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Disruption of the MacMARCKS gene prevents cranial neural tube closure and results in anencephaly

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ABSTRACT **MacMARCKS is a member of the MARCKS family of protein kinase C (PKC) substrates. Biochemical evidence demonstrates that these proteins integrate calcium and PKC-dependent signals to regulate actin structure at the membrane. We report here that deletion of the MacMARCKS gene prevents cranial neural tube closure in the developing brain, resulting in anencephaly. This suggests a central role for MacMARCKS and the PKC signal transduction pathway in the folding of the anterior neural plate during the early phases of brain formation, and supports the hypothesis that actin-based motility directs cranial neural tube closure.**

Protein kinase c (PKC) is a family of isozymes that has been implicated in a variety of biological processes including embryogenesis, morphogenesis, growth, and differentiation (1). The MARCKS proteins, MARCKS and MacMARCKS, are well-characterized PKC substrates (2, 3) and are likely effectors of PKC-induced responses. MARCKS is a peripheral membrane protein that binds to the sides of actin filaments and crosslinks them. This crosslinking activity is disrupted both by phosphorylation and by calcium/calmodulin (4). MARCKS is highly enriched in regions where actin filaments dynamically interact with the cytoplasmic surface of the plasma membrane (5, 6). These data suggest that MARCKS participates in regulating the structure of actin at the membrane, a notion reinforced by the observation that mutation of the PKC phosphorylation sites of MARCKS results in a defect in cellular movement in a wound-healing assay (7). MacMARCKS appears to be functionally related to MARCKS (8, 9) because both proteins share an almost identical effector domain which contains calmodulin and actin binding sites, as well as the PKC phosphorylation sites (4, 10). MacMARCKS also appears to regulate actin-based events, since mutation of its PKC phosphorylation sites causes dramatic cytoskeletal rearrangement in PC12 cells (S.C. and A.A., unpublished results). In addition, a MacMARCKS mutant lacking the effector domain causes a generalized defect in phagocytosis when transfected into a macrophage-like cell line (11).

Neurulation results in the formation of the neural tube, which ultimately gives rise to the brain and spinal cord (12). The cellular and molecular bases of neurulation are poorly understood (12, 13). Actin-based wedging of neuroepithelial cells is thought to be a major driving force in neural tube formation, although the role of extrinsic factors in these events are increasingly being appreciated (12, 13). Transmission electron microscopy demonstrates a dense circumferential microfilament band in the apices of neuroepithelial cells during neural plate bending, and disassembly of actin microfilaments by cytochalasin D prevents neural tube closure (14, 15).

Although there is ample evidence for the requirement of actin-based motility in neural tube closure, the signal transduction pathways and actin-binding proteins that control these events are unknown. We report here that deletion of the MacMARCKS gene prevents cranial neural tube closure, resulting in anencephaly. This implicates MacMARCKS and the PKC signal transduction pathway in neural tube closure and suggests a candidate gene, which when mutated, contributes to neural tube defects (NTDs).

MATERIALS AND METHODS

Construction of the MacMARCKS Targeting Vector. A 17-kb genomic clone encompassing the entire MacMARCKS coding region (16), as well as 8-kb upstream and 4-kb downstream sequences, was isolated from a 129/sv genomic library (Stratagene). The targeting construct was generated in a modified pPNT vector (17) as shown in Fig. 1*a*. The 1.3-kb *Eco*RI–*Xba*I fragment containing 3′ flanking sequences was inserted into corresponding sites 3′ of the pgk-*neo* gene in the pPNT vector and the 7.5-kb *Sac*I fragment containing the 5′ upstream sequences, including 17 bp of coding sequence, was inserted 5′ of the pgk-*neo* gene. Finally, an *Hin*dIII site was introduced 5′ to the pgk-*neo* gene to facilitate the identification of the required homologous recombination events that result in the deletion of the MacMARCKS coding sequence (Fig. 1*a*).

Manipulation of Embryonic Stem (ES) Cells. Maintenance, transfection, selection, and clonal expansion of J1 ES cells was performed as described (19). ES cells containing a mutant allele of the MacMARCKS gene were identified by PCR analysis using primers P1, (5′-GAGGAGTCTGACTACCTGAGAAGTC-3′) and P2, (5′-GCCA AGTTCTAATTCCATCAGAAGC-3′) (Fig. 1*a*). Genomic DNA samples that were positive for the 1.3-kb PCR product were digested with *Hin*dIII, and probed by Southern blot analysis with probes A, B, and Neo (Fig. 1*a*) to confirm the sites of recombination and the copy number of the integration.

Generation of MacMARCKS Null Mice. Chimeric and heterozygous mice were generated according to standard protocols (20). Heterozygous mice, and F2 mice or embryos, all in a C57BL/6J × 129/sv background, were analyzed by PCR using the following two independent reactions: A, the *neo* gene was detected using pgk-*neo* primers JM43 (5′-CTCTTCCTCATCTCCGGGCCTTTCG-3′) and N2 (5′-AATATCACGGGTAGCCAACGCTATG-3′); and B, the MacMARCKS gene was detected using intron primers IN1 (5′-TGAACTGCAGGACGAGGCAGCGCGG-3′) and IN2 (5′-AATATCACGG GTAGCCAACGCTATG-3′). For embryos over embryonic day (E) 8.5, the yolk sac DNA was used for PCR analysis. The sex of the null embryos or mice was determined by PCR analysis using primers for the male specific gene *sry*

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Abbreviations: PKC, protein kinase C; NTD, neural tube defect; wt, wild type; DLHP, dorsolateral hinge point; ES, embryonic stem; E, embryonic day.

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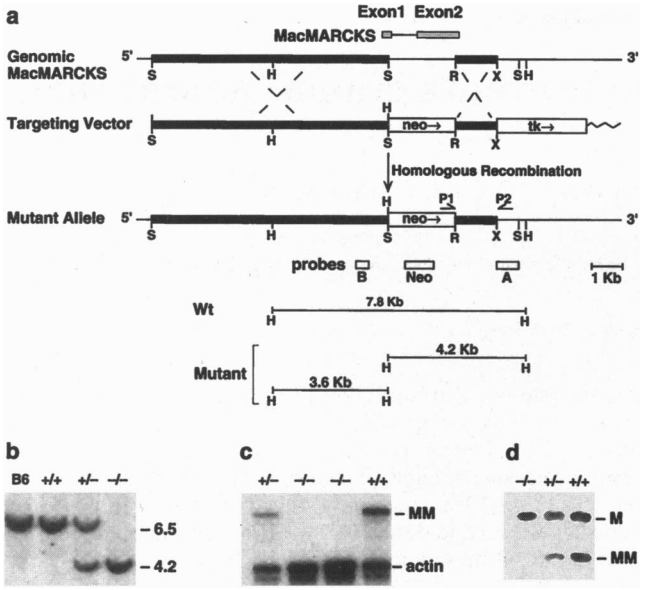

FIG. 1. Disruption of the MacMARCKS gene by homologous recombination. (*a*) Structure of the MacMARCKS gene and the gene targeting construct. Exon1, exon2, and the intervening intron of the MacMARCKS gene were replaced with a pgk-*neo* (*neo*) cassette and flanked by 7.5 kb of 5′ and 1.3 kb of 3′ MacMARCKS genomic sequences (thick black lines). The pgk-tk (tk; thymidine kinase) gene was used as a marker for negative selection. After homologous recombination in ES cells, the mutant allele was detected by a new *Hin*dIII (H) site. The expected sizes of the *Hin*dIII restriction fragments for both the wt (7.8 kb) and the mutant allele (4.2 kb for probe A and 3.6 kb for probe B) are indicated. P1 and P2 are the PCR primers for the identification of positive ES clones. Abbreviations: H, *Hin*dIII; R, *Eco*RI; S, *Sac*I; X, *Xba*I. (*b*) Germ-line transmission of the MacMARCKS mutant allele. Genomic DNAs from tails of F2 siblings were digested with *Hin*dIII and analyzed by Southern blot analysis using probe A. The presence of a single 4.2-kb fragment indicates a homozygous mutant (−/−). A DNA sample from a C57BL/6J (B6) mouse was included as a control for the *Hin*dIII fragment polymorphism between C57BL/6J (6.5 kb) and 129/sv (7.8 kb) mice. (*c*) Absence of MacMARCKS mRNA in MacMARCKS null mice. RPA was performed on 5 μg brain RNA samples from +/+ and +/−, and 10 μg from −/− E16.5 embryos. An actin riboprobe (Ambion, Austin, TX) was included in the RPA reaction as internal loading control. (*d*) Absence of MacMARCKS protein in MacMARCKS null mice. Fifty micrograms of heat-treated E16.5 embryonic brain lysate was used. Anti-MARCKS and MacMARCKS antiserum are as described (18). M and MM stand for MARCKS and MacMARCKS, respectively.

(21). Southern blot, RNase protection (RPA), and Western blot analysis were done according to standard protocols (22).

Analysis of Embryos. E8.5–E10.5 embryos were dissected, fixed with 4% paraformaldehyde in PBS, and photographed under dark-field luminescence. Histological analysis was performed on hemotoxylin/eosin-stained 5-μm horizontal sections of fixed embryos. E18.5 embryos were perfused transcardially before being processed for histology.

Whole Mount and Section *in Situ* Hybridization. Embryos were fixed in 4% paraformaldehyde for 2 hr at 4°C before being processed for whole mount *in situ* hybridization as described (23, 24). Riboprobes labeled with digoxygenin-UTP were detected using alkaline phosphatase coupled anti-digoxygenin antibody with purple blue as a substrate (Boehringer Mannheim). The embryos were photographed after being cleared in 80% glycerol/PBS. The MacMARCKS riboprobe was a 1.5-kb full-length cDNA (8). *Emx-2* and *En-2* riboprobes were as described (26, 27). Wild-type embryos were cryosectioned and processed for *in situ* hybridization as described (27, 28).

Immunohistochemistry of Whole Mount Embryos. MacMARCKS antibody staining of whole mount embryos was performed as described (21). Affinity-purified anti-MacMARCKS antibody was used at 1:50 dilution, and affinity-purified peroxidase-conjugated goat anti-rabbit secondary antibody (The Jackson Laboratory) was used at a 1:300 dilution.

RESULTS

Targeted Disruption of the MacMARCKS Gene. The MacMARCKS gene was disrupted with a 4.6% homologous recombination efficiency in J1 ES cells (19) as described in *Materials and Methods*. Site-specific integration of the targeting vector was confirmed by Southern blot analysis (data not shown). ES cells with the mutant allele were used to generate heterozygous (MacMARCKS$^{+/-}$) mice by standard techniques, and these mice were subsequently intercrossed to produce homozygous (MacMARCKS$^{-/-}$) mice (Fig. 1*b*). The MacMARCKS transcript was undetectable by RPA of total cellular RNA isolated from the brains of E16.5 MacMARCKS$^{-/-}$ fetal mice, whereas MacMARCKS$^{+/-}$ animals expressed approximately one-half the mRNA of their MacMARCKS$^{+/+}$ counterparts (Fig. 1*c*). Similarly, immunoblot analysis demonstrated the absence of the MacMARCKS protein in brains from MacMARCKS null animals, whereas MacMARCKS levels in brains of heterozygotes was approximately one-half that of their wild type (wt) counterparts (Fig. 1*d*). All three genotypes expressed identical levels of the MARCKS protein (Fig. 1*d*).

MacMARCKS Null Mice Are Born Anencephalic. MacMARCKS$^{+/-}$ heterozygous mice had no detectable abnormalities; however, MacMARCKS null mice were born anencephalic and died soon after birth (Fig. 2*a*). The empty cranial cavity showed no evidence of brain structures and the overlying skull and skin were absent (Fig. 2*a*). The eyes of the mutant animals also appeared to be abnormally displaced toward the midline. Non-Mendelian ratios were observed for live births (homozygous 11.4%, heterozygous 60.6%, and wild type 28%; $n = 132$), suggesting that a proportion of the MacMARCKS$^{-/-}$ fetuses were being resorbed prenatally. This resorption occurred after E10.5 because the genotypes of E8.5–E10.5 fetuses segregated with the expected Mendelian ratios of −/− 24.6%, +/− 50.8%, and +/+ 24.6% ($n = 236$). All homozygous embryos showed exencephaly with 100% penetrance, suggesting that the anencephaly observed at birth was due to a combination of *in utero* necrosis and birth-related trauma. There is no sex preference in exencephalic mice between E8.5 and newborn as determined by PCR using primers of the male specific gene *sry* (male 50.9%, female 49.9%; $n = 53$).

Brain Malformations in MacMARCKS Null Embryos. To further characterize the mutant phenotype, E18.5 embryos were examined. All mutant animals were alive and responded to tactile stimuli, but displayed grossly abnormal brains that were exposed to amniotic fluid. The skull and the overlying skin were completely absent (Fig. 2*b*). The brains of the mutants appeared small and flattened, with extensive necrosis throughout (Fig. 2*b*). E18.5 mutant brains displayed such extensive perturbations of normal architecture that firm identifications of brain structures were very difficult (Fig. 2 *c*, *e*, and *f*). Coronal sections of the E18.5 embryonic brains demonstrated that forebrain structures that were dorsal in the wt animal were lateral in the mutant (Fig. 2 *c* and *d*). Tissues displaying the distinctive lamination patterns of the cortex were displaced laterally and appeared to fold inside out in the mutant (Fig. 2 *c* and *e*), but laminations characteristic of the cortex were observed at higher magnification (Fig. 2*e*). Sagittal sections indicate that the midbrain was completely replaced with a fold of tissue that extended over the presumptive cortex (Fig. 2*f*). The cerebellum was absent, confirming that the disturbance extended into the metencephalon (Fig. 2*f*). However, identification of the pons indicated that not all metencephalic tissue was missing (data not shown). Moreover, the posterior choroid plexus was present (Fig. 2*f* and data not shown),

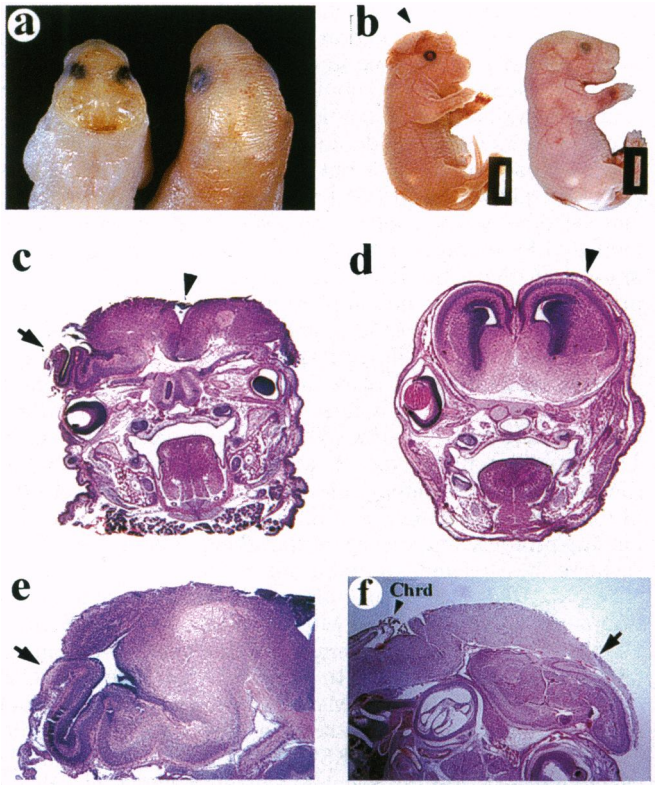

FIG. 2. Deletion of the MacMARCKS gene disrupts normal cranial neurulation and results in anencephaly. (*a*) Dorsal view of newborn MacMARCKS null (*left*) and wt (*right*) pups. MacMARCKS null mice are born anencephalic. (*b*) Side view of E18.5 MacMARCKS null (*left*) and wt (*right*) embryos. Arrowhead indicates the malformed brain. Hematoxylin/eosin staining of coronal sections through similar levels of the forebrain of E18.5 MacMARCKS null embryo (*c*) and wt embryo (*d*). The arrow in *c* indicates position of laterally displaced cortical rudiments. The arrowhead in *c* indicates the midline. The arrowhead in *d* indicates the skull of the wt embryo. (*e*) High magnification of laterally displaced cortical rudiments adjacent to the section shown in *c*. Note the laminar organization of the rudiments. (*f*) Sagittal section of E18.5 MacMARCKS null embryos. The arrow in *f* indicates the absence of a skull. The arrowhead indicates the choroid plexus (Chrd).

suggesting that disturbances due to MacMARCKS deletion lie rostral to the metencephalic/mylencephalic boundary.

Cranial Neural Tube Closure Is Disrupted in MacMARCKS Null Embryos. Exencephaly is defined as a failure of neural tube closure followed by outward expansion of neural tissue via the eversion of the neural plate (29). All MacMARCKS null embryos failed to close the cranial neural tube. Neural tube formation is a complex process, initially involving several events including shaping and bending of the neural plate (13, 30). Prior to E8.5 there was no discernible difference in neural plate shaping when comparing wt and mutant embryos: in both cases the neuroepithelium thickened and elevated around a median hinge point (data not shown). By E8.5 in wt embryos the neural plate began converging toward the midline around paired dorsolateral hinge points (DLHP), thereby initiating cranial neural tube closure (Fig. 3*a*, *right*). By contrast, in MacMARCKS null embryos, the DLHPs were absent, with the lateral edges of the neural folds splayed outward from the midline (Fig. 3*a*, *left*). In the absence of convergence of the neural folds the cranial neural tube failed to close.

By E9.5–E10.5, severe exencephaly involving the forebrain, midbrain, and hindbrain was apparent in MacMARCKS null embryos (Fig. 3*b*, *left* and *c*, *left*). Neurulation at the forebrain/midbrain boundary (closure point two), at the extreme rostral end of the neural plate (closure point three), and at the rostral hindbrain (closure point four) all failed to occur, resulting in a persistently open cranial neural tube (Fig. 3 *b* and *c*). Histological analysis of E9.5 and E10.5 embryos indicated that, whereas the neuroepithelial layer appeared normal, fusion of the neural folds at the forebrain and hindbrain failed to occur (Fig. 3 *d–g*; and data not shown). While closure never occurred at points two and four, it appeared to be delayed at closure point three. This was inferred from two observations: (*i*) in about 10% of the mutants, rudimentary closure occurred at point three by E9.5 (data not shown); (*ii*) initiation of closure point three appeared to have occurred in MacMARCKS null embryos by E11.5 (data not shown). The caudal aspect of the hindbrain up to the level of the otic vesicle appeared normal, indicating that initiation of closure point one had occurred (Fig. 3*c*, *left*). In contrast to the MacMARCKS null embryo, the cranial neural tube had completely closed in wt E9.5 (Fig. 3*b*, *right*). Interestingly, posterior neuropore closure appeared normal in MacMARCKS null embryos (data not shown).

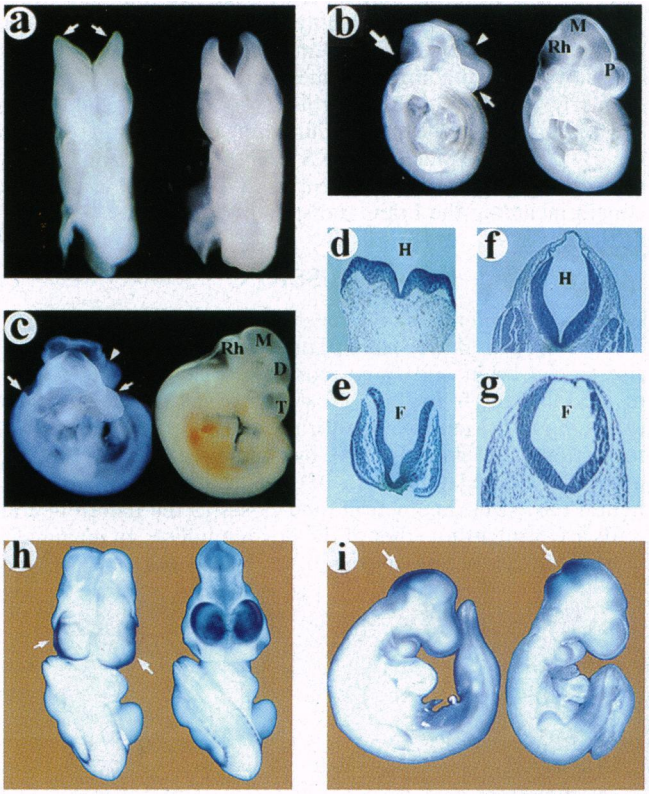

FIG. 3. Disruption of MacMARCKS prevents cranial neural tube closure and results in exencephaly. (*a*) Dorsal view of E8.5 MacMARCKS null (*left*) and wt (*right*) embryos. The neural folds of the wt embryo migrate toward the midline thereby closing the cranial neural tube. The neural folds of the MacMARCKS null embryos splay abnormally outward from the midline (arrows). (*b*) Side view of E9.5 MacMARCKS null (*left*) and wt (*right*) embryos. The MacMARCKS null embryo has an open cranial neural tube. The approximate closure points four (large arrow), two (arrowhead), and three (small arrow) are indicated. (*c*) Side view of E10.5 MacMARCKS null (*left*) and wt (*right*) embryos. Arrows and arrowhead designations are as in *b*. Coronal sections through the hindbrain (*d* and *f*) and forebrain (*e* and *g*) of E10.5 MacMARCKS null (*d* and *e*) and wt (*f* and *g*) embryos. In wt embryos DLHPs are present and neural tube closure has occurred. In contrast, in MacMARCKS, null mutant DLHPs are absent and neural tube closure has failed. (*h*) Whole mount *in situ* hybridization of the prosencephalon marker *Emx-2* to E10.5 mouse wt (*right*) and MacMARCKS null (*left*) embryos. Arrows indicate expression at the lateral edges of the presumptive telencephalon. (*i*) Expression of *En-2* in E10.5 MacMARCKS null (*left*) and wt (*right*) embryos. Arrows indicate expression at the midbrain/hindbrain junction. D, diencephalon; M, mesencephalon; P, prosencephalon; Rh, rhombencephalon.

The pattern of expression of two brain-specific homeobox genes were analyzed in MacMARCKS null embryos. While *Emx-2* was highly expressed in the rostral telencephalon in wt E10.5 embryos (Fig. 3*h*, *right*), it was localized to the lateral edges of the two unfused telencephalic hemispheres in mutant embryos (Fig. 3*h*, *left*). *En-2* expression occurred at the midbrain/hindbrain boundary in both wt and mutant E10.5 embryos (Fig. 3*i*). These results indicate that, whereas normal brain development is disrupted in the MacMARCKS null embryos, the patterns of expression of the region-specific homeobox genes still correctly reflect those seen in the normal embryos.

Expression of MacMARCKS During Embryogenesis Is Consistent with a Role in Neural Tube Closure. Whole mount *in situ* hybridization demonstrated that MacMARCKS mRNA was highly enriched at the lateral edge of the developing neural fold along the entire rostral–caudal length of the axis of normal E8.5 embryos, as well as in the medial hinge point (Fig. 4 *a* and *b*). This is consistent with it playing a role in the morphogenetic movements associated with neural tube closure. *In situ* hybridization of cross sections confirmed that MacMARCKS mRNA was highly enriched in the lateral edges of the neural plate, and also demonstrated that it was detectable in the surrounding head mesenchyme (data not shown). Similarly, the MacMARCKS protein was highly enriched along the folding edge of the entire neural tube in E9.5 embryos (Fig. 4*c*). By E12.5, the MacMARCKS mRNA (Fig. 4*d*) and protein (data not shown), was highly enriched in the central nervous system including the brain and spinal cord.

DISCUSSION

Neurulation refers to the transformation of a flat layer of ectodermal cells into the neural tube, which will ultimately give rise to the brain and spinal cord (12). Neurulation can be divided into the following four stages, which overlap spatially and temporally: neural plate formation, neural plate shaping, formation of the neural groove by bending of the neural plate, and fusion of the lateral edges of the neural groove to form the neural tube (31). The neural tube closes as the paired neural folds are brought together at the dorsal midline. In mammals, neural tube closure is initiated at several places along the anterior–posterior axis, and failure of closure at any of these points results in distinct neural tube defects (32, 33). Deletion of the MacMARCKS gene in mice by homologous recombination prevents cranial neural tube closure and results in exencephaly. Failure of cranial neural tube closure occurs because the neural folds do not bend toward the midline and approach each other, an event mediated by a pair of DLHPs (13). The cells within DLHPs are believed to increase in height and become wedge-shaped, an event that appears to be mediated by the actin cytoskeleton. Thus, treatment of embryos with the actin depolymerizing agent, cytochalasin D, prevents dorsolateral neural plate furrowing, wedging of dorsolateral neuroepithelial cells, and convergence of the neural folds toward the midline. It does not affect neural plate shaping, median neural plate furrowing, wedging of median neuroepithelial cells, or neural fold elevation (14). In other words, treatment of wt embryos with cytochalasin D results in a very similar phenotype to MacMARCKS null embryos, specifically in an apparent defect in actin-based shape change of the neuroepithelial cells of the DLHPs. The specific actin-binding proteins, and the signal transduction pathways, which regulate these events, are unknown.

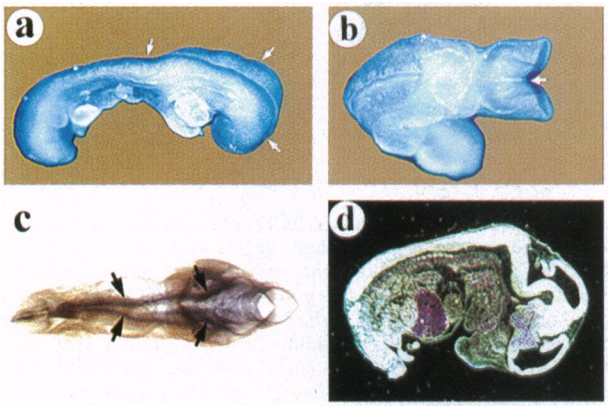

FIG. 4. Expression of MacMARCKS mRNA and protein in wt embryos is consistent with its role in neurulation. (*a*) Side view of whole mount *in situ* hybridization of E8.5 embryo using MacMARCKS antisense riboprobe. Enrichment is seen at the lateral edges (arrows) of the neural fold. (*b*) E8–E8.5 embryo showing that MacMARCKS expression is also enriched in the medial hinge point (arrow) of the neural folds. (*c*) Dorsal view of an E9.5 embryo stained with an antibody to MacMARCKS, indicating that the protein is present along the entire neural tube (arrows). (*d*) Sagittal section of E12.5 wt embryo hybridized with an antisense [^{35}S]UTP-labeled MacMARCKS riboprobe shows high levels of expression throughout the central nervous system. MacMARCKS null embryos and sense probes showed no signal above background (data not shown).

MacMARCKS has been implicated in regulating actin structure at the membrane. Like MARCKS, MacMARCKS binds F-actin in a PKC and calcium/calmodulin sensitive manner (A.A., unpublished results). In addition, transfection of PC12 cells with mutated forms of MacMARCKS result in profound alterations in the actin cytoskeleton (S.C. and A.A., unpublished results). MacMARCKS therefore represents a likely candidate as a specific regulator of actin-based cell-shape change at the DLHPs. This notion is reinforced by the high levels of MacMARCKS expression at the lateral edges of the converging neural folds. Because MacMARCKS activity is regulated by PKC and calcium/calmodulin, these important signal transduction pathways are similarly implicated in neural tube closure. Neural tube closure is a complex series of events involving factors that are both intrinsic and extrinsic to the neural epithelium (12, 13, 34, 35). MacMARCKS is highly enriched at the lateral edge of the neural fold, but also expressed in the lateral nonepithelial tissues that interact with the neural epithelium. Recently it has been demonstrated that *twist* null embryos fail to close the cranial neural tube because of a defect in the extrinsic pathway (36). Thus, although it is most plausible that MacMARCKS participates in neural tube closure via an actin-dependent mechanism intrinsic to the neuroepithelium, it is quite possible that it participates in cranial neural tube closure via an extrinsic, mesenchyme-dependent pathway.

Four sequential points of *de novo* neural tube closure occur in the developing brain (32, 37, 38). These closure points initiate sequentially at the hindbrain/spinal cord boundary (one), the midbrain/forebrain boundary (two), the rostral end of the forebrain (three), and the caudal end of the hindbrain (four), and then proceed in a zipper-like fashion to close the neural tube. It was apparent in MacMARCKS null embryos that *de novo* closure points two and four (12, 33, 37) were disrupted, and closure point three was retarded, leading to exencephaly extending from the rostral portion of the anterior forebrain to the rostral hindbrain. Interestingly, although MacMARCKS is highly enriched along the entire neural tube, only cranial neural tube closure is abrogated in MacMARCKS null embryos. This implies that redundant mechanisms exist for posterior neural tube closure. MARCKS is the most likely compensator of MacMARCKS function. Not only are the effector domains of MARCKS and MacMARCKS homologous, but both proteins appear to function as regulators of actin structure at the membrane. Functional deletion of the MARCKS gene generated a number of developmental defects in the mouse central nervous system, although not all homozygous embryos were similarly affected (39). A proportion of

MARCKS null embryos were exencephalic (25%), whereas others had defects such as decreased brain size, disrupted commissures, abnormal separation of the cerebral hemispheres, and poor cortical laminations (39). Thus, both MARCKS and MacMARCKS appear to have pivotal roles in brain development, and it seems likely that they may functionally compensate for each other in certain aspects of neurulation.

Several other mouse mutants with NTDs leading to exencephaly have been described including *p53*, *Gli-3*, *Pax-3*, *Hes-1*, *twist*, *apo-B*, and *jmj* (29, 36, 40-44). Many of these genes are homeobox genes or transcription factors, and the downstream effectors through which they control neurulation have not yet been defined. By contrast, MacMARCKS and MARCKS provide a plausible link between the actin cytoskeleton and the cell-shape changes required for cranial neural tube closure.

Regional markers of anterior–posterior position suggest that the MacMARCKS mutation does not perturb neural patterning. At E10.5, although evidence of failure of neural tube closure is already present, markers of anterior–posterior patterning appear normally at their appropriate positions. Specifically, we show that in MacMARCKS mutants *En-2* gene expression continues to demarcate the hindbrain/midbrain region of the brain. Similarly, *Emx-2* gene expression, although displaced laterally as a result of failure of neural tube closure, is still apparent in the dorsal diencephalon and dorsal telencephalon.

NTDs most commonly manifest as spina bifida and anencephaly, and the incidence of anencephalic births in the United States is about 0.3 per 1000 live births (45). Little is known about the etiology of NTDs in humans although complex multifactorial genetic and environmental components are involved (46). Recent studies have demonstrated that cranial neural tube closure during human neural development also occurs at the same four fusion sites as described for the mouse, and that anencephaly results from a failure of closure points two and four to initiate fusion (32, 45). Because the MacMARCKS null mouse also has a defect at closure points two and four and is born anencephalic, it promises to be a useful animal model for studying human anencephaly.

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