Phenotypic consequences of \(\beta_1\)-tubulin expression and MAP4 decoration of microtubules in adult cardiocytes

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The purpose of the present study was to directly evaluate these two plausible, but only inferentially implicated, bases for cardiac hypertrophic microtubule stabilization by overexpressing MAP4 and \(\beta_1\)-tubulin (37). Thus increased synthesis of \(\alpha\beta\)-tubulin heterodimers is one cause for the greater cardiac microtubule network density. However, given that the \(\alpha\beta\)-tubulin heterodimer-microtubule system is in dynamic equilibrium, enhanced microtubule stability is a potentially synergistic second mechanism for microtubule network densification. Indeed, we (34) found that the microtubules of pressure-hypertrophied cardiocytes demonstrate markedly enhanced stability, in which microtubule stabilization begins very shortly after cardiac pressure overload and persists indefinitely thereafter.

We then turned to two possible bases for this microtubule stabilization. The first of these was the role of microtubule-associated proteins (MAPs), which are known to regulate microtubule stability (11, 23, 26, 28, 44). Here, we focused on MAP4, the dominant structural MAP of the heart (29, 34), and found on both the mRNA and protein levels striking MAP4 upregulation in hypertrophied cardiocytes (34). This was associated with extensive microtubule decoration with MAP4, which was not seen in normal cardiocytes.

The second possible basis for this microtubule stabilization was differential transcriptional regulation of \(\beta\)-tubulin isoforms during cardiac hypertrophy, because MAPs and the expressed proteins of the \(\beta\)-tubulin multigene family exhibit coordinate developmental regulation (27) and the latter may, via their isoform-variable carboxy-terminal domain, confer differing MAP binding affinity and microtubule stability after assembly (22). Here, we found (25) that increased \(\beta\)-tubulin synthesis during cardiac hypertrophy is caused by transcriptional upregulation of the \(\beta_1\)-tubulin isoform and, to a much lesser extent, the \(\beta_2\)-tubulin isoform but not by upregulation of the predominant \(\beta_4\)-tubulin isoform. Furthermore, there was indirect evidence that the greater proportion of \(\beta_1\)-tubulin protein might have a causative role in the microtubule stabilization found in cardiac hypertrophy (25).

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both in normal isolated adult cardiocytes via adenovirus-mediated gene transfer and in murine hearts via transgenesis with the α-myosin heavy chain (MHC) promoter.

MATERIALS AND METHODS

Construction of recombinant adenoviruses. The pTG3602 plasmid system for generating replication-defective recombinant adenoviruses via homologous recombination with a bacterial system (6, 32) was used to construct adenoviruses for β1-tubulin, β4-tubulin, and MAP4 as well as for β-galactosidase (β-gal) as a control for nonspecific effects of adenovirus infection. For β1-tubulin, we used a 1.5-kb HindIII/NotI fragment of the BlskHAβ1 plasmid (15), a gift from F. Cabral (University of Texas, Houston, TX) containing the entire β1-tubulin coding sequence with 27 nucleotides encoding the 9-amino acid immunodominant epitope of influenza hemagglutinin antigen (HA) as an epitope tag at the 3′ end. The HA-tagged β1-tubulin cDNA was amplified with Phu polymerase (Stratagene, La Jolla, CA) and primers that inserted a HindIII site just 3′ of the stop codon after the HA tag. For β4-tubulin, the cDNA (43), a gift from N. Cowan (New York University), was cloned into the same vector in place of the β1-tubulin cDNA in-frame with the HA tag. The product in each case was gel purified, digested with HindIII, and ligated into an EcoRV/HindIII-digested pAD.CMV-Link.1 shuttle plasmid. The clones were then screened for the correct sequence. The MAP4 cDNA construct was generated by PCR with specific oligonucleotide primers and full-length human MAP4 cDNA, a gift from J. C. Bulinski (Columbia University), as a template (3). The primer for the amino terminus also contained a sequence for an epitope tag of amino acids 410–419 of human c-Myc, and KpnI restriction sites were included at the end of each primer. The sequence of the full-length MAP4 construct was confirmed by DNA sequencing. The KpnI fragment was subcloned into the pAD.CMV-Link.1 shuttle plasmid and sequenced to identify clones with correct orientation. The β1-tubulin, β4-tubulin, and MAP4 shuttle vector constructs were digested with NheI and ApaI and recombined with the ClaI-linearized viral DNA vector T63602 in Escherichia coli strain BJJ183. The recombinant adenovirus DNA containing the β1-tubulin (Adβ1-tubulin), β4-tubulin (Adβ4-tubulin), or MAP4 (AdMAP4) expression cassette was linearized and transfected into the HEK-293 human epithelial kidney cell line with Lipofectamine reagent. Virus expressing bacterial β-gal (Adβ-gal) was generated in the same way as described above. Each adenovirus plaque was plaque purified, expanded, purified by CsCl gradient centrifugation, dialyzed, and titered via the detection of visible plaque formation in HEK-293 monolayers.

Cardiocyte isolation and culture. Adult feline cardiocytes were isolated as described previously (20) via retrograde coronary artery perfusion with a collagenase solution (Liberase Blendzyme 1, Roche Molecular Biochemicals, Indianapolis, IN) and maintained at 37°C on dishes coated with laminin (Becton Dickinson, San Jose, CA) in serum-free M199 buffer with Earle’s salts (GIBCO-BRL, Grand Island, NY) supplemented with 0.2% (wt/vol) fraction V bovine serum albumin (Sigma, St. Louis, MO), 0.1 μM recombinant insulin (GIBCO BRL), 100 IU/ml penicillin, 100 μg/ml streptomycin, 100 μM ascorbate, 2 mM cysteine, 5 mM creatine, 20 μM phenylalanine, 5 mM taurine, and 10 μM cytosine arabinoside (Sigma). In some cases, cardiocytes were isolated separately from the right and left ventricles (10) of cats in which the right ventricle had previously been pressure overloaded via surgical banding of the pulmonary artery, a model with which we have had extensive experience (9). All studies were conducted in accordance with the “Guiding Principles for Research Involving Animals and Human Beings” of the American Physiological Society.

Adenovirus-mediated gene transfer. Replication-defective recombinant adenoviruses were added to cardiocyte suspensions at the time of plating in serum-free medium. The infections proceeded over 24 h at a multiplicity of infection (MOI) of 1–4 plaque-forming units (PFU)/cell, as determined by plaque assays. The cardiocytes were then rinsed in serum-free medium and incubated for 3 days to permit transgene expression.

Transgenic mice from FVB/N strain. To generate transgenic mice with cardiac-restricted overexpression of β1-tubulin, the cDNA encoding hamster β1-tubulin, confirmed by DNA sequencing, was the same as that used for constructing the Adβ1-tubulin virus. We used site-directed mutagenesis to remove the NotI site just 3′ of the HA tag and another round of site-directed mutagenesis to introduce a Sall site. After sequencing to ensure that no unwanted mutations had been introduced into the coding sequence, we subcloned the HA-tagged mouse cDNA into Sall site into pUC19-MHC-α promoter vector (36). Orientation of the insert was determined by sequencing. The α-MHC-β1-tubulin construct, containing the exon-intron organization of the α-MHC promoter linked to the entire β1-tubulin cDNA, was digested free of vector sequence with Sall, purified from agarose, and used to generate transgenic mice (33). Founder mice were identified by PCR and confirmed by genomic Southern blots. Two β1-tubulin mouse lines were made, both of which demonstrated homogeneous expression of the transgene in all ventricular cardiocytes via immunofluorescence microscopy of transmural frozen sections of the left ventricle. Third-generation or later adult mice heterozygous for the transgene were used for all studies.

Transgenic mice with cardiac-restricted overexpression of MAP4 were generated in an analogous fashion. The cDNA encoding human MAP4, confirmed by DNA sequencing, was the same as that used for constructing the AdMAP4 virus. The MAP4 cDNA was fused at the 5′ end to a sequence encoding an HA epitope tag. A 6-kb Sall–HindIII fragment containing the HA:MAP4 fusion cDNA was ligated into a plasmid containing the murine α-MHC promoter to generate a new plasmid, α-MHC clone 26. The final construct, containing the exon-intron organization of the α-MHC promoter linked to the entire MAP4 cDNA, was treated as above to generate transgenic mice. Founder mice were again identified by PCR and confirmed by genomic Southern blots. Two MAP4 mouse lines were made, both of which demonstrated homogeneous expression of the transgene in all ventricular cardiocytes via immunofluorescence microscopy of transmural frozen sections of the left ventricle. Here, too, third-generation or later adult mice heterozygous for the transgene were used for all studies.

MAP4 antibody. To make the MAP4 antibody, we used a bacterial expression construct having the 1–740 amino-terminal residues of human MAP4 (5). The recombinant protein, which had a hexahistidine tag inserted at the carboxy terminus, was overexpressed in E. coli, purified on a nickel-chelate affinity column, and submitted to SDS-PAGE. The purified protein band was excised, eluted from the gel, and sent to Lambert Biologica Laboratories (Pipersville, PA) for preparation of a rabbit polyclonal MAP4 antibody.

Separation of protein fractions for immunoblotting. Total tubulin and MAP4 were assayed in a total protein fraction. To prepare this fraction, cardiocytes were scraped in a 1% SDS buffer (in mM: 10 Tris·HCl, pH 7.4, 50 DTT, 1 Na3VO4).
boiled, and centrifuged at 16,000 g and 25°C for 10 min. This supernatant was saved as the total protein fraction. To prepare the tubulin heterodimer and microtubule fractions, cardiocytes were scraped in a microtubule stabilization buffer (1% NP-40, 50% glycerol, 5% DMSO, 10 mM Na2HPO4, 0.5 mM EGTA, 0.5 mM MgSO4, 25 mM Na2P2O7), homogenized, and centrifuged at 100,000 g and 25°C for 20 min. This supernatant was saved as the heterodimer fraction. The pellet was resuspended in 1% SDS buffer and boiled until the pellet was dissolved. This was saved as the microtubule fraction. For subsequent gel electrophoresis, to compare the amount of tubulin in the heterodimer vs. microtubule fractions of a given sample, an equal proportion of the two fractions from the same sample was applied. For between-sample comparisons, an equal amount of protein from each, based on a biocinchonic acid assay, was applied.

**Immunoblots.** The blots prepared with the protein fractions described in Separation of protein fractions for immunoblotting were incubated overnight with the primary antibody. After incubation with biotinylated secondary antibody, specific protein bands were detected with avidin-biotinylated horseradish peroxidase in conjunction with enhanced chemiluminescence.

**Microtubule stability assays.** Microtubule stability was assessed as before (34) in terms of resistance to nocodazole, a microtubule poison, and low temperature. For the former, cardiocytes were treated with 3 mM nocodazole for 1 h and either prepared for immunofluorescence microscopy or scraped in the microtubule stabilization buffer for subsequent immunoblot evaluation. For the latter, the same procedures were followed after the cardiocytes were incubated at 3°C for 30 min.

**Immunofluorescence confocal microscopy.** Cardiocytes were extracted in 1% Triton X-100 in microtubule stabilization buffer for 1 min, rinsed three times in this buffer, fixed in 3.7% formaldehyde for 30 min, and rinsed three times in PBS. Myocardium was perfusion fixed (10 mM NaIO4, 75 mM lysine, 37.5 mM phosphate buffer, and 2% paraformaldehyde) at a pressure of 60 cmH2O and 37°C for 1 h. The fixed tissue was cut into small pieces and placed in 100 mM phosphate-buffered 10% sucrose for 4 h, 15% sucrose for 4 h, and 20% sucrose overnight. It was then embedded in tissue freezing medium (Triangle Biomedical Sciences, Durham, NC), frozen in liquid nitrogen, and sectioned at 8 μm with a cryostat. The antibodies used for immunostaining of cells and tissue were specified in the figures.

**RESULTS**

**Microtubules, MAP4, and β1-tubulin in cardiac hypertrophy.** For MAP4, the micrographs in Fig. 1 show that in severe pressure-overload hypertrophy of the feline right ventricle (right ventricular systolic pressure ≥55 mmHg) induced by pulmonary artery banding (9), there is not only the expected increase in cardiocyte size but also marked densification of the microtubule network and extensive decoration of these microtubules by MAP4. These images were obtained at 1 mo after pressure-overloading, when cardiac mass after a fixed load increase is at a new steady state (37). The cardiac MAP4 immunoblot in Fig. 1 shows that when comparing same-animal normally loaded left ventricles with pressure-overloaded right ventricles, there is an increase in MAP4 at all time points examined, extending from 2 days, just before hypertrophic growth becomes apparent on the organ level, to 2 wk, when this growth process has just reached completion (37). Of interest, the upregulated isoform of MAP4 is the 220-kDa ubiquitous isoform rather than the muscle-specific 350-kDa isoform (24). For β1-tubulin, we have shown (25) that transcriptional upregulation of this gene, rather than of that for the constitutive β1-tubulin, accounts for almost all of the increase in β1-tubulin seen in cardiac hypertrophy and this β1-tubulin is incorporated into the microtubules of hypertrophied cardiocytes.

**Effects of MAP4 and β1-tubulin on microtubules of isolated cardiocytes.** To assess the importance of these two hypertrophically upregulated proteins to the increased density and stability of the microtubule network in the hypertrophied cardiocyte, we overexpressed them singly and together in normal cardiocytes via adenovirus-mediated gene transfer. As
controls, β-gal and constitutive β4-tubulin were overexpressed in the same way. Figure 2, prepared with an isofrom-common β-tubulin antibody, shows that for both β1- and β4-tubulin essentially all of the endogenous β-tubulin is replaced by the overexpressed protein at each of the viral titers used. Figure 3, left, shows first that there is very little MAP4 decoration of microtubules in the normal cardiocyte, whereas as in cardiac hypertrophy there is extensive MAP4 decoration of microtubules when the level of MAP4 is increased by an AdMAP4 moi of 4 pfu/cell. Figure 3, right, prepared with an α-tubulin antibody, shows that although increased levels of β4- or β1-tubulin do not cause an appreciable shift of tubulin dimers to the microtubule fraction, an increased level of MAP4 does so. However, this MAP4 effect is not augmented by a concurrent increase in the levels of either β4- or β1-tubulin.

In attempting to confirm this biochemical result and extend it to a consideration of microtubule stability in adenovirus-infected adult cardiocytes, we sometimes observed an apparent β1-tubulin-dependent increase in microtubule quantity and stability in immunoblots prepared with an isofrom-common β-tubulin antibody. However, the data in Fig. 4 show that this was an artifact caused by the formation of protein aggregates at higher levels of β4- and especially β1-tubulin overexpression. At an moi of 3 pfu/cell, ~2% of β4-tubulin-infected and ~10% of β1-tubulin-infected cardiocytes were found to contain aggregates, presumably of the overexpressed protein, that segregated to the microtubule fraction in immunoblots and were colchicine insensitive. Thus Fig. 4, A and B, prepared with a β-tubulin antibody, suggested that β4- and especially β1-tubulin overexpression causes an increase in colchicine-stable microtubules. However, Fig. 4, G and I, shows the presence of aggregates of the HA-tagged overexpressed β4- or β1-tubulin as green densities. These apparently do not contain tubulin dimers, because they do not show the coincident staining with the α-tubulin antibody that is seen in the microtubules of these same cardiocytes; Fig. 4, H and J, shows that these aggregates are colchicine stable. Figure 4, C and D, prepared with an α-tubulin antibody in these same samples, shows that in fact there is no effect of β4- or β1-tubulin overexpression on microtubule quantity or stability, because tubulin dimers in the microtubule fraction are not increased either before or after colchicine treatment in the adenovirus-infected cardiocytes.

Having next established that these protein aggregates do not form at a lesser Adβ4-tubulin or Adβ1-tubulin moi of 2 pfu/cell, we examined the effects of overexpressed MAP4, β4-tubulin, and β1-tubulin in more detail with an α-tubulin antibody for the immunoblots. Figure 5A shows that the microtubule network of the AdMAP4 + Adβ-gal-infected cardiocyte is much denser than that of the cell infected with Adβ-gal alone, with the expected redistribution in the immunoblots of tubulin dimers from the free to the microtubule fraction. The upper micrograph and the left two lanes of the immunoblot in Fig. 5A for the Adβ-gal-infected cardiocytes are identical to those of wild-type cardiocytes (34). Comparison of Fig. 5, B and C, to Fig. 5A shows that increases in β4- or β1-tubulin ( moi = 2 pfu/cell in each case) do not cause a micrographic increase in microtubule network density or any modification of the microtubule network densification caused by MAP4; the left two lanes of the immunoblots in Fig. 5, A–C, are in each case concordant with and confirmatory of those in Fig. 3. The remaining data in Fig. 5 show that only MAP4 has a positive effect on microtubule stability as gauged by resistance to nocodazole-induced depolymerization and that neither β4- nor β1-tubulin overexpression has any independent or additive effect on this variable. Use of low temperature (3°C for 30 min) as a probe of microtubule stability produced results that were qualitatively quite similar to those in Fig. 5, A–C (data not shown).

Effects of MAP4 and β1-tubulin on microtubules of myocardial cardiocytes. The extension of this work from isolated cells to the hearts of transgenic mice was driven by two considerations. First, the effects of a transient increase in a protein in terminally differentiated isolated cells do not necessarily mimic the effects of the long-term presence of that protein. Second, the effects of that protein in the same cell might well be quite different in the in vivo as opposed to the in vitro context. For the two types of transgenic mice made to address these considerations, the micrographs in Fig. 6

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**Fig. 2.** Effect of viral titer [multiplicity of infection (moi)] on expression levels of β4-tubulin, β3-tubulin, and GAPDH in feline cardiocytes infected with adenovirus expressing β-galactosidase (Adβ-gal, control), β1-tubulin (Adβ1-tubulin), or β4-tubulin (Adβ4-tubulin). The feline β1- and β4-tubulin constructs were hemagglutinin (HA) epitope tagged, such that in the top immunoblot, prepared with a β-tubulin antibody (DM1B) that binds to all β-tubulin isofoms equally, the β-tubulin protein expressed by the transgene runs at a higher molecular weight than that in the control lane expressed by the endogenous β-tubulin genes. At Adβ4-tubulin and Adβ1-tubulin moi ranging from 2.0 to 3.0 pfu/cell, there is equivalent expression of the 2 transgenes and each completely replaces the protein product of the native β-tubulin isoform genes. After being stripped, the immunoblot was probed with a GAPDH antibody (6C5); the bottom immunoblot shows that the level of GAPDH expression was unaffected by viral identity or titer.
show that the cardiac-restricted expression of both β1-tubulin and MAP4 is present at comparable levels in all of the ventricular cardiocytes. As in feline hearts with our isoform-specific β-tubulin antibodies (25), we found in murine hearts that virtually all of the native cardiac β-tubulin in the normal adult is the β4-tubulin isoform (data not shown). For the β1-tubulin transgenic mice, Fig. 6C shows that the HA-tagged β1-tubulin, which runs a bit higher than and replaces the endogenous protein, has a level relative to control that is closely comparable to that shown in Fig. 2 for Adβ1-tubulin-infected cells at an moi of 2.5 pfu/cell. For the MAP4 transgenic mice, Fig. 6F shows that the level of the ubiquitous MAP4 isoform in the lower band relative to control is closely comparable to that shown in Fig. 3 for AdMAP4-infected cells at an moi of 4 pfu/cell.

The data in Fig. 7 show first that, as in isolated cardiocytes, β1-tubulin overexpression in myocardial cardiocytes has no effect on the proportion of free versus polymerized tubulin dimers, whereas MAP4 overexpression shifts the tubulin dimers into the microtubule fraction. Again, as in isolated cardiocytes, concomitant overexpression of these two proteins in myocardial cardiocytes does not modify the effect produced by MAP4 overexpression alone. Second, whereas β1-tubulin overexpression has no appreciable effect on α-tubulin expression, MAP4 overexpression causes a marked increase in the levels of both α-tubulin and β-tubulin.

The data in Fig. 8 show that in terms of microtubule network density and stability, the effects of MAP4 overexpression in myocardial cardiocytes also mimic those seen in isolated AdMAP4-infected cardiocytes. There was very little MAP4 decoration of microtubules in control murine cardiocytes, and the microtubule network was quite labile on nocodazole exposure. In contrast, cardiocytes from the MAP4 transgenic mice showed a much denser microtubule network at baseline that was much more resistant to nocodazole.

The cardiocyte microtubule network in control vs. hypertrophied cells shown in Fig. 1 is strikingly similar to that in untreated normal vs. MAP4 transgenic cells shown in Fig. 8. Both for this reason and because the intent here was to gain insight into the operational significance of the microtubule alterations in hypertrophied myocardium, we next asked whether, in addition to the biochemical and morphological phenotype produced by MAP4 overexpression, this also produces the functional phenotype of microtubule-based frictional dissipation impeding sarcomere shortening (38) seen in hypertrophied cardiocytes. The laser diffraction
measurements of sarcomere motion (42) shown in Fig. 9 indicate that this is the case. The extent and velocity of sarcomere motion during 0.4-Hz contractions at 32°C in 1.2 mM Ca^{2+} buffer were reduced in both MAP4 transgenic and pressure-overload hypertrophied cardiocytes, and superimposition of pressure overloading on MAP4 overexpression accentuated these contractile abnormalities.

**DISCUSSION**

The intent of this study was to determine whether the upregulation found in severe pressure-overload cardiac hypertrophy of β₁-tubulin and MAP4 has etiologic significance, either singly or synergistically, for the microtubule network stabilization and densification and the associated contractile dysfunction characteristic of this pathological entity. In this data set, no such role for the upregulation of β₁-tubulin was found.

This lack of a β₁-tubulin effect is not terribly surprising in view of the preponderant notion that the β-tubulin isoforms are usually functionally equivalent in most eukaryotic cell types. However, this was for two reasons not an entirely expected result. First, data supporting the functional equivalence of β-tubulin isoforms are most compelling in relatively simple in vitro assay systems, single-celled organisms, and cultured cells (7, 21, 22). However, even in some cultured cell types, and certainly in multicellular organisms, there is clear evidence for distinct differences among the β-tubulin isoforms, whether in terms of the structural (31) and functional (17) features of microtubules or in terms of posttranslational β-tubulin modifications and affinity for nonmotor MAPs (22). Second, our own data from hypertrophied myocardium (25) show a segregation of the upregulated β₁-tubulin isofrom to a cold-stable, heavily MAP4-decorated microtubule network. However, the facts that β₁-tubulin was transcriptionally upregulated and that it selectively populated the cold-stable portion of the dense microtubule network of the hypertrophied cardiocyte (Fig. 1) did not constitute a causal linkage between these two observations. Indeed, the attempt to establish such a linkage between increased β₁-tubulin and microtubule network densification was a major impetus for the present study. Thus β₁-tubulin was expressed in normal cardiocytes at levels sufficient to replace the endogenous β₄-tubulin (Fig. 2), but this did not affect the proportion of the total β-tubulin found in the microtubule fraction (Fig. 3). Furthermore, although we initially thought on the basis of immunoblotting that high levels of β₁-tubulin overexpression in isolated cardiocytes caused an increase in microtubule quantity and colchicine stability, this was found to be an artifact produced by the formation of protein aggregates at high expression levels (Fig. 4). In fact, at expression levels sufficient to replace the endogenous β₄-tubulin without forming protein aggregates, β₁-tubulin caused neither a shift of tubulin heterodimers to the microtubule fraction nor microtubule stabilization (Fig. 5). Taking this question to the in vivo setting (Fig. 6), there was no effect of myocardial β₁-tubulin overexpression in transgenic mice on the proportion of tubulin dimers in the microtubule fraction (Fig. 7). Therefore, there is no selective role of β₁-tubulin in the normal heart and in muscle cells isolated from it either in the tendency of tubulin dimers to form microtubules or in the stability of these microtubules once formed.

![Image](http://ajpheart.physiology.org/DownloadedFrom/10.220.32.247/10.220.32.247.png)
In contrast, a major role for upregulated MAP4, such as that seen in cardiac hypertrophy, in the control of cardiac microtubule properties was found in this data set. In normal myocardium, both the muscle-specific and the ubiquitous isoforms of MAP4 are expressed but there is very little association of MAP4 with microtubules (Fig. 1). In hypertrophied myocardium, the ubiquitous isoform of MAP4 is persistently upregulated and there is extensive MAP4 decoration of the microtubules (Fig. 1). Similarly, in normal adult cardiocytes, adenovirus-mediated overexpression of β-gal causes no difference from control in the proportion of total tubu-

**Fig. 5.** Effects of adenovirus-mediated gene expression on microtubule stability in feline cardiocytes. A: top 3 panels show the effects of Adβ-gal expression (moi = 2 pfu/cell) on microtubule network density and proportion of free vs. polymerized tubulin before and after nocodazole. These and the other confocal micrographs (bar = 20 μm) were prepared with an α-tubulin antibody (B-5-1-2). In this and the other immunoblots, prepared with an α-tubulin antibody (12G10), lanes 1 are α-tubulin in the heterodimer fraction and lanes 2 are α-tubulin in the microtubule fraction. The top micrograph shows the microtubule network in the control state, and the bottom micrograph shows the microtubule network after nocodazole treatment (3 μM for 1 h). The immunoblot shows free and polymerized tubulin in the control state and, in triplicate experiments, after nocodazole treatment (1 μM for 30 min). It is evident that nocodazole treatment results in very extensive depolymerization of the microtubule network. The bottom 3 panels show the effects of combined Adβ-gal (moi = 2 pfu/cell) and AdMAP4 (moi = 4 pfu/cell) expression on these same variables. In these doubly infected cardiocytes, the microtubule network is both more extensive in the control state and more resistant to nocodazole-induced depolymerization. These findings were confirmed by densitometric analysis of microtubule fraction α-tubulin protein as % total α-tubulin protein [(lane 2)/(lanes 1 + 2) × 100] in these and additional immunoblots (n = 5): cardiocytes infected with Adβ-gal alone, 40.1 ± 4.3% (control) and 5.6 ± 0.7% (after nocodazole); cardiocytes infected with Adβ-gal + AdMAP4, 67.7 ± 5.8% (control) and 23.8 ± 4.0% (after nocodazole). Both before and after nocodazole exposure, P < 0.01 for the difference between singly and doubly infected cells by Student’s unpaired t-test. B: format identical to A except that Adβ-tubulin (moi = 2 pfu/cell) was used instead of Adβ-gal. It is clear that β1-tubulin has no effect on either microtubule network density or the amount of tubulin in the polymerized fraction in the control state, nor does it have any effect on microtubule stability in the cardiocytes infected with Adβ-gal; there are no β1-tubulin-dependent synergistic effects. These findings were again confirmed by densitometric analysis of microtubule fraction α-tubulin protein as % total α-tubulin protein in these and additional immunoblots (n = 5): Adβ-4 tubulin alone, 38.9 ± 5.8% (control) and 3.0 ± 0.2% (after nocodazole); Adβ1-tubulin + AdMAP4, 60.2 ± 5.7% (control) and 14.8 ± 1.5% (after nocodazole). Both before and after nocodazole exposure, P < 0.01 for the difference between singly and doubly infected cells by Student’s unpaired t-test. C: format identical to A except that Adβ1-tubulin (moi = 2 pfu/cell) was used instead of Adβ-gal. It is clear that just as for β1-tubulin, β1-tubulin has no effect on either microtubule network density or the amount of tubulin in the polymerized fraction in the control state, nor does it have any effect on microtubule stability on nocodazole exposure. Furthermore, concomitant MAP4 expression affects both of these variables just as in the cardiocytes infected with Adβ-gal or Adβ1-tubulin; there are no β1-tubulin-dependent synergistic effects. These findings were also confirmed by densitometric analysis of microtubule fraction α-tubulin protein as % total α-tubulin protein in these and additional immunoblots (n = 5): Adβ1-tubulin alone, 36.2 ± 5.7% (control) and 3.3 ± 0.9% (after nocodazole); Adβ1-tubulin + AdMAP4, 51.9 ± 1.2% (control) and 13.3 ± 1.1% (after nocodazole). Both before and after nocodazole exposure, P < 0.01 for the difference between singly and doubly infected cells by Student’s unpaired t-test.
lin in the microtubule fraction or in microtubule network density and MAP4 decoration, but overexpression of MAP4 causes a shift of tubulin into the microtubule fraction and densification of a heavily MAP4 decorated microtubule network, much as in hypertrophied cardiocytes (Fig. 1 vs. Fig. 3). Again, as in hypertrophy, the dense microtubule network of MAP4 overexpressing cardiocytes is resistant to nocodazole-induced depolymerization (Fig. 5). In the in vivo setting (Fig. 6), MAP4 overexpression caused increased expression of tubulin, with a higher proportion of tubulin dimers in the microtubule fraction (Fig. 7). Furthermore, the cardiocyte microtubule network in the MAP4 transgenic mice was strikingly similar to that in hypertrophied cardiocytes, both in terms of enhanced density and MAP4 decoration (Fig. 1 vs. Fig. 8) and in terms of enhanced stability (Fig. 8 vs. Fig. 5 in Ref. 34).

Because there was reason to think that differing affinities for structural MAPs of the carboxy-terminal isoform-variable region of the different β-tubulin isoforms might act synergistically with MAP4 overexpression to produce the microtubule phenotype characteristic of cardiac hypertrophy (22, 25), we looked for such an effect via combined overexpression of β1-tubulin and MAP4 as well as β1-tubulin and MAP4. No such effect was found, either during adenovirus-mediated overexpression in vitro (Figs. 3 and 5) or during transgenesis-mediated overexpression in vivo (Fig. 7).

Finally, because the models developed for this study were intended to provide mechanistic insight into the cytoskeletal changes characteristic of pressure-overload cardiac hypertrophy, we thought that it would be interesting to see whether the microtubule changes produced by MAP4 overexpression had the same functional consequences in terms of contractile abnormalities as those occurring during this pathological state (41, 42). This was indeed the case; surgical left ventricular pressure overloading and transgenic MAP4 overexpression caused similar decrements in the extent and velocity of sarcomere shortening in isolated cardiocytes, and the superimposition of these two interventions caused further decrements in these two measures of contractile function (Fig. 9).

Thus there is no selective role of β1-tubulin in the normal heart and in muscle cells isolated from it either in the tendency of tubulin dimers to form microtubules or in the stability of these microtubules once formed. We would therefore surmise that the β1-tubulin up-

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**Fig. 6.** Protein expression in transgenic mice via confocal micrographs and immunoblots of LV myocardium. A: wild-type FVB/N mouse myocardium stained with an α-tubulin (B-5-1-2) antibody (red) and an HA tag antibody (green). B: β1-tubulin transgenic mouse myocardium stained with the same antibodies. The HA-tagged β1-tubulin is present in all cardiocytes. C: immunoblot prepared from equally loaded total protein samples of wild-type (lane 1) and β1-tubulin transgenic (lane 2) mouse myocardium with an isoform-common β-tubulin antibody (DM1B). D: wild-type FVB/N mouse myocardium stained with an α-tubulin (B-5-1-2) antibody (red) and our MAP4 antibody (green). E: MAP4 transgenic mouse myocardium stained with the same antibodies. Markedly increased MAP4 expression is apparent in all cardiocytes. F: immunoblot prepared from equally loaded total protein samples of wild-type (lane 1) and MAP4 transgenic (lane 2) mouse myocardium with our MAP4 antibody.
regulation found in extensive pressure-overload cardiac hypertrophy is not causally linked to the formation of the dense, stable microtubule network characteristic of that entity. In common with many other protein isoforms normally expressed in the developing heart, downregulated in the adult heart, and reexpressed after hypertrophic stimulation, we found (25) that hypertrophic β-tubulin upregulation recapitulates that seen in the developing heart. However, in common with the questionable functional significance of many other cardiac protein isoform switches in which hypertrophy recapitulates phylogeny (8), no role for this tubulin isoform switch was established here, and the increase in β-tubulin protein per se is likely based via cotranslational regulation (39) primarily on the free tubulin dimers shifting into a stable microtubule network.

Although this data set does instead identify an important role for MAP4, it does not imply that the microtubule alterations found in hypertrophied myocardium are fully explained by the increase in MAP4 found there. That is, although it is clear from these data that increases in MAP4 protein itself, if extensive enough, cause microtubule network densification and stabilization, as well as the same MAP4 decoration of microtubules in cardiocytes as seen in other cell types (26), the physiological regulation of the MAP4-microtubule interaction is based less on the quantities of
The extent of MAP4 affinity is on the affinity of MAP4 for microtubules (4). The basis for the regulation of this affinity lies in the carboxy-terminal microtubule-binding domain of MAP4, which is closely homologous with the microtubule-binding domain of the other structural MAPs (30). It contains both a proline-rich region and a region containing four assembly-promoting sequences having KXGS motifs (16); both regions are important for MAP-microtubule affinity and binding (1). The phosphorylation state of Ser/Thr-Pro motifs within the proline-rich region clearly modulates MAP-microtubule binding (4, 19). In addition, the phosphorylation state of Ser residues within the KXGS motifs of the four highly conserved tandem repeats of the microtubule binding site appears to be quite interesting (12–14, 18, 35, 40), because phosphorylation of the Ser residue within the KXGS sequence of the first of these repeats abolishes microtubule binding, whether for MAP4 or for the homologous MAPs tau and MAP2 (13). This information, together with the facts that in early data not shown here we found in hypertrophied myocardium increased activity of protein phosphatase 2A, which regulates the phosphorylation state of the critical Ser-262 tau residue within the KXGS sequence of the first member of the four tandem repeats of the microtubule binding site (2), as well as decreased phosphorylation of the KXGS sequence Ser residue of MAP4, is the basis for our ongoing exploration of the

Fig. 9. Effects of MAP4 overexpression and pressure-overload hypertrophy on cardiocyte sarcomere mechanics. The extent (top) and velocity (bottom) of sarcomere shortening obtained via laser diffraction measurements are shown for representative isolated LV cardiocytes from a FVB/N control mouse, a FVB/N MAP4 transgenic mouse, a FVB/N mouse with surgically induced aortic stenosis, and a FVB/N MAP4 transgenic mouse with surgically induced aortic stenosis. In the latter 2 mice, the aortic arch was exposed and tightly constricted between the origins of the 2 carotid arteries via a silk suture tied over both the aorta and a juxtaposed 27-gauge needle. The needle was withdrawn, and the animals were allowed to recover for 6 wk. This resulted in marked LV pressure overloading, with an increase in LV-to-body wt ratio from a mean control value in this strain of 3.8 to 9.1 mg/g in the FVB/N banded mouse and 8.6 mg/g in the FVB/N MAP4 banded mouse. The increased microtubule network density, which imposes a viscous load on active myofilaments, is associated with similar decrements in contractile function in LV cardiocytes from both the MAP4 transgenic mouse and the pressure-overloaded mouse, with an additive effect when the MAP4 transgenic mouse is pressure overloaded.
reasons for the cytoskeletal alterations characteristic of pressure-overload cardiac hypertrophy.

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DISCLOSURES

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