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MARCKS deficiency in mice leads to abnormal brain development and perinatal death

(myristoylated, alanine-rich C kinase substrate/protein kinase C/*Macs* gene)

DEBORAH J. STUMPO*‡, CHERYL B. BOCK†, JANE S. TUTTLE*‡, AND PERRY J. BLACKSHEAR*†‡§

‡Howard Hughes Medical Institute Laboratories, Durham, NC; *Division of Endocrinology, Metabolism and Nutrition, Departments of Medicine and Biochemistry; and †Comprehensive Cancer Center, Duke University Medical Center, Durham, NC 27710

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ABSTRACT **The MARCKS protein is a widely distributed cellular substrate for protein kinase C. It is a myristoylprotein that binds calmodulin and actin in a manner reversible by protein kinase C-dependent phosphorylation. It is also highly expressed in nervous tissue, particularly during development. To evaluate a possible developmental role for MARCKS, we disrupted its gene in mice by using the techniques of homologous recombination. Pups homozygous for the disrupted allele lacked detectable MARCKS mRNA and protein. All MARCKS-deficient pups died before or within a few hours of birth. Twenty-five percent had exencephaly and 19% had omphalocele (normal frequencies, <1%), indicating high frequencies of midline defects, particularly in cranial neurulation. Nonexencephalic MARCKS-deficient pups had agenesis of the corpus callosum and other forebrain commissures, as well as failure of fusion of the cerebral hemispheres. All MARCKS-deficient pups also displayed characteristic lamination abnormalities of the cortex and retina. These studies suggest that MARCKS plays a vital role in the normal developmental processes of neurulation, hemisphere fusion, forebrain commissure formation, and formation of cortical and retinal laminations. We conclude that MARCKS is necessary for normal mouse brain development and postnatal survival.**

The myristoylated, alanine-rich C kinase substrate, or MARCKS protein, is the prototype of a small family of prominent cellular substrates for protein kinase C (PKC) (reviewed in refs. 1 and 2). It is an acidic, amphiphilic, myristoylated protein whose precise function in cells has not been determined. Known attributes of the protein include (*i*) multisite phosphorylation by PKC, as well as extremely high kinetic affinity for members of this family of kinases; (*ii*) high-affinity calmodulin binding, with calmodulin being displaced by PKC-dependent phosphorylation; (*iii*) apparent actin crosslinking and bundling activity, perhaps linking cortical actin with the plasma membrane; and (*iv*) myristoylation-dependent membrane association that can be at least partly reversed by phosphorylation in some systems. Most of these attributes are shared by the MARCKS-related protein, or MRP (also known as F52 or MacMARCKS), which nonetheless is clearly a distinct gene product.

Several types of experiments have suggested that MARCKS might be involved in development, particularly of the nervous system. First, it is highly expressed in the brain and spinal cord of adult animals (3–5). In the bovine brain, its concentration has been estimated to be about 12 μM (6). Second, its expression is much higher in fetal than in adult brain of rats (7). Finally, most members of the PKC family are expressed in brain (8, 9) and have been suggested to play important roles in brain development.

To elucidate possible roles of MARCKS in both development and normal cellular function, we disrupted its gene in mice by using the techniques of homologous recombination in embryonic stem (ES) cells. This gene, *Macs*, has been localized to mouse chromosome 10 in a region of synteny with human chromosome 6q21, where the human gene (*MACS*) has been mapped (10, 11). Our results indicate that expression of MARCKS during embryonic and fetal life in the mouse is necessary for normal development of the central nervous system as well as for extrauterine survival.

METHODS

Construction of Targeting Vector and Generation of Chimeric Mice. The *Macs* replacement targeting vector was created by using a 2.4-kb *Sst* I genomic fragment of the mouse genomic clone (10) that contains the 102-bp protein-coding region (exon I). A unique *Sst* I site was created by site-directed mutagenesis at bp 409 in this clone, and a unique *Bgl* II site was created at bp 1114, 18 bp past the initiator ATG. A 1.14-kg *Xho* I–*Bam*HI fragment of pMC1POLA (Stratagene) carrying a neomycin-resistance gene (*neo*) was blunted and subcloned into the blunted *Bgl* II site in the same orientation as the MARCKS protein-coding sequence. The *Macs/neo* construct was excised with *Sst* I, yielding a 3.2-kb fragment that was subcloned into pGEM-7Zf (Promega) in which the original *Sst* I site was destroyed and a new *Sst* I site was created by site-directed mutagenesis to replace the original *Eco*RI site in the multiple cloning site. A thymidine kinase gene (a 1.86-kb *Hin*dIII–*Xho* I fragment of pIC19R/MC1-TK; ref. 12) was subcloned into the blunted *Xba* I site of the pGEM-*Macs/neo* construct. Both selectable markers are under the control of the herpes simplex virus thymidine kinase gene promoter and a polyoma enhancer (13). The final targeting vector, pGEM/80K-Neo/TK, was linearized with *Cla* I prior to electroporation into ES cells.

Cell culture and electroporation of E14TG2a (14) ES cells and generation of chimeric mice were performed essentially as described (15). For Southern analysis of DNA from ES cells or tails, DNA (10 μl, 5–15 μg) was digested with *Hin*dIII and probed (16) with a 2.1-kb *Hin*dIII–*Sst* I fragment of the *Macs* genomic clone spanning exon I and most of the 5′ untranslated region and intron (10). This probe recognized both the 2.2-kb *Hin*dIII fragment of the endogenous gene and the 3.3-kb *Hin*dIII fragment of the *neo*-disrupted gene. To determine sex, Southern blots were hybridized with a Y chromosome-specific probe that was a generous gift from Beverly Koller, University of North Carolina, Chapel Hill.

Western Blot Analysis. For analysis of fetal mice, pregnant *Macs* +/− mice that had mated with male +/− mice were

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Abbreviations: MRP, MARCKS-related protein; PKC, protein kinase C; ES cells, embryonic stem cells; E*n*, embryonic day *n*.

§To whom reprint requests should be addressed at: Box 3897, Duke University Medical Center, Durham, NC 27710.

killed on embryonic day 18.5 (E18.5) by cervical dislocation; pregnancy dates were confirmed by the presence of a vaginal plug (E0.5). Fetal mice were rapidly removed and decapitated, and the heads were rapidly frozen in liquid N_2. These were pulverized under liquid N_2 and homogenized (13 strokes in a Teflon/glass homogenizer) in 4 volumes of an ice-cold homogenization buffer containing 100 mM β-glycerophosphate (pH 7.5), 20 mM EGTA, 20 mM dithiothreitol, and 0.6% (vol/vol) Triton X-100. The homogenates were placed on ice for 40 min and then centrifuged at 12,000 × *g* for 30 min. Heat-stable extracts were prepared by boiling these samples for 10 min, followed by cooling on ice and recentrifugation under the same conditions. Equal amounts of protein were subjected to electrophoresis in an SDS/12% polyacrylamide gel and transferred to a nitrocellulose filter; this was incubated with a rabbit antiserum (1:100 dilution) raised against an amino-terminal MARCKS peptide (17).

RNA Isolation and Analysis. Total cellular RNA was isolated from frozen heads of E18.5 pups by a modification of the single-step acid guanidinium thiocyanate/phenol/chloroform extraction (18). After the initial extraction and 2-propanol precipitation, the RNA pellet was washed twice with 70% (vol/vol) ethanol and resuspended in 100 μl of water. RNA (15 μg) was fractionated in a 2.2 M formaldehyde/1.2% agarose gel, transferred to Nytran (Schleicher & Schuell), and hybridized with a 1.6-kb *Pst* I fragment of pBS80K-4 as described (16).

Histological Analysis. Fetal tissues were immersed in Bouin's fixative for 48 hrs at room temperature, followed by several days of washes in 70% (vol/vol) ethanol at room temperature. Tissues were then embedded in paraffin; sections (5–7 microns) were used for hematoxylin and eosin staining by standard procedures. Histological sections were photographed with a Nikon Optiphot-2 photomicroscope and Kodak Ektar 100 film. Structural identifications were made using standard atlases (19, 20).

RESULTS

Disruption of *Macs* Results in Total MARCKS Deficiency. Nine chimeric founder males and one female had good frequencies of germline transmission when mated with mice of the C57BL/6J strain; to date, phenotypes have been identical in *Macs* −/− descendents of all 10 founders. Although the description of the phenotype presented here is restricted to studies in C57BL/6J mice, recent preliminary results indicate that the phenotype is identical in 129/SvJ mice, the strain from which the E14TG2a cells were derived (14).

Northern blot analysis of total cellular RNA samples prepared from heads of E18.5 fetal mice demonstrated that the normal MARCKS transcripts of 2.6 kb (spliced) and 4.5 kb (unspliced) were undetectable in the −/− animals and were expressed at about half (48% by densitometry) their normal intensity in the +/− animals compared with the +/+ animals (Fig. 1*a*). When the same Northern blot was probed with a cDNA probe for the MARCKS homologue MRP, its mRNA levels were essentially identical in the samples from all three genotypes (Fig. 1*a*).

By immunoblot analysis, MARCKS protein was not detectable in heads from E18.5 −/− animals (Fig. 1*b*); however, MARCKS was detected readily in extracts from the +/− and +/+ animals. As assessed by densitometry, extracts from the +/− animals contained 54% of the amount of immunoreactive MARCKS present in extracts containing equal amounts of protein from the +/+ animals (Fig. 1*b*). An identical gel of the same extracts stained with Coomassie blue confirmed equal loading of protein into the gel lanes (data not shown).

MARCKS Deficiency Results in Perinatal Lethality. Mice heterozygous for the disrupted gene (*Macs* +/−) appeared normal in all respects, and their reproductive ability was

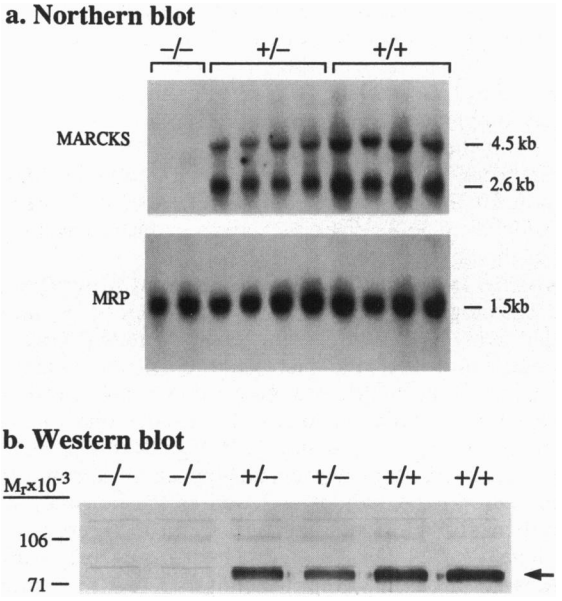

FIG. 1. Expression of MARCKS and MRP mRNAs and MARCKS protein. (*a*) Total cellular RNA was prepared from frozen heads of fetal mice at E18.5; 15 μg per lane was subjected to electrophoresis and Northern blot analysis. As indicated, the samples were from pups genotyped as *Macs* −/−, +/−, or +/+. The blot labeled MARCKS shows the two species of MARCKS mRNA, 4.5 and 2.6 kb. The same blot was stripped and hybridized with a MRP cDNA probe as indicated; shown is the single species of 1.5 kb representing MRP mRNA. (*b*) Western blot of protein extracts (about 315 μg per lane) from frozen heads of E18.5 pups; the *Macs* genotypes are shown at the top.

normal. However, the −/− state was associated with prenatal or perinatal lethality. Of 185 genotyped pups born that were recovered, 31 (17%) were *Macs* −/−, 52 (28%) were +/+, and 102 (55%) were +/−. The non-Mendelian ratios of these genotypes suggested loss of *Macs* −/− pups, due to prenatal death and resorbtion or postnatal cannibalization. Most of the 31 −/− pups were found dead on the day of birth; some of these pups survived briefly, but all died within several hours of birth. The few survivors were observed to breathe and move all four limbs, but none was ever observed to suckle or to have milk in the stomach.

MARCKS Deficiency Results in High Frequencies of Exencephaly, Omphalocele, and Runting. An additional 377 fetuses were delivered from E12.5 to E18.5. Data from these animals were combined with the natural births noted above, for a total of 574 (128 +/+, 336 +/−, and 110 −/−). There was a high frequency (25%) of exencephaly in the *Macs* −/− animals, compared with 0.9% of the +/− pups and 0% of the +/+ pups. Exencephaly is a severe birth defect in which the brain is malformed and in which the normal skin and skull covering the brain are absent. The *Macs* −/− pups also exhibited a high frequency of omphalocele, 19%, compared with 0.9% and 0% for the +/− and +/+ pups, respectively. Omphalocele is a large herniation at the umbilicus, in which portions of the liver and other abdominal contents protrude through the abdomen. Runting, defined as crown–rump length of less than about two-thirds that for most other pups in the litter, was also more frequent in the −/− animals, 29% compared to 5.4% and 3.9% in the +/− and +/+ animals, respectively. Fourteen percent of the pups with exencephaly also had omphalocele, 39% had runting, and 7% had both omphalocele and runting. Of pups with omphalocele, 19% also had exencephaly, 38% had runting, and 9% had both exencephaly and runting.

Because exencephaly is more common in female pups in other mouse models of exencephaly (21, 22), as is its human

equivalent, anencephaly (23), we determined the sex of the −/− pups. The frequency of exencephaly was indeed higher in females than in males with the −/− genotype: 31% of −/− females and 15% of −/− males exhibited this defect. In contrast, omphalocele was present in 14% of the −/− females and 15% of the −/− males.

Apart from omphalocele, no other apparent abnormalities were noted in the major extracranial organ systems of the *Macs* −/− animals at E18.5, by either gross or histological evaluation at the light microscopic level.

MARCKS Deficiency Results in Abnormal Brain Development. Histological analysis of brains of *Macs* −/− animals revealed many abnormalities, including decreased head size, decreased brain size, and increased ventricular volume. A more specific abnormality was observed in coronal sections at the level of the optic chiasm in three littermates, one +/+ (Fig. 2*a*) and two −/− (Fig. 2 *b* and *c*). In Fig. 2*a* can be seen the normal corpus callosum, the ventral hippocampal commissure, and the anterior commissure, as well as the normally fused cerebral hemispheres. In Fig. 2 *b* and *c*, all three of these commissures have been grossly disrupted, and there is abnormal separation of the cerebral hemispheres. These findings were observed in 13 of 14 brains examined from *Macs* −/− animals, and in none of the +/− or +/+ littermates; in the 14th case, the corpus callosum was partly intact, but the anterior commissure was disrupted. In Fig. 2 *b* and *c* can also be seen the abnormal accumulations of white matter on either side of the interhemispheric fissure that occur when axons cannot cross the midline; in the case of axons ordinarily destined to be within the corpus callosum, these have been termed "Probst's bundles" (24–26).

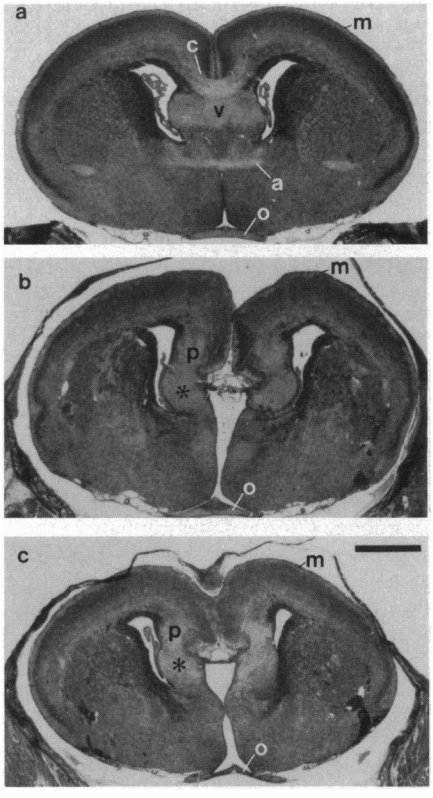

FIG. 2 Coronal sections of brains from E18.5 MARCKS-deficient mice. Shown are hematoxylin- and eosin-stained equivalent coronal sections of brains of three littermate fetal mice at E18.5; all three sections were taken at the level of the optic chiasm (o). The brain from a *Macs* +/+ animal is shown in *a*; brains from two of its −/− littermates are shown in *b* and *c*. m, Marginal zone; c, corpus callosum; v, ventral hippocampal commissure; a, anterior commissure; p, Probst's bundles. The asterisks indicate structures equivalent to Probst's bundles that have formed due to failure of formation of the ventral hippocampal commissures. (Bar = 0.5 mm.)

Another universal abnormality is shown in the higher-power coronal sections of a +/+ animal and its −/− littermate shown in Fig. 3. In addition to the disruption of forebrain commissures, both Figs. 2 and 3 show that the normal, smooth boundary of the cortical plate with the marginal zone is ruffled or irregular in the brains from the −/− animals. This and other abnormalities of cortical lamination were most prominent in the parietal cortex and hippocampal region and were seen in *every* brain examined microscopically from a total of 20 −/− animals, excluding those with exencephaly; the disrupted layering was not seen in any of the +/− or +/+ animals examined.

MARCKS Deficiency Results in Abnormal Retinal Layering. Grossly, eyes from the −/− animals appeared similar to those of their +/+ littermates. At E18.5, the developing retina can be divided into inner and outer nuclear layers and an intervening, nuclei-free layer known as the "transient fiber layer of Chievitz" (27, 28). This layer was prominent in normal retinas at E18.5 (Fig. 4 *a* and *c*); however, in all 14 retinas examined from −/− mice, this thick and discrete non-nuclear layer was nearly obliterated (Fig. 4 *b* and *d*).

DISCUSSION

The most important result of this study is that disruption of *Macs* and complete lack of MARCKS expression results in the failure of the mouse central nervous system to develop normally. Since we have so far failed to find obvious defects in other major organ systems, we postulate that it is this abnormality that leads to the death of all MARCKS-deficient mice at about the time of birth. These data suggest that MARCKS expression during embryogenesis in the mouse is necessary for the normal development of the central nervous system and, indeed, for extrauterine survival.

It should be emphasized that MARCKS is expressed in many extracranial tissues in the mouse (5) and is highly expressed in many developing neuronal tissues in which phenotypic alterations were not seen in the MARCKS-deficient mice (W. S. Lai, W. S. Young III, and P.J.B., unpublished data). It is possible that more subtle defects in these and the non-neuronal tissues will be detected by ultrastructural and/or immunological methods. However, it also seems possible that the high-level expression of the MARCKS homologue MRP in these tissues during development (W. S. Lai, W. S. Young III, and P.J.B., unpublished data) prevents them from exhibiting an abnormal phenotype. If MRP is truly a functional homologue of MARCKS (1, 2), then its continued high-level expression in the central nervous system in the MARCKS-deficient animals means that total "MARCKS equivalents" are not eliminated but are merely decreased, perhaps by less than 50%. In this case, only the most sensitive processes would be affected by this partial deficiency in MARCKS equivalents. These issues will be addressed when the "double-knockout" of MARCKS and MRP is generated; we predict that this animal should exhibit even more severe disruption of central nervous system development.

It is possible that the observed phenotype could be, at least in part, the result of abnormalities in putative MARCKS-binding proteins. For example, calmodulin, which binds to MARCKS with high affinity, could be present in the MARCKS-deficient animals as "free" calmodulin at inappropriate times or places; alternatively, failure of calmodulin to be tethered by MARCKS to specific membrane locations could also lead to developmental abnormalities.

Since the precise cellular function of MARCKS is unknown, it is of interest to try to correlate the rather complex phenotype resulting from its deficiency with the temporal and spatial patterns of MARCKS expression in the developing mouse. For

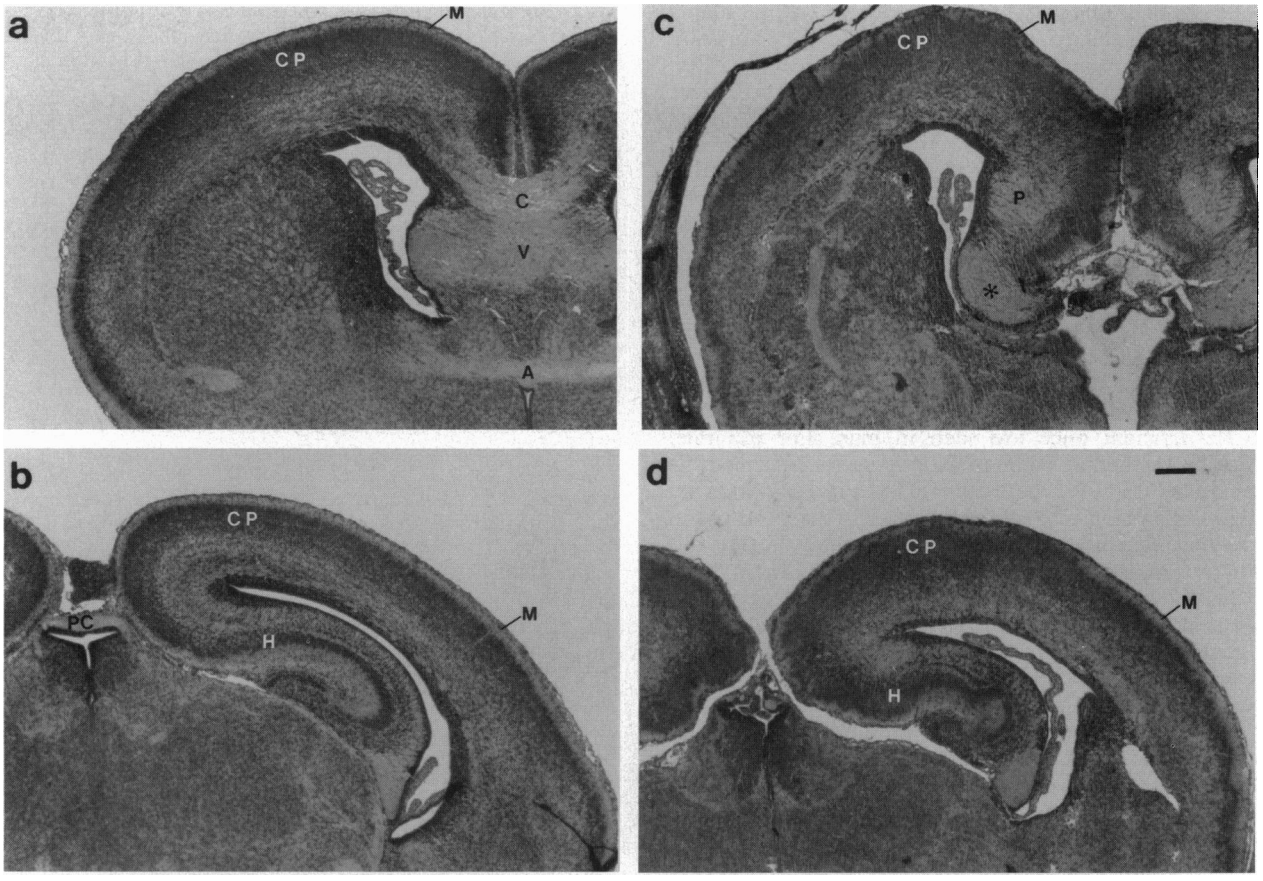

FIG. 3. Higher-power magnification of brains from E18.5 MARCKS-deficient mice. Shown are portions of hematoxylin- and eosin-stained coronal sections of brains from two littermates at E18.5, one *Macs* +/+ (*a* and *b*) and the other *Macs* −/− (*c* and *d*). In *a* is shown the normal anatomy in a plane at about the level of the optic chiasm; in *c* is shown a corresponding section from a MARCKS-deficient animal. *b* and *d* represent more posterior sections from the same normal and MARCKS-deficient animals, respectively. M, marginal zone; CP, cortical plate; H, hippocampus; C, corpus callosum; V, ventral hippocampal commissure; A, anterior commissure; P, Probst's bundles; PC, posterior commissure. The asterisk in *c* indicates an axon bundle that has formed due to the failure of formation of the ventral hippocampal commissure. (Bar = 200 μm.)

example, exencephaly results from the failure of complete cranial neuropore closure, which occurs in the mouse between E8 and late E9 (29, 30). The forebrain commissures in mice form between about E14 and E16 (31). The development of cortical laminations begins about E11 and proceeds during the rest of fetal life, as neuroblasts migrate from the ventricular zones to reach their final cortical destinations (32–36). In normal mice, MARCKS is highly expressed throughout the

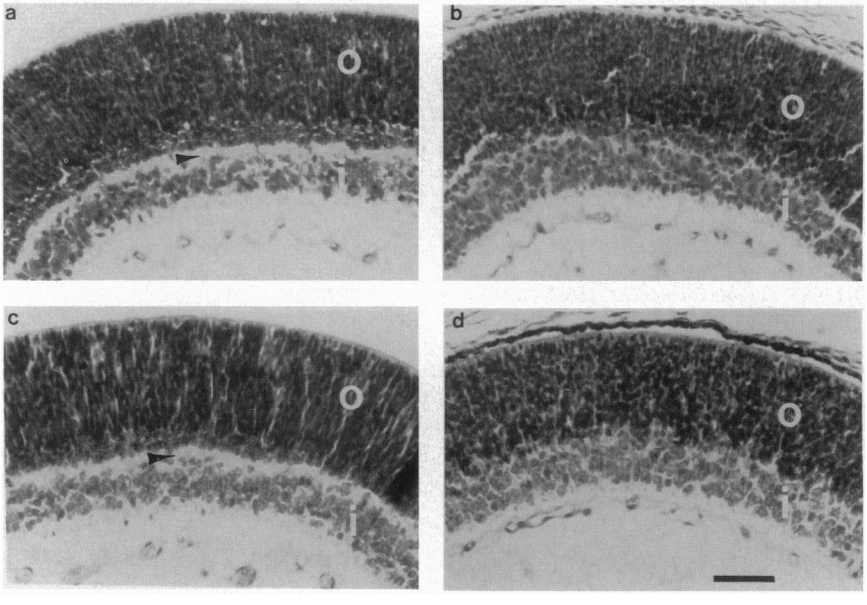

FIG. 4. Retinas from *Macs* +/+ and −/− mice. Shown are hematoxylin- and eosin-stained sections through the retinas of two *Macs* +/+ fetal mice from different litters at E18.5 (*a* and *c*), and retinas from their corresponding *Macs* −/− littermates (*b* and *d*). The vitreous humor is at the bottom of each micrograph. o, Outer nuclear layer; i, inner nuclear layer; arrowhead, transient layer of Chievitz. (Bar = 50 μm.)

brain and retina during these important developmental events (W. S. Lai., W. S. Young III and P.J.B., unpublished data), and its absence at appropriate times and anatomical locations apparently leads in some way to disruption of these processes.

In all cases, it seems possible that MARCKS and presumably PKC are involved in mediating a signal generated at the cell surface that is involved in cell–cell interactions, cell migration, or destination recognition. With the animal model described here, it should be possible ultimately to determine the cellular and biochemical nature of these interactions.

No human deficiency of MARCKS has been described to date, to our knowledge, except for a patient with a deletion of chromosome 6q21 (37) who is heterozygous for the *MACS* allele (10). We presume that total MARCKS deficiency in man would be an autosomal recessive condition, with likely fetal or early neonatal lethality. Many of the defects exhibited by the MARCKS-deficient mice are seen in man. For example, anencephaly, the human counterpart of mouse exencephaly, is a common neural tube defect in man, with a worldwide prevalence of about 1 in 1000 live births (23). Agenesis of the corpus callosum in man is also relatively common (38, 39), and several autosomal recessive syndromes that include this condition have been described (40, 41). Disorders of neural migration in man are also relatively common (42). In mice, many genetic syndromes have been identified that include a high frequency of exencephaly (29), agenesis of the corpus callosum (26), and abnormalities of neuronal migration (43); however, to our knowledge, no single mutant strain has been identified in which all three abnormalities coexist in a single heritable syndrome.

Considerable future work will be necessary to explain at the molecular and cellular levels the role of the MARCKS protein and presumably PKC in certain aspects of central nervous system development, especially neurulation, hemisphere fusion, and cortical and retinal lamination. Study of this animal model may shed some light on the mechanisms by which these processes take place during normal development.

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