Myocardial subproteomic analysis of a constitutively active Rac1-expressing transgenic mouse with lethal myocardial hypertrophy

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The progression of heart failure involves cardiac remodeling, a process comprising time-dependent alterations in ventricular shape, mass, and volume (22, 26). At the cellular level, cardiac remodeling involves myocyte hypertrophy, proliferation of cells in the extracellular matrix (ECM) and apoptosis (22, 26). Recently, several members of the Rho GTPases, monomeric GTP-binding proteins of the Ras superfamily including Rac1, have been implicated in development of hypertrophy and dilated cardiomyopathy (6). Rho, Rac1, and CDC42 are the most extensively studied Rho GTPases and are best known for their role in cytoskeletal organization although they are also known to regulate cellular kinase/phosphatase systems such as p21-activated kinase, Rho-activated kinase, and the MAPK pathway (3).

In isolated cardiac myocytes, short-term activation of Rac1 increases the expression of atrial natriuretic peptide and brain natriuretic peptide and promotes some morphological changes associated with early stages of hypertrophy (6). With chronic expression, constitutively active Rac1 transgenic mice develop severe dilated cardiomyopathy that correlates with early postnatal mortality, over 90% of which die between 14 and 17 days after birth from adverse dilated heart failure (36). This outcome suggests that the proteomic events underlying disease progression are precisely timed, an advantage as it should reduce proteome variation. This model, which was used in this study, underscores the important role of controlled cardiac hypertrophy for long-term survival.

An understanding of myocardial proteome changes occurring over time in response to the constitutive activation of Rac1 should provide insight into the processes and regulatory mechanisms underlying the development of dilated hypertrophy. Proteomics is an experimental strategy that allows for the analysis of a large number of proteins, simultaneously, with the goal of providing information about the breadth of cellular proteomic response (42). In the past, proteomic analysis of heart failure has been limited to the analysis of whole tissue obtained from human transplanted hearts and a canine fast-pacing model of heart failure (10, 11, 30) and has focused on metabolic and cytoskeletal/myofilament protein alterations associated with the disease. Conversely, the analysis of distinctive cellular subproteomes, defined by protein function or physical characteristics, allows for in-depth proteomic analysis of particular subsets of cellular proteins. In fact, subproteomic analysis when carried out using a robust method increases the depth of the proteome and provides information about the protein concentration at various cellular locations which may not be reflected with analysis of the whole tissue. In this study, a one-dimensional (1-DE) and two-dimensional gel electrophoretic (2-DE) analysis of two distinct subproteomes, the myofilament and cytoplasmic protein-enriched extracts of Rac1 transgenic and nontransgenic (NTG) control mouse ventricular tissue, was used to determine proteome changes at day 14 with the progression of heart failure. Additionally, a subset

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of these proteins representing three different functional classes was investigated at earlier stages of this phenotype.

MATERIALS AND METHODS

Animal Model

The Rac1 transgenic mouse was produced as described in Ref. 36. Hearts were isolated from Rac1 transgenic mice displaying the dilated phenotype at 7, 9, 11, and 13/14 days after birth and from their age-matched NTG mice, and the heart-to-body weight ratio of these animals was recorded. Hearts were frozen in liquid nitrogen and stored at −80°C until analysis. All experiments involving mice were approved by the Institutional Animal Care and Use Committees at Children’s Hospital Medical Center and San Diego State University.

Preparation of Cytoplasmic and Myofilament-Enriched Extracts

The sequential protein extraction technique “IN Sequence” was employed, as previously described (2, 23), for the production of two distinct subproteomes consisting of cytoplasmic and myofilament protein-enriched extracts of ventricular tissue from NTG and Rac1 transgenic mice (see Online Supplement).1

2-DE Analysis, Protein Visualization, Quantification, and Identification by Mass Spectrometry

Analyses of cytoplasmic and myofilament protein-enriched extracts were carried out as described in the Online Supplement. The reproducibility of the gel system was addressed in Buscemi et al. (5). Of note, it was often necessary to excise a given protein spot from multiple 2-DE gels (combining the equivalent spot from 5 to 10 2-DE gels) to obtain sufficient quantities of protein for mass spectrometric (MS) analysis. Nonetheless, for a number of protein spots of interest, sufficient protein for MS analysis was not obtained. The confidence of a correct protein identification by MS was based on the quality of the mass spectrum (signal to noise), the percentage of the amino acid sequence of the candidate protein encompassed by the fragments obtained by digestion and observed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) (peptide coverage), and whether amino acid sequence was obtained by tandem mass spectrometry (MS/MS) (see Table 1).

Selection of Candidate Protein Spots for Identification

The following criteria were employed for the selection of protein spots for protein identification: 1) the protein spot alteration was present in a large proportion of the sample population, 2) a sufficient amount of protein was available from the 2-DE gels for successful protein identification by MALDI-TOF and/or MS/MS, 3) there was an adequate degree of protein spot resolution on the 2-DE gel to allow for excision of only the spot of interest, and 4) the degree of resolution of the protein spot of interest on the 2-DE gel permitted confidence in its alignment with the same spot on multiple 2-DE gel images (see Online Supplement for more details). Specifically, the protein spot alteration was apparent in >75% of the 2-DE gels of each experimental group, mean spot integrated intensity was at least 25% that of the composite gel image in which it was present.

Western Blot Analysis and Immunostaining

Equal amounts of protein (2–5 μg) from ventricular homogenates or myofilament-enriched extracts of NTG and Rac1 mice at 7, 9, 11, and/or 14 days after birth were separated by one-dimensional gel electrophoresis (1-DE) or 2-DE. Western blotting was carried out as described in Refs. 2, 23, and 43. Immunostaining of left ventricles was carried out as described in Ref. 6 using the primary antibody (β-tubulin at 1:100) and the secondary antibody in FITC channel and Texas red phalloidin (see Online Supplement for details).

RESULTS

Proteomic Analysis of the Cytoplasmic Protein-Enriched Extract at Day 14

Fourteen days after birth, the heart-to-body weight was >2-fold greater in the Rac1 (14–17 mg/g) than the NTG control mice (5–6 mg/g), indicating myocardial hypertrophy occurs as part of the Rac1 phenotype. IN Sequence extraction was carried out on the ventricles to allow independent analysis of the soluble, low-abundant cytoplasmic and high-abundant myofilament proteins. The amount of protein obtained for cytoplasmic protein-enriched extract from a single ventricle (500–700 μg) was only sufficient for two to three large-format 2-DE gels, whereas over 50 large-format gels can be produced from the corresponding myofilament protein-enriched extract. The limited quantity of protein in the cytoplasmic protein-enriched extract restricted analysis to a single pH isoelectric focusing (IEF), done in triplicate (4). The pH 3–10 gradient was employed as it allows for a broad overview of the proteome, including proteins with a wide range of isoelectric points (pI); however, this is at a cost of reduced protein spot resolution. Loss of spot resolution can hinder, at least in the crowded areas of the gel, both assurance in spot image analysis (and hence protein quantification) and protein identification, due to potentially overlapping protein spots. Computer-derived composite images of the equally loaded silver-stained 2-DE gel images were created that represent the cytoplasmic extracts from NTG (n = 4) and Rac1 (n = 4) ventricles. Comparative analysis of protein spot mean integrated intensities among the NTG and Rac1 composite images for the cytoplasmic protein subtype revealed 23 potential protein spot alterations (Fig. 1). Twelve protein spots fulfilled the criteria set forth for robustness within and between experimental groups outlined in the method and had sufficient quantity for identification (Table 1). Examples of proteins with over a 1.5-fold mean integrated intensity difference between Rac1 and NTG composite images that increased in quantity are tubulin α- and β-chains and malate dehydrogenase (MDH) or that decreased in quantity are manganese superoxide dismutase (MnSOD) and a homolog to ATP synthase-δ. Additional proteins were present only in one experimental group. This includes endoplasmin precursor, which is detected only in Rac1 ventricles. Figure 2 shows a comparison of the composite images and a representative silver-stained gel for the region of the gels that both tubulin α- and β-chains are resolved as well as the verified increase in the β-chain by immunohistochemical analysis of ventricular tissue from NTG and Rac1 mice (Fig. 2C).

Proteomic Analysis of the Myofilament Protein-Enriched Extract at Day 14

Figure 3 shows the composite image of the silver-stained gels of the myofilament protein-enriched extract obtained from the ventricles of 14-day-old Rac1 mice. The arrows indicate key myofilament protein spots that were identified by MS. However, like several critical myofilament proteins, troponin I
Table 1. Identification of protein changes in cytoplasmic extract from Rac1 transgenic compared with NTG mice

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Protein Identified</th>
<th>Observed (O)</th>
<th>Theoretical and (T) pI/MM</th>
<th>MALDI-TOF Sequence Coverage, %</th>
<th>MS/MS Ion and Sequence Tag</th>
<th>Confidence of Correct ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Endoplasmic precursor (GRP94); present in Rac1, absent in NTG</td>
<td>O: 5.4/97 kDa</td>
<td>T: 4.7/92 kDa</td>
<td>43%</td>
<td>N/D</td>
<td>High</td>
</tr>
<tr>
<td>2</td>
<td>Serum albumin precursor; increased 1.8-fold in Rac1; (P &lt; 0.05)</td>
<td>O: 6.9/74 kDa</td>
<td>T: 5.8/69 kDa</td>
<td>51%</td>
<td>m/z 499.8 (2+): TPSEHVHTK (13C peak); m/z 575.3 (2+): LVQETDFAK; m/z 740.4 (2+): LGEYGFQNALLVR</td>
<td>Very High</td>
</tr>
<tr>
<td>3</td>
<td>Tubulin β-5 chain; increased 2.5-fold in Rac1 (P &lt; 0.05)</td>
<td>O: 5.9/55 kDa</td>
<td>T: 4.8/50 kDa</td>
<td>65%</td>
<td>m/z 539.3 (2+): LREEYDPR; m/z 565.8 (2+): FPGQLNADLR</td>
<td>Very High</td>
</tr>
<tr>
<td>4</td>
<td>Tubulin α-6 chain; increased 2.5-fold in Rac1; (P &lt; 0.05)</td>
<td>O: 6.0/58 kDa</td>
<td>T: 4.9/50 kDa</td>
<td>38%</td>
<td>m/z 851.9 (2+): VDLE</td>
<td>High</td>
</tr>
<tr>
<td>5</td>
<td>Creatine kinase M-chain; increased 1.6-fold in Rac1 (P &lt; 0.05)</td>
<td>O: 7.0/45 kDa</td>
<td>T: 6.6/43 kDa</td>
<td>63%</td>
<td>m/z 893.6 (2+); EVEQVQLVVDGVK; m/z 616.3 (2+); FDPLL</td>
<td>Very High</td>
</tr>
<tr>
<td>6</td>
<td>Aldose reductase; increased 2.5-fold in Rac1 (P &lt; 0.05)</td>
<td>O: 7.4/36 kDa</td>
<td>T: 6.8/36 kDa</td>
<td>14%</td>
<td>m/z 556.8 (2+): SPPGQVTEAVK</td>
<td>High</td>
</tr>
<tr>
<td>7</td>
<td>Malate dehydrogenase; increased 2.0-fold in Rac1 (P &lt; 0.05)</td>
<td>O: 6.4/34 kDa</td>
<td>T: 6.1/36 kDa</td>
<td>22%</td>
<td>N/D</td>
<td>High</td>
</tr>
<tr>
<td>8</td>
<td>gi21312004 RIKEN cDNA 0610009116 ISS-homolog to electron transfer flavoprotein β-subunit; present in NTG, absent in Rac1</td>
<td>O: 9.0/25 kDa</td>
<td>T: 8.6/27 kDa</td>
<td>19%</td>
<td>670.4 (2+): VLSVEE, c-term-R; 648.3 (2+): VP, ends with LR</td>
<td>High</td>
</tr>
<tr>
<td>9</td>
<td>RAB-11B; present in NTG, absent in Rac1</td>
<td>O: 5.5/24 kDa</td>
<td>T: 5.6/24 kDa</td>
<td>18%</td>
<td>N/D</td>
<td>Medium</td>
</tr>
<tr>
<td>10</td>
<td>Gi:12836768/dbj/BAB2 3806.1 homologous to ATP synthase δ-chain, mitochondrial precursor; decreased 1.8-fold in Rac1 (P &lt; 0.05)</td>
<td>O: 4.4/17 kDa</td>
<td>T: 4.7/16 kDa</td>
<td>24%</td>
<td>N/D</td>
<td>High</td>
</tr>
<tr>
<td>11</td>
<td>Manganese superoxide dismutase; decreased 2.1-fold in Rac1 (P &lt; 0.05)</td>
<td>O: 8.5/22 kDa</td>
<td>T: 8.8/25 kDa</td>
<td>22%</td>
<td>m/z 502.80 (2+): NVRPDYLK; m/z 514.80 (2+): GELLEALKR; m/z 720.90 (2+): VTTQVALQPALK</td>
<td>Very High</td>
</tr>
<tr>
<td>12</td>
<td>NADP⁺-specific isocitrate dehydrogenase; present in NTG, absent in Rac1</td>
<td>O: 9.4/49 kDa</td>
<td>T: 8.9/51 kDa</td>
<td>19%</td>
<td>N/D</td>
<td>Medium</td>
</tr>
<tr>
<td>13</td>
<td>Fibrinogen-β (N/C)</td>
<td>O: 6.6/53 kDa</td>
<td>T: 6.7/55 kDa</td>
<td>37%</td>
<td>N/D</td>
<td>High</td>
</tr>
<tr>
<td>14</td>
<td>β-Enolase (N/C)</td>
<td>O: 6.9/47 kDa</td>
<td>T: 6.8/47 kDa</td>
<td>58%</td>
<td>N/D</td>
<td>Very High</td>
</tr>
<tr>
<td>15</td>
<td>ATP synthase β-chain (N/C)</td>
<td>O: 5.1/58 kDa</td>
<td>T: 5.1/56 kDa</td>
<td>72%</td>
<td>N/D</td>
<td>Very High</td>
</tr>
<tr>
<td>16</td>
<td>Actin (N/C)</td>
<td>O: 5.3/43 kDa</td>
<td>T: 5.2/42 kDa</td>
<td>48%</td>
<td>N/D</td>
<td>High</td>
</tr>
<tr>
<td>17</td>
<td>Tropomyosin (N/C)</td>
<td>O: 4.6/34 kDa</td>
<td>T: 4.7/33 kDa</td>
<td>33%</td>
<td>N/D</td>
<td>High</td>
</tr>
<tr>
<td>18</td>
<td>Myosin light chain 1 (N/C)</td>
<td>O: 5.1/26 kDa</td>
<td>T: 5.0/23 kDa</td>
<td>70%</td>
<td>N/D</td>
<td>Very High</td>
</tr>
<tr>
<td>19</td>
<td>Myosin light chain 2 (N/C)</td>
<td>O: 4.9/20 kDa</td>
<td>T: 4.8/18 kDa</td>
<td>87%</td>
<td>N/D</td>
<td>Very High</td>
</tr>
</tbody>
</table>

* MALDI-TOF sequence coverage (%) refers to the extent of the amino acid sequence of the candidate protein that is encompassed by the fragments derived from the protein spot of interest and observed by matrix-assisted laser desorption ionization time of flight analysis. **MS/MS ion and sequence tag refers to the amino acid sequence of a given peptide obtained from tandem mass spectrometry and derived from the protein spot of interest that matches an amino acid region of a candidate protein.* For confidence of correct identification (ID), “very high” signifies that multiple sequence tags were used for identification; “high” signifies that one sequence tag was used for identification or peptide coverage over 20%; “medium” signifies that at least 2 of 3 of the following parameters have been determined: highly matched observed and theoretical pI/MM, MALDI-TOF sequence coverage (%) below 20%,” and/or MS/MS ion and sequence tag. **Confidence of correct ID denotes that the identification of the protein spot of interest could not be confirmed due to an insufficient amount of protein sample even when 5–10 gel spots from multiple gels were combined. **ND refers to “not determined” and signifies that the parameter could not be determined due to an insufficient amount of protein sample. **N/C refers to protein and signifies that no significant change was measured between Rac1 and nontransgenic (NTG) gels. pI/MM, isoelectric point/molecular mass; m/z, mass-to-charge ratio.
(TnI), troponin C (TnC), and troponin T (TnT) do not stain well with silver (16). Western blot analysis was carried out on the 2-DE (pH 3–10 and 12% SDS-PAGE) (Fig. 4) of the myofilament protein-enriched extract obtained from Rac1 (n = 3) and NTG (n = 3) mice ventricles. The single protein spots observed for TnT, TnI, and myosin light chain 1 (MLC1) were aligned in both the Rac1 and NTG hearts, indicating that the posttranslational modification (PTM) status of these myofilament proteins is unchanged in response to overactivation of active Rac1 (Fig. 4A). Interestingly, MLC2 (Fig. 3) and desmin (Fig. 4, B and C) were observed as two spots, suggesting that they are modified in cardiac tissue. Dephosphorylation of myocardium homogenate, using alkaline phosphatase, resulted in a shift in the intensity of the most acidic spot toward the most basic spot for desmin (data not shown), suggesting it is phosphorylated. For both proteins there was no change in quantity of either spot with the Rac1 vs. NTG hearts. To determine if differences exist in expression level of the myofilament proteins between the NTG and Rac1 mice, a quantitative Western blot analysis was preformed. Analysis of five myofilament proteins from whole tissue extracts revealed an increased abundance in the Rac1 mice, from 1.3-fold in TnI to 2.4-fold in TnT (Fig. 5).

Temporal Changes on Selective Proteins Change During Development of Dilated Cardiomyopathy

To provide insight on the temporal alterations associated with the development of the Rac1 phenotype, a time course analysis of protein expression was undertaken. Three proteins, β-tubulin, MnSOD, and MDH, were selected based on the following criteria: 1) changes observed at 14 days by 2-DE analysis, 2) representativeness of different protein classes found to change at 14 days, and 3) the availability of quality antibodies. All three proteins underwent their maximum increase in protein quantity from days 7 to 9 (Fig. 6, A–C). For example, at day 7 both β-tubulin and MDH are approximately the same quantity in the Rac1 and control ventricles, but by day 9, β-tubulin and MDH increase 5- and 2.5-fold, respectively, in the ventricles from the Rac1 compared with the NTG ventricles. These proteins remain elevated at day 11. The increased quantity of β-tubulin and MDH observed in whole tissue is in agreement with the increase in the amounts of these proteins observed in the 2-DE analysis of the cytoplasmic protein-enriched extract of the ventricle of the Rac1 and NTG hearts (Table 1) and the immunohistochemistry of tubulin (Fig. 2) at day 14. Taken together, these findings suggest that β-tubulin
and MDH increase early in the development of hypertrophy (between days 7 and 9) and remain elevated over the course of the disease.

Between days 7 and 9, the quantity of MnSOD also increased in the ventricle of the Rac1 heart. At day 9, where there is the largest difference in the quantity of MnSOD between the two experimental animals, there is also a subproteome distribution difference. Rac1 mice displayed a decrease in localization of MnSOD to the membrane-rich pellet extract, while showing an increase in the cytoplasmic protein-enriched extract compared with the NTG mice (Fig. 6D). These findings suggest that MnSOD may be modified during the progression of the phenotype and further highlight the importance of the 7- to 9-day period of development.

**DISCUSSION**

**Model and Analytical Method Issues**

With any study, one must balance the pros and cons when choosing an experimental model and the analytical strategy and technology. The choice of model was based on previous published data (36) in which the overexpression of Rac1 results in early and sudden mortality of the transgenic mice from extreme dilated phenotype at ~2 wk. This precise timing of phenotype is beneficial for proteomic analysis especially when analyzing longitudinal samples. Certainly, this time frame differs from the “typical progression” of heart failure in genetic and other experimental models that developed hypertrophy over a longer time window (6 wk or longer). One can speculate that in the Rac1 animals the heart was unable to sufficiently compensate to allow for survival. Comparisons of proteomic data from other heart failure models that evolve over a slower time period will allow us to tease apart the differential and compensatory mechanisms underlying the development of heart failure.

Proteomics is limited by the depth of the proteome (and consistency of the model) that can be annotated by any particular technical method, including 2-DE. Subproteome analysis is a well-documented strategy used to increase proteome depth. In the heart it is especially difficult to observe lower abundant proteins, given that the myocyte proteome is dominated by myofilament proteins. Subproteomic analysis is limited by the robustness of the extraction and the minimization of introduction of artificial PTM. The extraction method IN Sequence was designed specifically for cardiac muscle to meet these requirements (1, 2, 23), especially compared with more traditional methods for myofilament removal (31). 2-DE, although widely used, is limited in its ability to detect proteins with extreme pI, mass, and hydrophobicity. As such, 2-DE only detects a small fraction of the myocardial proteome. Therefore, further analysis of alternative subproteomes and use of other proteomic methods in combination with 2-DE will allow one to dig deeper into the proteomic landscape of the heart.

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**Fig. 3.** 2-DE analysis of myofilament subproteome. Composite image of silver-stained 2-DE gel images (pH 3–10, 12% SDS-PAGE) derived from analysis of myofilament protein-enriched extracts of ventricular tissue from Rac1 transgenic mice. Arrows indicate the positions of key myofilament protein spots; protein identifications are presented in Table 1. Procedures are outlined in MATERIALS AND METHODS.

**Fig. 4.** Posttranslational modification (PTM) analysis of myofilament proteins. Absence of change in extent of modification of troponin T (TnT), troponin I (TnI), MLC1, and desmin in Rac1 transgenic compared with NTG mice. A: Western blots of 2-DE (pH 3–10, 12% SDS-PAGE) of ventricular tissue derived from NTG and Rac1 transgenic mice for TnT (top), TnI (middle), and MLC1 (bottom). B: Western blots of 2-DE of ventricular tissue derived from NTG (top) and Rac1 transgenic (bottom) mice for desmin. C: equivalent region of a representative silver-stained 2-DE gel of the myofilament protein-enriched extract from an NTG heart. Cropped images show ~2 pH units; observed pIs for TnT, TnI, MLC1, and desmin are ~5, 5.5, 4.7, and 5, respectively. Procedures are outlined in MATERIALS AND METHODS.
deeper into the changes underlying this severe hypertrophy phenotype.

In addition to increasing the depth of proteome observed, subproteomic analysis also allows one to detect alterations in protein localization or physical characteristics as proteins moved between extracted fractions. This cannot be detected using whole tissue analysis. In the case of MnSOD, a distinct shift in solubility was observed at 9 days of development. In the Rac1 ventricles, the majority of MnSOD was found to be located in the cytoplasmic protein-enriched extract, whereas in the NTG mice, the protein was distributed between the cytoplasmic and pellet extracts (Fig. 6D). This observation would not be possible if only a whole tissue homogenate or a single subproteome was analyzed.

Numerous protein spots in the cytoplasmic protein-enriched extract were found to change by 2-DE but were eliminated from further analysis. In some instances, the protein changes were large but not consistent between the majority (75%) of Rac1 animals. This lack of a consistent, albeit robust, response across animals may be due to genetic variation between animals and/or the stage of disease. However, the majority of the proteins not meeting the selection criteria were eliminated due to the technical limitations of 2-DE, i.e., either poor resolution on 2-DE and/or low abundance, making protein quantification and/or identification difficult. The poor protein spot resolution is a well-known technical limitation of 2-DE, especially when using broad pH range IEF, such as pH 3–10. Protein spot resolution can be improved through the use of narrower pH (IEF) gradients (47). Narrow gels were not used in this study due to limitations in the quantity of protein obtained by IN Sequence extraction of the cytoplasmic protein-enriched extract. For several proteins, spots of sufficient quantity to allow MS identification could not be obtained even after combining the same protein spot from multiple gels (from 5 to 10 gels).

Additional issues arise with the analysis by 2-DE analysis of mixtures (subproteomes) containing a small number of proteins, and the mole ratio (or concentration) of these proteins differ with respect to each other over a small dynamic range (<10-fold); this is the case for the myofilament extract. Although 2-DE still allows PTM status determination, it is limited in the ability of quantification of changes between two experimental groups especially if the entire subproteome is...
altered to the same degree (so that the ratios between the various proteins remain the same). This can be the case in hypertrophy where the number of myocytes remains constant, while the myofilaments increase proportionally with the size of the expanding myocyte. The reason for the difficulty with 2-DE is that 1) total protein rather than number of myocytes is loaded onto the 2-DE and 2) the quantity of each protein spot is determined in relation to the total quantity (or intensity) of the subproteome. Hence, the myofilament subproteome on equally loaded gels of Rac1 or NTG ventricles will be quantitatively equal regardless of the relative amount of myofilament protein across conditions. To minimize but not eliminate this problem, quantitative analysis of the myofilament subproteome can be carried out on whole tissue homogenates of ventricular tissue, where observation of the subproteome occurs within the context of the entire cellular proteome. The limitation is that other cellular proteins will comigrate with the various myofilament proteins, adding to the intensity of each spot or band. To avoid this problem, Western blot analysis is required. The ventricles from the Rac1 hearts showed pronounced increases in MLC1, desmin, TnT, and TnI compared with the NTG hearts, relative to other cellular proteins (Fig. 5). Of note, the absolute fold change for the myofilament proteins comprising the thick and thin filaments was not equivalent.

Proteome Changes: End-Stage Dilated Cardiomyopathy

Twelve cytoplasmic proteins, along with the myofilament proteins, were found to be consistently altered in the Rac1 hearts at day 14 that were involved in 1) metabolism, 2) antioxidation, 3) structure, and 4) contraction. It is noteworthy that the majority of protein alterations reported herein have not been shown to occur in other proteomic studies of heart failure, and, furthermore, many have never been linked to heart failure.

Metabolism. Aldose reductase, a cytoplasmic protein that catalyzes the reduction of a wide variety of carbonyl-containing proteins to their alcohol form via an NADPH-dependent mechanism (reviewed in Ref. 18) is elevated in the myocardium of Rac1 transgenic compared with NTG mice. Although aldose reductase has not previously been implicated in heart failure, it has been shown to be increased in myocardium of animal models of cardiac ischemia-reperfusion injury, and inhibition of this enzyme enhances glycolysis and glucose oxidation (14). The enzyme NADP+-specific isocitrate dehydrogenase, which catalyzes the conversion of isocitrate to α-ketoglutarate for the production of ATP by the tricarboxylic acid (TCA) cycle (reviewed in Ref. 29), was not observed in the Rac1 composite image, suggesting a pronounced decrease in the amount of this protein in myocardium of Rac1 transgenic mice. Thus a decrease in NADP+-specific isocitrate dehydrogenase would (like that of an increase in aldose reductase) tend to decrease the rate of respiration and production of ATP by cardiac myocytes. Conversely, MDH, also part of the TCA cycle [catalyzes the conversion of malate to oxaloacetate (2)] and creatine kinase M-chain (which catalyzes the transfer of a phosphate moiety from phosphagens to form ATP) are increased in myocardium of Rac1 transgenic compared with NTG mice. These two proteins are present in both the cytoplasm and mitochondria, and although they are involved in conservation and preservation of ATP concentrations (21, 28, 44, 45), their exact roles in pathophysiology may differ depending on cellular location. MDH increased in whole tissue by day 9 and remained elevated throughout the course of disease development, suggesting a role in balancing ATP production and regeneration. It is noteworthy that in a patient population displaying dilated cardiomyopathy, there were significant increases in enzyme activities of creatine kinase and MDH in the myocardium, which correlated with ejection fraction (43).

Antioxidants. MnSOD, a protein primarily localized to the mitochondria and involved in the scavenging of superoxide radicals (32), had a dynamic expression pattern during the development of the Rac1 phenotype. Rac1 mice hearts demonstrated an increase at 9 days that was subsequently reduced through 11 and 14 days (Fig. 6B, Table 1). Our results are consistent with findings that during the progression of heart failure, antioxidant capacity, including MnSOD, increases in the compensated hypertrophic heart (8, 9) and then decreases once the failure becomes established (13). It has been suggested that an inadequate antioxidant reserve may play a role in the transition from hypertrophy to heart failure (8, 21, 33). This concept is supported by an SOD knockout mouse that was found to be lethal because of dilated cardiomyopathy (17).

At 9 days, a subproteome distribution difference was observed for MnSOD, such that its level was higher in the cytoplasmic protein-enriched extract compared with the pellet, and the difference was greater for Rac1 mice than NTG mice (Fig. 6D). This suggests that there is a change in the solubility and/or localization of MnSOD with progression of the dilated phenotype in Rac1 mice. It is possible that the enhanced solubility of MnSOD in the cytoplasmic extract may be due to a PTM. Indeed, MnSOD has been observed to be tyrosine nitrated in the oxidizing conditions of human kidney allograft rejection and human pancreatic ductal adenocarcinoma (18, 19), but it remains to be seen if this modification plays a role in heart failure.

Structural dynamics. Tubulin α- and β-chains, the monomer proteins of the tubulin heterodimer molecule, increased by day 9, early in the development of the Rac1 lethal phenotype, and remained elevated at day 14. The increase in tubulin in Rac1 compared with NTG mice was further confirmed by immunohistochemical analysis (Fig. 2C). Tubulin exists as a polymer forming microtubules (a major component of the cytoskeleton in the myocyte) and has been found to increase in human and animal models of cardiac hypertrophy and failure. The enhanced tubulin polymerization and microtubule formation correlated to a concomitant increase in cytoskeletal stiffness (12, 37, 41, 46). Interestingly, in several animal models of cardiac hypertrophy and failure, treatment of myocytes with colchicine, an inhibitor of tubulin polymerization, reversed myocyte stiffness and normalized contraction dynamics (15, 25, 27, 38, 39, 40). Moreover, treatment of myocytes with taxol, an agent that enhances tubulin polymerization and microtubule formation, resulted in a phenotype similar to that which is induced by pressure-overload hypertrophy (25, 27, 39, 40). Desmin, an intermediate filament protein (reviewed in Ref. 33), was increased in Rac1 compared with NTG mice. This is also consistent with previous studies of animal models in which an increase was observed during both the development of cardiac hypertrophy and the transition from cardiac hypertrophy to failure (7, 25, 27, 46). It is noteworthy that desmin appeared to be phosphorylated in the myocardium. Desmin is known to be
phosphorylated by a number of kinases (24), including p21-activated kinase, which is activated by Rac1 (5). Although this is the first in vivo report of desmin being potentially phosphorylated in cardiac muscle, the ratio between the two desmin spots was not different between the transgenic and NTG animals. It is possible that the functional abnormalities associated with the dilated phenotype of Rac1 mice may be related to the hyperpolymerization of structural proteins such as tubulin and desmin. Specifically, densification of the microtubule and intermediate filament networks of the myocyte would likely impose a greater load on the myocyte and act to reduce the compliance of the cell and impede sarcomere motion and force production. Thus increased tubulin and desmin protein levels in Rac1 mice may represent an important inciting mechanism for heart failure, especially because we observed tubulin to increase early in the development of the Rac1 phenotype.

**Force Production**

An unexpected finding of this study was the absence of additional modified forms of myofilament proteins in myocardium of Rac1 compared with NTG mice. Neither TnI nor MLC1 (proteins that are known to be phosphorylated under some conditions in cardiac myocytes) were found to be modified in Rac1 transgenic compared with NTG mice (2, 35). However, we provide evidence of a substantial increase in overall quantity of total myofilament proteins. The various myofilament proteins increased disproportionately perhaps because of the differential rates of processing of the various proteins. These proteins may differ in rates of translation, assembly, folding, turnover, and incorporation into sarcomeres, all of which would affect the probability of their detection by Western blotting. Indeed, the nature of cardiac muscle protein incorporation into sarcomeres of fully differentiated cardiac myocytes is quite varied (20).

In conclusion, a number of alterations in protein abundance occur in the myocardium of Rac1 transgenic compared with NTG mice, which encompass numerous cellular protein networks and functions. The profile of protein alterations in Rac1 transgenic mice suggests the involvement of cytoskeletal, myofilament, antioxidant, and metabolic proteins in mechanisms of heart failure. These proteins may act individually to either promote or compensate for the dilated phenotype. However, the phenotype of dilated cardiomyopathy likely results from interplay of cell signaling pathways comprising these protein alterations. The results of this study suggest an important role for structural abnormalities in the induction of cardiac hypertrophy and failure and, furthermore, highlight the potential relevance of Rac1 signaling as an inciting stimulus of structural alterations associated with cardiac hypertrophy and failure.

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