β-Adrenergic Pathway Induces Apoptosis through Calcineurin Activation in Cardiac Myocytes*

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Apoptosis of cardiac myocytes is one of the causes of heart failure. Here we examine the mechanism by which the activation of β-adrenergic receptor induces cardiomyocyte apoptosis. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling and DNA ladder analyses revealed that isoproterenol (Iso) induced the apoptosis of cardiac myocytes of neonatal rats through an increase in intracellular Ca2+ levels. The Iso-induced cardiomyocyte apoptosis was strongly inhibited by the L-type Ca2+ channel antagonist nifedipine and by the calcineurin inhibitors cyclosporin A and FK506. Iso reduced the phosphorylation levels of the proapoptotic Bcl-2 family member Bad and induced cytochrome c release from mitochondria to the cytosol through calcineurin activation. Infusion of Iso increased calcineurin activity by ~3-fold in the hearts of wild-type mice but not in the hearts of transgenic mice that overexpress dominant negative mutants of calcineurin. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling analysis revealed that infusion of Iso induced apoptosis of cardiac myocytes and that the number of apoptotic cardiomyocytes was significantly less in the hearts of the transgenic mice compared with the wild-type mice. These results suggest that calcineurin plays a critical role in Iso-induced apoptosis of cardiac myocytes, possibly through dephosphorylation of Bad.

Heart failure is the final clinical manifestation of a variety of human heart diseases, including idiopathic dilated cardiomyopathy, hypertrophic cardiomyopathy, and coronary artery disease. In these pathologic conditions, cardiomyocytes undergo apoptosis, and a loss of cardiomyocytes is postulated to cause heart failure (1–3). However, the mechanism by which cardiomyocytes fall into apoptosis is still unknown.

Recently, accumulating evidence has suggested that various factors such as angiotensin II, tumor necrosis factor-α, and oxidative stress induce apoptosis of cardiac myocytes (4–6). An adrenergic receptor agonist, norepinephrine, has been reported to be elevated in the plasma of heart failure patients (7) and to induce cardiomyocyte apoptosis (8, 9). It has recently been reported that isoproterenol (Iso)1 induces apoptosis of cardiac myocytes in vivo through β-adrenergic receptors (10) and that norepinephrine-induced cardiomyocyte apoptosis is suppressed by protein kinase A inhibitors (8–10). Selective overexpression of heterotrimeric GTP-binding proteins α, which transmit signals from β-adrenergic receptors to adenylyl cyclase, has also been reported to induce cardiomyocyte apoptosis in transgenic mice hearts (11). These results suggest that activation of protein kinase A through β-adrenergic receptors induces apoptosis of cardiac myocytes during the development of heart failure.

Activation of β-adrenergic receptors increases intracellular Ca2+ levels through voltage-dependent Ca2+ channels. Elevation of cytosolic Ca2+ has been reported to induce apoptosis in some cell types through activation of a Ca2+-dependent phosphatase calcineurin (12–14). Although calcineurin has recently attracted a great attention as a novel regulator of cardiomyocyte hypertrophy (15), it remains unknown whether calcineurin is involved in β-adrenergic stimulation-induced cardiomyocyte apoptosis.

EXPERIMENTAL PROCEDURES

Materials—Iso and nifedipine were obtained from Sigma, ionomycin was from Calbiochem, and cyclosporin A was from Wako Chemical Industries, Ltd. (Osaka, Japan). FK506 was the kind gift of Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan). Anti-Bad and anti-phospho-specific-Bad(serine 136) antibodies were purchased from New England Biolabs, Inc. (Beverly, MA). Anti-Bel-2 and anti-Bel-2-XL monoclonal antibodies were from Transduction Laboratories (Lexington, KY), and anti cytochrome c polyclonal antibody was from Santa Cruz Biotechnol.

Cell Culture—Primary cultures of cardiac myocytes were prepared from the ventricles of 1-day-old Wistar rats as described previously (16). Cardiomyocytes were plated onto 60-mm plastic culture dishes at a field density of 1 × 105 cells/cm2 and cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. Immunocytochemical study revealed that more than 90% of cells were cardiac myocytes.

Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick End Labeling (TUNEL)—Cardiomyocytes plated on a cover glass were fixed with 4% paraformaldehyde solution for 30 min at room temperature. After a rinse with phosphate-buffered saline, the samples were first incubated with phallidin-rhodamine for 1 h and with TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and fluorescein isothiocyanate-dUTP. In tissues, the 3-μm thick paraffin sections were deparaffinized by immersing in xylene, rehydrated, and incubated in phosphate-buffered saline with 2% H2O2 to inactivate endogenous peroxidases. Next, the sections were incubated with proteinase K (20 μg/ml), washed in phosphate-buffered saline, and incubated with terminal deoxynucleotidyl transferase for 90 min and fluorescein isothiocyanate-dUTP for 30 min at 37 °C using an apoptosis detection kit

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‡ The abbreviations used are: Iso, isoproterenol; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; dn, dominant negative; CnA, calcineurin A; CsA, cyclosporin A.
(Takara Biomedical). The sections were stained with diaminobenzidine for 10 min at room temperature, washed in phosphate-buffered saline, and mounted for light microscopic observations. The number of TUNEL-positive cardiac myocytes was determined by counting 3 × 10^6 cardiac myocytes. All morphometric measurements were performed by at least two independent individuals in a blinded manner.

**Agarose Gel Electrophoresis for DNA Fragmentation—**Cells (4 × 10^7) were lysed in 200 μl of lysis buffer (10 mM Tris-HCl (pH 7.4), 10 mM EDTA, 0.5% Triton X-100) followed by incubation with 40 μg of RNase (Roche Molecular Biochemicals) for 1 h at 37 °C and 100 μg of proteinase K (Roche Molecular Biochemicals) for 1 h at 37 °C, and only fragmented DNA was extracted. The pellet was resuspended in TEB buffer (10 mM Tris-HCl (pH 7.4), 1 mM EDTA) and treated with DNase-free RNase (Roche Molecular Biochemicals) for 1 h at 37 °C. DNA was ethanol-precipitated and finally resuspended in distilled water. The fragmented DNA was electrophoretically fractionated on 1.5% agarose gel and stained with ethidium bromide.

**Immunoblotting and Immunoprecipitation—**To detect phosphorylation of Bad, cells (4 × 10^7) were lysed in a buffer (10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA (pH 8.0), 150 mM NaCl, 1% Triton X-100, 0.2 mM sodium ortho-vanadate, 0.2 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40), and Bad was immunoprecipitated with anti-Bad antibody. Immunoprecipitates were recovered with protein A-agarose, separated by 15% SDS-polyacrylamide gel electrophoresis, and immunoblotted with anti-phospho-specific Bad (Ser-136) antibody (New England Biolabs). Total Bad content was assessed using anti-Bad monoclonal antibody.

To detect cytochrome c, cells were suspended in a buffer (20 mM HEPES (pH 7.5), 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) for 3 min on ice, homogenized by 10 strokes in a Dounce homogenizer, and centrifuged at 10,000 rpm for 15 min. The supernatant was the cytosolic fraction, and the pellet was resolated in lysis buffer as the membrane fraction.

**Generation of Dominant Negative (dn) Calcineurin Transgenic Mice—**A DNA encoding human calcineurin A (CnA) was obtained from a T cell-lg library using oligonucleotides as hybridization probes (12). ΔCnA lacking the autoinhibitory and the calmodulin binding domains was constructed by polymerase chain reaction to introduce a stop codon after N407. The catalytically inactive calcineurin mutant (dn calcineurin) was obtained from ΔCnA by mutating the histidine at position 160, a calcineurin active site, to glutamine (14). ΔCnA has been reported to prevent Bad redistribution as a trans-dominant negative mutant of CnA induced by an intracellular Ca^{2+} release agent (14). Hemagglutinin-tagged dn calcineurin was subcloned into the α-myosin heavy chain promoter-containing expression vector between the lamin A untranslated region (12) and simian virus 40 poly(A). The linearized DNA was injected into pronuclei of eggs from BDF1 mice, which were transferred into the oviducts of pseudopregnant ICR mice. The transgene was identified by polymerase chain reaction with transgene-specific primers and by Southern blot analysis using a 32P-labeled simian virus 40 sequence. Two lines of 16–18-week-old dn calcineurin transgenic mice and wild-type littermate mice were used in the present study. All protocols were approved by the guidelines of the University of Tokyo.

**Calcineurin Phosphatase Assays—**Determination of the calcineurin activity of hearts was performed as described previously (12). Briefly, murine hearts were homogenized in 100 μl of lysis buffer (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.5% Tween-20, 0.5 mg/ml bovine serum albumin, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin), and after cell debris had been removed by centrifugation at 10,000 × g for 5 min at 4 °C, calmodulin-binding calcineurin was separated from calmodulin and non-calmodulin-binding calcineurin with an ultrafilter-MC microcentrifuge filtertube (100,000 mMNML filter unit; Millipore Co.), and the supernatant was used for the phosphatase assay. A calcineurin substrate, glutathione S-transferase-RII peptide, which was fixed to glutathione-cellulose beads, was first phosphorylated by protein kinase A in the presence of [γ-32P]ATP (12). The 32P-labeled RII peptide was incubated in the extracts with 50 μl of phosphatase buffer (100 mM Tris-HCl (pH 7.4), 1 mM MnCl₂, 0.1 mM CaCl₂, 0.5 mg/ml bovine serum albumin, 100 mM calmodulin, 0.5 mM dithiothreitol, at 30 °C at 30 °C. 500 mM Naohedral acid was added to the reactions to suppress endogenous protein phosphatases PP1 and PP2A. Reactions were stopped by adding 500 μl of stop buffer (10% trichloroacetic acid, 0.1 μl sodium phosphate, 10 mg/ml bovine serum albumin). After centrifugation, the released 32P-phosphate was determined by Cheurenk methods.

**Statistical Analysis—**All results are expressed as mean ± S.E. Mul-

**RESULTS**

**Iso induces Cardiomyocyte Apoptosis through an Increase in Intracellular Ca^{2+} Levels—**To elucidate the mechanism by which β-adrenergic stimulation induces cardiomyocyte apoptosis, cultured cardiac myocytes of neonatal rats were incubated with a β-adrenergic receptor agonist, Iso, and subjected to TUNEL analysis. Less than 5% of cardiac myocytes were TUNEL-positive in the serum-free culture condition. Incubation with Iso for 48 h increased the number of TUNEL-positive cells in a dose-dependent manner (Fig. 1). A calcium ionophore, ionomycin (1 μM), which causes sustained elevation of intracellular Ca^{2+} concentration, also increased the number of TUNEL-positive cells (Fig. 1). Many nuclei of these TUNEL-positive cells were condensed and fragmented (data not shown), suggesting that Iso induces apoptosis of cardiac myocytes.

**Iso induces Cardiomyocyte Apoptosis through the Ca^{2+}-Calcineurin Pathway—**Activation of β-adrenergic receptors has been reported to increase intracellular Ca^{2+} levels by increasing Ca^{2+} influx through voltage-dependent Ca^{2+} channels (17, 18). When cardiomyocytes were treated with the selective L-type Ca^{2+} channel antagonist nifedipine (1 μM) for 1 h, Iso-induced cardiomyocyte apoptosis was significantly suppressed (Fig. 2A), suggesting that the Ca^{2+} influx through L-type Ca^{2+} channels plays a critical role in Iso-induced cardiomyocyte apoptosis. Elevation of cytosolic Ca^{2+} has been reported to induce apoptosis in some cell types through activation of a Ca^{2+}-dependent phosphatase calcineurin (12–14). We thus examined whether calcineurin is also involved in Iso-induced cardiomyocyte apoptosis. Treatment with the calcineurin inhibitors cyclosporin A (CsA) (1 μM) and FK506 (10 μg/ml) strongly suppressed Iso-induced apoptosis of cardiac myocytes (Fig. 2A and B). To confirm the role of calcineurin in Iso-induced apoptosis, we examined DNA fragmentation by gel electrophoresis. When cardiac myocytes were exposed to Iso (50 μM) for 48 h, extracted genomic DNA showed prominent ladder formation characteristic of apoptosis. The Iso-induced DNA ladder formation was almost completely suppressed by treatment with either CsA or FK506 (Fig. 2C). These results suggest that the Ca^{2+}-calcineurin pathway plays a critical role in β-adrenergic receptor-induced apoptosis in cardiac myocytes.

**Ios Dophosphorylates Bad through the Ca^{2+}-Calcineurin Pathway—**Apoptosis is determined by the relative balance be-
tween proapoptotic molecules such as Bad and Bax and anti-apoptotic molecules such as Bcl-2 and Bcl-xL (19–21). Bad promotes cell death by inhibiting Bcl-2 and Bcl-xL function. Phosphorylation of Bad at Ser-136 by protein kinase B (22) or mitogen-activated protein kinase cascades (21) promotes cell survival. Recently, Ca^{2+}-mobilizing agents have been reported to dephosphorylate Bad by activating calcineurin and to enhance Bad heterodimerization with Bcl-xL, leading to apoptosis (14). We thus examined the role of Bad in Iso-induced apoptosis of cardiomyocytes. Western blot analysis using anti-phospho-Bad antibody revealed that Bad was highly phosphorylated in control cardiac myocytes. Phosphorylation levels of Bad were transiently reduced at 2 h and 4 h after addition of Iso (50 μM) (Fig. 3A). A calcium ionophore, ionomycin (1 μM), also decreased phosphorylation levels of Bad in cardiac myocytes (Fig. 3A). When the Ca^{2+} influx and calcineurin activation were inhibited by treatment with nifedipine (1 μM) and FK506 (10 μg/ml), respectively, Iso-induced dephosphorylation of Bad was abolished (Fig. 3A). These results suggest that the Ca^{2+}-calcineurin pathway is necessary for Iso-induced dephosphorylation of Bad in cardiac myocytes. On the other hand, expression levels of Bcl-2 and Bcl-xL were not changed by Iso stimulation (Fig. 3B).

Iso Induces the Release of Cytochrome c from Mitochondria to the Cytosol through the Ca^{2+}-Calcineurin Pathway—A growing body of evidence indicates that cytochrome c release from mitochondria to the cytosol, which is tightly regulated by the Bcl-2 family proteins, induces activation of caspases, leading to apoptosis (23–25). We thus examined the subcellular distribution of cytochrome c in cardiomyocytes in the absence or presence of Iso. In unstimulated cardiomyocytes, cytochrome c existed abundantly in the membrane fraction and slightly in the cytosol fraction. From 8 h after exposure to Iso (50 μM), the cytosol fraction of cytochrome c was significantly increased, and the increase of cytochrome c in the cytosol continued until 24 h (Fig. 4A). To determine whether calcineurin is involved in the Iso-induced release of cytochrome c, the cells were pre-treated with CsA (1 μM) and FK506 (10 μg/ml). Both calcineurin inhibitors strongly reduced the release of cytochrome c from mitochondria to the cytosol induced by Iso (Fig. 4B).
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FIG. 3. Iso dephosphorylates Bad protein through calcineurin. A, cardiomyocytes were incubated with Iso (50 μM) or ionomycin (1 μM). Bad was immunoprecipitated with anti-Bad antibody, subjected to SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose filters. Blots were subsequently probed with anti-phospho-specific Bad antibody (rows 1–4) or anti-Bad antibody (row 5), followed by ECL-based detection. Rows: 1 and 5, Iso (50 μM); 2, ionomycin (1 μM); 3, Iso plus FK506 (10 μg/ml) pretreatment; 4, Iso plus nifedipine (1 μM) pretreatment. Representative blots of phosphospecific Bad (rows 1–4) and Bad (row 5) from three independent experiments are shown. B, cardiomyocytes were stimulated by Iso (50 μM) for the indicated periods of time, and Western blot analysis was performed using anti-Bcl-2 and -Bcl-xL antibodies. Representative blots of Bel-2 and Bel-XL from three independent experiments are shown.

FIG. 4. Iso induces the release of cytochrome c from mitochondria to the cytosol. Immunoblot analysis was performed using anti-cytochrome c antibody after separating the cytosol and membrane fractions. A, cardiomyocytes were treated with Iso (50 μM) for the indicated periods of time. B, after pretreatment of cardiac myocytes with CsA (1 μM) and FK506 (10 μg/ml) for 1 h, cardiac myocytes were stimulated by Iso (50 μM) for 24 h. Representative blots of cytochrome c from three independent experiments are shown. fr., fraction.

These results suggest that calcineurin plays an important role in the Iso-induced release of cytochrome c from mitochondria to the cytosol in cardiac myocytes.

Iso Induces Less Apoptosis in the Hearts of dn Calcineurin Transgenic Mice—We further examined the role of calcineurin in cardiomyocyte apoptosis of in vivo heart using transgenic mice that overexpress dominant negative mutants of calcineurin (dn calcineurin). The calcineurin activity was increased ~3-fold by infusion of Iso (20 mg/kg) in the hearts of wild-type mice but not of the transgenic mice (Fig. 5A). We next evaluated Iso-induced cardiomyocyte apoptosis in the heart using the TUNEL method. There was no TUNEL-positive cardiomyocyte in the hearts of wild-type mice before infusion of Iso. Infusion of Iso (20 mg/kg) induced apoptosis of many cardiac myocytes of wild-type mice (7.2 ± 2.5 of 105 cardiac myocytes) (Fig. 5, B and C). The number of TUNEL-positive cells was significantly smaller in the hearts of the transgenic mice (1.5 ± 1.3 of 105 cardiac myocytes) and wild-type mice administered with FK506 (1 mg/kg/day, intramuscular) for 3 days (1.3 ± 1.5 105 cardiac myocytes) than in the hearts of wild-type mice (Fig. 5B). These results suggest that calcineurin is also involved in β-stimulant-induced cardiomyocyte apoptosis of in vivo heart.

DISCUSSION

Many lines of evidence have suggested that activation of the sympathetic nervous system is observed in patients with heart failure and exerts deleterious effects on human hearts (7, 26, 27). Activation of the sympathetic nervous system provides the rationale for the use of β-adrenergic receptor antagonists to treat heart failure. In fact, therapeutic interventions by β-adrenergic receptor antagonists not only improve cardiac contractility but also improve the prognosis of heart failure (28–30). In the present study, we examined the mechanism by which β-adrenergic stimulation induces injury of cardiomyocytes.

Apoptosis has been demonstrated to occur in the myocardium in a variety of pathological situations. The number of apoptotic cardiomyocytes is increased in the myocardium obtained from heart failure patients (1, 2). Furthermore, prominent cardiomyocyte apoptosis was observed in the hearts of transgenic mice overexpressing GTP-binding proteins α, which transmit signals from β-adrenergic receptors to adenylly cyclase, and treatment with β-adrenergic receptor antagonists prevented cardiomyocyte apoptosis in mice (31). These observations suggest that β-adrenergic stimulation may induce deterioration of cardiac function by inducing cardiomyocyte apoptosis during the development of heart failure. Stimulation of β-adrenergic receptors activates adenylate cyclase, which increases intracellular cAMP. The cAMP-dependent protein kinase A activates L-type Ca2+ channels, leading to a significant increase in cytosolic Ca2+ levels. In the present study, Iso increased the number of TUNEL-positive cells. A calcium ionophore, ionomycin, which causes a sustained increase in intracellular Ca2+ concentration, increased the number of TUNEL-positive cells as much as did maximum Iso stimulation. Pretreatment with the L-type Ca2+ channel antagonist nifedipine and the selective calcineurin inhibitors CsA and FK506 strongly inhibited the Iso-induced increase in TUNEL-positive cells and Iso-induced DNA ladder formation. Taken together, these results suggest that the Ca2+-calcineurin pathway plays a critical role in β-adrenergic receptor-induced cardiomyocyte apoptosis.

The Bel-2 family proteins are important regulators of cell death in mammalian cells. Apoptosis is determined by the relative balance between proapoptotic molecules such as Bad and Bax and antiapoptotic molecules such as Bcl-2 and Bcl-xL. Phosphorylated Bad is sequestered in the cytosol by 14–3-3 proteins and is inactivated, thus promoting cell survival. De-phosphorylated Bad promotes cell death at least in part through heterodimerization with the antiapoptotic proteins Bcl-2 and Bcl-XL. The present study demonstrated that Iso as well as a calcium ionophore transiently reduced phosphorylation levels of Bad and that Iso-induced dephosphorylation of Bad was abolished when the Ca2+-calcineurin pathway was inhibited by calcineurin inhibitors and an L-type Ca2+ channel antagonist. Furthermore, we examined the subcellular distribution of cytochrome c in cardiomyocytes in the presence or absence of Iso. Iso significantly induced the release of cytochrome c from mitochondria to the cytosol, and the Iso-induced release of cytochrome c was reduced by pretreatment with calcineurin inhibitors. Bcl-2 and Bcl-XL function in the mitochondrial membrane as antiapoptotic molecules by preventing cell death.
the release of cytochrome c from mitochondria (24, 25). When Bad is dephosphorylated and translocated from the cytosol to mitochondria, Bad has been reported to form a complex with Bcl-2 and induce the release of cytochrome c (14). These results and observations collectively suggest that calcineurin plays an important role in the Iso-induced release of cytochrome c from mitochondria to the cytosol, possibly through dephosphorylating Bad in cardiac myocytes.

It has been reported that the calcineurin inhibitors prevent the development of cardiac hypertrophy and cardiomyopathy in rodent models (32). In the present study, to elucidate whether calcineurin is involved in the apoptosis of cardiac myocytes in vivo, we generated dn calcineurin transgenic mice under the control of cardiac myocyte-specific α-myosin heavy chain promoter. It has been reported that transfected ΔCnA induces the redistribution of Bad from the cytosol to mitochondria and the translocation of another calcineurin substrate, nuclear factor of activated T cells, from the cytosol to the nucleus. In contrast, dn calcineurin, a trans-dominant inhibitory mutant of calcineurin, prevented the Ca^{2+}-induced redistribution of Bad and nuclear factor of activated T cells and reduced apoptosis, indicating that dn calcineurin was effectively suppressing endogenous calcineurin function (14). Infusion of Iso increased the calcineurin activity and the number of TUNEL-positive cells in the heart. Iso-induced increases in the number of TUNEL-positive cells as well as in calcineurin activity were significantly less in the hearts of the transgenic mice or FK506-treated mice than in the hearts of wild-type mice. These results suggest that stimulation of β-adrenergic receptors also induces cardiomyocyte apoptosis through calcineurin in vivo.

Our results collectively suggest that the Ca^{2+}-calcineurin pathway plays a critical role in the progression of heart failure by regulating cardiomyocyte apoptosis and that inhibition of the Ca^{2+}-calcineurin pathway may be effective in the treatment of heart failure. To the contrary, De Windt et al. (33) recently reported that calcineurin activation prevents apoptosis of cardiomyocytes and that calcineurin transgenic mice hearts significantly increased TUNEL-positive cells of non-cardiomyocytes but not of cardiomyocytes. The reason for the discrepancy is unknown at present; however, it may come from the different stimuli. De Windt et al. (33) have reported that the activation of calcineurin protects cardiomyocytes against apoptosis induced by 2-deoxyglucose and staurosporine in vitro and by ischemia/reperfusion in vivo. We also observed that ischemia/reperfusion induced more apoptosis in the hearts of transgenic mice expressing dn calcineurin than in the hearts of wild-type mice.² It has been reported that calcineurin can activate opposing pathways that either suppress or induce apoptosis in the same cells (34). Further studies are necessary to elucidate in what situations calcineurin induces or suppresses cardiomyocyte apoptosis.

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