

PAM14, a Novel MRG- and Rb-Associated Protein, Is Not Required for Development and T-Cell Function in Mice

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PAM14 has been found to associate in complexes with the MORF4/MRG family of proteins as well as Rb, the tumor suppressor protein. This suggested that it might be involved in cell growth, immortalization, and/or senescence. To elucidate the in vivo function of PAM14, we characterized the expression pattern of mouse *Pam14* and generated PAM14-deficient (*Pam14*^{-/-}) mice. *Pam14* was widely expressed in all mouse tissues and as early as 7 days during embryonic development. Despite this ubiquitous expression in wild-type mice, *Pam14*^{-/-} mice were healthy and fertile. Response to mitogenic stimulation and production of interleukin-2 were the same in stimulated splenic T cells from *Pam14*^{-/-} mice as in control littermates. Cell growth rates of mouse embryonic fibroblasts (MEFs) from all three genotypes were the same, and immortalized cells were obtained from all cell cultures during continuous culture. There was also no difference in expression of growth-related genes in response to serum stimulation in the null versus control MEFs. These data demonstrate that PAM14 is not essential for normal mouse development and cell cycle control. PAM14 likely acts as an adaptor protein in nucleoprotein complexes and is probably compensated for by another functionally redundant protein(s).

Replicative senescence, or the state of terminal loss of cell division that normal cells in culture enter after a limited number of replications, is a well-established phenomenon (7, 26). This has been proposed to be representative of cellular aging in vivo, as well as a mechanism of tumor suppression. The latter derives from the fact that fusion of normal cells with various immortal cells (capable of indefinite division) yields hybrids that regain growth control and stop dividing, indicating that the senescent phenotype is dominant (21, 23). The immortal cell therefore arises from recessive defects in cell senescence-related genes, and the identification of four complementation groups for indefinite division (A to D) suggests that at least four such pathways exist (22). Microcell-mediated chromosome transfer has led to the identification of loci on human chromosomes 1, 4, and 7 for groups C, B, and D, respectively, as these chromosomes induce specific loss of proliferation in multiple immortal cell lines assigned to the complementation group and have no effect on cell lines assigned to the other groups (30). We cloned a gene on chromosome 4 (MORF4) that had the ability to cause proliferation loss as effectively as the intact chromosome (1) and determined that MORF4 was a member of a family of genes (seven members, three expressed) with motifs predictive of transcription

factor activity, most likely as partners in nucleoprotein complexes. To identify potential interacting proteins, we performed a yeast two-hybrid screen with the MORF-related expressed genes MRG15 and MRGX.

We obtained a novel cDNA PAM14 (for protein associated with MRG, 14 kDa) that interacts with all three MORF4/MRG proteins as well as Rb, an important tumor suppressor protein (13). Our laboratory has found that MRG15 and MRGX can activate the *B-myb* promoter in HeLa cells, most likely by disruption of the E2F/Rb/histone deacetylase complex known to interact with the E2F site in this promoter (12, 13, 29). Since MORF4 is a truncated version of MRG15 and MRGX, our working hypothesis is that MORF4 acts to replace and disrupt or inactivate complexes containing these proteins, with a resulting modulation of gene expression and loss of cell proliferation in a subset of immortal human cells. MORF4 is so similar to MRG15 (2) that it has been difficult to develop tools to study this gene and protein directly. To provide insights into the mechanism(s) of action of the MORF4/MRG proteins, we inactivated the *Pam14* gene, since PAM14 interacts with all three and is ubiquitously expressed. The results demonstrated that *Pam14* null (*Pam14*^{-/-}) mice were viable and appeared to have normal fertility. Analysis of the proliferative response of mouse embryonic fibroblasts (MEFs) and splenic T lymphocytes to mitogen stimulation demonstrated that *Pam14*^{-/-} cells were equivalent to cells from wild-type and heterozygous animals in growth response and expression of genes related to cell cycle initiation and progression. These results suggest that PAM14 is not required for either develop-

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ment or the cell proliferation response and that its function is likely compensated for by another protein(s).

MATERIALS AND METHODS

Isolation of the mouse *Pam14* gene. A total of 10^6 independent phage plaques of a Lambda FIX II mouse 129/SvEv genomic library (Stratagene, La Jolla, Calif.) were screened using the full-length human *Pam14* cDNA as a probe. The filters were hybridized at 65°C for 16 h in a solution of $5\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), $5\times$ Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), and 100 μ g of salmon sperm DNA/ml. Three clones were isolated, the inserts from these clones were subcloned into pBluescript II (Stratagene), and mapping and partial sequencing were performed.

Plasmids. A hemagglutinin (HA)-tagged mouse PAM14 (mPAM14)-encoding fragment was amplified with *Pfu* DNA polymerase, using the genomic clone as a template. PCR was achieved with the following primers: mPAM14-5', 5'-CGC GGA TCC GCC ACC ATG CGG CCC CTG GAC GCG GT-3' and mPAM14-3', 5'-CCG GAA TTC TCA AGC GTA ATC TGG AAC ATC GTA TGG GTA CGA AGA CTC GCT CTT TAT CC-3'. Construction of a V5-tagged mouse MRG15 (mMRG15) plasmid has been reported previously (31).

Immunoprecipitation and Western blot analysis. HeLa cells transfected with PAM14 and/or MRG15 expression plasmids were washed with phosphate-buffered saline and scraped into 1 ml of lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10% glycerol, 1% NP-40, and protease inhibitor cocktail set I [Calbiochem, San Diego, Calif.]) per 100-mm dish. The lysed cells were put into 1.5-ml tubes and kept for 30 min on ice. Following centrifugation at $17,000\times g$ for 15 min at 4°C, the supernatants were collected and protein concentrations were determined with the Bio-Rad protein assay, using bovine serum albumin as a standard. For immunoprecipitation, 500 μ g of protein was precleared for 1 h by addition of Bio-Mag beads (QIAGEN). The antibody was added to the precleared lysates and kept at 4°C for 1 h. Bio-Mag beads were added to each tube and kept at 4°C overnight. Beads were washed four times with 0.5 ml of lysis buffer, and $1\times$ loading buffer (25 mM Tris-HCl [pH 6.5], 5% glycerol, 1% SDS, 1% 2-mercaptoethanol, and 0.05% bromophenol blue) was then added. Samples were boiled for 3 min and run on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels. The separated proteins were blotted on nitrocellulose membranes (Bio-Rad), which were probed with antibody and subsequently with horseradish peroxidase-conjugated second antibody. To visualize the proteins, a standard enhanced chemiluminescence reaction was employed (ECL; Amersham Biosciences).

Nuclear extracts were prepared from two spleens of C57BL/6J females as described previously (27) and diluted 1:2 in 25 mM Tris-HCl (pH 7.5). The nuclear protein (300 μ g) was incubated with 4 μ g of either rabbit anti-Rb (M-153; Santa Cruz Biotechnology sc-7905), rabbit anti-MRG15 (our laboratory), or rabbit anti-HA (Y-11; Santa Cruz Biotechnology sc-805) antibody overnight, and protein A-agarose was then added for 1 h. After four washes with buffer (25 mM Tris-HCl [pH 7.5], 210 mM NaCl, 0.75 mM $MgCl_2$, 0.25 mM EDTA, and 12.5% sucrose), immunoprecipitates were run on SDS-PAGE gels followed by Western blot analysis, as described above.

Construction of *Pam14* targeting vector. A 5' homologous 3.2-kb BamHI-NotI fragment was blunted with T4 DNA polymerase (Gibco-BRL) and ligated to HindIII-EcoRI adaptors (Stratagene). After purifying the fragment, it was subcloned into a *PgkHPRT* selectable marker cassette. A 3' homologous 4.1-kb HincII-XhoI fragment was blunted and ligated to XbaI-XmnI adaptors (New England BioLabs, Beverly, Mass.), and this fragment was then subcloned into the *PgkHPRT* cassette vector containing the 5' homology arm region. Finally, the MC1tk expression cassette, for negative selection, was subcloned next to the 3' homologous region by using the NotI site.

Generation of mice harboring the *Pam14* mutation. Twenty-five micrograms of ClaI-linearized targeting vector was electroporated into 10^7 129/SvEv-derived AB2.1 embryonic stem (ES) cells. ES cells were then selected in medium containing hypoxanthine-aminopterin-thymidine and 1-(2'-deoxy-2'-fluoro- β -D-arabinofuranosyl)-5'-iodouracil (FIAU). For genomic Southern blot analysis, EcoRV- or BamHI-digested DNA was transferred to GeneScreen Plus nylon membranes (NEN Life Science Products, Boston, Mass.) and probed with an external ~500-bp SphI-BamHI fragment as 5' probe or an external ~650-bp NcoI-NcoI fragment as 3' probe, respectively. Mutant ES clones were injected into C57BL/6J blastocysts, and chimeric males were mated with C57BL/6J females. Germ line transmission was obtained from chimeras derived from two independent clones (16).

Northern blot analysis. RNA was isolated from tissues or MEFs by using the TRIzol reagent (Invitrogen). Frozen tissue samples were homogenized with a 1.5-ml pellet pestle, 1 ml of TRIzol was added to the tissue sample, and the

suspension was passed through a 21-gauge needle to shear the DNA. RNA was extracted according to the manufacturer's instructions. RNA was resolved on a 1% agarose gel and then transferred to a Hybond-N⁺ nylon membrane. Blots were hybridized in NorthernMax hybridization buffer (Ambion, Austin, Tex.) at 42°C overnight with a 320-bp SacI-SmaI probe from the mouse *Pam14* genomic clone. This fragment contains a 44-bp 5' noncoding region and most of the coding region. The fragment of *Mrg15* to be used as probe was amplified by PCR using MRG15-5' end primer, 5'-GGC AAA TCG CGC CCA GGA TGT AGA-3', and Exon 3R primer, 5'-CCA GCC ACT GTA ATG GAT GAA GTA-3'. This fragment contained 131 bp of the 5' untranslated region and 147 bp of the coding region of *Mrg15* and did not cross-hybridize with mouse *MrgX* (31). Plasmids which contained mouse cyclin E1 (*Cene1*), cyclin D1 (*Cend1*), *Mybl2*, and *Myc* fragments for probes were kindly provided by Nicholas J. Dyson. The blot was washed with $2\times$ SSC-0.1% SDS at 65°C twice for 10 min and then washed with $0.2\times$ SSC-0.1% SDS at 65°C twice for 15 min.

Antibody preparation. A bacterially expressed intein-human PAM14 (hPAM14) fusion protein (expression plasmid kindly provided by F. Quijcho) or glutathione *S*-transferase-human MRG15 was purified using chitin beads (New England BioLabs) or glutathione-Sepharose 4B (Amersham Biosciences), respectively, according to the instruction manuals. Rabbits were immunized with the purified proteins, and a polyclonal antibody was generated by Cocalico Inc. The antibodies against PAM14 and MRG15 were affinity purified using an antigen-binding Sepharose column (HiTrap; Amersham Biosciences).

Generation of MEFs. MEFs were derived from 13.5-day-old wild-type, *Pam14*^{+/-}, and *Pam14*^{-/-} embryos. After removal of the head and gastrointestinal tract, each embryo was washed with phosphate-buffered saline and minced, and the tissue was placed in a 15-ml conical tube. After centrifugation, 1 ml of trypsin solution (0.25% trypsin-0.005% EDTA) was added to the pelleted tissue and the tissue was digested on ice overnight. Trypsin was inactivated by the addition of Hank's minimum essential medium (Invitrogen) containing 10% fetal bovine serum (FBS), 2 mM glutamine, 0.1 mM nonessential amino acids, 28 mM HEPES, 100 U of penicillin G per ml, and 100 μ g of streptomycin per ml. After pipetting several times, the single-cell suspension was plated into one T75 tissue culture flask and incubated at 37°C for 2 to 3 days until the cells were confluent. We designated this culture as PD0. Using a 3T3 culture regimen (28), 3×10^5 cells were replated every 3 days onto 60-mm tissue culture dishes using Eagle's minimum essential medium (Invitrogen) containing 10% FBS, 2 mM glutamine, and 0.1 mM nonessential amino acids in a 5% CO₂ humidified chamber. Immortalized cells were occasionally obtained during continuous culture.

T-cell response assay. For analysis of T-cell response, single-cell suspensions were prepared from spleens of wild-type, heterozygous, and null mice at 4 weeks of age. Splenic tissue was homogenized between frosted glass slides in T-cell medium (RPMI 1640 containing 10% FBS, 0.1 mM nonessential amino acids, 100 U of penicillin per ml, and 100 μ g of streptomycin per ml). A total of 2×10^4 splenocytes were plated into each well of a 24-well plate and stimulated with various concentrations of concanavalin A (ConA) or 10 ng of phorbol myristate acetate (PMA) per ml plus 100 ng of ionomycin per ml for 48 h. The culture supernatants were centrifuged at $2,700\times g$ for 10 min, and the supernatants were stored at -80°C until assayed. Interleukin-2 (IL-2) was measured using an IL-2 enzyme-linked immunosorbent assay (ELISA) kit (BD Pharmingen).

RESULTS

Isolation and characterization of *mPam14* genomic clones. Three genomic clones from a 129/SvEv mouse genomic library were isolated using a human *Pam14* cDNA as a probe. Partial sequencing indicated that they encoded the same gene and had a similar genomic structure to human *Pam14*, as both have only one intron in the 3' untranslated region that is 250 bp in length (Fig. 1A). mPAM14 and hPAM14 proteins shared 86% amino acid identity (Fig. 1B).

We determined the expression of *Pam14* in mouse tissues and during development by dot blot analysis and found that it was expressed ubiquitously, with highest expression in brain, heart, and testis (Fig. 2). This was very similar to what our group had observed previously with MRG15 and MRGX (1, 31). Interestingly, *Pam14* was expressed at high levels in the 7-day embryo, but following this time expression was constant during development.



FIG. 1. Structure of mouse *Pam14* genomic DNA and protein sequence similarity of mouse and human PAM14. (A) Schematic diagram of the mouse *Pam14* gene. (B) Alignment of amino acid sequences of mPAM14 and hPAM14. Identical amino acids are marked with black boxes, and similar amino acids are shown with gray boxes.

mPAM14 interacts with MRG15. We had originally isolated hPAM14 as an MRG-interacting protein in a yeast two-hybrid screen. mMRG15 protein is 100% identical at the amino acid level to hMRG15 (2, 31). To confirm that mPAM14 isolated here was indeed an hPAM14 ortholog, we constructed an HA-tagged mPAM14 expression plasmid and transfected this with or without a V5-tagged MRG15 expression plasmid into HeLa cells. After 24 h, cell lysates were prepared and interaction between mPAM14 and MRG15 proteins was determined by immunoprecipitation and immunoblot analysis. mPAM14 was found to interact with MRG15 in the cells (Fig. 3A), providing evidence that mPAM14 is an ortholog of hPAM14.

mPAM14 is present in a complex with Rb. Our investigators have previously reported that hPAM14 associates with Rb

and MRG15 or MRGX in immortal human cell lines (13, 20, 29). We therefore investigated whether a similar association occurred in normal mouse cells. Splenocyte nuclear extracts from C57BL/6J females were immunoprecipitated with rabbit anti-Rb, rabbit anti-MRG15, or rabbit anti-HA (negative control) antibody, and immunoprecipitated proteins were separated on PAGE and transferred to nitrocellulose membranes. Using an anti-PAM14 antibody, PAM14 was detected in the anti-Rb and anti-MRG15 but not anti-HA immunoprecipitate (Fig. 3B). We have thus demonstrated that PAM14 interacts with Rb and MRG15 in normal mouse cells, similar to what is observed in human cells. Additionally, Rb and MRG15 were present in the Rb and MRG15 but not HA immunoprecipitates.

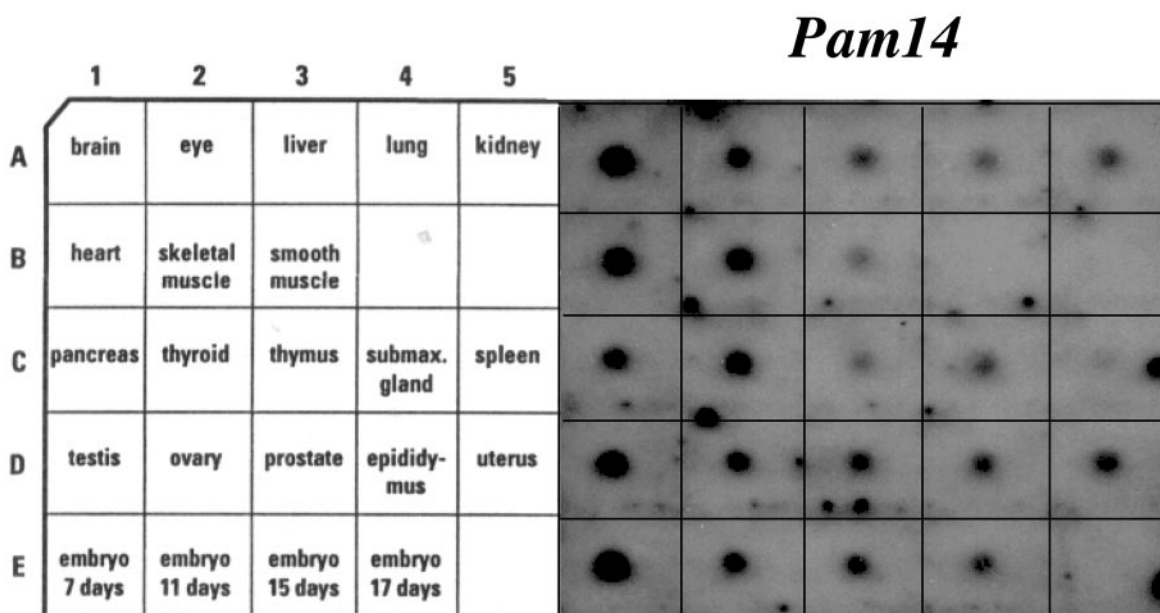


FIG. 2. PAM14 is expressed ubiquitously in mouse tissues. A dot blot of poly(A)⁺ RNA from multiple mouse tissues (mouse RNA master blot; no. 7771-1; Clontech) was probed with the mouse *Pam14* coding region.

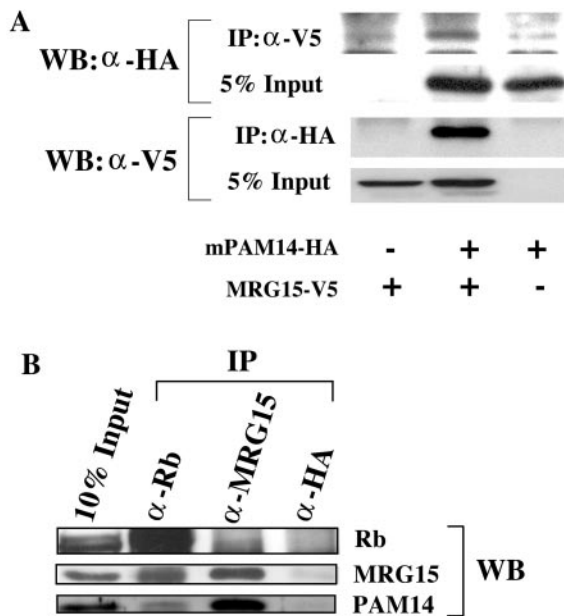


FIG. 3. mPAM14 interacts with MRG15 and Rb. (A) HeLa cells were cotransfected with expression vectors, and lysates were subjected to immunoprecipitation (IP) and Western blot (WB) analysis with antibodies as indicated. (B) Nuclear extracts from splenocytes of C57BL/6J females were immunoprecipitated using anti-Rb, anti-MRG15, or anti-HA (negative control) antibodies, and detection of the presence of Rb, PAM14, and MRG15 proteins in the immunoprecipitates by Western blot analysis was done using anti-Rb, anti-PAM14, and anti-MRG15 antibodies.

Targeted disruption of the *Pam14* gene. To disrupt the *Pam14* gene, the entire coding region was replaced with a *PgkHPRT* cassette (Fig. 4A). Forty of 144 (30%) hypoxanthine phosphoribosyltransferase (HPRT)-positive and FIAU-resistant clones, screened for homologous recombination by Southern blot analysis using 5'- and 3'-specific probes, were found to have correct targeting events. Injection of mutant ES clones was performed, and successful germ line transmission from two independent cell lines was achieved. Mice from the two independent ES cell lines were bred and maintained separately. There was no difference in the gross phenotype between the two lines, and the results reported here were observed with both lineages.

Expression of *Pam14* RNA and protein. Mice were examined for the expression of *Pam14* mRNA to confirm that the targeted disruption of *Pam14* had occurred successfully in the *Pam14*^{-/-} mice. Northern blot analysis indicated that there was no expression of *Pam14* mRNA in various tissues from the null mice (Fig. 4B) and an intermediate level of expression in *Pam14*^{+/-} mice. PAM14 protein in the lysates from splenocytes (corresponding to 3 × 10⁵ cells) was detected by Western blotting using a rabbit anti-PAM14 antibody against full-length PAM14 that we generated (Fig. 4C). We prepared lysates from three mice per genotype. As expected, no expression was detected in lysates from homozygous mice, and intermediate expression of PAM14 protein was detected in lysates from heterozygous mice. As a control, the amount of MRG15 protein was found to be the same in all three genotypes (Fig. 4C).

TABLE 1. Genotype analysis of F₂ mice at 3 to 4 weeks of age from heterozygous × heterozygous intercrosses

Sex	No. (%)		
	Wild type	Heterozygous	Homozygous
Male	61 (26.4)	129 (55.8)	41 (17.8)
Female	67 (27.1)	110 (44.5)	70 (28.4)
Total	128 (26.8)	239 (50.0)	111 (23.2)

PAM14 is not required for normal development and has no effect on the aging phenotype. Heterozygous mice were intercrossed, and F₂ offspring were genotyped at 3 weeks of age by Southern blotting; all three genotypes were detected (Fig. 4D). Wild-type, *Pam14*^{+/-}, and *Pam14*^{-/-} offspring from heterozygous crosses were obtained in the expected Mendelian ratio (Table 1). *Pam14*^{-/-} mice developed normally and were not grossly different from wild-type and heterozygous littermates. This indicated that PAM14 is not required for normal development. Both male and female *Pam14*^{-/-} mice were fertile. We have maintained *Pam14*^{-/-} mice for over 2 years and observed no differences with aging compared with control littermates.

T-cell function is unaffected by loss of expression of PAM14. Following our identification of PAM14, we found that the human sequence had already been deposited in the GenBank database as a gene related to T-cell activation (PGR1; GenBank accession number AF116272). Moreover, Rb and its family members are involved in the control of T-cell proliferation (17, 25, 32, 35). We therefore investigated whether there was a difference in T-cell differentiation in the spleen and thymus of wild-type, *Pam14*^{+/-}, and *Pam14*^{-/-} mice by using fluorescence-activated cell sorter analysis. Splenocytes and thymocytes from each genotype were stained with anti-CD4 and anti-CD8 antibodies. The ratio of CD4 to CD8 cells in *Pam14*^{-/-} mice in both tissues was the same as that in wild-type and *Pam14*^{+/-} mice (data not shown), indicating that loss of PAM14 expression did not affect T-cell development. Next, we examined IL-2 production by splenic T cells from *Pam14*^{-/-} mice and compared them to wild-type and *Pam14*^{+/-} littermates. Splenic T cells were isolated and stimulated with ConA for 48 h, and IL-2 production was measured by ELISA. IL-2 production was the same for all the genotypes (Fig. 5). A similar result was obtained when T cells were stimulated with PMA plus ionomycin (data not shown).

Proliferation assays and analysis of expression of cell cycle genes by using MEF cultures. MEFs were isolated from 13.5-day wild-type, heterozygous, and null embryos, and the conventional 3T3 protocol was performed to determine cell cycle time, number of divisions prior to entry into senescence, and ability to immortalize. No significant difference in growth rate of mass cultures was observed, and all cells, regardless of genotype, entered senescence after six to seven passages (i.e., 10 to 12 population doublings) (Fig. 6A). Immortalized cell lines were obtained in all cases.

We then determined whether *Pam14*^{-/-} MEFs responded to serum stimulation with the expected changes in gene expression of cell cycle-related genes. We examined the genes cyclin E1 (*Ccne1*), cyclin D1 (*Ccnd1*), B-myb (*Mybl2*), and *c-myc* (*Myc*), which are known to be expressed in a cell cycle-

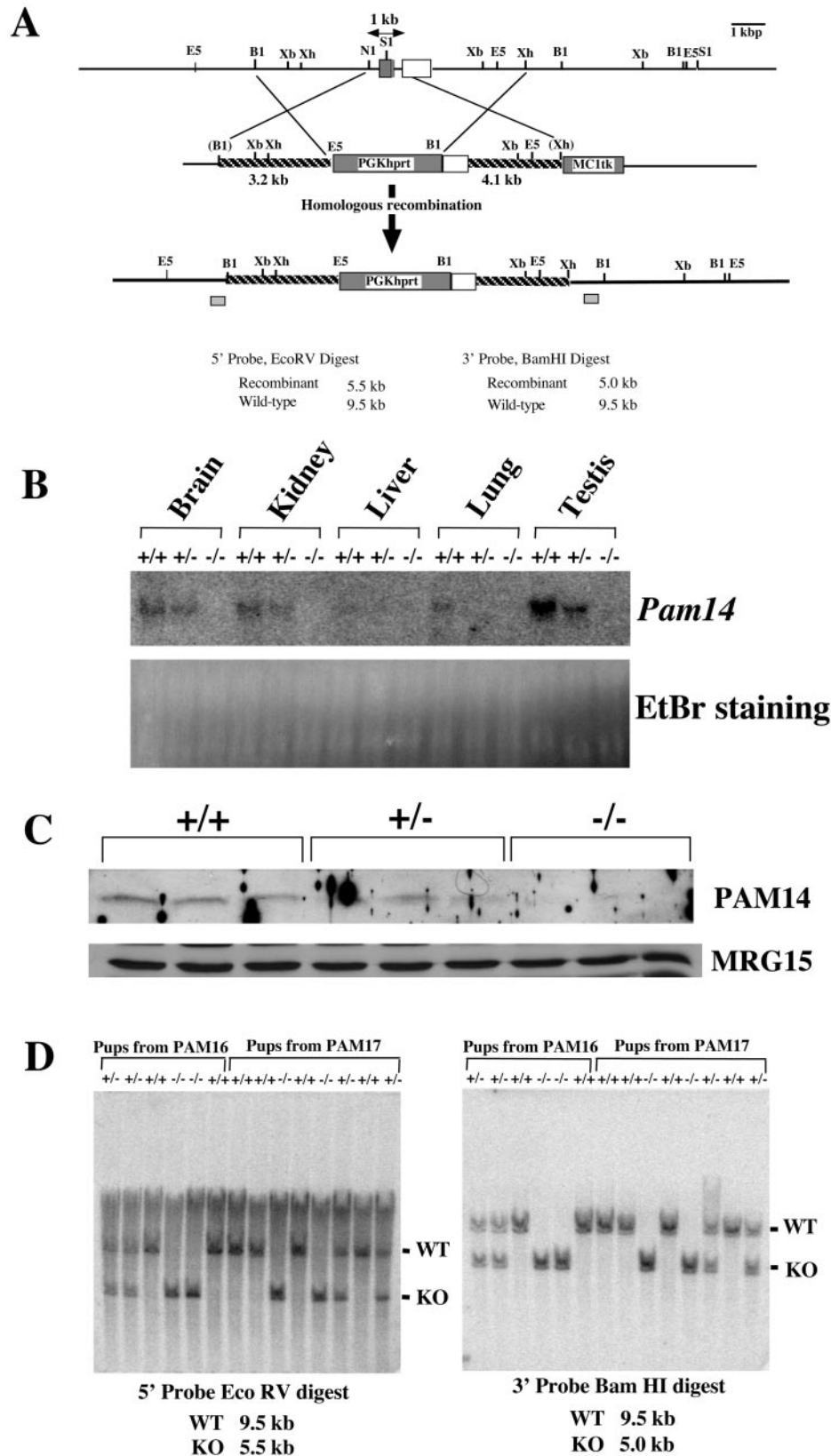


FIG. 4. Disruption of *Pam14* by gene targeting. (A) Structure of the *Pam14* targeting construct. Abbreviations: E5, EcoRV; B1, BamHI; Xb, XbaI; Xh, XhoI; N1, NotI; S1, SmaI. (B) Northern blot of mRNA isolated from brain, liver, lung, spleen, and testis. The lower panel shows 18S and 28S rRNA stained with ethidium bromide as a loading control. (C) Western blot of whole-splenocyte lysates from mutant and control mice, using rabbit anti-PAM14 antibody against full-length hPAM14. (D) Southern blot analysis of tail DNAs from 3-week-old progeny of a *Pam14*^{+/-} intercross. These results were obtained from two independent litters.

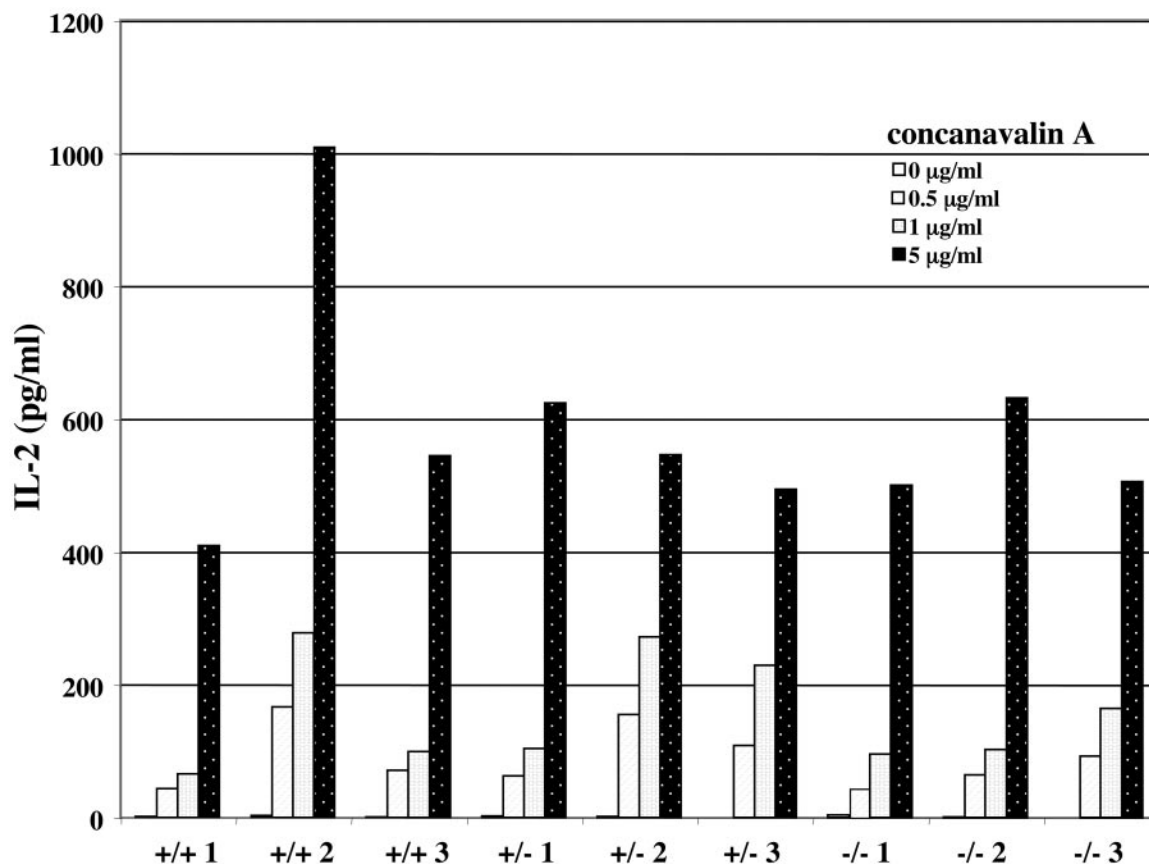


FIG. 5. IL-2 production by wild-type, *Pam14*^{+/-}, and *Pam14*^{-/-} T cells. Splenocytes from 8-week-old wild-type, heterozygous, and homozygous mutant *Pam14* mice were stimulated with various concentrations of ConA for 48 h. The production of IL-2 was determined by ELISA. The results are representative of two independent experiments.

dependent manner. The expression of all four genes in wild-type, *Pam14*^{+/-}, and *Pam14*^{-/-} MEFs was low in the quiescent state (0 h) and increased in a time-dependent manner after serum stimulation (Fig. 6B), and the expression patterns were the same in the three genotypes. The expression levels of *Mrg15* and *Gapd* remained constant during this time. We confirmed that no *Pam14* transcript was expressed in *Pam14*^{-/-} MEFs, using the same membrane.

DISCUSSION

In this study, we used gene targeting technology to attempt to determine the in vivo function(s) of the novel protein, PAM14, which is present in nucleoprotein complexes with the MORF4/MRG family of proteins and with Rb. Although PAM14 is expressed ubiquitously, homozygous mutant mice lacking *Pam14* showed no obvious abnormalities and no gross differences from wild-type and heterozygous littermates under normal conditions. The most obvious explanation is that another unknown protein(s) compensates for PAM14 functions in the null mice.

PAM14 was previously isolated by our group as an MRG15/MRGX-interacting protein in a yeast two-hybrid screen (13). We have previously shown that PAM14 is present in a complex with MRG15 and Rb in various human cells by sucrose gradi-

ent separation, coimmunostaining, and coimmunoprecipitation (20). A large amount of the total MRG15 protein appears to associate with this complex, and we hypothesize that this may form the basic complex found in most cell types and that PAM14 may help to stabilize this complex by acting as an adaptor protein.

Our laboratory has also identified another MRG15-containing complex (MAF2) by sucrose gradient separation (20). The MAF2 complex contains MOF, one of the MYST family histone acetyltransferases (18), and corresponds to the budding yeast NuA4 histone acetyltransferase complex, which has been purified and contains the MRG15 ortholog Eaf3p (6). The MAF2 complex does not include PAM14, and a PAM14 homolog does not exist in yeast. Thus, PAM14 and PAM14-related proteins probably evolved in mammalian systems, which are more complex.

Similarly, Rb, which is another component of the MRG15/PAM14 complex, has no homolog in yeast. However, it is one of the important tumor suppressor proteins that negatively control the cell cycle (4). *Rb*^{-/-} MEFs are not more easily immortalized than wild type because other Rb family members, p130 and p107, compensate for this function (5, 24); however, cyclin E1 expression in the G₀ state is maintained at high levels and time to entry into S phase is shortened (8, 11). Cyclin E1 expression in *Pam14*^{-/-} MEFs is not upregulated in

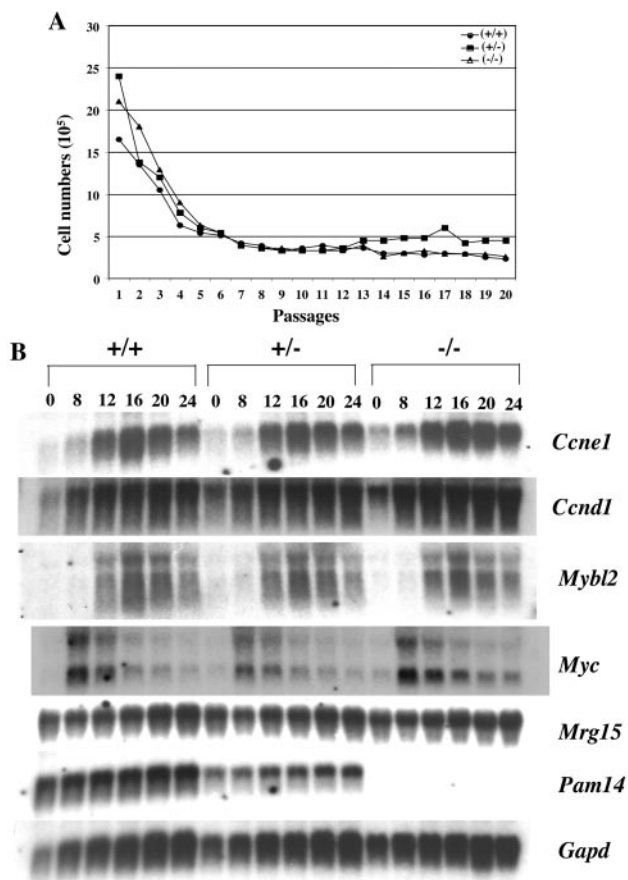


FIG. 6. MEF analyses. (A) Cell proliferation was measured by the 3T3 protocol. At 3-day intervals, the total number of cells per 60-mm culture dish was counted prior to redilution to 3×10^5 cells per dish for the next passage. The results are representative of at least three independently derived lines of MEFs per genotype. (B) Expression patterns of cell cycle-regulated genes by Northern blot analysis. Quiescent MEFs were stimulated by adding 10% FBS-containing medium, and total RNA was isolated at the indicated time points (hours after stimulation). The expression of cyclin E1 (*Ccne1*), cyclin D1 (*Ccnd1*), *Mybl2*, *Myc*, *Mrg15*, *Pam14*, and *Gapd* mRNAs was detected by Northern blotting.

G₀ phase. Moreover, the mRNA expression pattern of *cyclin E1* as well as that of the genes *cyclin D1*, *Mybl2*, and *Myc*, which are known to be regulated by the Rb family proteins and E2F, are the same in *Pam14*^{-/-} and *Pam14*^{+/-} MEFs as well as in wild-type MEFs. The Rb family proteins are involved in maintaining peripheral T lymphocytes in a quiescent state. Although p130 is primarily responsible for this in resting T lymphocytes, it is known that Rb and p107 can functionally compensate for p130 in these cells (17). *Pam14*^{-/-} splenic T lymphocytes produced IL-2 in response to ConA and PMA plus ionomycin to the same levels as wild-type and *Pam14*^{+/-} T cells and were not hypo- or hyperresponsive to these stimulants. Finally, *Rb*^{+/-} mice develop pituitary and thyroid tumors within 1 year (9, 15, 33), but *Pam14*^{-/-} mice appear to be healthy at that time. These data suggest that PAM14 is not required for Rb function or inactivation because there may be redundant molecules or pathways that compensate for it.

A database search using the *Pam14* cDNA sequence did not

find homologs with high sequence similarity. However, PAM14 is a small protein (14 kDa), and the only known protein motif is a coiled-coil region. Many proteins, including transcription factors, have this motif, which is known to function in mediating protein-protein interactions (3, 10, 14). In budding yeast, it is predicted that 9% (1 in every 11) of proteins contain a coiled-coil sequence (19, 34), and many associations between coiled-coil regions of proteins have been confirmed by the yeast two-hybrid assay (19). Therefore, a functionally redundant molecule(s), with a coiled-coil motif, similar to that of PAM14 must exist in mammalian cells and compensate for PAM14 function(s) in null mice. The result is a lack of a definitive phenotype in null versus wild-type mice.

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