A mutation in the human phospholamban gene, deleting arginine 14, results in lethal, hereditary cardiomyopathy

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The sarcoplasmic reticulum Ca^{2+}-cycling proteins are key regulators of cardiac contractility, and alterations in sarcoplasmic reticulum Ca^{2+}-cycling properties have been shown to be causal of familial cardiomyopathies. Through genetic screening of dilated cardiomyopathy patients, we identified a previously uncharacterized deletion of arginine 14 (PLN-R14Del) in the coding region of the phospholamban (PLN) gene in a large family with hereditary heart failure. No homozygous individuals were identified. By middle age, heterozygous individuals developed left ventricular dilation, contractile dysfunction, and episodic ventricular arrhythmias, with overt heart failure in some cases. Transgenic mice overexpressing the mutant PLN-R14Del recapitulated human cardiomyopathy exhibiting similar histopathologic abnormalities and premature death. Coexpression of the normal and mutant-PLN in HEK-293 cells resulted in sarcoplasmic reticulum Ca^{2+}-ATPase superinhibition. The dominant effect of the PLN-R14Del mutation could not be fully removed, even upon phosphorylation by protein kinase A. Thus, by chronic suppression of sarcoplasmic reticulum Ca^{2+}-ATPase activity, the nonreversible superinhibitory function of mutant PLN-R14Del may lead to inherited dilated cardiomyopathy and premature death in both humans and mice.

dilated cardiomyopathy | heart failure | calcium cycling | mutation | phosphorylation

Heart failure is the leading cause of morbidity and mortality worldwide. Therapies targeting the neurohormonal axis in heart failure with β-adrenergic receptor blockers and angiotensin-converting enzyme inhibitors have improved the overall prognosis, but the overall 5-year mortality rate is still ≈50% (1). These therapies are aimed at treatment of the symptoms of the disease and are most effective when they target underlying alterations in subcellular mechanisms in the cardiomyocyte that lead to cardiac dysfunction, pathological remodeling, and heart failure. A common clinical hallmark and characteristic of failing cardiomyocytes is abnormal Ca^{2+} homeostasis, manifested by a prolonged time course of intracellular Ca^{2+} transients and changes in systolic and diastolic Ca^{2+} levels (2) that, at least in part, may be linked to depressed sarcoplasmic reticulum (SR) Ca^{2+} cycling (3, 4). The prolonged Ca^{2+}-transient decay and the increased diastolic Ca^{2+} levels may reflect impaired ryanodine receptor function and decreases in sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) levels, as well as increased SERCA2a inhibition by phospholamban (PLN) (5–8). PLN is a crucial Ca^{2+} regulatory protein of Ca^{2+} cycling and a primary mediator of the β-adrenergic effects resulting in enhanced cardiac output (9). The levels of PLN and/or its degree of phosphorylation profoundly influence the activation state of SERCA2a. In the dephosphorylated state, PLN interacts with SERCA2a and shifts its Ca^{2+} activation toward lower apparent Ca^{2+} affinity. Upon PKA-mediated phosphorylation, the inhibitory interaction between PLN and SERCA2a is abolished and the apparent Ca^{2+} affinity is raised. Thus, regulation by PLN is the rate-determining factor for Ca^{2+} reuptake by SERCA2a to induce relaxation and decrease diastolic Ca^{2+} levels.

Recent searches for human PLN genetic variants that may be associated with cardiomyopathy in families identified two mutations in the coding region of the *PLN* gene and one in its promoter region. One of the coding region mutations harbors a stop codon for lysine 39 (PLN-L39stop) and was discovered in two large Greek families (10). Although heterozygous individuals indicated a variable clinical phenotype, the inheritance of two copies of the mutant *PLN* gene led to dilated cardiomyopathy (DCM) and heart failure during the teenage years. In a cardiac explant, no PLN was detected. Thus, in contrast to the enhanced contractility and a normal life span observed with *PLN* ablation in mice, humans lacking PLN developed a lethal cardiomyopathy. The second mutation, R9C, in the coding region of the human *PLN* gene had no effects on SERCA2a activity under basal conditions but appeared to trap PKA, preventing phosphorylation of even wild-type PLN molecules (11). The detrimental effects of such chronic inhibition of SERCA2a activity led to DCM, and the clinical findings were supported by studies in transgenic mice overexpressing the PLN-R9C mutant. Thus, it was proposed that the chronic, specific inhibition of PLN phosphorylation, with its attendant effects on Ca^{2+} transients, may be sufficiently deleterious to cause the onset of DCM in humans.

In addition to increased PLN inhibition by mutations in the coding region, alterations in PLN expression, arising from regulation of gene transcription, are associated with different degrees of inhibition of SERCA2a activity and cardiac functional properties. Indeed, a recent study reported a mutation in this region (A to G at −77 bp) in 1 of 87 hypertrophic patients (12). *In vitro* infections of neonatal rat myocytes indicated that this mutation increased PLN promoter activity by 1.5-fold, suggesting that the mutation may play a significant role in depressed Ca^{2+} cycling, leading to hypertrophy.

In this study, we report a fourth naturally occurring mutation (Arg14Del) in the human *PLN* gene, associated with the deletion of Arg-14 in the coding region. The mutation was identified in a large family pedigree in which heterozygous carriers exhibited inherited DCM and death by middle age. A mouse model with cardiac-specific expression of the heterozygous PLNArg14Del mutant recapitulated the human phenotype. The mechanism under-

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Abbreviations: DCM, dilated cardiomyopathy; ECG, electrocardiogram; PLN, phospholamban; SERCA2a, sarcoplasmic reticulum Ca^{2+}-ATPase; SR, sarcoplasmic reticulum.

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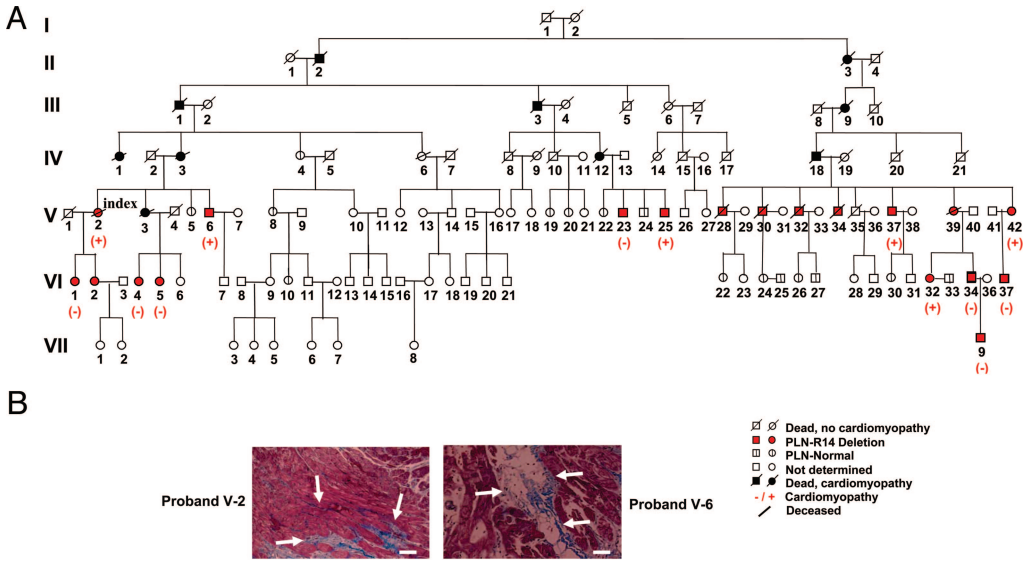

Fig. 1. Analysis of inheritance of the PLN-R14Del mutation and the cardiomyopathic phenotype and cardiac histology of affected individuals. (*A*) Pedigree for the presence or absence of AGA (R14) in the PLN gene. Squares represent males, and circles represent females. Slash denotes deceased. (*B*) Histological analysis of cardiac biopsies from probands V-2 and V-6 heterozygous for the R14 deletion mutation, stained with Masson's-Trichrome, illustrating the massive interstitial fibrosis and myocardial disarrangement (arrow). All images are at equal magnification. (Scale bar, 50 μm.)

lying the detrimental effects of this human mutation appears to involve molecular synergism with normal PLN, resulting in super-inhibition of SERCA2a activity, likely mediated through a disturbance in the structure of PLN. These findings also add to the accumulating evidence that mutations in the human *PLN* gene that increase its inhibitory function predispose to DCM and early death.

Results

Clinical History. A total of 1,203 patients with DCM and 658 normal subjects were recruited from the Cincinnati Heart Failure/Transplant Program and the Onassis Cardiac Surgery Center. Available clinical data from these patients indicated that the average age of the patients was 44.4 ± 12.3 years. The average ejection fraction, determined by echocardiography, was 23 ± 10%. Comorbid conditions in the cohort included as follows: hypertension (8%), diabetes (6%), hypercholesterolemia (12%), and atrial fibrillation (12%).

Identification of a Mutant *PLN* Gene in Cardiomyopathy Pedigrees. *PLN* gene was sequenced in 1,203 unrelated individuals with DCM and heart failure. Sequencing of the *PLN* gene coding region from these unrelated individuals identified one patient heterozygous for the deletion of nucleic acids 39, 40, and 42, which resulted in the deletion of arginine 14 (Fig. 6, which is published as supporting

Table 1. Clinical history of the subjects carrying the PLN-R14Del mutation in the pedigree

Subject	Age, yrs	IVEDT	LVEDD	PWEDT	LVESD	LVEF	Comments
V-2	Deceased at age 56	6.1	56	6.0	47	27	Heart failure symptoms, abnormal characteristic ECG, sustain VT episodes (ACID)
V-6	47	7.1	56	7.1	41	41	Heart failure symptoms, abnormal characteristic ECG, non-sustain VT episodes
V-23	55	9.7	47	9.2	34	67	Asymptomatic, normal screening tests
V-25	52	7.4	57	8.4	38	42	Heart failure symptoms, abnormal characteristic ECG, sustain VT episodes (ACID)
V-37	62	8.6	54	8.3	42	52	Palpitations, abnormal characteristic ECG, frequent ventricular extra systoles
V-42	56	7.3	55	8.2	33	48	Palpitations, abnormal characteristic ECG, frequent ventricular extra systoles
VI-1	32	9.7	50	9.7	35	56	Asymptomatic, abnormal characteristic ECG
VI-2	31	ND	ND	ND	ND	ND	No screening tests available
VI-4	27	6.2	45	6.6	30	65	Asymptomatic, normal screening tests
VI-5	34	6.5	47	6.5	28	64	Asymptomatic, normal screening tests
VI-32	35	6.3	53	6.3	39	45	Palpitation, abnormal characteristic ECG, frequent ventricular extra systoles
VI-34	37	8.2	45	7.3	28	62	Asymptomatic, abnormal characteristic ECG
VI-37	30	11	54	9	36	57	Asymptomatic, abnormal characteristic ECG
VII-9	12	7.4	37	8.5	23.4	62	Asymptomatic, normal screening tests

IVEDT, interventricular septum end diastolic thickness; PWEDT, posterior wall end diastolic thickness; LVEDD, left ventricular end diastolic dimension; LVESD, left ventricular end systolic dimension; LVEF, left ventricular ejection fraction; VT, Ventricular tachycardia; AICD, Automatic Implantable Cardioverder Defibrillator; ND, not done.

information on the PNAS web site). This mutation was not found in 76 unrelated normal subjects matched in age and ethnic background or in 582 unrelated normal subjects. Arginine 14 is highly conserved among species (13), fulfilling the requirement for basic amino acids upstream of the PKA phosphorylated site at Ser 16. Consequently it was hypothesized that its deletion might alter PLN phosphorylation and the regulation of SERCA2a.

To assess the significance of this deletion mutation, the extended pedigree of the index case, Proband V-2, who had recently died of DCM (Fig. 1*A*), was examined. Proband V-2 presented heart failure symptoms and DCM, diagnosed at age 50. At age 52, an Automatic Implantable Cardioverder Defibrillator was implanted, because of sustained ventricular tachycardia episodes. In subsequent years, the heart deteriorated progressively toward failure (NYHA class III–IV). The patient died while awaiting cardiac transplantation at age 56 with incessant sustained ventricular tachycardia and ventricular fibrillation episodes (Table 1). The index case belongs to a large family, which consists of three related subfamilies with a long clinical history of DCM. The mother (IV-3), the aunt (IV-1), and the sister (V-3) of the index case had also died of DCM (≈48 yrs), but no sample was available for DNA analysis. Further clinical history analysis of the subfamilies revealed that in generations III, IV, and V, probands III-1, III-3, III-9, IV-12, IV-18, V-28, V-30, V-32, V-34, and V-39 died of DCM, but no sample was available for DNA analysis. No data are available for generations I and II. However, family members recollect that probands II-2 and II-3 died of sudden death at a young age.

The brother of the index patient (V-6) was also found to be heterozygous for PLN-R14Del. He was diagnosed with DCM at age 42 and has presented frequent nonsustained VT episodes during his 5-year followup. A distant cousin (V-25), who was heterozygous for PLN-R14Del, was also diagnosed with DCM at age 46 when an Automatic Implantable Cardioverder Defibrillator was implanted because of sustained ventricular tachycardia episodes.

All of the family members carrying the PLN-R14Del mutation, and the other live family members who participated in this study, underwent a medical interview, rigorous clinical evaluation by using a physical examination, 12-lead electrocardiogram (ECG) echocardiogram and 24-h Holter ECG monitoring. Three heterozygotes carrying the PLN-R14Del deletion members, V-37 (62 years), V-42 (56 years), and VI-32 (35 years), presented mild left ventricular systolic dysfunction and dilation in echocardiograms and frequent (>1,000 per 24 h) ventricular extrasystoles in 24-h Holter ECG monitoring. Available clinical evaluations and medical records of the heterozygous members are summarized in Table 1. VI-1 (32 years), VI-4 (27 years), V-5 (34 years), VI-34 (37 years), VI-37 (30 years), and VII-9 (12 years) are also heterozygous for the PLN-R14Del mutation but are asymptomatic to date, indicating that the onset of the disease may be age-dependent.

Histopathologic examination of Proband V-2 and V-6 biopsies showed significant replacement fibrosis and myofibrillar disarrangement (Fig. 1*B*). Interestingly, most of the probands carrying the PLN-R14Del mutation, regardless of the absence or presence of echocardiographic abnormalities, exhibited similar abnormal characteristic ECGs with low QRS complex potentials and decreased R wave amplitude, mainly in anterior-lateral precordial leads (Fig. 7, which is published as supporting information on the PNAS web site). History, physical examinations, and screening tests of the other family members who did not carry the PLN-R14Del did not reveal any findings of cardiomyopathy.

The analysis of the genomic DNA of all available individuals from the three subfamilies indicated that the PLN-R14Del mutation was inherited in this pedigree and suggested that this mutation caused disease, because it cosegregated with the affected status. Furthermore, PLN-R14 Del altered a highly conserved residue, and it was absent from 1,316 normal chromosomes. However, the overall difficulty in phenotype/genotype correlation has made it impossible to obtain a logarithm of odds score at this stage of the investigation.

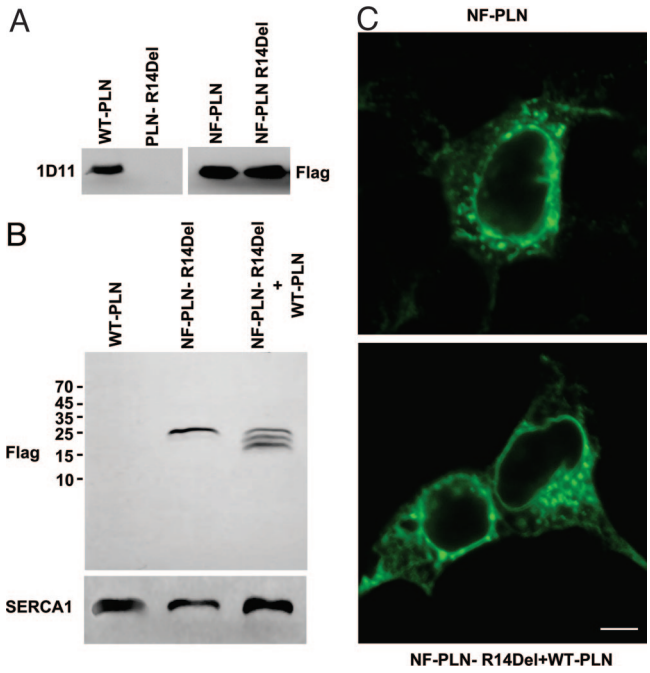

Fig. 2. Expression and localization of WT-PLN and PLN-R14Del mutant in HEK-293 cells. (*A*) Immunoblot analyses of WT-PLN and PLN-R14Del in transfected cell lysates. (*Left*) Immunoblots of boiled microsomes from cells transfected with WT-PLN and PLN-R14Del and detected by using anti-PLN antibody, 1D11. (*Right*) Immunoblots of boiled microsomes isolated from cells transfected with NF-PLN or NF-PLNR14Del and detected with anti-flag antibody, M2. (*B*) Immunoblots of unboiled microsomes of NF-PLN-R14Del (homozygote) and NF-PLN-R14Del plus WT-PLN (heterozygote) and probed with anti-flag antibody. (*B Lower*) Blots were striped and reprobed with anti-SERCA1 antibody. (*C*) Immunofluorescence of NF-PLN and NF-PLN-R14Del (heterozygote) transfected cells analyzed by confocal microscopy 48 h after transfection. In both cases, immunofluorescence is exclusively in the ER. (Scale bar, 30 μm.)

***In Vitro* Analysis of PLN-R14Del.** We transfected HEK-293 (human embryonic kidney) cells with cDNAs encoding WT-PLN and PLN-R14Del in expression constructs. Immunoblot analyses of whole-cell lysates confirmed the expression of WT-PLN, although we could not visualize expression of the PLN mutant (Fig. 2*A Left*). This result is likely due to the fact that the PLN-R14Del mutation is within the epitope region for the 1D11 antibody, which is from amino acid 7–17 (14). Therefore, we generated the PLN-R14Del mutation on the N-flag-tagged PLN expression backbone (NF-PLN) and repeated the immunoblot analyses by using the flag antibody. In this case, NF-PLN and mutant NF-PLN expression were observed (Fig. 2*A Right*). To mimic the heterozygous patients, which contain one copy of the WT-NF-PLN and one copy of the NF-PLN-R14Del, we transfected HEK-293 cells with either WT-NF-PLN alone, the NF-PLN-R14Del alone, or with equivalent amounts of WT-PLN and NF-PLN-R14Del (Fig. 2*B*). Immunoblot analyses by using flag antibody and unboiled cell lysates showed that the pentameric structure of PLN appears to be destabilized, because, in addition to the large PLN pentamer, smaller distinct bands corresponding to different-sized oligomers were observed. Similar results in the heterozygous cultures were obtained by using the 1D11 antibody (results not shown).

Confocal microscopy in HEK-293 cells transfected with either NF-PLN or NF- PLN-R14Del coexpressed with WT-PLN showed flag immunoreactivity to be located in the ER in both cases, with

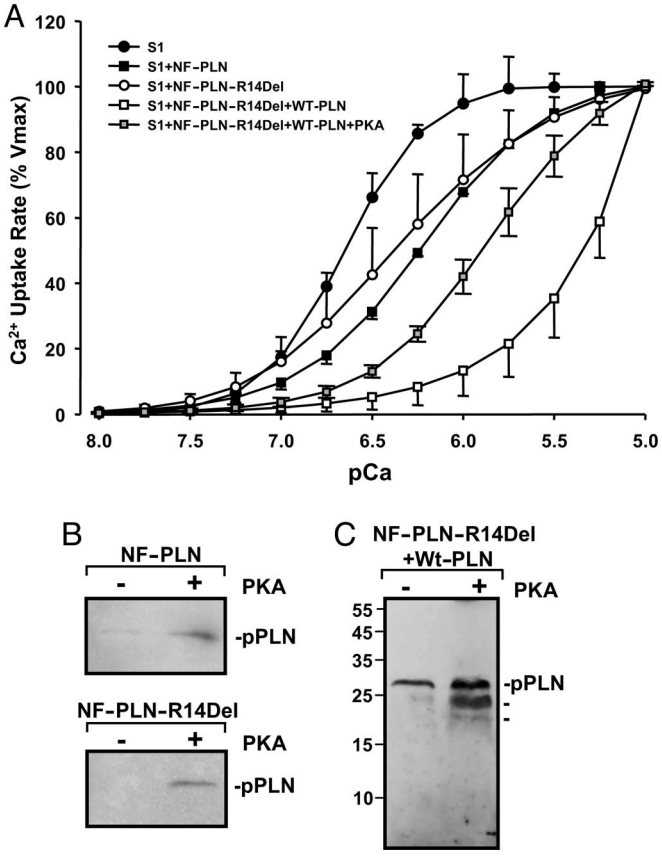

Fig. 3. Effect of PLN and mutant PLN on the Ca^{2+} affinity of SERCA1a in HEK-293 cells. Cells were cotransfected with wild-type, homozygous, or heterozygous mutant PLN cDNA and SERCA1 cDNA, and the rates of Ca^{2+} uptake were measured. V_{max}, maximum velocity of Ca^{2+} uptake. (*A*) SERCA1 Ca^{2+} affinity is highly inhibited when cells are cotransfected with heterozygous mutant PLN-R14Del cDNA. PKA phosphorylation partially relieves the inhibitory effect of NF-PLN-R14Del plus WT-PLN (heterozygote) mutant, leaving it in the superinhibitory range. Phosphorylation of NF-PLN and NF-PLN-R14Del mutant (pPLN, phosphorylated PLN) was detected in boiled (*B*) and unboiled (*C*) microsomes in the presence and absence of PKA.

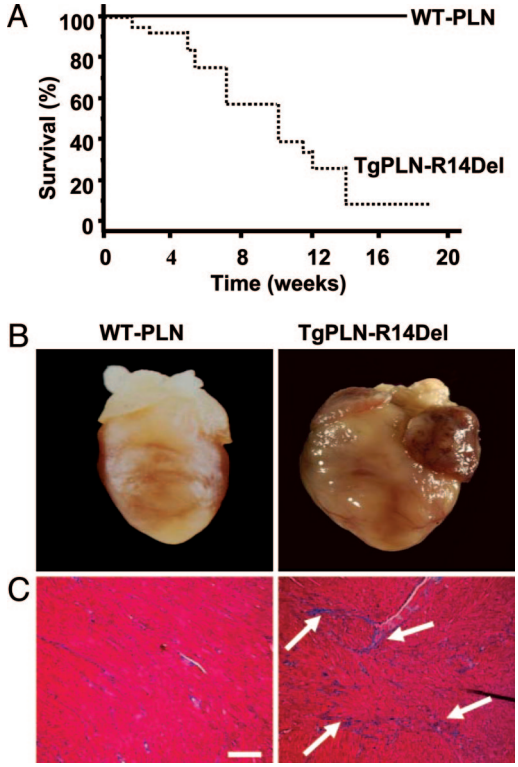

Fig. 4. Mortality and cardiac morphology of a mouse model expressing the PLN-R14Del mutant PLN. (*A*) Mice carrying the PLN-R14Del mutant exhibited mortality at a young age. (*B*) A comparison of the hearts of strain and age-matched WT-PLN and TgPLN-R14Del overexpression mice at 6 weeks of age showed enlargement for TgPLN-R14Del only. (*C*) A comparison of the histopathology of left ventricular heart tissue from 6-week-old, strain-matched WT-PLN and TgPLN-R14Del mice by using Masson's trichrome for collagen showed massive interstitial fibrosis in the PLN-R14Del mouse only (arrow). All images are at equal magnification. (Scale bar, 50 μm.)

perinuclear and defined cellular-staining patterns that were typical of normal ER-staining patterns (Fig. 2*C*).

We compared the effect of the PLN-R14Del mutant protein on the Ca^{2+} affinity (K_{Ca}) of SERCA1 in an assay of the Ca^{2+} dependence of Ca^{2+} transport in microsomes from HEK-293 cells transfected with SERCA1, or SERCA1 cotransfected with either NF-PLN or NF-PLN-R14Del or both constructs together. SERCA1 alone had an apparent K_{Ca} of 6.81 $\pm$ 0.04 pCa units (n = 3) (Fig. 3*A*), whereas WT-PLN resulted in a significantly lower ($P <$ 0.05) apparent K_{Ca} of 6.28 $\pm$ 0.04 pCa units (n = 4). The NF-PLN-R14Del mutation showed a significantly lower affinity of SERCA for Ca^{2+} (K_{Ca} of 6.62 $\pm$ 0.05; n = 4; $P < 0.05$). This affinity was also significantly different ($P < 0.05$) from the values observed with NF-PLN alone. However, when the NF-PLN-R14Del mutant was coexpressed with WT-PLN and SERCA1, there was a significant ($P < 0.05$) supershift in the pCa value (5.46 $\pm$ 0.16; n = 5; $P <$ 0.05), indicating a superinhibitory effect compared to WT-PLN or PLN-R14Del (Fig. 3*A*). This observation suggests that the PLN-R14Del mutant exerts a dominant negative effect on PLN-WT.

To reverse the inhibition of SERCA by the heterozygous PLN condition (NF-PLN-R14Del plus WT-PLN), we treated microsomes with protein kinase A (PKA) before Ca^{2+} uptake assays. In these experiments, the highly superinhibitory effect of the heterozygous complex was diminished by PKA, although it could not be fully relieved (K_{Ca} of 6.12 $\pm$ 0.33; n = 4; $P < 0.05$; Fig. 3*A*).

To measure PKA-dependent phosphorylation, microsomes were treated with PKA and subjected to immunoblotting by using the PLN phospho-specific antibody (A285) (Fig. 3*B*). In boiled samples, we determined that the PLN-R14Del mutant can be phosphorylated to levels similar to full-length PLN. To examine the effect of the mutation on pentamer formation and stability in the heterozygous NF-PLN-R14Del plus WT-PLN cultures, PKA-treated microsomes were subjected to SDS/PAGE without boiling (Fig. 3*C*). In this case, we observed that the higher molecular mass PLN band, representing the pentamer, was the most phosphorylated. However, we were able to verify the ability of the mutant to be phosphorylated by using boiled microsomes, ^{32}P-labeling, and autoradiography. In these experiments, the low molecular mass PLN monomers are clearly phosphorylated to a similar extent in the presence of PKA for all constructs and conditions (Fig. 8, which is published as supporting information on the PNAS web site).

Transgenic Mouse Model of Human PLN-R14Del Mutant. To assess the physiological significance of the PLN-R14Del mutant *in vivo*, transgenic mice with cardiac specific expression of mutant PLN-R14Del were generated. Mice harboring the mutant PLN-R14Del transgene were identified by PCR analysis. PLN-R14Del mutant mice died between 2 and 16 weeks of age, whereas there were no deaths in wild-type controls during this period (Fig. 4*A*). To further assess the pathological alterations in the mutant PLN-R14Del transgenic hearts, histological analysis was performed at 6 weeks of

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age. Every mutant PLN-R14Del mouse analyzed showed a dramatic increase in heart size relative to wild-type controls (Fig. 4*B*). Furthermore, histological analysis revealed ventricular dilation, myocyte disarray, and myocardial fibrosis in hearts of transgenic mice expressing the cardiac specific mutant PLN-R14Del, similar to the findings in the human patients (Fig. 4*C*).

Sarcoplasmic Reticulum Ca^{2+} Uptake Assay in Transgenic Mouse Hearts Overexpressing PLN-14Del. The initial rates of SR Ca^{2+} transport, assessed over a wide range of [Ca^{2+}], indicated that overexpression of mutant PLN-R14Del resulted in a significant increase in the EC_{50} value for Ca^{2+} (0.471 μM, $n = 1$), compared with WT-PLN (0.326 $\pm$ 0.013 μM, $n = 3$; Fig. 5). These data suggest that the PLN-R14Del mutation, combined with WT-PLN, resulted in superinhibition of the Ca^{2+} affinity for SERCA2a. When phosphorylation by PKA preceded the SR Ca^{2+} uptake studies, the inhibitory effects of WT-PLN were relieved (Fig. 5). However, the superinhibitory effects of the PLN-R14Del were not fully relieved (Fig. 5), consistent with the *in vitro* studies in HEK-293 cells. Unfortunately, overexpression of mutant PLN-R14Del had deleterious effects and caused early death in this transgenic model, which made it impossible to have a greater number (n) for this assay.

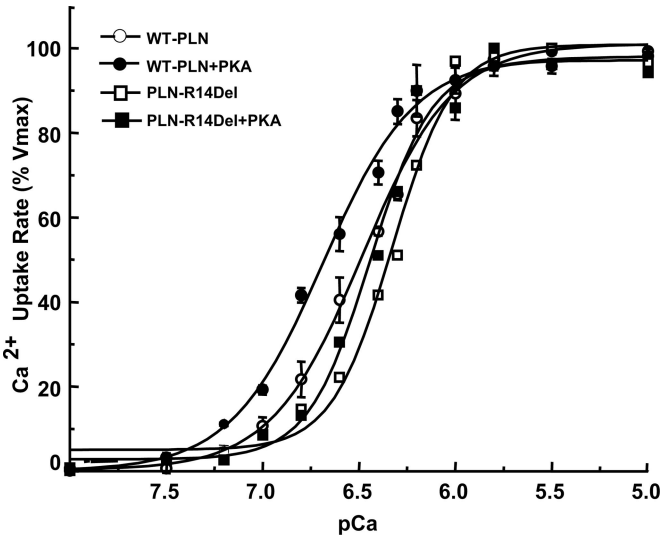

Fig. 5. Effect of wild-type and mutant PLN-R14Del on the Ca^{2+} affinity of SERCA2a. Cardiac homogenates from WT-PLN and TgPLN-R14Del (heterozygote) were incubated in the presence or absence of ATP and PKA catalytic subunit, and then the initial rates of SR Ca^{2+} transport were measured. V_{max}, maximum velocity of Ca^{2+} uptake.

Discussion

We identified herein a previously uncharacterized human PLN mutation, the deletion of PLN Arg-14, that is associated with inherited human DCM and premature death. Some of the heterozygous individuals develop mild left ventricular dilation and dysfunction with frequent ventricular extra systolic beats and ventricular tachycardia episodes, leading to progressive left ventricular dilation and heart failure.

Transgenic PLN-R14Del overexpressing mice recapitulated human DCM with abnormal histopathology and premature death. In accordance, expression of the heterozygous mutant PLN in HEK-293 cells resulted in superinhibition of SERCA1, which was greater than any of the previously reported superinhibitory PLN-mutants (6, 15, 16). The dominant effect of the PLN-R14Del mutation could not be reversed fully upon phosphorylation by PKA. Thus, mutant PLN could no longer function as a key effector of β-adrenergic stimulation to facilitate augmentation of cardiac function. Previous transgenic studies on PLN mutations indicate that life is not threatened if the inhibitory function of PLN can be reversed by endogenous β-agonists, but DCM occurs if the mutation is highly inhibitory and is not reversible by endogenous β-agonists (14–16).

Interestingly, some of the heterozygous human subjects for the PLN-R14Del mutation presented DCM with ventricular extra systolic beats and ventricular tachycardia episodes, which progressed to congestive heart failure by middle age. Some of the probands carrying the mutation were asymptomatic and presented normal echocardiography at a young age, indicating that the complete mutation expression may be age-dependent. However, even at an early age, some of the patients presented abnormal ECG characteristics that may be associated with signs of disease. These clinical phenotypes could be attributed to the superinhibitory effects of the PLN-mutant on SR Ca^{2+} cycling and cardiac function. Increased inhibition over a period of years could, conceivably, lead to ventricular remodeling, which may then progress to ventricular failure by later years. Indeed, there are several examples of an association between the development of heart failure and superinhibition of SERCA2a by PLN mutants in animal models (14–16). Although these findings suggest that the PLN-R14Del mutation may initiate human DCM, other factors, including environmental perturbations, may also modify the PLN-R14Del human phenotype and contribute to the functional effects associated with this mutation.

Evidence from human and experimental heart failure studies indicated that SR Ca^{2+} cycling is critical to the progression of heart failure and point to the central regulatory role of SR Ca^{2+} uptake, storage, and release in this process. Consistent with this notion, adenoviral-mediated myocardial-targeted expression of SERCA2a (17) and antisense suppression (18) or inhibition (19) of PLN activity resulted in enhanced SR Ca^{2+} cycling, improved energetics, survival, and cardiac function. PLN inhibition also attenuated heart failure progression in cardiomyopathic hamsters (20), whereas RyR inhibition resulted in improved cardiac function (21), suggesting that abnormal SR Ca^{2+} cycling represents a root cause of heart failure. Support for this hypothesis has been provided by studies in model systems. Cardiac overexpression of superinhibitory PLN mutants resulted in increased inhibition of the Ca^{2+} affinity of SERCA2a and cardiac contractility, leading to cardiac hypertrophy and premature death (14–16). Furthermore, human calsequestrin mutations (22) and human RyR mutations (23) have been identified, which result in ventricular arrhythmias and cardiac polymorphic ventricular tachycardia. Thus, the SR Ca^{2+}-cycling proteins have been suggested to be key regulators of cardiac contractility, and disturbances in their function may lead to cardiac pathology.

The mechanisms by which the PLN-R14Del mutant exerts its superinhibitory effects is likely to be related to the structure of PLN in both its unphosphorylated and phosphorylated forms. For instance, the deletion of Arg-14 results in a partial disruption of the stability of the PLN pentamer, leading to enhanced monomer concentration. Several dominant-acting mutants of PLN (L37A, I40A, and V49G) gain inhibitory function by inducing monomer formation, associated with dramatic changes in the apparent affinity of SERCA2a for Ca^{2+}. An enhanced association between PLN and SERCA would be consistent with the inability of PKA-mediated phosphorylation to relieve fully the PLN-R14Del mutant superinhibitory effects, similar to findings in ref. 15.

Our studies support the view that chronic suppression of either basal SERCA2a activity (PLN-R14Del mutant) or the stimulatory effect of the β-adrenergic signaling pathway (PLN-R9C mutant) (11) result in human cardiomyopathy and heart failure. On the other hand, absence of PLN inhibition by the PLN-L39stop mutant, associated with the lack of regulatory inhibition of SERCA2a and increased cardiac work, may also result in heart failure. Thus, the mutation reported in this study, together with the two previously reported human PLN mutations, point to the paramount impor-

tance of maintaining normal homeostatic mechanisms for calcium cycling in the human heart.

Materials and Methods

Mutation Identification and EarI Restriction Endonuclease Screening. Written informed consent was obtained from participating subjects. All protocols were approved by the institutional review board of the Onassis Cardiac Surgery Center or the University of Cincinnati College of Medicine. Genomic DNA was isolated either from whole blood or from paraffin blocks containing heart tissue. A 348-bp fragment of the *PLN* gene containing the entire PLN coding region was amplified by PCR with 60 ng of genomic DNA and a high-fidelity *Taq* polymerase. The primers were as follows: sense, 5′-TCTCATATTTGGCTGCC-3′, and antisense, 5′-ATTGTTT-TCCTGTCTGC-3′ tagged with M13 forward and reverse primer sequences, respectively. The conditions were as follows: one cycle at 94°C for 3 min, linked to 30 cycles at 94°C for 1 min, 46°C for 1 min, and 72°C for 1 min, followed by one cycle at 94°C for 1 min, 53°C for 1 min, and 72°C for 10 min. *PLN* DNA was sequenced by using automated dye-primer chemistry. The generated sequences were compared with the reported human *PLN* sequences by a computational method, and the electropherograms were inspected individually for confirmation. The mutation resulted in a loss of an EarI restriction endonuclease site. Thus, for rapid screening, the PCR products were digested with EarI, which provided two fragments of 136 and 212 bp from wild-type templates and only one fragment of 345 bp for AGA homozygotes after 2% agarose gel electrophoresis.

Echocardiography. Comprehensive 2D and Doppler echocardiography was performed according to the recommendations of the American Society of Echocardiography (24). Left ventricular dimensions (interventricular septum end-diastolic thickness, left ventricular posterior wall end-diastolic thickness, left ventricular end systolic and diastolic diameter) were measured with M-mode echocardiography by using the left parasternal window. Left ventricular volumes and ejection fraction were determined by apical two- and four-chamber views with the modified Simpson rule (25).

Creation of Mutant Phospholamban Mice. The site-specific deletion of AGA was introduced into mouse *PLN* cDNA by PCR (26). The entire expression construct was composed of the cardiac specific α-myosin heavy chain promoter (α-MHC_P, 5.5 kb; a gift from J. Robbins, Children's Hospital, Cincinnati), the PLN coding region with the AGA deletion mutation (0.65 kb), and the human growth hormone polyadenylation signal. Microinjection and identification of transgenic mice were performed as described in ref. 26.

Ca^{2+} Transport and Immunofluorescence in HEK-293 Cells. Mutagenesis of cDNAs and transfections were carried out as described in refs. 26 and 27. Cells were harvested 44–48 h after transfection, and microsomes were prepared and assayed for Ca^{2+} transport activity in the presence or absence of PKA or immunoblotting with the PLN monoclonal antibody ID11 (a gift from Robert Johnson, Merck Research Laboratories) (27).

For immunofluorescence experiments, HEK-293 cells were grown on 18 × 18 mm glass coverslips and transfected with 400 ng of PLN cDNA by using calcium phosphate. At 48 h after transfection, cells were washed, fixed, and processed for immunofluorescence by using the PLN antibody ID11 and an FITC-conjugate secondary antibody (Jackson ImmunoResearch). Images were collected by using a Leica DM IRBE inverted microscope equipped with a Leica TCS SP laser scanning confocal system. Images were assembled by using Adobe Systems (San Jose, CA) PHOTOSHOP 7.

Biochemical Assays. Quantitative immunoblotting of cardiac homogenates was used to determine the levels of PLN and SR Ca^{2+} handling proteins, as described in ref. 28. Oxalate-supported Ca^{2+} uptake in cardiac homogenates was measured in the presence or absence of PKA phosphorylation by a modified Millipore filtration technique (16).

Histology. Right ventricular biopsy samples were collected from patients during heart catheterization, fixed overnight in 10% formalin, buffered with PBS, dehydrated in 70% ethanol, and transferred to xylene and then into paraffin. Paraffin-embedded heart samples were sectioned at 4 μm and stained with Masson's Trichrome.

Statistics. Data are presented as mean ± SEs. Comparisons were by Student's *t* test as appropriate. A P value of <0.05 was considered statistically significant.

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