In Vivo Modeling of Myosin Binding Protein C Familial Hypertrophic Cardiomyopathy

Qinglin Yang, Atsushi Sanbe, Hanna Osinska, Timothy E. Hewett, Raisa Klevitsky, Jeffrey Robbins

Abstract—Myosin binding protein C (MyBP-C) is an integral part of the striated muscle sarcomere. As is the case for other sarcomeric genes in human populations, multiple mutations within the gene have been linked to familial hypertrophic cardiomyopathy. Although some MyBP-C lesions are the result of missense mutations, most show truncated polypeptides lacking either the myosin or myosin and titin binding sites. Previously, we generated transgenic (TG) mice with cardiac-specific expression of a MyBP-C mutant lacking the myosin and titin binding domains. Surprisingly, the mutant protein was stable and made up a majority of the MyBP-C species, with concomitant reductions in endogenous MyBP-C such that overall MyBP-C stoichiometry was conserved. In the present study, we created a second series of TG mice that express, in the heart, a mutant MyBP-C lacking only the myosin binding site. In contrast to the previous data for the MyBP-C lacking both titin and myosin binding sites, only very modest levels of protein were found, consistent with data obtained from human biopsies in which mutated MyBP-C could not be detected. Despite normal levels of wild-type MyBP-C, there were significant changes in the structure and ultrastructure of the heart. Fiber mechanics showed decreased unloading shortening velocity, maximum shortening velocity, and relative maximal power output. (Circ Res. 1999;85:841-847.)

Key Words: transgenic ■ myosin ■ cardiac disease ■ muscle

Myosin binding protein C (MyBP-C) is a major protein component of the striated muscle sarcomere. Initially isolated as a contaminant during myosin purification, it can make up as much as 2% to 4% of the myofibril’s protein complement. Three isoforms of MyBP-C, fast skeletal, slow skeletal, and cardiac, exist and are encoded by unique genes. Although the protein’s function(s) remain obscure, it appears likely that it plays important structural and signaling roles in each of these muscle types. MyBP-C can participate in thick filament assembly through several protein-protein interactions and thus may play a role in maintaining sarcomere stability. MyBP-C binds with myosin subfragment-2 and light meromyosin and also interacts with titin (or connectin). Recently, it was shown that fragments of the protein can bind to subfragment-2 of myosin and that the regulatory function of MyBP-C may be mediated by this interaction. As is the case for other myosin binding proteins and titin, MyBP-C belongs to the intracellular immunoglobulin (Ig) superfamily and is composed of repeated Ig and fibronectin domains. The last domain at the carboxyl terminus contains a myosin binding site whereas the last three domains are responsible for mediating binding to titin. The cardiac isoform is of particular interest, both structurally and functionally. It is composed of 11 Ig and fibronectin domains with an extra N-terminus Ig domain compared with the skeletal isoform. Near the amino terminus, there is a 9-amino acid insertion that can be phosphorylated and a 28-amino acid linker region (Figure 1). The cardiac isoform is expressed only in the heart at all developmental stages in both humans and mice. It is also one of the eight sarcomeric proteins in which mutations have been identified as causing familial hypertrophic cardiomyopathy (FHC). More than 30 mutations in the cardiac MyBP-C gene have been found in various FHC patients, with most of them resulting in truncated forms of MyBP-C missing either the carboxyl myosin binding site or the myosin and titin binding domains. In addition to its hypothesized role in maintaining overall sarcomere stoichiometry, cardiac MyBP-C may play a role in modulating the heart’s short-term responses to external stress, given that the protein can be phosphorylated under adrenergic stimulation. These posttranslational modifications can, via alterations in myosin crossbridge architecture, modify force production.

The MyBP-C class of FHC mutations is relatively benign, with most affected individuals remaining asymptomatic into their fifties and sixties. Compared with the patient populations displaying FHC caused by mutations in the other sarcomeric proteins, studies indicate that the MyBP-C patients have later onset, lower penetrance, a better prognosis, and longer life expectancies. Because MyBP-C FHC can be completely asymptomatic, it is possible that it is more prevalent than previously thought, and, thus, it becomes...
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protein showed normal stability and made up 40% to 60% of
the normal and the truncated MyBP-C (MyBP-C.mu2) missing the 240 nucleo-
tides at the 3’ end of the cDNA that constitute the myosin binding
domain were inserted into a vector containing the α-myosin heavy
chain (α-MyHC) promoter.25 The human myc epitope was placed
after the initiator methionine residue in both constructs. The final
constructs were digested free of vector sequence with Nol, purified
from agarose gels, and used to generate TG mice as described.26

Materials and Methods
DNA Constructs and TG Mice
A cDNA clone encoding the murine cardiac MyBP-C (GenBank
accession No. AF059576) was used to prepare TG constructs with
polymerase chain reaction. The full-length MyBP-C (MyBP-C.wt)
and the truncated MyBP-C (MyBP-C.mu2) missing the 240 nucleo-
tides at the 3’ end of the cDNA that constitute the myosin binding
domain were inserted into a vector containing the α-myosin heavy
chain (α-MyHC) promoter.25 The human myc epitope was placed
after the initiator methionine residue in both constructs. The final
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Protein and Phenotypic Analyses
For SDS-PAGE electrophoresis, the left ventricular apex and atrial
flaps were obtained from TG mice and nontransgenic (NTG)
littermates. FVB/N mice were obtained from Taconic (Germantown,
NY) and were housed in germ-free barrier facilities that met or
exceeded AAALAC regulations. Samples enriched for the myofibril-Protein and Phenotypic Analyses

Using transgenesis, we previously attempted to model the
disease by replacing ~50% of the wild-type cardiac protein
with a mutant lacking the myosin and titin binding sites.23
Although a single clinical study reported no detectable
protein from an FHC MyBP-C patient,24 the transgenic (TG)
protein led to decreased levels of endogenous MyBP-C. Thus,
we were unable to distinguish whether the phenotype was due
to the presence of a "poison peptide," a lack of normal
amounts of the wild-type protein (haploinsufficiency), or a
combination of both. In any case, the mice displayed a
striking pattern of sarcomere disorganization and dysgenesis.

In the present study, we generate TG mice in which a
different class of MyBP-C FHC mutants is expressed speci-
ically in the heart. In contrast to the MyBP-C mutant lacking
both the myosin and titin binding sites, only very low levels
of the TG protein could be detected in the heart, consistent
with the clinical report, in which no detectable mutant protein
could be found. The low levels of mutant MyBP-C protein
could, however, be detected in the cardiac sarcomeres and
caused structural and functional alterations. Because the very
low levels of mutant polypeptide resulted in conservation of
essentially wild-type levels of normal protein, we conclude
that the mutant protein mediates its effect by acting as a
poison polypeptide.

Results
Expression of Mutated MyBP-C
TG mice that express the normal, full-length cardiac MyBP-C
(line 2/1:MyBP-C.wt) in the heart have been described

important to model the disease in an animal such that its
development and progression can be rigorously studied.

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Although a single clinical study reported no detectable
protein from an FHC MyBP-C patient,24 the transgenic (TG)
protein showed normal stability and made up 40% to 60% of
the total MyBP-C protein pool. The transgenically encoded,
truncated protein was, however, not incorporated efficiently
into the sarcomere. Consistent with the clinical presentation,
the population appeared outwardly normal, but TG muscle
fibers showed a leftward shift in the pCa2

Figure 1. Transgene structure and expression. A, Schematic
representation of the TG constructs used. Two MyBP-C
transgenes, encoding either the mouse cardiac wild-type (MyBP-
C.wt) or truncated (MyBP-C.mu2) proteins were linked to the
cardiac-specific α-MyHC promoter. hGH indicates human
growth hormone polyadenylation signal sequence. B, MyBP-
C.mu2 transcript levels from the different lines were quantitated
using total cardiac RNA derived from 8-week-old animals. The data were corrected for loading varia-
tions using the glyceraldehyde-3-phosphate transcript as refer-
ence and are expressed as fold increases over that of the en-
dogenous MyBP-C transcript as described previously.26
Transcript levels varied from 1.5- to 5-fold. The data are com-
pared with that obtained from the MyBP-C.wt line (wt), which
was used as a control throughout the present study. C, TG pro-
tein analyses. To detect the TG protein, Western blots using
anti-myc mAb were performed. MyBP-C.mu2 protein was pres-
ent in very low amounts in all of the TG lines, despite robust
transcript expression. Note that despite essentially equal tran-
script levels for line 133 and the MyBP-C.wt line, the normal
transgenically encoded protein (wt) is present in much higher
concentrations than found for the MyBP-C.mu2 species.
previously and were used, along with NTG littermates, in control cohorts throughout these studies. Although MyBP-C.wt mice have ~5-fold increases in MyBP-C transcript, overall MyBP-C protein stoichiometry is rigorously maintained, and the mice are indistinguishable from NTG mice in all respects when analyzed at the molecular, biochemical, cellular, whole organ, and whole animal levels. The full-length construct served as the starting point for construction of the truncated MyBP-C (Figure 1A). The truncated protein is normal in all respects except that it lacks the terminal 80 amino acids that encode a myosin binding domain. This construct, termed MyBP-C.mu2, was used to generate multiple lines of TG mice (Figure 1B). Founders were subsequently outbred to NTG littermates to confirm germline transmission and establish stable lines. Hearts from the F1s were used to isolate RNA and determine expression levels of the TG MyBP-C RNA, relative to the endogenous message. These are compared with the expression level in the MyBP-C.wt line (Figure 1B). Transcript levels of MyBP-C.mu2 varied between 1.5- to 5-fold of the endogenous MyBP-C; these levels were consistent with levels observed previously for a related construct.

To detect the TG protein, Western blots using anti-myc mAb were performed on total cardiac protein. Surprisingly, very little TG protein could be detected in the TG-derived material (data not shown). In an attempt to increase sensitivity, myofilament protein preparations were then subjected to analysis. In contrast to the mice expressing MyBP-C.wt, none of the newly generated TG lines showed significant levels of the truncated protein (Figure 1C). This is in direct contrast to our previous results obtained with a construct lacking both the myosin and titin binding sites. It is, however, consistent with the clinical finding that mutated protein could not be detected in endomyocardial biopsies derived from the affected patient’s left ventricle. Because levels of the TG protein were so low, endogenous MyBP-C levels were essentially unaffected across all of the TG lines, and normal MyBP-C stoichiometry was observed for all of the TG lines (data not shown).

The steady-state levels of the mutant MyBP-C protein were significantly less than what was expected on the basis of transcript levels. Western blot analyses using both the total and myofilament protein complements confirmed that the little protein that was present copurified with the myofilament proteins (Figure 1C). To precisely localize the mutant protein’s location within the sarcomere, confocal analyses were performed (Figure 2). Staining with anti-myc resulted in a doublet-staining pattern in the A band, a pattern characteristic of endogenous MyBP-C (Figure 2A). Doublet staining using anti-desmin (located at the Z line) confirmed that the transgenically encoded MyBP-C is incorporated into the correct sarcomeric location (Figure 2C) and that incorporation of the endogenous MyBP-C protein is unaffected in these mice.

**Figure 2.** Immunofluorescent microscopy. Hearts from both MyBP-C.mu2 and MyBP-C.wt mice (line 2/1) as well as NTG littermates were perfused with relaxing buffer, fixed with 4% paraformaldehyde, embedded in O.C.T. compound, and snap-frozen. All experiments were carried out on multiple, adult (8- to 16-week-old) animals (n=4 to 7). Multiple lines showed similar patchy staining patterns; a representative section (5 μm) from a line 133, 12-week-old MyBP-C.mu2 mouse is shown. The sections were stained with antibody before being subjected to confocal microscopy. A, Positive staining could be detected in the MyBP-C.mu2 heart section using anti-myc mAb. A doublet-staining pattern localized to the A band is consistent with the location of endogenous MyBP-C. B, The same heart section is stained for desmin, which is localized at the Z line of the sarcomere. C, Overlay of the anti-myc and anti-desmin antibodies confirms that the small amount of MyBP-C.mu2 present is correctly incorporated into the A band of the sarcomere. D, Normal staining pattern for wild-type MyBP-C. Magnification: A through D, ×80; D, ×100.

**TQ Protein Expression Leads to Structural and Biochemical Alterations in the Sarcomere**

Cardiac sarcomeres from multiple mice from lines 19, 24, and 133 were examined using transmission electron microscopy, as similar studies carried out on our previous FHC MyBP-C mutants revealed significant deficits in sarcomere organization. Sarcomeres from each of the lines showed similar abnormalities: a representative section from an MyBP-C.mu2 mouse (line 24, 35 weeks old) illustrates the significantly altered sarcomere arrangements that could be commonly observed (Figure 3). Normally, sarcomeres are in exact register with one another, as displayed in sections derived from either the NTG or MyBP-C.wt animals (Figure 3A and 3B). In contrast, sarcomeres derived from the MyBP-C.mu2 animals were consistently out of register and the normal pattern disrupted, as evidenced by the inability of the Z lines to retain their normal arrangements between the sarcomeric rows (Figure 3C). Normally, because of the rigid definition of the sarcomere, the entire sarcomere remains visible within the section plane. Yet, in the TG-derived material, oftentimes the thick-thin filament array appears to disappear because it is no longer held within the plane of the section (Figure 3C).

Mitochondrial abnormalities were also observed, with both the numbers and organization showing significant alterations from the patterns observed in either the NTG or MyBP-C.wt mice. These changes were reproducibly seen in all lines studied, as early as 12 weeks.
Histological studies of the lines also revealed changes in overall cardiac morphology. The adult TG hearts appeared to be globose, and mild hypertrophy in the papillary muscle and the left ventricular free wall was noted (Figure 4). There were, however, no remarkable changes in the overall cardiomyocyte and interstitial tissue morphology in these hearts. To confirm that mild cardiac hypertrophy was present, the heart weight/body weight ratios in mature animals (50 weeks old) were measured; the mice did show increased heart weight/body weight ratios (Table).

The lack of any visible morphological deficit at the fiber level led us to question whether alterations in function could be detected. Mechanical measurements on skinned fibers derived from the left ventricular papillary muscles of young adult animals (before any cardiac remodeling was apparent) showed that unloading shortening velocity was significantly decreased (Figure 5A). Similarly, fibers from the MyBP-C.mu2 mice show significantly reduced maximal unloading velocity (Figure 5B), and their maximal relative power output is also significantly decreased compared with NTG control littermates (Figure 5C). Clearly, expression of the mutated MyBP-C impairs fiber mechanics, a result that is consistent with data obtained for the cardiac MyBP-C missing both the titin and myosin binding sites.23

TG Animals Show an Impaired Exercise Response
MyBP-C mutations, when present, are oftentimes asymptomatic and heart function is essentially normal.16,21,33,34 Consistent with the disease’s presentation in human populations, we were unable to detect any differences in whole heart hemodynamics, as measured using either the Langendorff or working heart preparations, in any of the lines at any developmental time. We measured cardiac work, time to peak pressure, relaxation, and contractility35–37; all were unremarkable in these mice (data not shown). A striking characteristic

![Figure 3. Transmission electron microscopy of MyBP-C.mu2 sarcomeres. All experiments were carried out on multiple, adult (8- to 12-week-old) animals (n=2 to 4), and samples shown are from the left ventricular freewall. Multiple lines showed similar alterations in their ultrastructure. A, Cardiomyocyte ultrastructure of an NTG, age-matched littermate. B, Similar section taken from a 16-week-old, MyBP-C.wt heart. C, Representative section derived from a 35-week-old, line 24 animal. Noticeable sarcomere disarray and loss of the rigid planar geometry present in panel A can be discerned. Magnification was $\times 17000$. D, High magnification showing details of the sarcomere disarray and lack of general organization between the sarcomere tiers. Magnification: $\times 103500$.]

![Figure 4. Light microscopy of NTG and TG hearts. All experiments were carried out on multiple, adult (16- to 35-week-old) animals (n=2 to 4). Multiple lines showed similar alterations in overall cardiac structure. Shown are representative, longitudinal sections, stained with trichrome blue, derived from a 35-week-old MyBP-C.mu2 mouse (line 24) and an age-matched NTG littermate. Note the globular shape of the MyBP-C heart compared with the NTG control. There is, however, no obvious fibrosis and gross disarray. Magnification: $\times 8$.]
of FHC in the human population is its ability to cause sudden death, oftentimes during significant cardiac stress brought on by exercise. Exercise regimens are routinely used in the clinical setting as a tool to detect functional alterations in cardiac reserve, and we previously established a defined stress exercise regimen that allows us to measure an animal’s exercise capabilities in a quantitative fashion. When coupled with an implantable telemetry device (see Materials and Methods), exercise performance can be correlated in real time with an animal’s heart rate, and arrhythmias can also be detected. The MyBP-C.mu2 mice exhibited a significant impairment of their exercise capacity relative to the NTG and MyBP-C.wt cohorts (Figure 6). Heart rates of the MyBP-C.mu2 mice in response to the exercise were significantly decreased in comparison to the NTG and MyBP-C.wt cohorts (623 ± 27, 725 ± 18, and 743 ± 19 bpm, respectively; P < 0.001). However, detailed examination of the ECG records did not reveal any discernible differences when the animals were at rest, and no significant arrhythmias could be detected during or after exercise.

Discussion

FHC can be caused by multiple mutations in a number of different contractile proteins and because of this, has been termed a “disease of the sarcomere.” Although the genetic pathogenesis has thus been precisely defined, the altered structure-function relationships and the resultant pathogenic processes remain obscure. Creation of suitable animal models may allow determination of these relationships and could offer considerable insight into the disease’s progression over the animal’s lifetime. MyBP-C.mu2 mice may be valuable for determining the normal functions of a protein whose role in the sarcomere is obscure, as well as the short- and long-term

<table>
<thead>
<tr>
<th>Line</th>
<th>TG or NTG (n)</th>
<th>Body Weight, g</th>
<th>Heart Weight, mg</th>
<th>Hwt/Bwt, mg/g</th>
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<tbody>
<tr>
<td>71</td>
<td>NTG (4)</td>
<td>37.23 ± 4.34</td>
<td>157.77 ± 22.31</td>
<td>4.23 ± 0.15</td>
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<tr>
<td></td>
<td>TG (4)</td>
<td>30.7 ± 4.96</td>
<td>157.2 ± 20</td>
<td>5.14 ± 0.17*</td>
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<tr>
<td>24</td>
<td>NTG (5)</td>
<td>28.24 ± 2.57</td>
<td>122.8 ± 9.2</td>
<td>4.42 ± 0.16</td>
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<tr>
<td></td>
<td>TG (4)</td>
<td>25.93 ± 2.85</td>
<td>148.38 ± 27.2</td>
<td>5.68 ± 1.06*</td>
</tr>
<tr>
<td>MyBP-C.wt</td>
<td>NTG (4)</td>
<td>33.6 ± 5.78</td>
<td>141.6 ± 15.1</td>
<td>4.28 ± 0.63</td>
</tr>
<tr>
<td></td>
<td>TG (8)</td>
<td>31.9 ± 5.94</td>
<td>136.29 ± 20.74</td>
<td>4.31 ± 0.51</td>
</tr>
</tbody>
</table>

Shown are heart weight/body weight ratios (Hwt/Bwt) for 2 lines expressing MyBP-C.mu2 and a control cohort expressing MyBP-C.wt. NTG littermates were maintained under identical caging conditions for each of 3 experimental cohorts. Preliminary data indicated no significant bias for sex, and animals of both sexes were used for all experimental cohorts. All mice were 50 weeks old. Values are mean ± SD. *P < 0.05.

Figure 5. Skinned fiber mechanics of left ventricular papillary muscle. The kinetics of shortening and force and power development of NTG and line 133 TG fibers were measured in isolated skinned fibers. Each fiber set (n = 5) was derived from five 10-week-old animals. In each panel, the points obtained on the apparatus and the resultant histograms are shown. A, Unloaded shortening velocity was determined using the slack test. B, Maximum shortening velocity was determined as described in Materials and Methods. C, Determination of the maximum relative power that the fiber is able to develop. Statistical significance was determined using paired t test. *P < 0.05.

Figure 6. Treadmill exercise. Three cohorts of age-matched animals, derived from NTG, MyBP-C.wt (line 2/1), and MyBP-C.mu2 (line 71 and 133) were used. After determining that there were no differences between lines 133 and 71, the data were grouped. Thirty-week-old animals were subjected to a series of runs on a motorized treadmill as detailed in Materials and Methods. An inability to maintain speed at the predetermined pace resulted in the animal being carried to the back of the apparatus where “bar touches” were registered. MyBP-C mice exhibit a striking impairment of exercise capacity relative to both MyBP-C.wt and NTG animals, as evidenced by the increase in bar touches.
effects the mutation has on the ultrastructural integrity and biochemical function(s) of the heart.

It is useful to compare these results with our previous data obtained for a series of TG lines that overexpressed MyBP-C, FHC-linked mutations in which the protein lacks both the titin and myosin binding sites.23 The protein described in that report behaves in a fundamentally different way. First, unlike MyBP-C.mu2, that protein is stable and, although it is incorporated into the sarcomere, is not restricted to the A band where it is normally located. Immunofluorescent localization showed diffuse staining and/or focal staining in the I band.23 Because of weak binding, the protein did not copurify with the other multifilament proteins. In those mice, TG-encoded protein accumulated to significant levels. Consequently, endogenous MyBP-C decreased proportionally, in order to maintain overall contractile protein stoichiometry.28,29 Thus, it was not possible to distinguish whether the resultant phenotype was due to the presence of the mutated protein, decreased levels of the endogenous protein, or a combination of the two. In contrast to those results, the data from the present study allow a more unambiguous interpretation. Although mRNA levels were comparable between the various lines generated with the two constructs, protein levels were not. On the basis of our previous work with other TG lines, we think it likely that all of the TG mRNA is translated efficiently, but that only a very small (≤5%) amount of the nascent protein is incorporated stably into the sarcomeres with the unincorporated protein being degraded rapidly.42 That is, only incorporated protein is being detected in our assays. Because steady-state levels of the mutated protein are very low, endogenous MyBP-C protein levels remain essentially normal. Therefore, any resultant phenotype is almost certainly not due to haploinsufficiency effects but rather reflects the dominant negative effects of a poison polypeptide. It is, however, striking that both classes of MyBP-C mutations result in a sarcomere dysgenesis that is detectable at the ultrastructural level.

Consistent with disease presentation in the human population, the phenotype at the whole organ and whole animal levels is quite subtle and is difficult to detect before ~1 year. Although mild hypertrophy is present (Table), we were unable to detect any changes in cardiac hemodynamics in the isolated heart preparations. However, changes could be detected in isolated fibers and in cardiomyocyte ultrastructure. Expression of the mutated protein also eventually resulted in functional deficits, as detected under conditions of cardiac stress in the whole animal. The mice are compromised in their exercise capabilities, and the normal chronotropic response to running is significantly reduced with detectable bradycardia. It may be that reduced adrenergic responsiveness underlies the depressed exercise response. In light of these data, it is interesting to note that the hypertensive response in exercised FHC patients is well documented.43 Another suggested mechanism for this effect is stress activation of the mechanoreceptor on the myocardial wall. In a hypertrophic heart under severe stress, sympathetic tone is downregulated while vagal tone is reciprocally upregulated. Although there was no reduction of heart rate response observed in the FHC patients under exercise stress, this was attributed to a catecholamine surge during the exercise.44 Further studies to dissect the detailed mechanisms involved in these responses are being initiated.

We have not been able to detect any increases in morbidity and/or mortality in mice housed under barrier conditions, and no sudden deaths have been observed in animals up to 15 months old. These results are consistent with clinical observations for patients with analogous MyBP-C gene mutation. These patients display only a very mild phenotype and are often completely asymptomatic. They also generally have a better prognosis than those patients suffering from FHC that is caused by mutations in the other sarcomeric proteins such as β-MyHC or troponin T.31,22 As is the case in the human patient population, we were able to detect in the older animals a mild but significant hypertrophy. By 12 months, the heart weight/body weight ratio increased by ~20% relative to normal. Thus, the hypertrophic response is not marked and is attenuated relative to some of the other FHC models that have been reported.37,45 The MyBP-C.mu2 animals should serve as a useful model for uncovering the pathogenic processes that evolve gradually over the entire lifespan of the animal.

Acknowledgments

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