice with 50 mmol/L H3 PO4 and placed in scintillation vials with 10 mL of liquid scintillation cocktail. Radioactivity was determined with a liquid scintillation counter.

Northern Blot Analysis

Total RNA was isolated by acid guanidinium thiocyanate–phenol–chloroform methods.25 Murine ANF cDNA was synthesized from RNA obtained from murine ventricles by reverse transcription and polymerase chain reaction amplification using oligonucleotide primers (5’ primer, 5’-CTCTAGAGAGACGGCATGCT-3’ and 3’ primer, 5’-TATGGAGATGGGAGAGGGA-3’) according to the nucleotide sequence reported by Seidman et al.26 ANF and c-fos cDNAs were labeled by [32 P]dCTP with a PRIME IT labeling kit. Total RNA (10 µg) was separated on a 1% formaldehyde-agarose gel and transferred to a nylon membrane in the presence of 20×SSC (300 mmol/L sodium chloride and 300 mmol/L sodium citrate, pH 7.0). Prehybridization was performed at 42°C for 4 to 6 hours in 650 mmol/L sodium chloride; 100 mmol/L sodium PIPES, pH 6.8; 5×Denhardt’s solution; 0.1% SDS; 10 mg/L of denatured salmon sperm DNA; and formamide at a final concentration of 50%. After hybridization for 12 to 24 hours at 42°C, membranes were washed twice with 2×SSC and 0.1% SDS and three times with 1×SSC and 0.1% SDS at 60°C. They were then exposed to x-ray film for 3 to 24 hours at −70°C. The filters were washed and rehybridized with human β-actin cDNA (Takara). The intensity of the bands was analyzed by densitometry (Image Quant, Molecular Dynamics).

Leucine Incorporation

Two days after viral infection, cardiac myocytes were starved for 12 hours and stimulated with 1×103 U/mL of LIF in the presence or absence of 20 µmol/L PD98059 for 24 hours. [3H]leucine (1 µCi/mL) was added to the culture medium at the same time. Thereafter, they were washed three times with PBS, incubated with 5% trichloroacetic acid for 10 minutes at 4°C, and lysed with 0.5 mol/L NaOH. Six volumes of scintillation fluid were applied to the lysates, and the mixtures were counted in a liquid scintillation counter.

Figure 2. Tyrosine phosphorylation of STAT3 and STAT1 in cardiac myocytes stimulated with LIF. A, Cardiac myocytes were transfected with AD, AD/WT, or AD/DN and cultured for 2 days. They were starved for 6 hours and incubated for 15 minutes in the presence or absence of LIF (1×106 U/mL). Cells were pretreated with PD98059 for 30 minutes and then stimulated with LIF for 15 minutes. Cell lysates were immunoprecipitated with anti-STAT3 antibody for 4 hours at 4°C, separated by 7.5% SDS-PAGE, and transferred onto an Immobilon-P membrane. Blot was probed with anti-phosphotyrosine antibody (4G10) (top). Blot was reprobed with anti-STAT3 antibody (bottom). B, Cardiac myocytes were treated as described above except with somatostatin for cell lysis. Cell lysates were separated by 5% SDS-PAGE and transferred onto an Immobilon-P membrane. Blot was probed with phospho-specific STAT1 antibody (top). Blot was reprobed with anti-STAT1 antibody (bottom).

Statistical Analysis

Statistical analysis was performed by use of Student’s t test. A value of P<0.05 was considered significant.

Results

Tyrosine Phosphorylation of STAT3 and STAT1 in Cardiac Myocytes Transfected with AD, AD/WT, or AD/DN

Recently, we reported that LIF-induced maximal phosphorylation of STAT3 was observed within 15 minutes and dephosphorylated by 60 minutes in cardiac myocytes.14 In the present study, we first examined the activation level of STAT3 in cardiac myocytes transfected with AD, AD/WT, or AD/DN. As shown in Figure 2A, tyrosine phosphorylation of STAT3 was observed in AD-transfected cardiac myocytes 15 minutes after stimulation with 1×103 U/mL of LIF. Although STAT3 phosphorylation was not observed in AD/WT-transfected cardiac myocytes before stimulation, augmented phosphorylation of STAT3 was observed after the stimulation (Figure 2A, top, lanes 2 and 5). In contrast, phosphorylation of STAT3 was not detected in AD/DN-transfected cardiac myocytes either with or without LIF stimulation (Figure 2A, top, lanes 3 and 6). PD98059 pretreatment did not change the levels in tyrosine phosphorylation of STAT3 in cardiac myocytes stimulated with LIF (Figure 2A, top, lanes 7 to 9). The amounts of immunoprecipitated STAT3 were greater in AD/WT- and AD/DN-transfected cardiac myocytes than in AD-transfected cells (Figure 2A, bottom).
These results indicate that activation of the JAK-STAT pathway, especially the STAT3-dependent pathway, was enhanced in AD/WT-transfected cardiac myocytes with LIF stimulation. In contrast, in AD/DN-transfected cardiac myocytes, this pathway was not fully activated by LIF stimulation. Therefore, AD/DN was demonstrated to act as a dominant negative STAT3 in cultured cardiac myocytes.

STAT1, which is also known to be phosphorylated after LIF stimulation, was activated in AD-, AD/WT-, and AD/DN-transfected cells after LIF stimulation. Although the level of STAT1 phosphorylation was slightly increased in AD/WT- and AD/DN-transfected cells, there was no difference between these two types of cells (Figure 2B, top). The amount of STAT1 protein was the same in all types of cells (Figure 2B, bottom).

MAPK Activity in Cardiac Myocytes Transfected With AD, AD/WT, or AD/DN

MAPK is known to be a key molecule in promoting cardiac hypertrophy through a G protein–coupled receptor. We compared the MAPK activities in these three different types of cardiac myocytes after LIF stimulation. MAPK activity from anti-ERK1 and anti-ERK2 antibody-immunoprecipitated proteins were measured 5 minutes after LIF stimulation. As shown in Figure 3, MAPK activity was almost equal among the three groups under unstimulated conditions (open bars). MAPK activity was comparably elevated to 7 times the level in the unstimulated period after LIF stimulation (shaded bars) (P<0.05). There were no significant differences in MAPK activity among AD-, AD/WT-, and AD/DN-transfected cells after LIF stimulation.

We next compared the MAPK activity in the cardiac myocytes pretreated with the MAPK kinase inhibitor PD98059. MAPK activity in the cardiac myocytes pretreated with PD98059 for 30 minutes was significantly inhibited even after LIF stimulation (closed bars). In addition, there were no significant differences in MAPK activity among these three groups.

Although MAPK activity was inhibited by PD98059 in cardiac myocytes even after LIF stimulation, the level of STAT3 tyrosine phosphorylation was not interfered with (Figure 2A). In addition, MAPK inhibitor did not interfere with the phosphorylation of STAT1 (data not shown). Therefore, in the present series of experiments, MAPK activities were almost equal despite the distinct activation patterns of STAT3 in AD-, AD/WT-, and AD/DN-transfected cardiac myocytes after LIF stimulation.

c-fos mRNA Expression in Cardiac Myocytes Stimulated With LIF

Induction of the c-fos gene, which is an immediate early gene, was reported to precede hypertrophic responses by stimulation with PE28 or angiotensin II, which utilize G protein–coupled receptors, and LIF or CT-1, which utilize gp130, in cardiac myocytes. To investigate whether the expression of c-fos mRNA induced by LIF in cardiac myocytes was mediated by the JAK/STAT pathway, c-fos mRNA expression was examined in AD-, AD/WT-, and AD/DN-transfected cells by Northern blot analysis (Figure 4). Although c-fos mRNA expression was not detected before
stimulation, rapid induction of c-fos mRNA was observed 30 minutes after LIF stimulation in all types of cells. Augmented expression of c-fos mRNA was observed in myocytes transfected with AD/WT, and induced expression was inhibited in those transfected with AD/DN, compared with that induced in AD-transfected cells. PD98059 pretreatment significantly suppressed the induction of c-fos mRNA in these three types of cardiac myocytes after LIF stimulation.

**ANF mRNA Expression in Cardiac Myocytes Stimulated With LIF**

Reactivation of embryonic phenotype genes, especially the ANF gene, is known to be associated with hypertrophic responses in cardiac myocytes. gp130 activation in cardiac myocytes after LIF or CT-1 stimulation causes induction of ANF mRNA, with a maximum at 24 hours and subsequent gradual decline. We examined the contribution of the STAT3-dependent signaling pathway to the induction of ANF mRNA expression after LIF stimulation. As shown in Figure 5, expression of ANF mRNA was detected in embryonic murine cardiac myocytes. Cardiac myocytes transfected with AD, AD/WT, or AD/DN were cultured with or without LIF for 24 hours. Expression of ANF mRNA in AD/WT-transfected cardiac myocytes was slightly increased without stimulation and significantly augmented after 24 hours of LIF stimulation. AD- and AD/DN-transfected cardiac myocytes showed little increase in ANF mRNA expression even after LIF stimulation. Pretreatment with PD98059 significantly suppressed ANF mRNA expression in all types of cells after LIF stimulation.

**Leucine Incorporation in Cardiac Myocytes Stimulated With LIF**

We examined protein synthesis in these three types of cardiac myocytes by measuring [3 H]leucine incorporation after stimulation with 1×10^3 U/mL LIF for 24 hours (Figure 6). Without LIF stimulation, protein synthesis was slightly decreased in AD/DN-transfected cells compared with that in AD-transfected cells (open bars). AD- and AD/WT-transfected cardiac myocytes exhibited significantly increased [3 H]leucine incorporation after 24 hours of LIF stimulation, by 116% and 128%, respectively (shaded bars) (P<0.05). The increase was greater in AD/WT-transfected cells than in AD-transfected cells (P<0.05). However, little increase in protein synthesis was observed in AD/DN-transfected cells. Protein synthesis after LIF stimulation appeared to be enhanced mainly through the JAK/STAT signaling pathway in cardiac myocytes. Pretreatment with PD98059 significantly inhibited the protein synthesis in all types of cells after LIF stimulation (solid bars). These results resembled those observed in ANF mRNA expression.

Therefore, maximal activation of transcription by STAT3 would require MAPK activation.

**Discussion**

Because the efficiency of gene transfer in cardiac myocytes is very low with conventional transfection methods, evaluating the function of transfecting proteins is considered to be difficult. Therefore, we used replication-deficient adenovirus-mediated gene transfer to obtain high levels of expression. The efficiency of expression examined by the Lac-Z gene in cardiac myocytes infected by adenovirus was reported to exceed 90%.
Activation of gp130 is reported to transduce hypertrophic signals both in vivo and in vitro.15,33 The signaling pathway from gp130 to the nucleus was reported to consist of two major pathways: one a MAPK pathway, the other a JAK-STAT pathway.17,18 With regard to the former pathway, there are many reports concerning cardiac hypertrophy in the G protein–coupled receptor system. In contrast, activation of the MAPK pathway after gp130 phosphorylation was reported to be important in inhibiting apoptosis induced by serum depletion but was thought not to be necessary to induce hypertrophy in cardiac myocytes.19 The underlying molecular mechanisms of gp130-dependent cardiac myocyte hypertrophy have not yet been elucidated.

In the present study, the significance of the STAT3-mediated pathway in cardiac hypertrophy was examined. MAPK is also activated with LIF stimulation in cardiac myocytes.34 Therefore, MAPK activation at various levels of STAT phosphorylation was examined, and little difference was found among three types of cardiac myocytes with or without LIF stimulation. In addition, pretreatment with PD98059, a specific MAPK kinase inhibitor, did not affect STAT3 phosphorylation after LIF stimulation, although MAPK activity was significantly suppressed. The augmented c-fos and ANF mRNA expression and protein synthesis observed in wild-type STAT3–transfected cardiac myocytes appears to result mainly from increased STAT3 phosphorylation.

c-Fos protein was reported to provide a link between short-term signals elicited at the membrane and long-term cellular response.34 Induced c-fos mRNA expression was observed in all cell types after LIF stimulation. The upexpression level of c-fos mRNA by LIF was enhanced in AD/WT- and reduced in AD/DN-transfected cardiac myocytes compared with AD-transfected cells. These results are consistent with those of a previous study concerning the transcriptional regulation of the c-fos gene by GM-CSF.35 Binding of GM-CSF to its receptor activates JAK2, STAT1, STAT3, and MAPK. STAT proteins bind to the sis-inducible element of the c-fos gene promoter, and MAPKs activate ternary complex factor serum response factor to increase the transcription of the c-fos gene through binding to the serum response element of its promoter. These results suggest that both the JAK-STAT and MAPK cascades downstream of the GM-CSF receptor contribute to the regulation of c-fos gene transcription. The induction of c-fos mRNA by LIF in AD/DN-transfected cardiac myocytes might take place mainly through the MAPK cascade, not through STAT3, and this would account for the partial activation. When MAPK activity was inhibited by PD98059, the transcriptional activation of the c-fos gene was significantly suppressed even after LIF stimulation.

ANF mRNA is highly expressed in embryonic cardiac myocytes and decreases rapidly after birth.35 The expression of embryonic phenotype genes was reported to be reactivated in the heart because of pressure overload17 or in cultured neonatal rat ventricular myocytes stimulated with PE.36 Ang II,29 ET-1,38 or CT-1.15 In the present study, a slight increase in ANF mRNA expression was observed in AD- and AD/DN-transfected cardiac myocytes after LIF stimulation, whereas the induction of ANF mRNA was significantly augmented in cells transfected with AD/WT. These findings demonstrate that ANF mRNA induction by the STAT3-dependent signaling pathway may occur through a distinct mechanism compared with G protein–mediated induction of the ANF gene. The transcriptional regulation of the ANF gene by PE and CT-1 has been examined by use of a 3.0-kb promoter region. Although both PE and CT-1 were reported to upregulate ANF mRNA in neonatal rat cardiac myocytes, only PE increased the 3.0-kb promoter activity of the ANF gene.15

Although the expression level of ANF mRNA was reduced after PD98059 pretreatment, there were substantial differences in the expression level among AD-, AD/WT-, and AD/DN-transfected cells. This would be explained by the cross talk between JAK/STAT and MAPK cascades. Without serine phosphorylation, which is induced by activated MAPK, the transcriptional activity of tyrosine phosphorylated STAT is reported to be reduced.59 In addition, gene activation by STAT3, which obligatorily requires tyrosine phosphorylation to become active, is reported to depend on maximal activation on serine phosphorylation.40

Both JAK/STAT and MAPK signalings through gp130 were necessary in protein synthesis. We examined the effect of LIF on the expression level of the MHC genes. LIF induced β-MHC mRNA expression and decreased α-MHC mRNA in neonatal rat cardiac myocytes (unpublished data). This regulation of MHCs followed the same pattern as that induced by PE stimulation.61 Not only may the underlying molecular mechanisms of LIF-induced hypertrophic changes be explained by the regulation of MHCs, but also, it is possible that the expression of other cardiac sarcomeric proteins is regulated through the STAT3-mediated signaling pathway.

In summary, the induction of cardiac myocyte hypertrophy and c-fos and ANF mRNA expressions induced by LIF were amplified by STAT3 overexpression, whereas these were attenuated under conditions that inhibited STAT3 signaling. Furthermore, when MAPK activation was inhibited, gene expression and protein synthesis were significantly suppressed even in the cells that overexpressed STAT3. The JAK-STAT pathway, especially the STAT3-mediated pathway, appears to be essential in the induction of cardiac myocyte hypertrophy through gp130.

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References
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