The actin filament system is essential for many cellular functions, including shape, motility, cytokinesis, intracellular trafficking, and tissue organization. Tropomyosins (Tms) are rod-like components of most actin filaments that differentially affect their stability and flexibility. The Tm gene family consists of four genes, αTm, βTm, γTm (Tm5 NM, where “NM” indicates “nonmuscle”), and δTm (Tm4). Multiple isoforms of the Tm family are generated by alternative splicing of three of these genes, and their expression is highly regulated. Extensive spatial and temporal sorting of Tm isoforms into different cellular compartments has been shown to occur in several cell types. We have addressed the function of the low-molecular-weight Tms encoded by the γTm gene by eliminating the corresponding amino-terminal coding sequences from this gene. Heterozygous mice were generated, and subsequent intercrossing of the F1 pups did not result in any viable homozygous knockouts. Genotype analysis of day 2.5 morulae also failed to detect any homozygous knockouts. We have failed in our attempts to delete the second allele and generate in vitro double-knockout cells, although 51 clones displayed homologous recombination back into the originally targeted locus. We therefore conclude that low-molecular-weight products from the γTm gene are essential for both embryonic development and cell survival.
Establish that embryonic development requires nonmuscle products from this gene as early as day 2.5. In addition, we show that ES cells require nonmuscle γTm products for cell growth. We conclude that products from the γTm gene are essential for both embryonic development and cell survival.

MATERIALS AND METHODS

Construction of a targeting vector. Genomic clones of the mouse γTm gene were isolated by screening a 129-mouse genomic library (lambda DASH II vector; Stratagene, La Jolla, Calif.), with oligonucleotide probes directed towards the γTm exons 1a to 2b and a flankning sequence between the NcoI sites, replacing the 132-bp coding sequence of exon 1b as well as 64 bp of the intronic sequence 5’ to exon 1b.

Generation of ES cells heterozygous for γTm. The targeting vector (40 μg) was linearized with NdeI and transfected into R1 (32) ES cells by electroporation (0.24 kV; 500 μF; time constant of 11.0 ms). ES cells were subsequently cultured for 10 days in knockout Dulbecco’s Modified Eagle’s medium (Invitrogen, Carlsbad, Calif.) containing 10% fetal bovine serum, 2 mM glutamine, 10 mM nonessential amino acids, 100 μM β-mercaptoethanol, and 1,000 U of ESGRO (Chemicon International, Temecula, Calif.) per ml, with selection done using 300 μM ganciclovir (Roche Products, Dee Why, New South Wales, Australia). Surviving ES cell clones were isolated, expanded, and screened by PCR (as described below), and sequenced for the presence of a single LoxP and for the absence of the coding region of exon 1b.

Generation of heterozygous mice. ES cell clones that had undergone both homologous and subsequent Cre-mediated recombination were injected into cultured BALB/c blastocysts as described by Lemkert et al. (26), followed by transfer into pseudopregnant foster mice. Chimeric males were bred against both BALB/c and 129/SvJ females. All animal experimentation was performed in accordance with institutional guidelines and guidelines of the National Health and Medical Research Council, Canberra, Australia.

Genomic tail DNA was isolated from the tails of F2 pups and genotyped by PCR for the presence of both wild-type and knockout alleles. An oligonucleotide primer set common to both alleles (For. 9341, 5′-GGCTACAACGC CGGACCGG GAGCAG-3′, and Rev. 9342, 5′-CGGGGCTGATTTTTCCAGCAG-3′) was used for either side of the deletion and used in the PCR, based on the prediction that the For. 9341 and Rev. 9342 primers would generate a 345-bp fragment for the wild-type allele and a 355-bp fragment for the mutated allele. A primer further upstream (For. 9399, 5′-AGAGCCACCCGATAAGAGAGG-3′) was used in an alternative PCR and gave identical results. The PCR products were analyzed on a 2% agarose gel in 1× Tris-acetate-EDTA.

Breeding and genotyping of embryos and morulae. Mouse γTm gene heterozygous (F1) pups were intercrossed in order to generate null pups. F2 progeny were genotyped by PCR. As no live births were determined as −/−, the presence of 2 days to 12 embryos was isolated, and genomic DNA was prepared for genotyping by PCR as described above. Day 2.5 morulae were also isolated and cultured in M16 embryo medium (Sigma, St. Louis, Mo.) for a further 1.5 days to increase cell numbers. Individually cultured morulae were collected in minimum-volume M16 embryo medium, and DNA was prepared by lysing morulae with 10 μl of 50 mM KOH at 95°C for 10 min, followed by the addition of 10 μl of 50 mM Tris-HCl, pH 7.5. Morula DNA was genotyped by PCR as described above.

Western blot analysis of mouse tissue and cell lines. Protein was prepared from the brain tissue of adult mice heterozygous for the γTm gene and control littermates. Protein was also prepared from wild-type ES cells and primary mouse embryonic fibroblasts (PMEF) that were cultured as described above. Brain tissue, ES cells, and PMEF pellets were collected and homogenized in a sodium dodecyl sulfate (SDS) solubilization buffer (10 mM Tris-HCl [pH 7.6], 2% SDS, 2 mM dithiothreitol), and the protein concentration was determined by using a bichinchoninic acid protein assay kit (Pierce, Rockford, Ill.). Total protein (10 μg) was denatured and reduced in sample buffer containing β-mercaptoethanol. Proteins were analyzed by SDS–15% polyacrylamide gel electrophoresis containing a low level (0.9%) of bisacrylamide. Gels were transferred to an Immobilon-P membrane (Millipore, Bedford, Mass.) via electroblotting techniques. The membranes were blocked in 5% skim milk powder in Tris-buffered saline (TBS), pH 7.5, and washed in TBS with 0.1% Tween 20 for 5 min each.

The primary antibodies used for Western analysis were mouse monoclonal antibody CG3 (20), a kind gift provided by J. Lin, University of Iowa; rabbit antisemir SWS/9d (36); and rabbit antisemir WD4/9d (19). The CG3 antibody recognizes an epitope within the 1b exon of the γTm gene, and therefore all 11 known nonmuscle products were detected. The SWS/9d rabbit polyclonal antisemir recognizes a single exon, 9d, containing product from the δTm gene. Primary antibodies were diluted at a ratio of 1:1,000 in TBS and were incubated with the membranes at room temperature for 1 h. The membranes were then washed in TBS with 0.1% Tween 20 (as described above) prior to addition of a secondary antibody at a 1:5,000 dilution (goat anti-mouse immunoglobulin [lg] or goat anti-rabbit Ig conjugated to horseradish peroxidase; Jackson ImmunoResearch, West Grove, Pa.). Western bands were detected using a Western Lighting kit (Perkin-Elmer Life Sciences, Boston, Mass.) followed by exposure to Fuji (Tokyo, Japan) Super RX X-ray film.

Screening for double-knockout ES clones. Two ES cell clones that had undergone both homologous and Cre-mediated recombination were reelectroporated with the targeting construct, followed by growth and selection in G418 as described above. Clones were expanded and selected for analysis by either PCR or Southern techniques as described above.

RESULTS

Targeting of the γTm gene. In order to eliminate all nonmuscle Tm isoforms encoded by the γTm gene (Fig. 1) and to study the developmental impact in mice, the first coding exon (1b) and donor splice junction were knocked out of the γTm...
gene (Fig. 2A). The coding sequences and splice donor site of exon 1b were replaced by a 4-kb cassette containing Neo r and thymidine kinase genes flanked by LoxP sites (Fig. 2B). Homologous recombination events in multiple ES cell clones were identified by Southern analysis (Fig. 2C), where both the parental allele at 7.9 kb and the mutated allele at 5.0 kb were observed. The two ES cell clones shown in Fig. 2C were expanded, and the selection cassette was removed by transient transfection with a cytomegalovirus-Cre recombinase plasmid to yield the mutated gene structure shown in Fig. 3A.

**Generation of heterozygous animals.** One of the heterozygous cell lines was used to generate a male chimera that was backcrossed to a 129/SvJ female. Germ line transmission was assessed by the PCR screening strategy shown in Fig. 3, and the identities of the two products obtained at 405 and 355 bp were verified by DNA sequence analysis to be the wild-type and disrupted alleles following Cre-mediated (Cre') recombination are shown. (B) PCR screening of both F_1 and F_2 progeny yielded products with the expected sizes of 405 and 355 bp, respectively. −ve, negative; +ve, positive.

**TABLE 1. PCR genotype analysis of F_1 and F_2 mouse pups**

<table>
<thead>
<tr>
<th>Generation</th>
<th>No. of mice that are:</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>F_1</td>
<td>+/+</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>+/−</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>−/−</td>
<td>N/A</td>
</tr>
<tr>
<td>F_2</td>
<td>17</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>11</td>
</tr>
</tbody>
</table>

* N/A, not applicable.
levels derived from the /H9253 locus. We therefore conclude that both ES clones tested were competent for homologous recombination into this locus. Indeed, a total of 51 pups. More than 300 ES clones were assayed, and we were not able to detect any homozygous-knockout ES cells in either of the two ES lines (Table 2). From the originally observed recombination rate, we expected to detect multiple clones targeted in the remaining wild-type allele. Indeed, a total of 51 clones showed targeting of the originally targeted allele, indicating that both ES clones tested were competent for homologous recombination into this locus. We therefore conclude that the activity of the γTm gene is required for ES cell viability.

Haploinsufficient mice. Western blot analysis on whole adult brain tissue of wild-type and heterozygous mice was performed. The Western blot was exposed to an antibody, CG3, that recognizes products from the coding region of exon 1b and therefore will detect all 248-amino-acid γTm nonmuscle isoforms (Fig. 1). The results in Fig. 4A show no difference in the amounts of γTm gene nonmuscle products expressed between wild-type and heterozygous mouse brains. We therefore conclude that the haploinsufficiency of this gene does not result in a reduced accumulation of protein.

Western blot analysis of ES clones. The failure to generate any double-knockout ES cells was surprising since Tms are encoded by four different genes. The results therefore suggest that none of the remaining Tm genes are capable of rescuing the deletion of the exon 1b-containing products of the γTm gene although two of the other genes make similar 248-amino-acid Tms. In order to confirm that these genes are active in ES cells, we performed Western blot analysis on ES cell protein. The expression of products from all four Tm genes was observed by comparing wild-type ES cells to PMEF by using Tm antibodies. Figure 4B shows that there is substantial expression of the other Tm gene products in ES cells, and it is therefore apparent that these Tm isoforms were not able to rescue the phenotype of the γTm homozygous knockouts.

DISCUSSION

The γTm gene is not functionally redundant. We have shown in this paper that one or more products from the mouse γTm gene are absolutely required for ES cell survival and early embryogenesis. This result occurs despite the finding that three other Tm genes are active in both ES cells and early mouse embryogenesis (8, 31) (Fig. 4). The essential function provided by the γTm gene, therefore, cannot be rescued by the products expressed by the other Tm genes in ES cells and early embryos.

However, it does remain a formal possibility that there is a Tm isoform not expressed in ES cells or in early embryos, which could rescue the γTm gene knockout if it were expressed in these cells.

The essential function provided by the γTm gene most likely involves the γTm NM1 and/or NM2 isoforms because the exon 9d carboxy terminus accounts for most if not all products from this gene in ES cells (B. Vrhovski and P. Gunning, unpublished observation), and these two isoforms are the only isoforms which use this carboxy terminus (Fig. 1). Neither the 9a nor the 9c alternative carboxy terminus can be detected in ES cells (J. Hook, B. Vrhovski, and P. Gunning, unpublished observation). The isoforms most similar to γTm NM1 and NM2 are Tm4 from the δTm gene and Tm5a and Tm5b from the αTm gene. Since all three isoforms are expressed in ES cells (Fig. 4B), it seems unlikely that the failure to rescue the γTm gene knock-
out is due to a lack of expression of the most closely related isoforms. Rather, it appears more likely that γTm isoforms are sorted to specific intracellular sites in neurons (18, 19, 36, 42), the brain (41), epithelial cells, and fibroblasts (33) and that their location is subject to developmental (43) and cell cycle (33) regulation. In particular, a number of γTm isoforms have been shown to be associated with the cell cortex (28), stress fibers (7), areas of polarized growth (18, 36), and the Golgi apparatus (20). A number of isoforms from the other Tm genes are also found in the cell cortex (28, 29, 39), stress fibers (7, 33), and areas of polarized growth (18, 36), but γTm products are the only ones thus far detected in the Golgi apparatus (20). It is therefore possible that the elimination of γTm NM1 and/or NM2 from the Golgi apparatus cannot be rescued by another isoform because no other isoform may be sorted to that structure. Alternatively, Clayton and Johnson (8) have shown that Tm isoforms in yeast leads to altered head morphogenesis, shorter sarcomeres, and disruptions to thick and thin filament packing (23, 38, 40). However, a rescue experiment with a nonflight muscle Tm isoform showed that the phenotype of the mutant indirect flight muscle Tm could be restored (30). More recently, mutations of the TmII gene were shown to impact the regulation of dendritic growth (27). In mice, targeted elimination of only the striated muscle isoform (4) or all isoforms (35) from the αTm gene leads to embryonic lethality at embryonic day 9.5 (E9.5) to E13.5 or E8 to E11.5, respectively. It is therefore quite unequivocal that Tms perform essential cellular functions, and where multiple isoforms are generated, there is little evidence for functional equivalence of isoforms.

The αTm gene findings together with the γTm gene findings presented in this paper show evidence for three different stages of development requiring different isoforms or groups of isoforms. The difference between the αTm striated muscle isoform knockout (4) and the knockout of all αTm isoforms (35) suggests that one or more nonstriated isoforms are required at approximately E8 to E9.5 for normal development and that the striated isoform is required at E9.5 to E13.5. It is notable, however, that the elimination of all αTm isoforms is not as severe as that of just the low-molecular-weight γTm isoforms.

The impact of Tm elimination on different organisms also indicates that Tms are required for a number of different cellular functions. The yeast Tm knockout and mouse γTm knockout results suggest a role for Tm in cell survival possibly related to vesicle transport and/or cell division. Results from the C. elegans, Drosophila, and αTm studies of mice indicate a role in development. Finally, the αTm knockout also indicates a role in cardiac function, as might be expected because of its pivotal role in regulating myosin head engagement with the actin filament. The data are therefore consistent with the proposal that Tm isoforms are responsible for a diverse set of biological functions and that this is underpinned by the temporal and spatial regulation of isoform expression.

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