

Research article

Apical accumulation of MARCKS in neural plate cells during neurulation in the chick embryo

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Published: 24 April 2001

Received: 20 March 2001

BMC Developmental Biology 2001, 1:7

Accepted: 24 April 2001

This article is available from: <http://www.biomedcentral.com/1471-213X/1/7>

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Abstract

Background: The neural tube is formed by morphogenetic movements largely dependent on cytoskeletal dynamics. Actin and many of its associated proteins have been proposed as important mediators of neurulation. For instance, mice deficient in MARCKS, an actin cross-linking membrane-associated protein that is regulated by PKC and other kinases, present severe developmental defects, including failure of cranial neural tube closure.

Results: To determine the distribution of MARCKS, and its possible relationships with actin during neurulation, chick embryos were transversely sectioned and double labeled with an anti-MARCKS polyclonal antibody and phalloidin. In the neural plate, MARCKS was found ubiquitously distributed at the periphery of the cells, being conspicuously accumulated in the apical cell region, in close proximity to the apical actin meshwork. This asymmetric distribution was particularly noticeable during the bending process. After the closure of the neural tube, the apically accumulated MARCKS disappeared, and this cell region became analogous to the other peripheral cell zones in its MARCKS content. Actin did not display analogous variations, remaining highly concentrated at the cell subapical territory. The transient apical accumulation of MARCKS was found throughout the neural tube axis. The analysis of another epithelial bending movement, during the formation of the lens vesicle, revealed an identical phenomenon.

Conclusions: MARCKS is transiently accumulated at the apical region of neural plate and lens placode cells during processes of bending. This asymmetric subcellular distribution of MARCKS starts before the onset of neural plate bending. These results suggest possible upstream regulatory actions of MARCKS on some functions of the actin subapical meshwork.

Background

Major tissue movements during neurulation include neural plate bending as well as neural folds elevation and its convergence to fuse and close the neural tube. These movements of the neural plate result from the actions of extrinsic and intrinsic forces [1], and the latter are believed to be mainly driven by the actin cytoskeleton [2,3,4,5]. Neural plate cells are polarized cells; actin and

myosin are mainly restricted to regions of cell narrowing, especially to the apical border of the epithelium [2,6]. In the apical region, cells are joined together by extensive actin-associated *zonula adherens* cell junctions, which are thought to be important in invagination processes [7,8]. Knockout analyses in mice have shown that some actin binding or *adherens* junction proteins are important for neural tube formation. Examples of these pro-

teins are: vinculin [9], shroom [10], and the two closely related actin cross-linking proteins MARCKS (Myristoylated Alanine-Rich C Kinase Substrate) [11] and MacMARCKS (also called F52 and MRP) [12].

MARCKS is a ubiquitous protein substrate for different PKC family kinases and proline directed kinases such as MAPK and Cdks [13,14,15,16,17]. Its PKC-phosphorylation domain or PSD (Phosphorylation Site Domain) is highly conserved and it is also the site for interaction with other molecules, such as calcium-calmodulin, negatively charged membrane phospholipids and F-actin [13,14]. Binding to calcium-calmodulin and plasma membrane, as well as actin filament cross-linking activity, are antagonized by PSD phosphorylation [13,14,18]. Conversely, calcium-calmodulin binding inhibits PSD phosphorylation and actin crosslinking. In addition to neural tube closure, MARCKS and MacMARCKS have been implicated in several other events related to actin cytoskeleton, such as cell motility, cell spreading, membrane ruffling, phagocytosis, exocytosis and neurite outgrowth [13,19,20,21,22,23,24].

To examine possible anatomical relationships between MARCKS and actin during bending movements, we double labelled chick embryo cryosections at levels showing cranial and spinal neurulation. To compare with other invaginating epithelia we also analyzed the localization of these proteins in the lens placode finding that, in both cases, MARCKS is transiently accumulated in the apical border of the bending epithelia, in a position very close to the apical actin belt. In our knowledge, this is the first report showing a polarized distribution of MARCKS towards an apical cell border, as well as its association with the progression of an essential morphogenetic movement.

Results and Discussion

We performed all our fluorescence microscopy analysis by double labeling serial chick embryo transverse sections (H-H stages 6-15 [25]) with a polyclonal anti-carboxy terminal chicken MARCKS antibody [26] and with rhodamine-conjugated phalloidin (for F-actin labeling).

MARCKS in the open neural plate

At stage 6 chick embryo cephalic neural plate was still flat, although the prospective neuroepithelium was clearly visible as a relatively broader region of the ectoderm (Fig. 1A). We detected MARCKS immunoreactivity in all the embryonic tissues, and a noticeably high signal in the mesoderm, including the notochord (Fig. 1A). In the lateral ectoderm, MARCKS immunolabeling was homogeneously distributed through the epithelium, but in the neural plate region, we observed a higher signal in the apical border of the cells (Fig 1A). By double labeling

the sections with rhodamine-conjugated phalloidin, we could compare MARCKS and actin filaments distribution. We found that F-actin was also highly concentrated in the apical regions of the prospective neuroepithelial cells, where the apical actin meshwork is usually formed (Fig. 1B and 1C). In favorable sections, actin was also seen clearly apicalized in the non-neural ectoderm (see, for example, the right portion of ectoderm in figure 1B). However, we never observed this phenomenon in MARCKS immunolabelled embryos (Figure 1A and 1C). In addition, we observed a higher concentration of F-actin in the basal region of the neuroepithelium around the prospective median hinge point, that seemed to be accompanied by a relatively higher MARCKS signal (Figure 1). Nevertheless, careful analysis of these regions at higher magnifications, exploring consecutive microscopic fields parallel to the section plane, revealed that the amount of MARCKS in the basal regions of neuroepithelial cells was not different to the amount present in the lateral cell regions. The phenomenon of apical MARCKS accumulation, coinciding with an actin filament-dense region, was even more important in slightly more advanced embryos (stage 6+), where the bending at the median hinge point has already started in the cephalic neural plate (data not shown).

Neural tube closure at the presumptive prosencephalic region

At stage 8, neural folds are about to contact at the level of the midbrain, but are still apart in the rest of the neural plate. The presumptive prosencephalic region was characterized by an extended V transversal shape, where the median hinge point was very well marked and the dorso-lateral ones were just beginning to form (Figure 2A-C). There was a high MARCKS immunolabeling throughout the neuroepithelium, but a much higher signal, colocalizing with the apical actin belt, was evident at the edge of the neural plate (Figure 2A,B and 2C). A similar apical co-distribution of MARCKS and actin was evident at stage 8+, when fusion of the neural folds started at the midbrain, and the dorso-lateral hinge points were markedly bent in the prosencephalic primordium (Figure 2D-F). Remarkably, we found here no apparent differences in MARCKS localization or immunolabeling intensity between the hinge point and non-hinge point areas. Later, at stage 9, cephalic neural tube closure is almost complete, and the prosencephalon shows an elongated shape in the transversal plane due to the initial protrusion of the optic vesicles (Figure 2G-I). The most striking result was to find a homogeneous MARCKS distribution in nearly all the neuroepithelial cells, while actin filaments remained highly concentrated in their apical borders (Figure 2G-I). No other change in MARCKS immunolabeling pattern was observed, and the overall signal intensity appeared to be the same than at earlier stages. Thus,

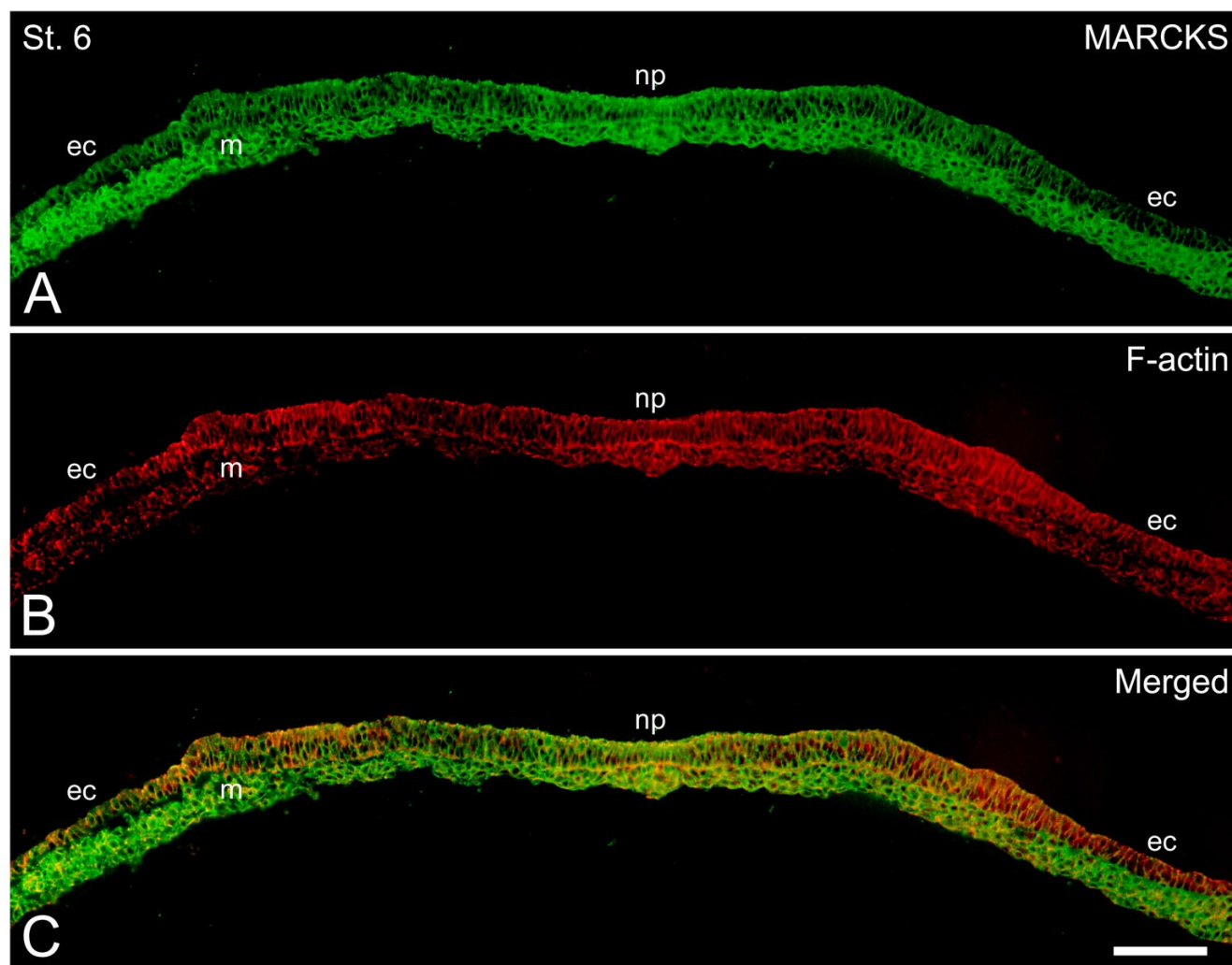


Figure 1
MARCKS and F-actin distribution in the open neural plate. Stage 6 chick embryo transverse cryosection, at the presumptive cephalic region. Double labeling of MARCKS, using a polyclonal antibody, and actin filaments, using phalloidin. In order to show all the neural plate and the adjacent ectoderm, these pictures were reconstructed from five consecutive microscopic fields photographed for each fluorochrome. ec, non-neural ectoderm; m, mesoderm; np, neural plate. Scale bar: 120 μ m.

MARCKS seems to be differentially distributed during cephalic neural plate bending. Blackshear and coworkers also reported a non-homogeneous distribution of MARCKS in the open cephalic neural plate, in mouse embryos, where higher amounts of immunoreactive protein were found near the tips of the neural folds [27]. They did not describe, however, heterogeneity in the subcellular distribution of the protein. Further experiments should be conducted in order to ascertain whether these disparate results were due to differences in the analyzed species, or in the experimental procedures employed (i.e., the anti-MARCKS antibodies used, etc.).

Neural tube closure at the presumptive rhombencephalic and spinal regions

At the rhombencephalic and truncal levels, we observed exactly the same phenomenon than in the prosencephalic region (Figure 3). By stage 8, these regions of the neural plate were still open, and V-shaped. MARCKS was highly present in the apical portion of the neuroepithelium (Figure 3A and 3G), as were actin filaments (Figure 3B and 3H). Again, we observed extensive co-localization of these proteins in the apical borders (Figure 3C and 3I). Once the neural tube was completely closed at these regions (stage 9), we found that MARCKS was homogeneously distributed, while the actin filaments maintained their apical localization (Figure 3D-F and 3J-L). These

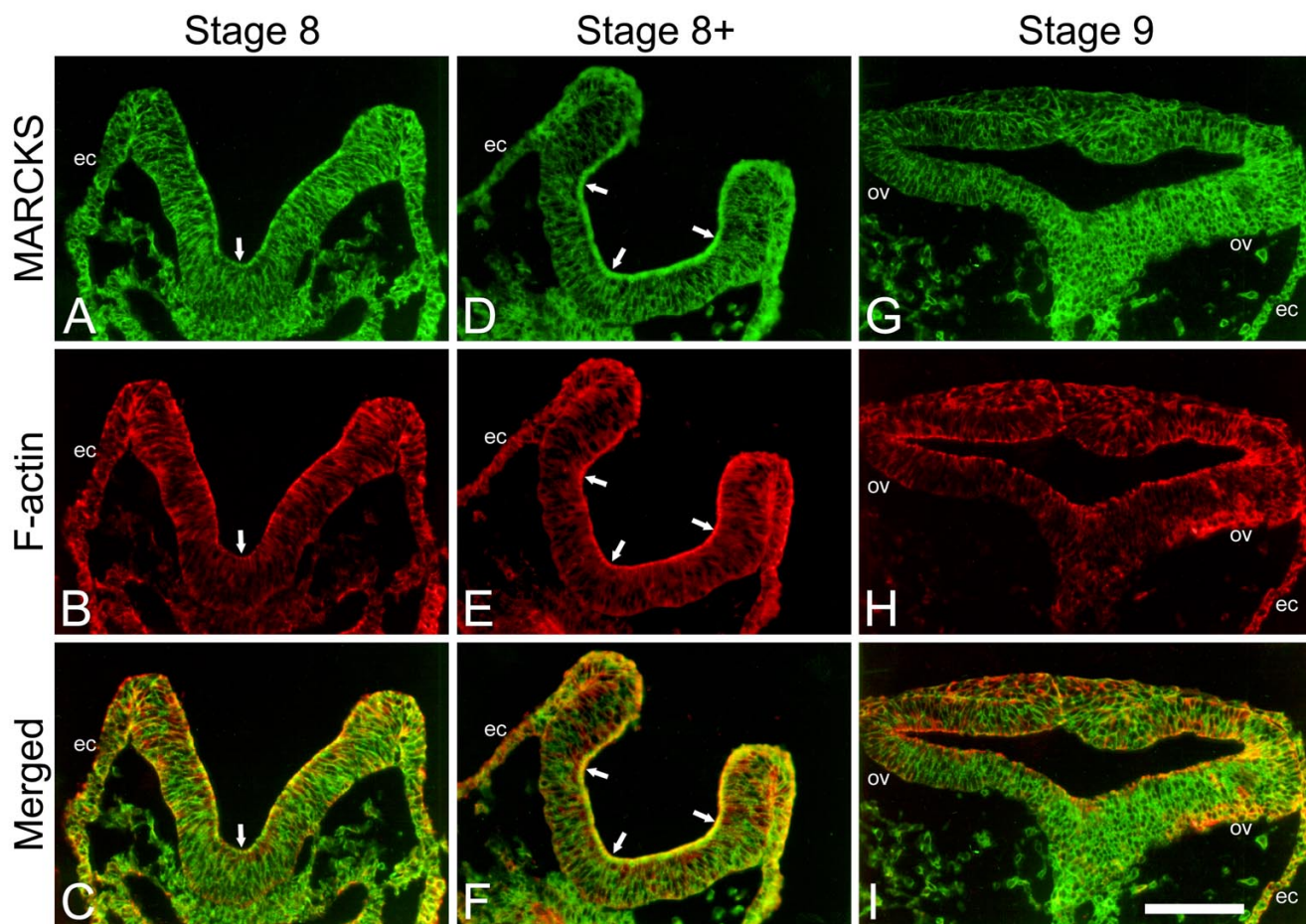


Figure 2
 MARCKS and F-actin distribution during prosencephalic neural tube closure. Neurulating chick embryos transverse cryosections, at the level of the presumptive prosencephalon. Double labeling of MARCKS, using a polyclonal antibody, and actin filaments, using phalloidin. Arrows, median and dorsolateral hinge points. ec, ectoderm; ov, optic vesicles primordia. Scale bar: 60 μ m.

results are very intriguing, since the knockout mice bore no apparent defects in truncal neural tube closure [11], suggesting that MARCKS could be important to, but not essential for caudal neural plate bending. Moreover, only 25% of the null mice showed neural tube defects [11]. In this respect, the authors recognized several different patterns of neurulation failure, where different regions of the cranial neural tube, from the forebrain to the hind-brain, remained open [27]. One possibility is that the absence of MARCKS is complemented by another protein. Remarkably, 100% of the MacMARCKS deficient mice were exencephalic [12]. Another explanation could be that MARCKS function in this process is mainly to regulate actin filaments dynamics, and, as it has been recently shown, F-actin is essential for cranial but not for truncal neural tube closure [28]. This suggestion is supported by the phenotype of the MacMARCKS and vinculin mutant mice, which also failed to close the cranial,

but not the spinal neural tube [9,12]. We did not observe intensity differences in MARCKS labeling between any of the hinge point regions and the rest of the neural plate, and the same happened, as previously described, with the actin filaments [28]. Hence, these data result in a new unanswered question: what is the meaning of this apical distribution of MARCKS in the spinal cord region, if neither MARCKS nor F-actin seem to be essential for its closure? As in the more cephalic sections, all tissues were labeled with the anti-MARCKS antibody, but a much stronger signal was detected in the truncal mesoderm, especially in the somites and notochord (Figure 3G and 3J).

Subcellular localization of MARCKS and F-actin during neural tube bending

By analyzing sections at higher magnification, we observed that, although MARCKS was highly enriched in

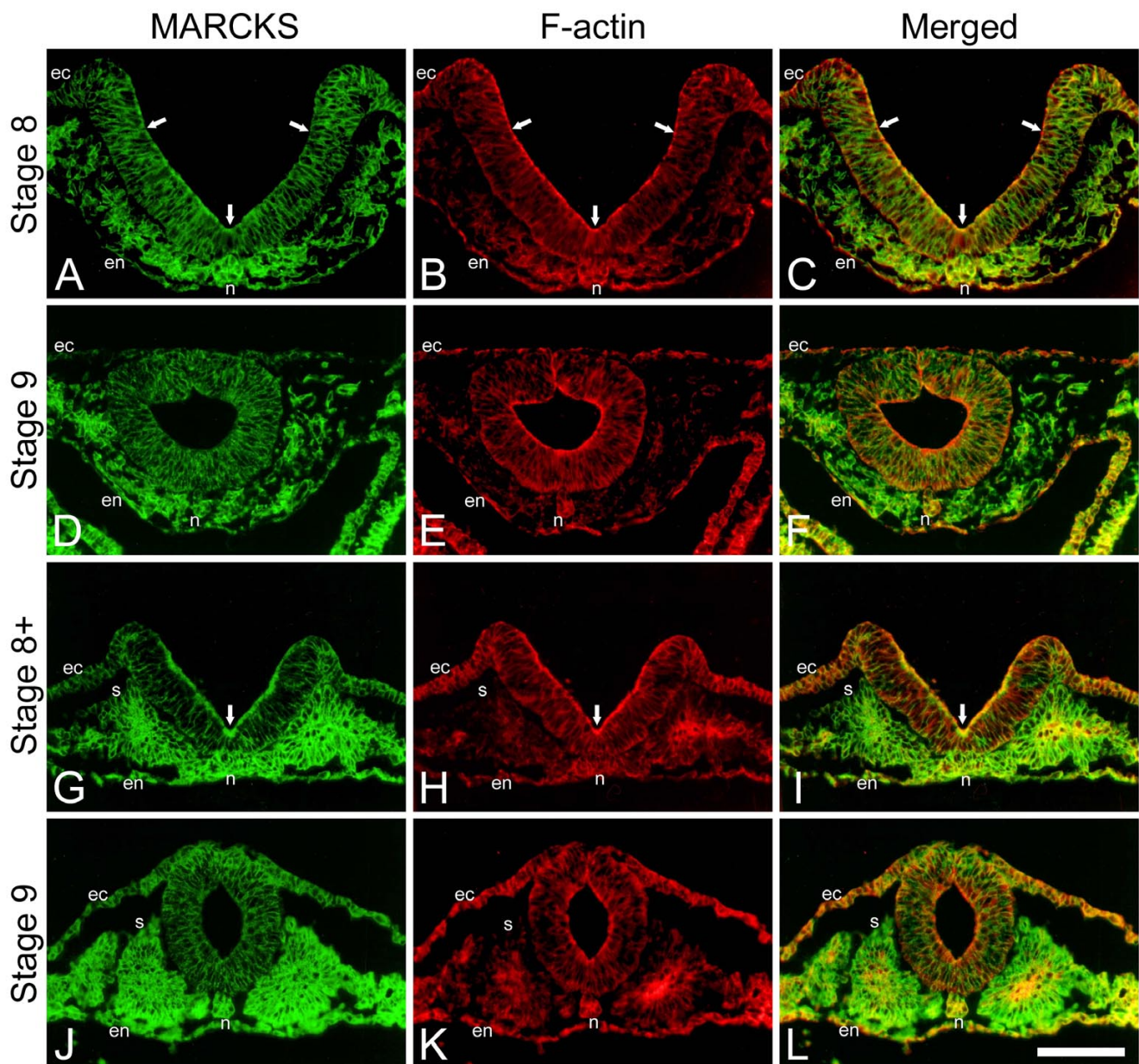


Figure 3
 MARCKS and F-actin distribution during rhombencephalic and spinal neural tube closure. Neurulating chick embryos transverse cryosections, at the level of the presumptive rhombencephalon (A-F) and spinal cord (G-L). Double labeling of MARCKS, using a polyclonal antibody, and actin filaments, using phalloidin. Arrows: median and dorsolateral hinge points. ec, ectoderm; en, endoderm; n, notochord; s, somite. Scale bar: 60 μ m.

the apical portion of neural plate cells, its fine distribution was different to that of actin filaments. In perfectly transverse sections, MARCKS positive region appeared broader and extended more basally respect to that of actin (not shown). Moreover, the differences were especially evident in slightly oblique sections, where the apical borders of neuroepithelial cells appeared in diagonal section (Figure 4). Here actin filaments formed polyhedral

arrays whereas MARCKS was distributed in small, irregular, spots that only in some cases were associated with the actin filaments. This peripheral, patchy distribution of MARCKS has been already described in other cell types [13,29]. In particular, this and other related PKC substrates, such as GAP43 and CAP23, were shown to be associated to phosphatidylinositol(4,5)bisphosphate in cholesterol-rich membrane rafts, and this association

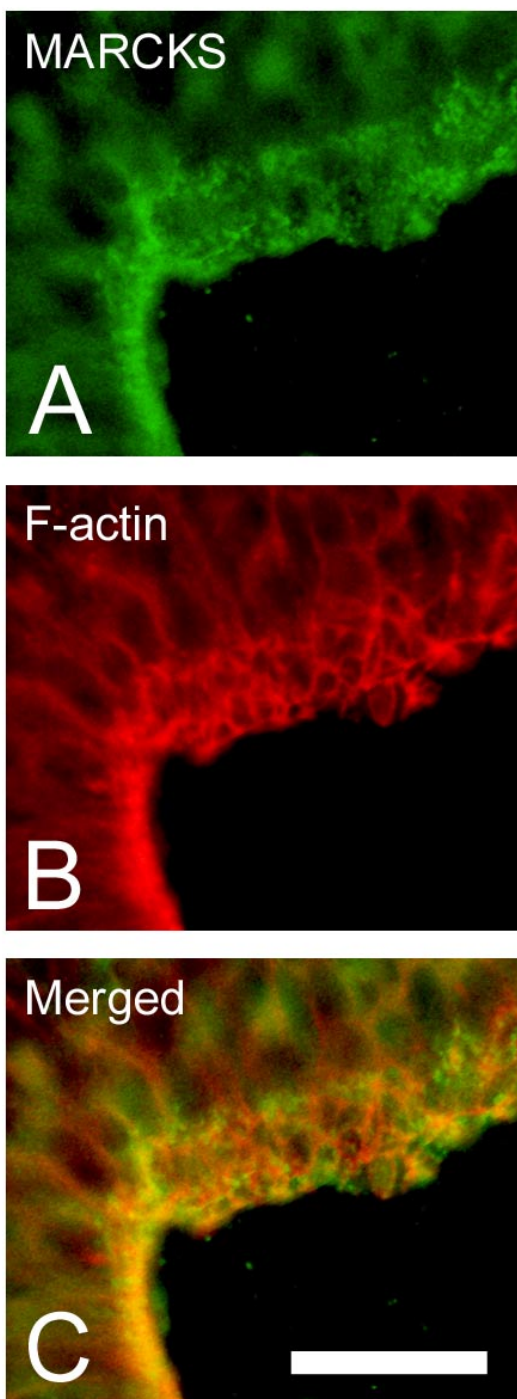


Figure 4
MARCKS and F-actin fine localization in the apical border of the closing neural plate. Stage 8+ chick embryo slightly oblique cryosection, at the level of the presumptive rhombencephalon. Double labeling of MARCKS, using a polyclonal antibody, and actin filaments, using phalloidin. Scale bar: 30 μ m.

would be essential for the regulation of cortical actin dynamics [24]. Thus, the distribution of MARCKS in peripheral patches is not in contradiction with its possible role in regulating actin filaments, but it only shows that it is not included into the apical actin meshwork. A suggestive additional information comes from the experiments showing that a failure of cranial neural tube closure occurs in mice lacking squalene synthase, the enzyme responsible for the generation of squalene, which is the first specific intermediate in the cholesterol biosynthesis [30]. These animals, having cholesterol metabolism alterations, could develop defects in the functions of some peripheral membrane proteins, such as MARCKS, associated with the cholesterol-rich membrane rafts.

Formation of the lens vesicle

As the morphogenesis of the lens vesicle is similar in several aspects to the formation of the neural tube, we were interested in comparing the distribution of MARCKS and F-actin also during this process. At stage 15 the optic cup has just formed and is laterally invaginating, as well as the lens placode (Figure 5). The lens placode cells exhibited the same relative distribution of MARCKS and filamentous actin as has been described above in the closing neural tube cells. Again, although MARCKS was ubiquitous and highly present in all the observed tissues, including ectoderm, mesenchyme, diencephalon, optic cup and lens placode, only the latter showed a preferentially apical localization of the protein (Figure 5A). As in the neural plate, the site of highest MARCKS immunosignal corresponded to the localization of the apical actin belts (Figure 5B and 5C). The amount of apical MARCKS was reduced to become the same as the one present in the other cell regions, once the lens vesicle was closed. As far as we know, defects in the formation of the lens vesicle have been reported neither in MARCKS, nor in MacMARCKS defective mice. Lens placode invagination could involve a putative substitute protein as suggested for the spinal cord closure in the null mutants mentioned above.

Although evidence is lacking, the changes in the subcellular localization of MARCKS described here could be explained, in principle, by two mechanisms: MARCKS could be either re-distributed from a formerly homogeneous peripheral distribution, or newly synthesized protein could be directly driven to the apical region of the epithelial cells. At the opposite, the change observed after neural tube or lens vesicle closure could be explained by a redistribution of the protein, or by a specific degradation in the apical region of the epithelia. Northern-blot and reporter gene expression in neurulating mice embryos showed an important rise in MARCKS expression in the closing cranial and caudal neural plate [27].

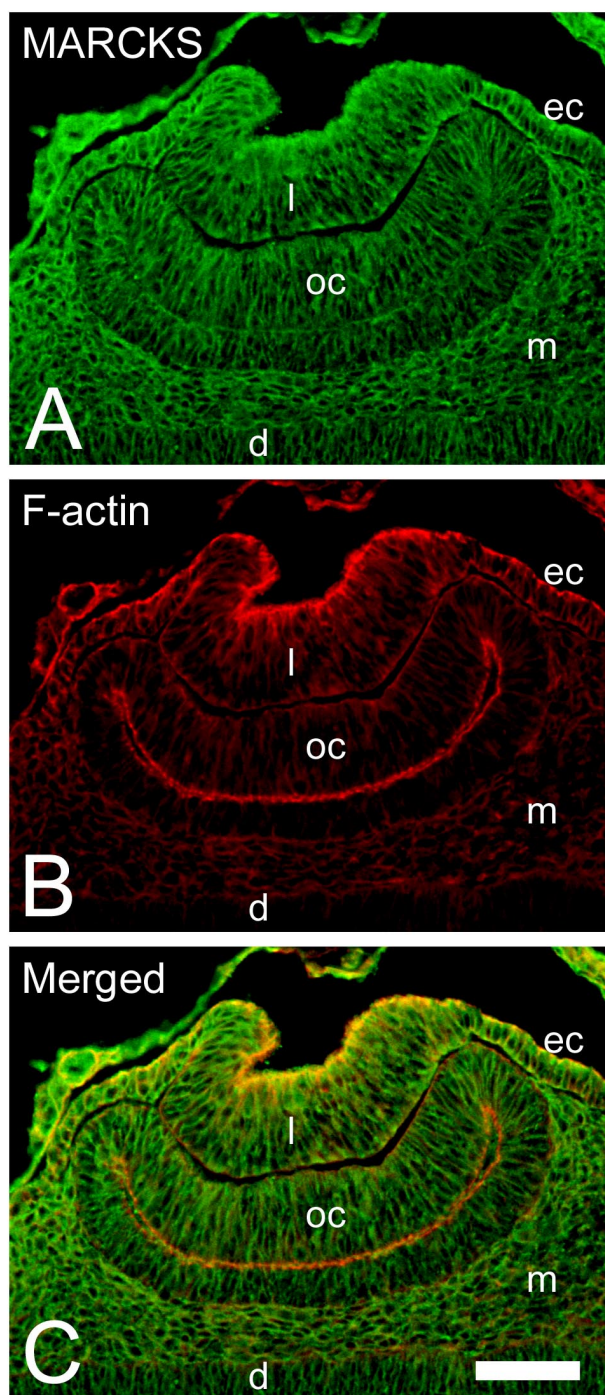


Figure 5
MARCKS and F-actin distribution during the formation of the lens vesicle. Stage 15 chick embryo cryosection, at the head level, showing the optic cup (oc) and lens placode (l). Double labeling of MARCKS, using a polyclonal antibody, and actin filaments, using phalloidin. d, diencephalon; ec, ectoderm; l, closing lens placode; m, head mesenchyme; oc, optic cup. Scale bar: 60 μ m.

In polarized, confluent MDCK cells, MARCKS localization at the lateral membranes depended on PKC activity and was correlated with the subcellular localization of other membrane and actin interacting protein, fodrin [31]. For MacMARCKS, a basolateral membrane targeting determinant has been found into the PSD [32], this domain is almost identical to MARCKS PSD [33] therefore, it is tempting to speculate that a similar mechanism could determine the subcellular localization of MARCKS in some polarized cells. In the case of the neural plate described here, apical localization could be explained by a singular signal, perhaps related to other phosphorylation site(s). Defects in neural tube closure, from the truncal to the cranial region, and in eye formation were reported after incubating neurulating rat embryos in the absence of methionine [34]. In these experiments, some cytoskeletal proteins, including actin, were hypomethylated, and a change of the actin and tubulin distribution was observed in the neural plate.

Another question remains: is the maintenance of apical MARCKS dependent on an interaction with the plasma membrane, the actin cytoskeleton, or both? MARCKS association to the plasma membrane is known to be reversible [35], and a permanent pool of cytosolic MARCKS has been described [36]. Our observations consistently showed a peripheral distribution of MARCKS in the closing neural plate and lens placode. Similar results were obtained by Blackshear's group in neurulating mice [27]. Altogether, these observations suggest that MARCKS is mainly associated to the plasma membrane during neural plate and lens placode bending. MARCKS can bind to membranes by at least two different sites: a, the myristoyl group at its amino-terminus, that inserts into the lipid bilayer, and b, the positively charged PSD, that, while unphosphorylated, electrostatically interacts with acidic head groups of phospholipids [13]. However, the transgenic expression of modified forms of the protein in MARCKS null mice suggest that membrane association is not necessary for neural tube closure promoting activity. Either the expression of a nonmyristoylatable [37] or a nonmyristoylatable and pseudophosphorylated (where serines in the PSD were substituted by glutamic acid residues)[38] forms of MARCKS in knockout mice resulted in the complete rescue of the neural tube closure phenotype.

Conclusions

Our results show that MARCKS protein is transiently accumulated to the apical border of neural plate and lens placode cells, in close apposition to the apical actin meshwork, during the processes of neural tube and lens vesicle formation. These observations provide additional structural counterparts to the knockout and transgenic mice analyses, although they also generate new prob-

lems, as respect to the role of MARCKS in spinal neural plate bending. In addition, these new results concerning an apical concentration of MARCKS open new questions about the mechanisms able to generate and transiently maintain its asymmetric distribution.

Materials and methods

Fertilized hen eggs were kindly supplied by Prodhin (Uruguay) and incubated in our laboratory until the desired stages. Whole embryos were fixed by immersion in 3.7% paraformaldehyde in PBS for 12-36 hrs and cryo-protected in 5% and 20% sucrose in PBS. They were then gelatin embedded [39] and quickly frozen in liquid N₂. Transverse cryosections (4-5 μm) were made on a Reichert-Jung Cryocut E cryostat and adhered to gelatin-subbed slides. At least two embryos were analyzed at each stage. Anti carboxy-terminal chick MARCKS antibody (a kind gift of Dr. Pico Caroni, Friedrich Miescher Institute, Basel, Switzerland [26]) was diluted 1:2000-1:3000 in blocking solution (PBS/1% BSA). Secondary antibody: FITC-conjugated goat anti mouse IgG (Gibco BRL, UK), 1:200. TRITC-conjugated phalloidin (Molecular Probes Inc., USA) was diluted 1:4000. Labeled sections were observed and photographed using a Nikon Microphot FXA microscope equipped with epifluorescence.

Acknowledgements

Supported in part by the programs PEDECIBA and Investigación Científica (CSIC), Universidad de la República. Uruguay. We greatly acknowledge Drs. P. Caroni and S. McLoon for kindly gifting of antibodies. We are indebted to Prof. H. Trenchi for continuous support supplying us fertilized avian eggs.

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