Normal Development of Mice Lacking Metablastin (P19), a Phosphoprotein Implicated in Cell Cycle Regulation*

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MATERIALS AND METHODS

Gene Targeting—A rat metablastin cDNA (36) was used to screen a DBA/2 mouse genomic library constructed in EMBL-3 (obtained from Clontech). Several clones were isolated, subcloned in plasmid vectors, mapped by Southern blot analysis, and sequenced (41). Two isolates were identified as pseudogenes by their lack of introns and presence of missense mutations. Another clone was found to contain all five exons of the metablastin gene as deduced from comparisons with the rat (36, 38) and human cDNAs (37) and confirmed by RNase protection analysis (data not shown). To prepare an isogenic gene targeting construct, the metablastin gene was rescued from a 129SV mouse genomic library constructed in Charon 35 (provided by Oliver Smithies). A 2-kb Sall-EcoRI fragment containing exon I and a 10-kb EcoRI-Sall fragment containing exons II-V were each subcloned in Bluescript (Stratagene). The targeting construct (Fig. 1b) was assembled in the plasmid pSP72 (Promega), which had been modified by inserting a NotI linker at the EcoRV site. A 1649-base-pair EcoRI-KpnI fragment containing exons II and III was replaced by a PGKneo cassette, in which the neomycin phosphotransferase (neo) gene of the phage transposon Tn5 (42) is driven by the murine phosphoglycerokinase (pgk-1) gene promoter (43). The promoters for the metablastin and neo genes are in the same transcriptional orientation. At the 3′ end of the metablastin gene sequences, a PGKIIK cassette was inserted, in which the herpes simplex thymidine kinase gene (44) is under the control of the pgk-1 promoter. Prior to transfection, the targeting construct was linearized at the unique NotI site.

E14-1 embryonic stem (ES) cells were maintained on mitotically inactive STO feeder layers (45) and transfected with the linearized targeting vector described above. Electropermeation and selection of clones resistant to G418 and gancyclovir was performed as described previously (46). Southern blot analysis demonstrated that a homologous...
recombination event had occurred in 7 of 69 ES cell clones analyzed (data not shown). Four of these metablastin \(1^{-/-}\) clones were injected into C57BL/6 donor blastocysts followed by surgical transfer into the uterus of pseudopregnant CD1 foster mothers. Thirty-two 129SV/C57BL/6 chimeric pups were obtained from six foster mothers. As assessed by coat color, their range of chimerism was 30–90%. When several chimeric (metablastin \(1^{-/-}\)) males were mated to C57BL/6 females, 57 of 98 offspring were agouti. Their metablastin \(1^{-/-}\) genotype was confirmed by Southern blot analysis of tail DNA (see below). Chimeric males derived from 3 of the 4 ES cell clones injected gave rise to metablastin \(1^{-/-}\) offspring.

Genomic Southern Blot Analysis—Genomic DNA was isolated from ES cells and from mouse tail fragments, digested with restriction enzymes as specified, resolved by electrophoresis in 0.8% agarose gels and transferred to Genescreen membranes (DuPont NEN) using standard procedures (41). Conditions for hybridization were as specified by the manufacturer. HindIII-restricted ES cell DNA was hybridized to \(32^P\)-labeled probes derived from metablastin gene sequences either outside or contained within the targeting construct. To identify clones in which random integration of the targeting construct had occurred, blots were also rehybridized using a probe derived from the neo gene. Mouse tail DNA was digested with either BamHI or SacI, and the blots were hybridized to the same probes used for analysis of ES cell DNA.

Northern Blot Analysis—Total RNA was extracted from brains of 2-day-old and testes of 2-month-old mice and analyzed by Northern blotting as described previously (25). The blots were sequentially probed using \(32^P\)-labeled rat metablastin (P19) and rat SCG10 cDNA as described previously (23, 25, 47).

Immunoblot Analysis—Extracts of 2-day-old mouse brains and 2-month-old mouse testes were prepared and subjected to immunoblot analysis as described previously (21, 25). The antisera used was previously shown to react with the carboxyl terminus of metablastin (36), which is encoded by a region not altered in the targeted metablastin gene locus. FACScan analysis—Single cell suspensions were prepared from mouse lymphoid tissues (thymus, spleen, and lymph nodes). The number of cells in each organ was determined by counting samples in a Neubauer hemocytometer. The cells were triple-stained as described previously (48) using fluorescein isothiocyanate-labeled anti-T-cell receptor (clone 57-S597), Tricolor labeled anti-CD4 (clone L3T4) purchased from CALTAG, San Francisco, CA, and biotinylated anti-CD8 monoclonal antibody (clone 53.6.72). The biotin label was revealed by streptavidin-R-phycocerythrin (Sigma). 20,000 cells were acquired for analysis in the FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Histological Analysis—Brains and testes of 2-month-old mice were snap-frozen in liquid N2 and stored at –86°C. Cryostat sections were stained with cresyl violet.

RESULTS AND DISCUSSION

A recombinant vector was constructed so as to yield, by homologous recombination, a rearranged metablastin locus in which exon I and exon III are replaced by a PGK-neo cassette (Fig. 1, b and c). Such a rearrangement would be predicted to abolish the expression of a functional gene product from this locus because exon I contains the translation initiation codon and exon III encodes a region of metablastin containing two critical phosphorylation sites as well as a putative coiled-coil domain considered important for protein-protein interactions. Murine embryonic stem (ES) cells were transfected with this replacement type construct and subjected to positive-negative selection as described (49). Southern blot analysis was used to distinguish between ES cell clones in which a homologous recombination event had occurred from those exhibiting random integration of the targeting construct. Four of seven correctly targeted clones were injected into blastocystcs derived from C57BL/6 mice and used to generate chimeric mice. When mated with C57BL/6 females, chimeric males derived from three different ES cell lines transmitted the disrupted metablastin allele to their offspring (data not shown). Mice derived from two of these ES cell lines were analyzed in the subsequent studies and gave identical results.

Metablastin \(1^{-/-}\) mice were crossed to obtain mice in which both metablastin alleles are disrupted (metablastin \(1^{-/-}\)). Because of the high degree of evolutionary conservation of the metablastin gene and its widespread expression during embryonic development, we expected to see developmental defects, possibly resulting in embryonic lethality. However, viable metablastin \(1^{-/-}\) offspring were obtained (Fig. 1d) with mendelian frequency (Table I). To confirm that the homozygous knockout mice lacked metablastin expression, we examined neonatal brain and testis, the two tissues that most abundantly express metablastin in wild-type (metablastin \(1^{+/+}\)) mice, for RNA transcripts and for the presence of immunoreactive metablastin. Using for hybridization a genomic probe derived from metablastin sequences that had been deleted in the metablastin \(1^{-/-}\) line (Fig. 1d, e and f), two RNA transcripts were detected in wild-type neonatal brains. The resulting autoradiogram is shown (overnight exposure). The genotype of the mice, shown above each lane, was determined by genomic Southern blot analysis as shown in d. The position of the ribosomal RNA bands, visualized by ethidium bromide fluorescence, is shown at the right margin, f, immunoblot. Extracts (50 μg of protein) of brains and testes of neonatal and 2-month-old mice, respectively, were subjected to SDSPolyacrylamide gel electrophoresis, followed by electrotransfer to a nitrocellulose membrane and probing with metablastin antisera. The signals were visualized with ECL reagents. The genotype of the mice, shown above each lane, was determined as shown in d. The arrow indicates the location of the 19-kDa band of metablastin.

**Fig. 1** Targeted disruption of the metablastin gene. a, metablastin gene; b, targeting vector; c, rearranged gene locus after homologous recombination with the targeting construct. Thick horizontal lines, genomic sequences; thin horizontal lines, vector sequences; tall open boxes, untranslated exon regions; tall stippled boxes, translated exon sequences; checkered box, PGKneo gene cassette; striped box, PGKtk gene cassette; the position of the probe used for the Southern blot shown in d and the sizes of the predicted restriction fragments are also indicated. The pertinent restriction enzyme sites are N, NotI; S, SacI; R, EcoRI; K, KpnI, d, genomic Southern blot of metablastin \(1^{-/-}\) offspring. Mouse tail DNA was digested with SacI, resolved by agarose gel electrophoresis, transferred to a Nylon membrane, and hybridized to the probe indicated in a. The sizes of hybridizing fragments (kb) are indicated at the right border. The deduced genotypes \(1^{+/+}\), \(+/-\), and \(-/-\) at the top of each lane. e, Northern blot. Total RNA (10 μg) extracted from neonatal brains was resolved by denaturing agarose gel electrophoresis, transferred to a nylon membrane, and hybridized to a \(32^P\)-labeled 1.6-kb genomic EcoRI-KpnI fragment (containing exon II and exon III of the metablastin gene as shown in a). The resulting autoradiogram is shown (overnight exposure). The genotype of the mice, shown above each lane, was determined by genomic Southern blot analysis as shown in d. The position of the ribosomal RNA bands, visualized by ethidium bromide fluorescence, is shown at the right margin, f, immunoblot. Extracts (50 μg of protein) of brains and testes of neonatal and 2-month-old mice, respectively, were subjected to SDS-polyacrylamide gel electrophoresis, followed by electrotransfer to a nitrocellulose membrane and probing with metablastin antisera. The signals were visualized with ECL reagents. The genotype of the mice, shown above each lane, was determined as shown in d. The arrow indicates the location of the 19-kDa band of metablastin.
metablastin<sup>−/−</sup> mice (Fig. 1e). Immunoblotting using an antibody that binds to the carboxyl terminus of metablastin revealed a reduced steady-state level of metablastin in brain of metablastin<sup>−/−</sup> mice and no detectable metablastin in brain or testis of metablastin<sup>−/−</sup> mice (Fig. 1f).

The abundance of metablastin mRNA in brain was reduced in metablastin<sup>−/−</sup> mice to approximately 50% of that observed in wild-type mice (Figs. 1e and 2a). Interestingly, when using a hybridization probe containing exonic sequences that were retained in the disrupted metablastin locus, a low level of transcript, smaller than the major transcript observed in wild-type mice, was detected in the metablastin<sup>−/−</sup> mice (Fig. 2a). The data suggest that the PGKneo cassette and genomic intervening sequences are spliced out from a transcript read through the rearranged metablastin locus, and that this RNA is less stable or is generated at a lower rate, than wild-type metablastin mRNA. This apparently truncated transcript does not appear to be translated, inasmuch as no protein band was detected (Fig. 1f). The data demonstrate that the knockout mice we have generated are functional null mutants (metablastin<sup>−/−</sup>).

Grossly, the metablastin<sup>−/−</sup> mice are normally developed at birth, have no overt neurological or behavioral deficits, and grow at a rate that is indistinguishable from that of both their metablastin<sup>−/+</sup> and wild-type litter mates. Inspection of the gross anatomy of metablastin<sup>−/−</sup> mice, examined at different ages, has revealed no detectable abnormality. On closer examination of brain and testis, the granular cells of the dentate gyrus and the germ cells of the testis, both of which abundantly express metablastin in wild-type mice, appear phenotypically normal in metablastin<sup>−/−</sup> mice, and are present in numbers comparable with those of wild-type litter mates (data not shown). Because of the abundant expression of metablastin in male germ cells during spermatogenesis (23), we considered the possibility that a homeotic disruption of this gene might result in reduced fertility in males. However, the metablastin<sup>−/−</sup> mice reproduce at a normal rate with litters of normal size.

Metablastin expression is highly abundant in a variety of human leukemia cells (25, 50–53) and strongly induced during mitogenic activation of normal human T lymphocytes (3, 25–27). We therefore assessed its potential role in T cell development. Surface antigen expression of T cells was studied in metablastin<sup>−/−</sup> mice and wild-type litter mates using FACS analysis. As shown in Table II, we observed a decrease in the absolute number of T cells in thymus and spleen and a slight increase in lymph nodes of metablastin<sup>−/−</sup> mice. The significance of this observation is not clear, in view of the finding that the relative numbers of CD4<sup>+</sup>, CD8<sup>+</sup>, and CD4<sup>+</sup>/CD8<sup>+</sup> (double positive) lymphocytes in thymus, spleen, lymph nodes, and peripheral blood, as well as their expression of T cell receptors, did not differ significantly between metablastin<sup>−/−</sup> mice and their wild-type litter mates (Fig. 3 and Table II). The data suggest that T cell maturation, which includes positive and negative selection and apoptotic events, proceeds normally in metablastin<sup>−/−</sup> mice. However, it should be noted that the metablastin<sup>−/−</sup> mice are being maintained in a pathogen-free isolation facility. It will be of interest to test their ability to respond to a variety of immune challenges such as exposure to antigens, mitogens, ionizing irradiation, or infectious agents.

SCG10, a protein of unknown function that is exclusively expressed in the nervous system (54), closely resembles metablastin in its primary amino acid sequence (36). The genes encoding metablastin and SCG10 have been mapped to different chromosomes (4, 55, 56) and have presumably arisen from duplication of a common ancestral gene. Because of their closely related amino acid sequence, the two proteins are likely to perform similar functions. We have therefore tested whether the expression of SCG10 is altered in metablastin<sup>−/−</sup> mice. If SCG10 were able to substitute the function normally served by metablastin, one might expect, in metablastin<sup>−/−</sup> mice, the expression of SCG10 to be up-regulated in cells that, in wild-type mice, show abundant metablastin but no SCG10 expression. As shown in Fig. 2, the steady-state level of SCG10 mRNA in newborn brain is essentially identical in wild-type and metablastin<sup>−/−</sup> mice. Furthermore, the testes, which do not express SCG10 in wild-type mice, lack not only metablastin but also SCG10 transcripts in the metablastin null mutants (Fig. 4). The data demonstrate that SCG10 expression is not altered in the metablastin<sup>−/−</sup> mice and rule out up-regulation of this gene as a compensatory mechanism for lack of metablastin. Our studies cannot exclude the possibility that other, more distantly related and not yet characterized, genes may compensate for the lack of metablastin in metablastin<sup>−/−</sup> mice.

### Table I

| Genotype of F<sub>1</sub> offspring of metablastin<sup>+/−</sup>/metablastin<sup>+/−</sup> mice | Number of mice |
|---|---|
| +/− | 89 |
| +/+ | 42 |
| −/− | 42 |

### Table II

| Phenotype | Cells per organ × 10<sup>6</sup> | 100 |
|---|---|---|
| Thymus | CD4<sup>+</sup> | 9.0 | 5.5 | 11.2 | 10.9 |
| | CD8<sup>+</sup> | 2.5 | 1.3 | 3.1 | 2.6 |
| | DP<sup>+</sup> | 67 | 42 | 83.3 | 84.9 |
| | DN<sup>+</sup> | 1.9 | 1.6 | 2.4 | 0.8 |
| Spleen | CD4<sup>+</sup> | 38 | 24 | 20 | 21 |
| | CD8<sup>+</sup> | 25 | 10 | 13.2 | 9.2 |
| | Lymph node | CD4<sup>+</sup> | 7.0 | 9.5 | 50.5 | 50.0 |
| | | CD8<sup>+</sup> | 3.5 | 5.5 | 24.7 | 28.8 |

* Double positive (CD4<sup>+</sup>/CD8<sup>+</sup>).
* Double negative (CD4<sup>+</sup>/CD8<sup>+</sup>).
leukemia cells in G2. They have proposed that metablastin phosphorylationsitemutations resulted in arrest of human cells in G2/M cell cycle checkpoint. Gullberg and co-workers (33, 35) have demonstrated that forced overexpression of metablastin carryin cell cycle regulation. Moreover, recent studies suggest that metablastin plays a role in cell cycle regulation. In view of the high degree of evolutionary conservation and an essential role in mammalian development, this is surprising in view of the observation that forced expression of phosphorylation site mutants of metablastin in leukemia cells resulted in arrest of human leukemia cells in G2. It is of interest, in this regard, that forced overexpression of metablastin carrying phosphorylation site mutations resulted in arrest of human leukemia cells in G2. They have proposed that metablastin normally participates in a G2/M cell cycle checkpoint, and that transition through this checkpoint requires phosphorylation of metablastin. The apparently normal growth and development of the metablastin−/− mice are not necessarily incompatible with this hypothesis. The absence of an inhibitory component of a cell cycle checkpoint would not be expected to prevent transition through the cell cycle. It is of interest, in this regard, that targeted disruption of at least two other genes known to play a role in cell cycle control, those encoding p53 and p21CIP1/WAF1, did not affect normal mouse development (57, 58). Moreover, a growing body of evidence indicates that checkpoints of a process as critical as the cell cycle are under the control of multiple genes. Therefore, loss of one component may not suffice to deregulate this process and to perturb normal development. A study of cell cycle kinetics of metablastin−/− mouse-derived cells in primary culture should provide important insights regarding the potential role of metablastin in cell cycle regulation.

Considering that several somatic mutations in critical control genes can lead to cell transformation, the disruption of a gene encoding a component of a cell cycle checkpoint could result in a predisposition to tumor formation. This is borne out in p53 knockout mice, which have an increased incidence of tumors (57). However, at the time of this report, at 13 months of age, the metablastin−/− mice have not developed tumors and have remained indistinguishable from their metablastin+/+ and wild-type litter mates. Studies are in progress to explore this question in more depth. Of particular interest will be to study the tumor susceptibility of the metablastin−/− mice upon exposure to carcinogens or ionizing irradiation.

In view of the observation that forced expression of phosphorylation site mutants of metablastin in leukemia cells resulted in failure of the cells to enter mitosis (33, 35), it is of interest that p53 has been shown to participate in a mouse spindle checkpoint (59). Therefore, crossing the metablastin−/− genotype into the p53−/− background may lead to a more severe tumor susceptibility in the double knockout mutants.

In conclusion, we have generated a metablastin knockout mouse. Although we cannot exclude subtle functional abnormalities in these mice, their apparently normal growth, development, reproduction, and behavior suggest that metablastin is not essential for any of these processes. However, in view of the available evidence implicating metablastin in cell cycle regulation, these mice will be valuable for experimentally testing predictions that follow from this hypothesis.

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Addendum—After submission of this manuscript, it was reported that metablastin interacts with tubulin dimers and increases the catastrophe rate of microtubules in vitro (60). The metablastin−/− mice will be of great value in determining the significance of this finding in vivo.

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