Dilated cardiomyopathy in transgenic mice expressing a mutant A subunit of protein phosphatase 2A

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Brewis, Neil, Kim Ohst, Katherine Fields, Antonio Rapacciulo, Danny Chou, Colin Bloor, Wolfgang Dillmann, Howard Rockman, and Gernot Walter. Dilated cardiomyopathy in transgenic mice expressing a mutant A subunit of protein phosphatase 2A. Am J Physiol Heart Circ Physiol 279: H1307–H1318, 2000.—The protein phosphatase 2A (PP2A) holoenzyme consists of a catalytic subunit, C, and two regulatory subunits, A and B. The PP2A core enzyme is composed of subunits A and C. Both the holoenzyme and the core enzyme are similarly abundant in heart tissue. Transgenic mice were generated expressing high levels of a dominant negative mutant of the A subunit (ΔA5) in the heart, skeletal muscle, and smooth muscle that competes with the endogenous A subunit for binding the C subunit but does not bind B subunits. We found that the ratio of core enzyme to holoenzyme was increased in ΔA5-expressing hearts. Importantly, already at day 1 after birth, ΔA5-transgenic mice had an increased heart weight-to-body weight ratio that persisted throughout life. Echocardiographic analysis of ΔA5-transgenic hearts revealed increased end-diastolic and end-systolic dimensions and decreased fractional shortening. In addition, the thickness of the septum and of the left ventricular posterior wall was significantly reduced. On the basis of these findings, we consider the heart phenotype of ΔA5-transgenic mice to be a form of dilated cardiomyopathy that frequently leads to premature death.

protein phosphatase 2A holoenzyme; protein phosphatase 2A core enzyme; heart weight-to-body weight ratio; muscle-specific gene expression

THE FUNCTIONS OF SERINE/THREONINE KINASES in cardiac gene expression and hypertrophy have been studied extensively, in particular those of protein kinase C, protein kinase A, and members of the mitogen-activated protein (MAP) kinase pathways (37). However, the functions of serine/threonine phosphatases in these processes are less well understood. Recently, calcineurin (protein phosphatase 2B) has been shown to play a role in the induction of cardiac gene expression and hypertrophy through a mechanism that involves dephosphorylation of the transcription factor NF-AT3 (nuclear factor of activated T cells-3) (24, 40). Other serine/threonine phosphatases are also involved in cardiac function. This became apparent when it was discovered that okadaic acid, an inhibitor of protein phosphatase 1, protein phosphatase 2A (PP2A), and related serine/threonine phosphatases, induces a shortening in contraction time of isolated cardiac muscle (13). The effects of okadaic acid are similar to those induced by β-adrenergic receptor agonists. It has been suggested that they are mediated by increased phosphorylation of the cardiac-specific proteins phospholamban and troponin I (27, 39). Because okadaic acid inhibits several serine/threonine phosphatases (6, 7), it is difficult to correlate the biological effects of okadaic acid with the inhibition of a specific phosphatase. However, there is biochemical evidence that protein phosphatase 1 is mainly responsible for the dephosphorylation of phospholamban (18), whereas dephosphorylation of troponin I might be carried out by PP2A (25). PP2A is also involved in regulating ATP-sensitive potassium channels, as demonstrated by single channel recordings on inside-out membrane patches from ventricular myocytes (16). Furthermore, PP2A may modulate MAP kinase pathways, which are activated in response to treatment with phorbol ester, endothelin-1, or phenylephrine (3, 30, 43). As shown for cardiomyocytes (4) and other systems (38, 45), PP2A can either enhance or reduce signaling through the MAP kinase pathway.

PP2A exists in cells as two forms: the heterodimeric core enzyme, composed of the catalytic subunit C and the regulatory subunit A, and the heterotrimeric holoenzyme, composed of the core enzyme and a second regulatory subunit, subunit B. The A subunit polypeptide consists of 15 nonidentical repeats. The B subunit binds to repeats 1–10, and the C subunit binds to repeats 11–15 of the A subunit (Fig. 1, see Ref. 26 for review). The core enzymes and holoenzymes are ex-

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pressed in approximately equal quantities (15), and they differ in substrate specificity (26). B subunits fall into three families, designated B (10, 20, 29, 46), B’ (or B56) (8, 22, 23, 41, 42), and B’’ (5), which are unrelated by primary sequence. The B’ family consists of numerous isoforms and splice variants that determine the specificity and intracellular localization of the holoenzyme (22, 42). In addition, there is tissue-specific expression of B’ subunits (8, 22, 23, 42). Importantly, B56α- and B56γ-subunits are preferentially expressed in heart and skeletal muscle, suggesting that they direct the holoenzyme to a specific subcellular location in these tissues and/or toward a specific substrate.

We have previously demonstrated that expression of the Δ5 subunit, a NH2-terminal mutant of the A subunit of PP2A with a deletion of repeat 5 which binds the catalytic C subunit but none of the regulatory B subunits (Fig. 1), causes an increase in core enzymes and a decrease in holoenzymes in tissue culture cells (32). As a result of this change in the core enzyme-to-holoenzyme ratio, the Tat protein-stimulated transduction of the human immunodeficiency virus-1 (HIV-1) long-terminal repeat and HIV-1 virus production are strongly inhibited. On the basis of this result, we asked whether expression of the mutant AΔ5 subunit in animals would alter PP2A activity and provide information about the function of PP2A. Here we show that mice expressing the mutant AΔ5 subunit under the combined control of the chicken β-actin promoter and the cytomegalovirus (CMV) enhancer, yielding high gene expression in muscle tissue, have an increased heart weight-to-body weight ratio early in life and develop a form of dilated cardiomyopathy.

MATERIALS AND METHODS

DNA construction. PCR was used to engineer a sequence encoding the nine amino acid epitope tag EEKEYMPME (EE) at the 3’ end of the human A subunit-cDNA open reading frame (33). The construct was subcloned into the ecdysone-responsive expression plasmid pIND (Invitrogen) using EcoRI and XhoI. This vector was termed pINDΔ5EE. To create an EcoRI site at the end of the Δ5 sequence, the vector pINDΔ5EE was digested with XhoI, treated with Klenow, and blunt-end ligated to an EcoR I linker. The EcoR I-EcoR I insert of Δ5EE was ligated into the EcoR I site of the pCAGGS vector (28) resulting in pCAGGSΔ5EE (Fig. 2). The pCAGGS vector containing the CMV enhancer, the chicken β-actin promoter, and the rabbit β-globin polyadenylation signal was used because this vector yields high gene expression in muscle tissue (19).

Generating transgenic mice. The vector pCAGGSΔ5EE was cut with Ssp I and BamHI. The 3.7-kb fragment (shown in Fig. 2) was purified from the gel using a Qiagen kit and then concentrated with an Elutip (Schleicher & Schuell). The DNA was ethanol precipitated and dissolved in 5 mM Tris (pH 7.5) and 0.1 mM EDTA at a concentration of 2.0 μg/ml.

To develop transgenic mice, pronuclei of fertilized eggs from CB6 F1 mice were microinjected with the 3.7-kb fragment at a concentration of 2.0 μg/ml. Surviving embryos were transferred into the oviducts of pseudopregnant ICR mice. Litters were delivered after 19–20 days of gestation. After the mice were 3 wk old, tail clippings were taken, and the DNA was extracted and digested with ApaI. The digested DNA (15 μg) was loaded on a 0.7% agarose gel in Tris-borate-EDTA and transferred to Hybond-N nylon membrane (Amersham). The blot was then hybridized with a 32P-labeled probe. The probe used was the entire injected fragment from Ssp I to BamHI. Of the 40 offspring born, 8 had integrated the transgene and were considered the founders. The founders were bred with mice of the same strain, and the resultant litters were also analyzed by Southern blotting as described above. Transgenic lines were established with the two founders that expressed the protein in their tissue lines I1 and I4) in addition to being positive by Southern blotting. The approximate transgene copy numbers in the I1 and I4 lines, as determined by Southern blots, were 7 and 25, respectively (data not shown). Heterozygous and homozygous I1- and I4-transgenic mice were distinguished by Southern blotting.

Analyzing mouse organs for expression of Δ5EE protein. The mice were euthanized by CO2 inhalation for 30 s. The different tissues were removed, frozen on dry ice, and weighed. Protein was extracted by grinding the defrosting tissue with a pestle and then grinding with 10 μl of SDS sample buffer [2% SDS, 5% β-mercaptoethanol, 5% glycerol, 10 mM Tris (pH 6.8), 10 mM dithiothreitol, and 0.025% bromophenol blue] per milligram of tissue. An 18-gauge needle was used to further dissociate the tissue, and the lysate was then boiled for 5 min, diluted, and boiled again. For protein determination, aliquots of solubilized tissues were precipitated with trichloroacetic acid. Protein (10–20 μg) was analyzed on a 10% acrylamide gel and transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore). Western blotting was done using the 6G3 antibody (15).

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Heart weight-to-body weight ratios and histology. Mice were euthanized using CO₂ inhalation as described in Analyzing mouse organs for expression of AA516ER protein, and the mice were weighed. Their hearts were removed, blotted on filter paper, and weighed. For histology, hearts were fixed in 10% buffered Formalin for at least 48 h, cut longitudinally or transversely, dehydrated, and infiltrated with paraffin. Sections (5 μm) were cut after embedding the hearts in paraffin. Sections were floated onto slides and dried overnight. The sections were stained with hematoxylin and eosin or with Masson trichrome stain.

Immunoprecipitation of PP2A. Mice were euthanized by 30 s of CO₂ inhalation, and their hearts were removed and weighed. Sections of 100–150 mg of heart were frozen in liquid nitrogen and ground with a mortar and pestle and lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 3 mM MgCl₂, 1 mM Pefabloc SC (Boehringer), 50 μM leupeptin (Sigma), 0.2 mg/ml soybean trypsin inhibitor (Calbiochem), 1 μg/ml aprotinin, and 1 mM dithiothreitol). The lysate was spun at 16,000 × g at 4 °C, and the supernatant protein concentration was determined with Pierce’s Coomassie Plus protein assay. Over 95% of PP2A was solubilized under these conditions, as determined by Western blotting. Addition of 0.5% Triton X-100 to the lysis buffer did not increase the amount of PP2A in the supernatant. For immunoprecipitation, 300 μg of lysate was incubated for 2.0 h at 4 °C with 360 μg of anti-EE, 300 μg of 6F9, or 200 μg of 5H4 monoclonal antibodies coupled to Gamma Bind Plus sepharose beads (Pharmacia) (15). After centrifugation, we incubated the supernatant with the next antibody. The immunoprecipitates were washed three times with 1 ml of wash buffer (above lysis buffer with 0.5% Triton X-100 without Pefabloc, aprotinin, and soybean trypsin inhibitor) and boiled in SDS sample buffer. One-half of the immunoprecipitate was loaded on a 10% high-bis gel (19 × 16 cm). The protein was transferred to the polyvinylidene difluoride membrane, and antibody detection was carried out with rat monoclonal anti-A subunit (6G3), rabbit anti-Bo-subunit, and mouse monoclonal anti-C subunit antibodies, as described (32).

Culturing and immunostaining of cardiomyocytes and heart fibroblasts. For the preparation of fibroblasts, hearts from 4-mo-old animals were rinsed in cold PBS, the atria and aorta were removed, and the ventricles were cut into small pieces and placed into 37°C buffer containing 18.3 mM HEPES, 0.8 mM MgSO₄, 1.0 mM NaH₂PO₄, 116.4 mM NaCl, 5.5 mM d-glucose, and 5.4 mM KCl. The hearts were digested six times for 20 min at 37°C with 100 U/ml collagenase type 2 (Worthington Biochemicals) and 0.6 mg/ml pancreatin (GIBCO) in the same buffer (5.6 ml/heart). The cells dissociated from the last five digestions were pooled and plated in DMEM, 10% FCS, and 1% penicillin-streptomycin solution. Cells were transferred once and plated on tissue culture plates or coverslips. Cells were fixed for 7 min with 4% paraformaldehyde and permeabilized with 1% NP-40/PBS for 1 min. The cells were blocked for 30 min in 1% BSA/PBS, incubated for 1 h with anti-glutamic acid-glutamatic acid (EE) tag antibody (50 μg/ml of 1% BSA/PBS), washed with PBS, incubated for 1 h with anti-mouse horseradish peroxidase (1:250 Jackson Immuno Research), washed with PBS, and incubated with diaminobenzidine substrate (Vector). Cardiomyocytes were prepared from five 1-day-old mouse hearts, as described above except that 0.3 ml of enzyme solution (0.8 mg/ml pancreatin and 0.55 mg/ml collagenase type 2) was used per heart. The myoblasts were plated into media containing 13.9 g/l DMEM, 3.02 g/l medium 199, 3.4 g/l sodium bicarbonate, 10% horse serum, and 5% fetal calf serum (11, 14). The reason why cardiomyocytes were prepared from 1- to 5-day-old mice and not from adults was because adult cardiomyocytes are difficult to isolate and to maintain in culture for immunostaining.

Phosphatase assays. Phosphorylase A and Rb peptide were used as substrates and labeled as described (32). Heart extracts were made as described for Immunoprecipitation of PP2A, and the protein contents of the heart extracts were immediately determined and adjusted to be equal. Extracts containing equal amounts of protein were diluted 1:40 in 0.1% BSA, 50 mM Tris-HCl (pH 7.5), 0.1 mM EGTA, and 0.1% 2-mercaptoethanol. Extracts (5 μl) containing 0.8 μg of total protein were assayed in the presence of protein phosphatase 1 inhibitor 2 as described (32). The reaction time was 10 min. The assay was linear for at least 10 min and directly dependent on the amount of extract used. The amounts of catalytic C subunit in the wild-type and I1 heart extracts used for the assay were identical as determined by Western blotting.

Transcardiac echocardiography. Echocardiography was performed in intact anesthetized mice (2.5% Avertin, 14 μl/g ip) using an Apogee CX echocardiograph (Interspec-ATL, Bothell, WA) as previously described (31). Wild-type and homozygous I1-transgenic mice were studied at age 4–7 mo. The average heart weight-to-body weight ratios of the I1-transgenic and wild-type mice were 6.7 and 5.0, respectively (data not shown). The operator who performed and measured the echocardiograms was blinded to the genotype of the animals. The following parameters were measured: left ventricular end-diastolic dimension (LVEDD), left-ventricular systolic dimension (LVESD), percent fractional shortening [calculated as (LVEDD – LVESD)/LVEDD × 100], septal wall thickness, posterior wall thickness, and heart rate.

Arterial blood pressure. Mice were anesthetized with ketamine (100 mg/kg) and xylazine (5 mg/kg ip). A polyethylene-50 flame-stretched, fluid-filled catheter was introduced through a cervical incision into the carotid artery and attached to a modified P50 Statham transducer. Systolic and diastolic blood pressure were recorded.

Northern blot analysis. Two- to three-month-old wild-type and homozygous I1-transgenic mice were euthanized for 30 s with CO₂ inhalation. The hearts were removed, frozen quickly in liquid nitrogen, and kept on dry ice until homogenized. To avoid RNA degradation, the heart weight was not determined. Total RNA was isolated using the RNeasy Midi Kit (Qiagen) following the manufacturer’s instructions. The RNA concentration was determined by measuring optical density at 260 nm. RNA (10 μg) was loaded onto a 1% agarose gel under denaturing conditions, as described by Qiagen. The RNA was transferred onto a Magna Graph nylon membrane (MSI) overnight with 20× saline-sodium citrate buffer and hybridized with probes. Myosin heavy chain-α (α-MHC) and myosin heavy chain-β (β-MHC) oligonucleotides were end labeled with deoxynucleotide transferase. Hybridization was carried out overnight at 65°C. The mouse α-MHC oligonucleotide had the sequence AAGTGTATTTTATTGTGGTTG- GCCACAGCGAGGTTCT derived from the 3′ untranslated region that does not show homology to the β-form. The oligonucleotide from rat β-MHC was purchased from Calbiochem (cat. no. ON366, sequence unknown). Atrial natriuretic factor, calsequestrin, sarco(endo)plasmic reticulum Ca²⁺-ATPase, phospholamban, and the ryanodine receptor RNAs were labeled with Amersham’s multiprime kit. Hybridization was carried out overnight at 42°C, as described by He et al. (9).
RESULTS

Expression of AΔΔ5EE subunit in muscle tissue of transgenic mice. As previously described (11), transgene expression controlled by the CMV enhancer and the chicken β-actin promoter combined, as in the vector pCAGGS (28), is very high in muscle tissue and low or undetectable in other tissues. We chose this promoter because the endogenous level of the A subunit of PP2A is very high (~0.1% of total protein) in all cells and tissues that have been studied (15, 17, 36). Therefore, a dominant negative mutant of the A subunit, such as the AΔΔ5 subunit (35), can only compete with the endogenous A subunit if the mutant subunit is expressed as high or higher than the endogenous protein. We found two transgenic founder mice that expressed a high amount of the AΔΔ5EE protein in the heart, skeletal muscle, and smooth muscle (stomach). These mice were bred to generate the lines I1 and I4. As shown in Fig. 3, the approximate ratios of the AΔΔ5EE subunit to the endogenous A subunit for the I1 line are 4:1 for heart, 8:1 for skeletal muscle, and 1:1 for stomach. The approximate ratios for the I4 line are 1:1 for heart, 1:2 for skeletal muscle, and 1:8 for stomach. Note that the amounts of the A subunit and the AΔΔ5EE subunit cannot be compared between lanes because unequal amounts of protein were loaded. In addition, to optimize visualization and quantitation of the ratio of the wild-type A subunit to the AΔΔ5EE subunit, different exposure times were chosen for different lanes. Importantly, expression of the AΔΔ5EE subunit had no significant effect on the level of the endogenous A subunit in the heart (data not shown). Only trace amounts of the AΔΔ5EE subunit were found in the kidney, liver, and brain (data not shown). During the first year after these two mouse lines were established, the expression of the AΔΔ5EE subunit dropped. In hearts from I1-transgenic mice, which were used for the majority of our experiments, the ratio of the AΔΔ5EE subunit to the endogenous A subunit reached a level of 2:1. The AΔΔ5EE and endogenous A subunits were evenly distributed between the ventricles and atria of I1- and I4-transgenic mouse hearts (data not shown). Importantly, the AΔΔ5EE subunit was only expressed in cardiomyocytes but not in heart fibroblasts, as shown in Fig. 4.

Fig. 3. Expression of mutant AΔΔ5EE protein in transgenic mice. Western blot of wild-type A subunit and mutant AΔΔ5EE subunit in the heart (He), skeletal muscle (Sk), and stomach (St) from two independent lines of transgenic mice, I1 and I4. Monoclonal antibody 6G3 was used as described in MATERIALS AND METHODS.

Transgenic expression of AΔΔ5EE subunit causes increase in heart weight-to-body weight ratio. We discovered that the hearts of transgenic animals were significantly larger than normal hearts and that there was no sign of interstitial fibrosis (Fig. 5). Furthermore, the heart weights and heart weight-to-body weight ratios of the I1-transgenic mice were already increased on day 1 after birth and continued to be high throughout life. These data are presented in Figs. 6 and 7, showing the values of individual animals in the different age groups (plus signs, closed triangles, and open squares...
for I1-transgenic, I4-transgenic, and wild-type mice, respectively), and in Table 1, presenting the average values. Similar results to the I1-transgenic mice were obtained for the I4-transgenic mice, although the increase in heart weight and heart weight-to-body weight ratio was generally smaller than for I1-transgenic mice. This latter finding is consistent with the observation that I4-transgenic mice expressed less AΔ5EE subunit than I1-transgenic mice. Heterozygous I1- and I4-transgenic mice had lower heart weight-to-body weight ratios than the corresponding homozygous mice (as shown in Fig. 8), further indicating that the heart weight and heart weight-to-body weight ratio correlate with the level of AΔ5EE subunit expression. These results strongly suggest that the AΔ5EE subunit is the cause for the increased heart weight of the AΔ5EE-transgenic mice. There was no apparent sex difference in heart weight and heart weight-to-body weight ratio. Interestingly, starting at 3–4 mo of age, I1-transgenic mice with extremely large hearts and heart weight-to-body weight ratios (between two- and fivefold above the norm) were observed. These mice represented ~25% of all animals in the 7- to 12-mo age group. They were characterized by general weakness, slow movements, and increased respiratory rate, and they died within a few weeks after showing these symptoms. A summary of all data is presented in Fig. 7 and Table 1. Note that the P values for the heart weights and the heart weight-to-body weight ratios of I1-transgenic mice in the 3- to 4-, 5- to 6-, and 7- to 12-mo-old age groups are skewed by the mice with extremely high ratios. For example, the increased ratio of 3- to 4-mo-old mice appears statistically nonsignificant due to the presence of two animals with extreme ratios, although the increase is highly significant, as illustrated in Fig. 6.

I1-transgenic mice have a general tendency toward a lower body weight compared with wild-type mice of the same age group. This was most pronounced at 7–12 mo of age, when some control mice became very large (while maintaining a constant heart weight-to-body weight ratio), whereas I1-transgenic mice never surpassed a weight of 30 g (Fig. 6). However, despite the decrease in body weight, it is clear that the increase in heart weight is by far the predominant factor for the increased heart weight-to-body weight ratio of AΔ5EE-transgenic mice. An exception was 1- to 2-mo-old AΔ5EE-transgenic mice whose hearts were not significantly larger than control hearts, whereas their body weights were reduced by 19%, and this gave rise to an increase in the heart weight-to-body weight ratio. These results suggest that between 10 days and 1–2 mo, control mice grew slightly more than transgenic mice. During the same period, transgenic hearts, which are significantly larger than the controls at 1 and 10 days, grew slightly less than the controls. After 1–2 mo, the development of progressive cardiomyopathy began and eventually led to death.

Heart function of AΔ5EE-transgenic mice is impaired, and blood pressure is unchanged. Echocardiography was used to evaluate cardiac chamber size and function in vivo in wild-type and homozygous AΔ5EE-transgenic mice. As shown in Table 2, LVEDDs for I1-transgenic mice were significantly greater compared with wildtype mice (4.5 vs. 3.5 mm, respectively), indicating chamber enlargement of the I1-transgenic mouse hearts. Furthermore, a significant increase in LVESD was found in the I1-transgenic mouse hearts compared with wild-type mouse hearts (3.5 vs. 2.2 mm, respectively). Calculated fractional shortening (end-diastolic dimension – end-systolic dimension × 100%/end-diastolic dimension), a measure of systolic function, indicates depressed cardiac performance in vivo in the I1-transgenic mice (21%) compared with wild-type mice (36.5%). Another important result from the echocardiographic analysis was that the thickness of the septum and of the left ventricular posterior wall was significantly reduced in transgenic mice. Taken together, these data of chamber enlargement, reduced systolic function, and wall thinning are consistent with a phenotype of dilated cardiomyopathy.

The results from echocardiography appear to contradict the histological data (Fig. 5) showing a mild increase in wall thickness and normal chamber size in AΔ5EE-transgenic mice. This difference between echocardiography and histology is due to a fixation artifact. Because the purpose of the histology was only to look for fibrosis, the hearts were not arrested in diastole by

![Fig. 5. Increase in heart size of AΔ5EE-transgenic mice and lack of interstitial fibrosis. Hearts from a normal (A) and a AΔ5EE-transgenic mouse (B) were sectioned tranversely and stained with Masson trichrome stain. The scale is 2 mm.](http://ajpheart.physiology.org/doi/10.22033/ajp/2017/8/AJ/411)
Fig. 6. Increase in heart weight of AΔ5E-transgenic mice on postnatal day 1 (A) and throughout life. Body weights and heart weights of WT (○), AΔ5E I1-trangenic (■), and AΔ5E I4-transgenic (▲) mice were determined at different ages [10-day-old mice (B), 1- to 2-mo-old mice (C), 3- to 4-mo-old mice (D), 5- to 6-mo old mice (E), and 7- to 12-mo-old mice (F)]. G and H: comparison of body weight to heart weight in male (G) and female (H) WT, I1-, and I4-transgenic mice. I: comparison of body weight to heart weight in all of the mice. This figure illustrates the general increase in heart weight of transgenic compared with WT animals and the appearance of animals with extremely large hearts beginning at 3–4 mo of age. It also illustrates the general tendency of transgenic hearts toward a slightly decreased body weight compared with WT hearts.
Fig. 7. Increase in heart weight-to-body weight ratio of AΔ5EE-transgenic mice. The heart weight-to-body weight ratio was plotted for the different age groups. This figure illustrates the general increase in the ratio for transgenic animals and the extremely high ratio for a fraction of older animals. Also note that the ratio is constant for WT mice at all ages. □, WT; †, I1-transgenic mice; ▲, I4-transgenic mice.

Fig. 8. Homozygous AΔ5EE-transgenic mice have a larger heart weight-to-body weight ratio than heterozygous mice. The P values (means ± SD) for homozygous (Homo) I1- and I4- and heterozygous (Hetero) I1- and I4-transgenic mice compared with WT mice are 0.0005, 0.0054, 0.0011, and 0.24. The P value for I1 Homo-compared with I4 Homo-transgenic mice is 0.0178; for I1 Hetero compared with I4 Hetero, 0.0077; for I1 Homo compared with I1 Hetero, 0.0062; and for I4 Homo compared with I4 Hetero, 0.0235.

Table 1. Increase in heart weight and heart weight-to-body weight ratio in AΔ5-transgenic mice

<table>
<thead>
<tr>
<th>Age</th>
<th>Strain*</th>
<th>Body Weight, g†</th>
<th>P value</th>
<th>Heart Weight, mg†</th>
<th>P value</th>
<th>Heart Weight: Body Weight ratio, mg/g†</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 day</td>
<td>WT(20)</td>
<td>1.4 ± 0.1</td>
<td>2.8 × 10⁻³</td>
<td>7.0 ± 1.5</td>
<td>2.4 × 10⁻⁴</td>
<td>5.1 ± 0.8</td>
<td>2.6 × 10⁻⁹</td>
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<tr>
<td></td>
<td>II(27)</td>
<td>1.2 ± 0.1(92)</td>
<td></td>
<td>8.4 ± 1.1(121)</td>
<td>4.8 × 10⁻⁴</td>
<td>6.8 ± 0.8(133)</td>
<td>1.1 × 10⁻⁶</td>
</tr>
<tr>
<td>10 days</td>
<td>WT(5)</td>
<td>5.1 ± 0.3</td>
<td></td>
<td>24.6 ± 2.6</td>
<td></td>
<td>4.8 ± 0.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>II(5)</td>
<td>5.5 ± 0.5(107)</td>
<td></td>
<td>44.9 ± 3.6(182)</td>
<td>6.9 × 10⁻⁶</td>
<td>8.2 ± 0.5(171)</td>
<td>1.1 × 10⁻⁶</td>
</tr>
<tr>
<td></td>
<td>I(14)</td>
<td>5.9 ± 0.3(116)</td>
<td>3.5 × 10⁻³</td>
<td>42.3 ± 3.7(127)</td>
<td>2.3 × 10⁻⁵</td>
<td>7.1 ± 0.3(148)</td>
<td>2.9 × 10⁻⁵</td>
</tr>
<tr>
<td>1–2 mo old</td>
<td>WT(8)</td>
<td>23.8 ± 1.4</td>
<td></td>
<td>124.5 ± 9.6</td>
<td></td>
<td>5.2 ± 0.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>II(9)</td>
<td>19.2 ± 1.7(81)</td>
<td>3.4 × 10⁻⁵</td>
<td>120.0 ± 9.6(96)</td>
<td>7.9 × 10⁻³</td>
<td>6.3 ± 0.4(119)</td>
<td>2.1 × 10⁻⁵</td>
</tr>
<tr>
<td></td>
<td>I(14)</td>
<td>17.8 ± 1.1(75)</td>
<td>1.1 × 10⁻⁴</td>
<td>103.4 ± 7.3(83)</td>
<td>7.9 × 10⁻³</td>
<td>5.8 ± 0.2(111)</td>
<td>2.4 × 10⁻²</td>
</tr>
<tr>
<td>3–4 mo old</td>
<td>WT(6)</td>
<td>27.0 ± 2.6</td>
<td></td>
<td>129.0 ± 13.9</td>
<td></td>
<td>4.8 ± 0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>II(22)</td>
<td>22.2 ± 3.3(82)</td>
<td>3.3 × 10⁻³</td>
<td>167.8 ± 59.7(130)</td>
<td>NS</td>
<td>7.9 ± 4.0(165)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>I(14)</td>
<td>26.6 ± 2.1(99)</td>
<td>NS</td>
<td>173.7 ± 15.1(135)</td>
<td>3.1 × 10⁻³</td>
<td>6.5 ± 0.4(137)</td>
<td>4.8 × 10⁻⁵</td>
</tr>
<tr>
<td>5–6 mo old</td>
<td>WT(16)</td>
<td>27.4 ± 4.9</td>
<td></td>
<td>130.7 ± 28.9</td>
<td></td>
<td>4.8 ± 0.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>II(18)</td>
<td>24.5 ± 4.0(89)</td>
<td>NS</td>
<td>182.0 ± 33.2(139)</td>
<td>3.8 × 10⁻⁵</td>
<td>7.5 ± 1.8(159)</td>
<td>9.5 × 10⁻⁷</td>
</tr>
<tr>
<td></td>
<td>I(14)</td>
<td>26.0 ± 2.2(95)</td>
<td>NS</td>
<td>168.3 ± 6.8(129)</td>
<td>4.2 × 10⁻²</td>
<td>6.5 ± 0.3(137)</td>
<td>3.4 × 10⁻⁶</td>
</tr>
<tr>
<td>7–12 mo old</td>
<td>WT(5)</td>
<td>35.6 ± 8.5</td>
<td></td>
<td>158.8 ± 44.6</td>
<td></td>
<td>4.4 ± 0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>II(37)</td>
<td>21.7 ± 3.4(61)</td>
<td>2.9 × 10⁻⁸</td>
<td>192.3 ± 56.6(121)</td>
<td>NS</td>
<td>9.2 ± 3.7(207)</td>
<td>6.5 × 10⁻³</td>
</tr>
</tbody>
</table>

*Numbers in parentheses indicate the number of animals used; †numbers in parentheses indicate the percent body weight, heart weight, or heart weight-to-body weight ratio compared with the wild-type (WT); means ± SD are given, with the P value for the Student t-test comparing each transgenic group to the respective control; NS, not significant. AΔ5-transgenic mice were mice with a deletion of repeat 5 in the A subunit of protein phosphatase 2A; I1 and I4 are the two lines of transgenic mice generated.
**Table 2. Left ventricle function is impaired in AΔ5E-transgenic mice**

<table>
<thead>
<tr>
<th>Strain</th>
<th>LVEDD, mm</th>
<th>LVEDD, mm</th>
<th>FS, %</th>
<th>SWth,m</th>
<th>PWth,m</th>
<th>HR, beats/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>2.2 ± 0.66</td>
<td>3.5 ± 0.50</td>
<td>36.3 ± 10.1</td>
<td>0.98 ± 0.18</td>
<td>0.95 ± 0.18</td>
<td>428 ± 60</td>
</tr>
<tr>
<td>I1</td>
<td>3.5 ± 0.60</td>
<td>4.5 ± 0.43</td>
<td>21.2 ± 7.3</td>
<td>0.66 ± 0.14</td>
<td>0.65 ± 0.09</td>
<td>377 ± 56</td>
</tr>
</tbody>
</table>

All values (means ± SD) are from four or more mice. LVEDD, left ventricle end-diastolic dimension; LVEDD, left ventricle end-systolic dimension; FS, fractional shortening; SWth,m, septal wall thickness; PWth,m, posterior wall thickness, HR, heart rate. P < 0.05 for all values except HR, transgenic (I1) vs. WT mice.

As shown in Fig. 9 (lanes 1 and 3), more C subunit was bound to the AΔ5EE subunit (precipitated with anti-EE antibody) than to the endogenous A subunit (precipitated with 5H4), demonstrating that the AΔ5EE subunit effectively competes with the wild-type A subunit for binding of the C subunit. Importantly, there was no B subunit bound to the AΔ5EE-C (lane 1) and A-C core enzymes (lane 3). To determine whether the ratio of core enzyme (AΔ5EE-C plus A-C subunits) to holoenzyme was changed in transgenic heart tissue, heart extracts were first precipitated with anti-EE (lanes 1 and 2) and 5H4 (lanes 3 and 4) antibodies to remove all core enzyme, followed by precipitation of holoenzyme with the 6F9 antibody (lanes 5 and 6). As a control, extracts from wild-type hearts were precipitated with the 5H4 antibody (lanes 7 and 8) followed by the 6F9 antibody (lanes 9 and 10). In AΔ5EE-transgenic heart extracts, there was more C subunit in the core enzyme [AΔ5EE-C (lanes 1 and 2) and A-C (lanes 3 and 4) core enzymes combined] than in the holoenzyme (lanes 5 and 6). In contrast, in extracts from wild-type hearts, there was more holoenzyme (lanes 9 and 10) than core enzyme (lanes 7 and 8), on the basis of the amount of C subunit. A similar result was obtained when the I1-transgenic heart extract, first precipitated with the 5H4 antibody (lanes 11 and 12) followed by the 6F9 antibody (lanes 13 and 14), was compared with wild-type heart extract, which was precipitated with the 5H4 (lanes 7 and 8) and 6F9 antibodies (lanes 9 and 10). These experiments demonstrate that the core enzyme and holoenzyme are similarly abundant in the heart and that AΔ5EE subunit expression increases the level of core enzyme and decreases the level of holoenzyme, as expected. Because precise quantitation of Western blots is difficult (in particular when using the enhanced chemiluminescence system), we also carried out phosphatase assays (see below).

If the AΔ5EE subunit can only bind the C subunit, one would expect that the 5H4 antibody removes all the AΔ5EE-C subunits from cell extracts. However, this was not the case. As shown in Fig. 9 (lanes 13 and 14), the 6F9 antibody precipitated ~30% of the AΔ5EE subunit despite exhaustive prior precipitation with the 5H4 antibody (lanes 11 and 12). This indicates that, in a fraction of the AΔ5EE subunits, the 5H4 epitope was covered up by another protein. This protein could not have been the Ba-subunit because there was no Ba-subunit in precipitates of the AΔ5EE subunit with the anti-EE antibody. It also could not have been one of the other known forms of B subunit (B’ or B’’) because none of these binds to the AΔ5EE subunit, as shown.

**ÁΔ5EE subunit binds C subunit but not B subunit: similar abundance of core enzyme and holoenzyme in hearts.** The underlying assumptions on which the present work is based are that 1) the ÁΔ5EE mutant subunit competes with the endogenous A subunit for binding of C subunit, 2) the ÁΔ5EE subunit does not bind B subunits, 3) the ratio of core enzyme to holoenzyme increases in ÁΔ5EE-expressing tissue, and 4) the phosphatase activity changes due to the increase in the core enzyme-to-holoenzyme ratio. To test these assumptions, we carried out immunoprecipitations with normal and transgenic heart extracts, using monoclonal antibodies against the EE tag (anti-EE) and monoclonal antibodies 5H4 and 6F9 directed against the wild-type A subunit. These latter antibodies distinguish between the core enzyme and holoenzyme. Whereas 5H4 recognizes the core enzyme but not the holoenzyme, 6F9 precipitates both forms (Fig. 1) (15). To achieve quantitative precipitations, two consecutive precipitations were carried out with each antibody. The amounts of the A, B, and C subunits in immunoprecipitates were determined by Western blotting with antibodies against the individual subunits as described in MATERIALS AND METHODS.
previously (33, 34). Alternatively, there is a remote possibility that the 5H4 epitope was denatured and therefore not recognized by the 5H4 antibodies in a fraction of the AΔ5βD subunits. However, because the 6P9 epitope was intact in AΔ5βD subunits, the latter possibility seems less likely. Reduction of holoenzyme activity in transgenic hearts. To determine whether a decrease in holoenzyme activity and an increase in core enzyme activity in fact occurs as a result of AΔ5βD subunit expression, we used phosphorylase a, phosphorylated by phosphorylase kinase, and Rb peptide, phosphorylated by cdk1 kinase/cyclin B, as substrates. The holoenzyme is 100-fold more active than the core enzyme in dephosphorylating Rb peptide (1), whereas the core enzyme activity toward phosphorylase a is ~2.5-fold higher than that of the holoenzyme (44). As shown in Table 3, the phosphatase activity toward the Rb peptide was reduced by 29% in extracts from I1-transgenic mouse hearts compared with extracts from control hearts, whereas the activity toward phosphorylase a increased by 24%. This change in activity is reflected in a drop in the ratio of the Rb peptide phosphatase activity to the phosphorylase a phosphatase activity from 3.7 to 2.1. Because the activity toward the Rb peptide comes almost exclusively from the holoenzyme, these results indicate a 29% drop in the level of the holoenzyme; and, because holoenzyme represents approximately two-thirds (66%) of the total PP2A in the wild-type heart extract (Fig. 9), a 29% drop would result in a final amount of 42% holoenzyme in I1-transgenic mouse heart extract. Furthermore, because the decrease in holoenzyme leads to a corresponding increase in the core enzyme, the level of core enzyme is expected to rise from ~33% in wild-type heart extract (Fig. 9) to 58% in I1-transgenic mouse heart extract. Because the core enzyme is 2.5 times more active than the holoenzyme, one would expect a 25% increase toward phosphorylase a, which is very close to the experimental value of 24%. Thus the results obtained from phosphatase assays confirm those obtained by immunoprecipitation.

Increased β-MHC gene expression in AΔ5-transgenic hearts. A characteristic of cardiac hypertrophy is the reactivation of genes whose expression is normally restricted to fetal development, e.g., the β-MHC and the atrial natriuretic factor gene. On the other hand, the transcription of genes encoding sarco(endo)plasmic reticulum Ca2+-ATPase, phospholamban, and α-MHC has been reported to be suppressed in hypertrophic hearts (see Refs. 2 and 14 for review). As demonstrated by Northern blotting, the level of β-MHC transcripts in homozygous AΔ5-transgenic hearts was significantly increased, whereas no change in atrial natriuretic factor, sarco(endo)plasmic reticulum Ca2+-ATPase, phospholamban, and α-MHC expression was observed (Fig. 10A). The increase in β-MHC transcripts in AΔ5-transgenic hearts was fourfold compared with control animals (Fig. 10B). Elevated levels of β-MHC were also observed in transgenic mice that developed cardiac hypertrophy and ventricular dilation due to overexpression of the calcium-dependent phosphatase calciineurin in the heart (24). We also found no change in calsequestrin and ryanodine receptor gene expression.

DISCUSSION

In this report, we have shown that transgenic expression of a dominant negative mutant (ΔΔ5) of the regulatory A subunit of PP2A, which is defective in binding regulatory B subunits but normal in binding the catalytic C subunit, leads to a significant increase in heart weight and heart weight-to-body weight ratio. Previous experiments with tissue culture cells (32) have demonstrated that expression of the AΔ5 subunit causes an increase in the amount of the core enzyme and a decrease in the amount of the holoenzyme, resulting in a change in PP2A activity. In the heart, expression of the AΔ5 subunit also caused an increase in the ratio of the core enzyme to the holoenzyme and a change in activity, and this change is responsible for the observed increase in heart weight and the depressed cardiac function. At present, we do not know what causes the dramatic increase in heart weight at older age. It is conceivable that this is due to genetic or environmental factors in addition to expression of the AΔ5 subunit. The increase in heart weight was proportional to the expression of the AΔ5 subunit, because I4-transgenic mice, expressing less of the AΔ5 subunit than I1-transgenic mice, had smaller hearts than the I1-transgenic mice. Furthermore, heterozygous I1-transgenic mice had smaller hearts than homozygous mice. These observations further indicate that the AΔ5 subunit is the actual cause for the observed phenotype. Echocardiographic analyses revealed that transgenic hearts were significantly dilated, the walls of the hearts were thinner, and the capacity of the hearts to contract was considerably reduced. These findings are consistent with a phenotype of dilated cardiomyopathy. The AΔ5-transgenic hearts show only a modest increase in β-MHC expression and normal atrial natriuretic factor expression. These findings are compatible with the echocardiography results showing no increase in wall thickness. In addition, no significant interstitial fibrosis occurred.

We have shown that the heart weight of the AΔ5-transgenic animals was significantly increased at birth. Because up to this time in development the heart grows by an increase in the number of cardiomyocytes, this finding might suggest that expression of the AΔ5 subunit stimulates cardiomyocyte proliferation (hypertrophic

Table 3. Altered phosphatase activity in heart extracts from AΔ5-transgenic mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Rb Peptide*</th>
<th>Phos a*</th>
<th>Rb:Phos a</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>1,605 ± 89.0</td>
<td>434 ± 17.6</td>
<td>3.7</td>
</tr>
<tr>
<td>I1</td>
<td>1,145 ± 27.5 (71)</td>
<td>536 ± 3.2 (124)</td>
<td>2.1</td>
</tr>
</tbody>
</table>

*Numbers (means ± SD) represent picomoles per minute per milligrams of phosphate released. The values are from triplicate assays with the background subtracted. Numbers in parentheses represent % of WT. Phos a, phosphorylase a.
plasia) during fetal development. Alternatively, the cardiomyocytes in AΔ5-transgenic mice may be abnormally large at birth, whereas the number of cardiomyocytes is unchanged. Transgenic overexpression of the c-myc proto-oncogene also causes cardiac enlargement, which in this case is due to myocyte hyperplasia during fetal development (12).

We have demonstrated that the core enzyme and holoenzyme are both similarly abundant in the heart, as shown previously for bovine hearts (25) and for several lines of cultured fibroblasts (15). This was achieved by quantitative immunoprecipitation of the core enzyme and holoenzyme with specific monoclonal antibodies. The conditions of extract preparation and immunoprecipitation were chosen such that proteolysis and dissociation of the holoenzyme into the core enzyme and B subunit were minimized. Our data contradict the commonly held view that the holoenzyme is the only form of PP2A in intact cells and that the core enzyme is an artifact of enzyme purification (for discussion, see Ref. 15). A simple calculation shows that the change in the amounts of the core enzyme and holoenzyme, which would result from a twofold AΔ5 subunit overexpression, are relatively small (in the order of 30%) and fall within close range of the observed values of 20–30%. If core enzymes were not initially present in cardiomyocytes, the expected increase in core enzyme activity due to the formation of the AΔ5-C subunit would be dramatic (50-fold). That this formation is, in fact, only 24% further supports the notion that the core enzyme is as similarly abundant as the holoenzyme. Our findings are important because the core enzyme differs significantly in substrate specificity from the holoenzyme. In addition, the core enzyme may serve as a reservoir for the binding of various regulatory B subunits. Besides the core enzyme and Bα-subunit-containing holoenzyme, at least three additional heart-specific forms of the holoenzyme can be inferred from the fact that three additional B subunits are expressed in the heart. The B56α- and B56γ-subunits (members of the B′ family) are preferentially expressed in the heart and may have cytosolic and nuclear functions, respectively (22, 42). The 74-kDa B′′ subunit is highly expressed in the heart and brain (5). It is likely that these B subunits do not exist as free monomers but are bound to excess core enzymes forming distinct holoenzymes. The phenotype of AΔ5-transgenic mice may be attributed either to an increase in core enzymes or a decrease in one or several forms of holoenzyme. Because the AΔ5 mutant protein does not bind any form of the B subunit, we assume that transgenic expression of the AΔ5 subunit caused a decrease in all forms of holoenzyme. Hence, it is not possible to link the phenotype of the AΔ5-transgenic mice to the decrease of a particular form of the holoenzyme. To identify the heart-specific substrates of the core en-

![Fig. 10. Cardiac gene expression in I1-transgenic mice. A: Northern blot showing cardiac expression in both I1-transgenic and WT mouse hearts. RNA was isolated and hybridized with specific probes as described under MATERIALS AND METHODS. B: quantitative comparison of transcripts from I1-transgenic and WT mice. WT gene expression was set to 100%. Numbers (means ± SD) in arbitrary units were obtained from 3 animals. MHC, myosin heavy chain; CSQ, calsequestrin; SERCA, sarco(endo)plasmic reticulum Ca2+-ATPase; PLB, phospholamban; RYR2, ryanodine receptor 2; ANF, atrial natriuretic factor. The measured values (in arbitrary units) for WT mice are the following: β-MHC, 4.6 ± 1.3; ANF, 260 ± 79; CSQ, 19.4 ± 1.6; α-MHC, 310 ± 49.6; SERCA, 141.9 ± 8.5; PLB, 793.8 ± 50.9; RYR2, 1.6 ± 0.4. The measured values for I1-transgenic mice are the following: β-MHC, 18.2 ± 6.3; ANF, 350 ± 170; CSQ, 21.2 ± 3.8; α-MHC, 324.5 ± 95.5; SERCA, 120.4 ± 20.6; PLB, 705.6 ± 101.8; and RYR2 1.3 ± 0.7. Note that the level of expression varies greatly between the different genes. The exposure times shown in A were chosen to best visualize the WT and I1 signals in each case.
zyme and the various holoenzymes is an important goal. One candidate is MAP kinase, a known substrate of PP2A (38), which is involved in the hypertrophic response of cardiomyocytes to a variety of factors and stimuli. Interestingly, there is an inverse relationship between increasing MAP kinase activity and decreasing PP2A phosphatase activity in cultured cardiomyocytes upon treatment with phorbol ester, an inducer of protein kinase C and of hypertrophic response (4).

It is intriguing that the genes encoding the B56α- and B56γ-subunits have been mapped to regions on the human chromosome linked to heart disease (21). The B56α-subunit gene is located on chromosome 1q41, a region linked to rippling muscle disease and ventricular cardiomyopathy, and the gene for the B56γ-subunit is located on 3p21, a region linked to a form of familial cardiomyopathy. However, mutations in the B56α- or B56γ-subunit genes have not been reported in patients with heart disease. It is conceivable that mutations of the A subunit, which impair binding of the Bo-, B56α-, B56γ-subunits, or the 74-kDa B’’ subunit to the core enzyme, are genetically linked to heart disease, in particular because a large number of point mutations in the A subunit abolish binding of specific B subunits (33). We recently constructed mutants that do not bind B’ (B56) subunits but bind Bo- and B’’ subunits normally (33). The expression of these mutants in the heart is expected to shed light on the function of B’’ subunits, including the B56α- and B56γ-subunits.

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