REVIEW ARTICLE

Cross-talk unfolded: MARCKS proteins

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The proteins of the MARCKS (myristoylated alanine-rich C kinase substrate) family were first identified as prominent substrates of protein kinase C (PKC). Since then, these proteins have been implicated in the regulation of brain development and postnatal survival, cellular migration and adhesion, as well as endo-, exo- and phago-cytosis, and neurosecretion. The effector domain of MARCKS proteins is phosphorylated by PKC, binds to calmodulin and contributes to membrane binding. This multitude of mutually exclusive interactions allows cross-talk between the signal transduction pathways involving PKC and calmodulin. This review focuses on recent, mostly biophysical and biochemical results renewing interest in this protein family. MARCKS membrane binding is now understood at the molecular level. From a structural point of view, there is a consensus emerging that MARCKS proteins are 'natively unfolded'. Interestingly, domains similar to the effector domain have been discovered in other proteins. Furthermore, since the effector domain enhances the polymerization of actin *in vitro*, MARCKS proteins have been proposed to mediate regulation of the actin cytoskeleton. However, the recent observations that MARCKS might serve to sequester phosphatidylinositol 4,5-bisphosphate in the plasma membrane of unstimulated cells suggest an alternative model for the control of the actin cytoskeleton. While myristoylation is classically considered to be a co-translational, irreversible event, new reports on MARCKS proteins suggest a more dynamic picture of this protein modification. Finally, studies with mice lacking MARCKS proteins have investigated the functions of these proteins during embryonic development in the intact organism.

Key words: actin, calmodulin, membranes, PIP_2, PKC.

INTRODUCTION

In 1982 it was demonstrated that protein kinase C (PKC) regulates the phosphorylation of an '87 kDa' substrate in brain synaptosomes, and that this phosphorylation can be inhibited by calmodulin (CaM) [1]. PKC is now known to be a major mediator of G-protein-coupled receptor signalling, which is involved in biological events such as growth control, differentiation, secretion and metabolism (reviewed in [2,3]). CaM is the prototype mediator of calcium signalling, and is involved in the control of gene expression, cell growth, cell cycle progression and muscle contraction (reviewed in [4,5]; see also [5a]). This review will focus on the '87 kDa' protein known today as MARCKS (myristoylated alanine-rich C kinase substrate). MARCKS proteins have been implicated in the regulation of brain development and postnatal survival, cellular migration and adhesion, as well as endo-, exo- and phago-cytosis, and neurosecretion [6,7]. Work on MARCKS has provided information about mechanisms of reversible membrane binding, mechanisms of cross-talk between different strains of signalling, the structural basis for cross-talk and, more recently, regulation of the actin cytoskeleton and control of lipid second messengers.

There are two known members of the MARCKS family: MARCKS, a 32 kDa ubiquitously expressed protein, and MARCKS-related protein (MRP, also known as MacMARCKS, F52 or MLP), a 20 kDa protein expressed mainly in brain, reproductive tissues and macrophages [6,7]. MARCKS has been found in *Torpedo californica*, *Xenopus laevis*, chicken, mice, rat, cow and human. A distant MARCKS relative, DAKAP200, has been identified in *Drosophila melanogaster* [8]. DAKAP200 contains, in addition to regions similar to MARCKS, a protein kinase A binding domain. An alternatively spliced variant lacking the protein kinase A region is the *Drosophila* protein most closely resembling MARCKS [8].

This review will focus on recent developments in the MARCKS field, with particular emphasis on biochemical and biophysical studies performed at the molecular level. Classical studies of MARCKS that have been reviewed previously [6,7,9–12] will first be summarized briefly.

CLASSICAL CONCEPTS

MARCKS proteins possess three highly conserved regions. The N-terminus represents a consensus sequence for myristoylation, a co-translational lipid modification attaching myristic acid, the C_{14} saturated fatty acid, via an amide bond to the amino group of the N-terminal glycine residue. The MH2 domain, of unknown function, resembles the cytoplasmic tail of the cation-independent mannose-6-phosphate receptor and is also the site of the only intron-splicing event. Finally, the phosphorylation site domain contains all serine residues known to be PKC phosphorylation sites. This domain has been shown to be central to the function of MARCKS proteins, and is therefore called the effector domain (ED) in the more recent literature. The ED is highly basic (see Figure 2), in contrast with the rest of the highly acidic protein. MARCKS binds to membranes because these basic residues interact electrostatically with acidic lipids and the myristate

Abbreviations used: CaM, calmodulin; ED, effector domain; GFP, green fluorescent protein; MARCKS, myristoylated alanine-rich C kinase substrate; MD, molecular dynamics; MLCK, myosin light chain kinase; MRP, MARCKS-related protein; PH, pleckstrin homology; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLC, phospholipase C.

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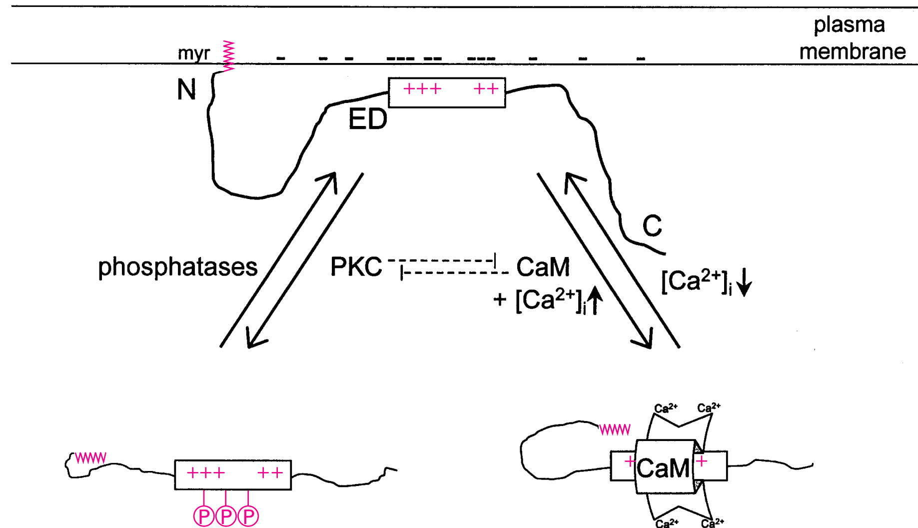

Figure 1 Myristoyl/electrostatic switch model

MARCKS bound to the plasma membrane through the N-terminal myristoyl moiety and the ED can translocate reversibly into the cytosol, either through cycles of phosphorylation by PKC and dephosphorylation, or through temporary increases in the intracellular calcium concentration ($[Ca^{2+}]_i$) leading to activation of CaM. Note that the interactions of PKC and CaM with MARCKS are mutually exclusive (broken lines). Highlighted in red are the myristoyl moiety (myr) and the positive charges contributed by the basic residues within the ED (+), as well as the phosphate groups that are attached by PKC (P).

inserts hydrophobically into the core of the membrane. Neither of these two interactions on its own is sufficient for significant membrane binding (myristoyl/electrostatic switch [9,13–15]).

MARCKS interacts, for example, with the plasma membrane of macrophages [16,17], neurons [18] and fibroblasts [19,20]. Phosphorylation by PKC (which attaches negatively charged phosphate groups to the serine residues) abrogates membrane binding of MARCKS in many cell types [16,19,21], since neutralization of the positive charges of the basic residues by the phosphoserine residues abolishes the electrostatic contribution of the ED to membrane binding, and myristoylation on its own is not sufficient to anchor the protein to the membrane. A recent study using a MARCKS–green fluorescent protein (GFP) fusion protein showed in living cells that plasma membrane-bound MARCKS is phosphorylated by activated membrane-bound PKC [21], indicating the importance of correct absolute and relative subcellular localization of the kinase and its substrate. If the phosphoserine residues are subsequently dephosphorylated by protein phosphatase 1, protein phosphatase 2A or calcineurin [22–24], MARCKS returns to the membrane (Figure 1). This molecular model explains the observed reversible translocation of MARCKS from the plasma membrane to the cytosol. However, phosphorylation of MARCKS can also result in translocation to intracellular membranes (for example, of lysosomes in fibroblasts [20]), which might be explained by particular, as yet unidentified, protein–protein interactions.

Besides PKC, the MARCKS ED is also a target for Ca^{2+}/CaM. After activation by increased intracellular Ca^{2+} concentrations, calcium-bound CaM binds to MARCKS with nanomolar affinity and is thus able to pull MARCKS off membranes *in vitro* as well as *in vivo* (reviewed in [12]). This initiates another cycle of reversible membrane binding of MARCKS, since CaM will release MARCKS once the intracellular Ca^{2+} concentration has returned to normal levels (Figure 1).

Importantly, the interactions of PKC and CaM with the MARCKS ED are mutually exclusive: phosphorylation of MARCKS by PKC significantly decreases its affinity for CaM [25]. Also, CaM bound to MARCKS sterically hinders access of PKC to MARCKS and thus its phosphorylation. If CaM and PKC are both active, they can compete for their common substrate MARCKS. MARCKS can therefore mediate cross-talk between the PKC and CaM signal transduction pathways. Experimental evidence for this has not only been gathered from biochemical and cell biological studies, but can also be obtained *in vivo*. Initiation of forward swimming of the protozoan *Paramecium* requires CaM, and could be inhibited by injection of a MARCKS ED peptide [26]. This inhibition was reversed by activation of PKC with phorbol esters, indicating that, in a living organism, a complex between CaM and the MARCKS ED can be disrupted by PKC-induced phosphorylation of the ED.

Several possible scenarios resulting from this cross-talk between PKC and CaM via MARCKS proteins have been suggested (for reviews, see [7,10,11]). For example, if PKC is activated first, MARCKS will become phosphorylated and thus will be removed from the pool of potential CaM-binding proteins. Subsequent activation of CaM would then lead to a stronger activation of CaM substrates such as the important signalling molecules NO synthase, myosin light chain kinase (MLCK), CaM kinase II and the phosphatase calcineurin, since phospho-MARCKS cannot compete with them for CaM. In contrast, if CaM is activated first and forms a complex with MARCKS, subsequent activation of PKC will lead to a more pronounced phosphorylation of PKC substrates other than MARCKS.

NEW INSIGHTS INTO MECHANISMS OF MEMBRANE BINDING

Analysis of membrane binding of MARCKS

As emphasized above, membrane binding is crucial for the biology of MARCKS proteins. In recent years, more details of the underlying molecular mechanisms have been revealed. As mentioned, the myristoyl/electrostatic switch was suggested to regulate MARCKS–membrane interactions: both the hydrophobic incorporation of the myristic chain into the lipid bilayer and the electrostatic interaction of the basic ED with acidic lipids

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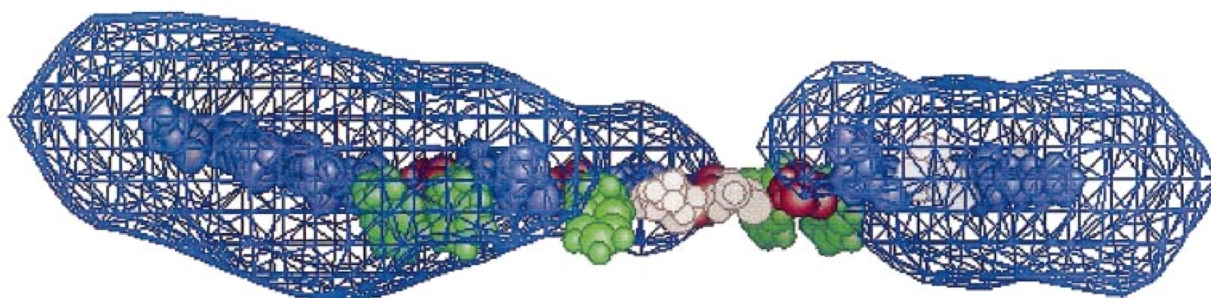

MARCKS ED: LysLysLysLysLysArgPheSerPheLysLysSerPheLysLeuSerGlyPheSerPheLysLysAsnLysLys

MRP ED: LysLysLysLysLys---PheSerPheLysLysProPheLysLeuSerGlyLeuSerPheLysArgAsnArgLys

Figure 2 Molecular model of the ED

An atomic model of the MARCKS ED, built in agreement with all known structural data, shows the molecule in an extended conformation. This peptide model was built using the Insight-II/Biopolymer package (MSI) and was kindly provided by Dr Diana Murray, Cornell University. The 13 basic lysine and arginine residues are coloured blue, the five aromatic phenylalanine residues are coloured green, and the four serine residues containing the PKC-phosphorylated sites are coloured brown. Shown as a blue mesh is the +25 mV equipotential surface, as calculated in a 100 mM univalent salt solution using GRASP [40]. Note the strongly positive field around the N- and C-terminal lobes of the ED. Below, the primary sequences of the bovine MARCKS and MRP EDs are aligned using the same colour code. The proline residue of MRP that replaces the second serine residue of MARCKS is underlined.

in the plasma membrane are necessary for significant membrane attachment [9]. MARCKS binding to membranes has already been discussed in detail in several reviews [12–15,27].

The enzyme myristoyl-CoA:protein N-myristoyltransferase recognizes the consensus sequence for myristoylation (H_2N-GXXXS) and catalyses the co-translational attachment of the myristoyl chain to the N-terminal glycine residue [13,28–30]. The C_{14} saturated carbon fatty acid chain inserts into the hydrophobic core of the phospholipid membrane [31] and thus contributes to the peripheral membrane binding of the protein. The myristoyl chain can contribute energy, corresponding to an effective dissociation constant of 10^{-4} M [32]. Lipid modifications of proteins have recently been reviewed elsewhere [13,29,30].

Intensive *in vitro* studies with MARCKS [33,34] and peptides corresponding to the ED [35–37], as well as cell fractionation studies using mutant proteins [38,39], support the myristoyl/electrostatic switch model, showing that hydrophobic and electrostatic contributions act together to achieve membrane binding. In particular, these studies demonstrate that the binding of the ED to phospholipid membranes is due mainly to non-specific electrostatic interaction of basic residues with acidic lipids in the membrane [33,35,36,38]. In contrast, the ED has only a low affinity for neutral membranes [36], which can be explained by hydrophobic contributions of its phenylalanine residues (see below).

Figure 2 presents a molecular model of the MARCKS ED, built in accordance with all known structural data, showing the ED in an extended conformation (see below). The 13 basic lysine and arginine residues are coloured blue, the five hydrophobic phenylalanine residues are coloured green, and the four serine residues, including the PKC phosphorylation sites, are coloured brown. The electrostatic field surrounding the peptide was calculated using GRASP [40]. The +25 mV equipotential surface is shown as the blue mesh in Figure 2, clearly indicating the basic nature of the ED. Note that the ED contains no acidic residues, explaining the absence of any negative charges under physiological conditions. Underneath the model, the primary sequences of the MARCKS and MRP EDs are shown.

The electrostatic components of the binding free energy can be calculated by solving the non-linear Poisson–Boltzmann equation for atomic models of protein (peptide)/membrane systems [14, 41,42]. The energy minimum is then determined by the balance of the electrostatic Coulomb attraction and the Born/desolvation repulsion of the peptide from the low dielectric interface, as reviewed in [15]. The theoretical predictions of a strong dependence of membrane binding on the mol % acidic lipid in the membrane, as well as on the salt concentration in the electrolyte solution, could be confirmed experimentally [35,36]. Incorporation of 20 % acidic lipid in the membrane increased the binding of the MARCKS ED peptide 10000-fold, as predicted theoretically. The chemical structure of the univalent lipid did not matter, indicating the importance of the non-specific electrostatic interaction. However, binding of MARCKS protein was increased only 100-fold, which might be explained by the acidic residues adjacent to the ED which partially compensate for the basic ED and repulse the negatively charged membrane [34].

These *in vitro* results are in agreement with cellular studies. For example, it was shown that non-myristoylatable (N-terminal glycine residue changed to alanine) and pseudo-phosphorylated (all four serine residues in the ED changed to aspartic acid) mutants of MARCKS bind significantly less strongly, and that a protein containing both mutations binds negligibly to membranes [38,39]. Recent studies using chimaeric MARCKS conjugated to fluorescent proteins confirmed these observations in living cells. Wild-type MARCKS–GFP was located at the plasma membrane of CHO-K1 cells, and was translocated rapidly to the cytosol and perinuclear region when phosphorylated [21]. Also, a fusion protein of the myristoylated non-phosphorylatable MARCKS mutant with GFP was located predominantly at the plasma membrane of HEK293 cells [43]. Finally, non-myristoylatable and double mutants fused to GFP were distributed throughout the cytoplasm in CHO-K1 and HEK293 cells [21,43].

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In addition to the 13 basic lysine and arginine residues, the MARCKS ED contains five aromatic phenylalanine residues (shown in green in Figure 2). The contribution of hydrophobic residues to the binding of model peptides to neutral membranes has been studied in detail [44]. Regarding MARCKS, it was found that the five phenylalanine residues of the MARCKS ED penetrating into the lipid head-group region [36,45,46] are contributing enough energy to account for the weak binding of the ED peptide to neutral membranes that is predicted theoretically and observed experimentally [15,36,47]. A peptide in which the five phenylalanine residues were changed to alanine did not bind to neutral membranes, as judged from EPR, NMR and monolayer experiments [36,46]. This peptide binds less strongly to the negatively charged membranes and is located further away from the membrane/water interface than the wild-type peptide [36,46]. In summary, the interplay between these three forces (long-range Coulomb electrostatic attraction, short-range hydrophobic attraction and Born/desolvation repulsion) determines the specific location of the peptides at the membrane/water interface [15].

Analysis of membrane binding of MRP

Whereas the interactions of MRP with CaM and PKC are very similar to those of MARCKS [48,49], the picture is much less clear with regard to differences in the interactions of MARCKS and MRP with membranes.

In agreement with the model proposed for MARCKS, a myristoyl moiety and the MRP ED combined are sufficient to target a protein to the plasma membrane *in vivo*, as was demonstrated in MDCK cells using a myristoylated GFP–MRP-ED construct [50]. Also, a chimaera of yellow fluorescent protein with MRP translocates rapidly from the plasma membrane to the cytosol and the perinuclear region of macrophages when phosphorylated, similarly to the typical behaviour of MARCKS [51]. However, differences in subcellular localization and membrane binding have been reported for MARCKS and MRP. For example, while MARCKS is localized to phagosomes, MRP is found only at phagocytic cups [17,52]. Also, at least in some cases, phosphorylation does not cause significant translocation of membrane-bound MRP to the cytosol [50,53]. Furthermore, *in vitro* measurements of MRP binding to sucrose-loaded large unilamellar vesicles or membrane-coated glass beads showed that the co-operativity between the ED and myristoylation is very weak in the case of MRP: incorporation of 20 % negatively charged lipids into the membrane increases MRP binding by only 5-fold [54,55], in contrast with the 100-fold increase observed for MARCKS [34]. Finally, neither phosphorylation nor CaM interferes significantly with the binding of myristoylated MRP to negatively charged vesicles [54].

A series of studies used optical waveguide light-mode spectroscopy to investigate the binding of MRP to planar membranes. With this sensitive set-up, the importance of the myristoyl moiety for interaction with both neutral and acidic membranes was confirmed [56]. The authors observed significant binding of unmyristoylated MRP to neutral membranes, but less than to acidic membranes. Only a mutant lacking both the myristoyl moiety and the ED did not bind to acidic membranes. However, in the case of neutral membranes a very low amount of residual binding was still detectable. These results might point to the importance of hydrophobic interactions in addition to those of the myristoyl moiety in MRP membrane binding [56]. Similar studies with wild-type MRP failed to reveal a significant increase in membrane binding upon addition of phosphatidylserine [57]. Finally, the competition between membranes and CaM for the MRP ED was investigated, revealing that membrane-bound MRP could not be removed by CaM [54,58], but that CaM could significantly retard the membrane binding of MRP [58].

The lack of endogenous tryptophan residues in MARCKS proteins was exploited to engineer single tryptophan residues into MRP at various positions (see also below). Use of tryptophan fluorescence to monitor the interaction of vesicles with myristoylated MRP containing a single point mutation within the ED (Phe-93 $\rightarrow$ Trp) demonstrated directly that the ED is involved in the interaction of MRP protein with phospholipid membranes. The occurrence of an isobestic point in the fluorescence spectra indicated that the binding of MRP to membranes is a simple equilibrium between free and membrane-bound protein, confirming an assumption on which the quantitative description of the membrane binding of MARCKS proteins through partition coefficients is based [59].

The differences in the membrane binding of MARCKS and MRP might be caused by the intrinsic differences in the size and/or amino acid composition of the two proteins. In particular, sequences adjacent to the conserved membrane-binding motifs of myristate and ED might influence the overall behaviour of the proteins. For example, the net charge calculated as a function of pH differs significantly between MARCKS and MRP (G. Vergères, unpublished work). Furthermore, while the myristoyled N-terminus and the ED are almost 100 % conserved between the two proteins [6,7], and peptides corresponding to the ED of MARCKS and MRP exhibit qualitatively similar membrane binding [47], some differences should be noted (see Figure 2). The second serine residue in the MARCKS ED is replaced by a proline residue in the MRP ED, potentially influencing the structural and functional properties of the ED within the protein. Furthermore, only two serine residues are phosphorylated in the MRP ED, compared with three in MARCKS. This lower degree of phosphorylation might account for the membrane binding of phospho-MRP after phosphorylation by PKC. The observed differences in subcellular localization might result from additional, protein-specific interactions. Finally, one should note that different experimental methods, such as cell lines, peptide/protein samples and model membrane systems, were used. Although many aspects of the biology of MARCKS proteins have been investigated for both proteins, reports in which MARCKS and MRP were compared in the same experiment and in the same laboratory are rare. Such comparative studies would clearly highlight differences between these proteins, and consequently increase our understanding of their respective functions.

STRUCTURE

The previous sections have demonstrated the flexibility of MARCKS proteins and their EDs in binding to diverse proteins as well as to lipid membranes. According to the design principle of 'form follows function', the question of the structural basis of these interactions arises. In particular, a simple helical wheel projection, not taking charge effects into account, suggested that the ED can adopt an amphipathic α-helical conformation, with the charged residues and the hydrophobic residues segregated to different sides [6].

Structure of MARCKS proteins in solution

Electron microscopy has shown rod-shaped, elongated molecules with dimensions of approx. 4.5 nm $\times$ 36 nm for MARCKS (32 kDa) [60], while analytical ultracentrifugation measurements gave dimensions of approx. 2 nm $\times$ 13 nm for unmyristoylated

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MRP (20 kDa) [49]. CD studies of recombinant MARCKS proteins revealed a high percentage of unfolded sequence, together with α-helical and very few β-sheet regions [49,61]. CD spectra of myristoylated MRP in the presence of trifluoroethanol or hexafluoroisopropyl alcohol (organic solvents known to induce α-helices) displayed, however, a higher degree of helicity, indicating the structural flexibility of the protein [49].

CD and EPR measurements also revealed that peptides corresponding to the ED are random coils in aqueous solution [45]. Molecular dynamics simulations based on NMR results gave a non-helical, extended conformation [62]. This extended conformation is due to the repulsion of the positively charged residues at both the N- and C-termini of the ED (see Figure 2). A recent study addressed the structure of the ED within myristoylated MRP. Taking advantage of the fact that wild-type MRP does not contain tryptophan residues, the authors generated mutants in which single residues within the ED were changed to tryptophan [63]. Three different parameters of fluorescence, namely emission maximum, anisotropy and quenching by acrylamide, were determined for each of the ten mutants. In aqueous solution, all mutants exhibited properties typical of a polar environment, suggesting that the ED is exposed to the aqueous phase. In view of the rod-shaped form of the protein, one can conclude that the ED can act as a flexible linker between the N- and C-termini of the protein [63].

Regarding the potential impact of phosphorylation on the structure of the protein, CD spectra of phospho-MARCKS purified from brain again revealed a high percentage of unfolded sequence [64]. Molecular dynamics simulations showed that the phosphorylated MARCKS ED adopts a bent, more compact structure [62]. This can be explained by the negative charges of the phosphoserine residues in the middle of the ED attracting the positive charges of the basic residues at both ends of this domain. This result is also in agreement with electron microscopy photographs showing a compaction of MARCKS molecules after phosphorylation [60].

Structure of CaM-bound MARCKS proteins

Both MARCKS and MRP bind *in vitro* to Ca^{2+}/CaM with nanomolar affinity. The same high affinity is found for MARCKS and MRP ED peptides. There is also evidence that this interaction is important *in vivo* (see above). While classical CaM substrates such as MLCK are α-helical before binding to CaM, other substrates might only become α-helical upon CaM binding. CaM itself does not gain secondary structure through ligand binding, but the central α-helix connecting the two terminal lobes is bent [25]. As discussed above, the ED is not α-helical in the free protein, but shows sufficient flexibility to become α-helical. The structure of the complex of MARCKS proteins with CaM has therefore evoked considerable interest.

Surprisingly, CD spectroscopy showed no significant changes in the overall secondary structures of MARCKS proteins upon CaM binding [49,61]. EPR spectroscopy of spin-labelled peptides was employed to investigate this question in more detail. A series of peptides corresponding to the MARCKS ED containing single cysteine mutations was synthesized, spin-labelled and analysed [65]. The middle of the ED was buried in the CaM molecule, while both termini of the ED showed high mobility and were accessible to the aqueous environment. In this respect, the complex resembled the CaM·MLCK complex [65], one of the few CaM complexes whose high-resolution structure has been solved. The structures show the MLCK peptide in an α-helical conformation buried between the two lobes of CaM [66,67], and therefore it was concluded that MARCKS ED also adopts an α-helical conformation when bound to CaM. However, this question is still unresolved, as a different study employing CD and NMR measurements of CaM·ED complexes concluded that the peptide bound to CaM is, rather, non-helical [25].

The CaM·MRP complex was recently studied using the series of single tryptophan mutants of myristoylated MRP [63]. Emission maximum, anisotropy and quenching by acrylamide were determined for each of the ten MRP mutants bound to CaM. All three parameters showed changes indicative of a more hydrophobic environment when MRP was bound to CaM compared with that of free MRP. However, the tryptophan residues were not completely shielded from the environment through complexion of MRP by CaM. Since the data showed no periodicity typical of an α-helix, it can be concluded, at least for the C-terminus of the ED, that this region is not in an α-helical conformation within the CaM·MRP complex [63]. The authors also found that the N-terminal and C-terminal lobes of CaM interact with the C-terminal and N-terminal parts respectively of the ED in full-length MRP, the same relative orientation as in the CaM·MLCK complex. Finally, the use of a tryptophan residue close to the N-terminus showed that the N-terminus of MRP interacts with CaM if it is myristoylated [63]. Based on the similar nanomolar affinities of unmyristoylated and myristoylated MRP for CaM [49], and the relatively small shift in the emission maximum, the conformational change in the complex induced by myristoylation can be considered as peripheral and does not contribute significantly to CaM binding. In contrast, myristoylation of 22 kDa neuronal tissue-enriched acidic protein (NAP-22), a protein with physico-chemical properties and cellular function similar to MARCKS proteins, is required for CaM·NAP-22 complex-formation [68].

Structure of membrane-bound MARCKS proteins

CD measurements of wild-type and EPR measurements of spin-labelled MARCKS ED peptides each showed a non-helical structure when the peptides were bound to a charged membrane [45]. A model was deduced from the EPR spectra of the membrane-bound peptides showing the major part of the membrane-bound ED in an extended, non-helical conformation parallel to the surface of the membrane, with the hydrophobic side chains of the phenylalanine residues reaching into the head-group region of the membrane. In contrast, the N-terminus of the ED with the pentalysine sequence is directed away from membrane and reaches out into the solution [45]. This can be explained by Born repulsion/desolvation effects (see above and recent reviews [14,15]).

The tryptophan mutants of MRP (see above) were also used to investigate membrane binding. An N-terminal point mutation (Ser-4 $\rightarrow$ Trp) proved that the N-terminus of MRP interacts with phospholipid membranes only if it is myristoylated [59]. This result is in agreement with the analysis of the N-terminal amino acid sequence of MRP, which does not reveal any motifs predicting interactions with membranes (or CaM), and with the observation that the myristoyl moiety inserts into the hydrophobic core of the membrane bilayer [31].

Finally, a quantitative kinetic analysis of the binding of unmyristoylated and myristoylated MRP to negatively charged planar membranes using optical waveguide light-mode spectroscopy concluded that myristoylation causes compaction of membrane-associated MRP. The calculated areas occupied on the membrane surface by unmyristoylated and myristoylated MRP were 17 and 11 nm^2 respectively [69].

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MARCKS proteins are 'natively unfolded'

The structure of MARCKS proteins and their various complexes has been investigated mainly by spectroscopic techniques, as described above. The general conclusion can be drawn that MARCKS proteins, and in particular their EDs, exist not as classical, global proteins with a defined, folded structure, but as randomly folded chains in non-classical, extended conformations. Further experimental evidence supports this claim. For example, one might be puzzled that no detailed structural analysis via X-ray crystallography has been performed. Despite extensive trials, MARCKS proteins could not be crystallized (G. Vergères, unpublished work), indicating an absence of defined structures. Some unusual properties of MARCKS proteins, such as their anomalous behaviour on SDS/PAGE and very high thermal stability, should also be noted. On SDS/PAGE, MARCKS proteins show an anomalous migration behaviour, resulting in widely overestimated molecular masses. This is due to their non-globular, elongated forms and the weak binding of SDS molecules to the highly acidic proteins. Also, MARCKS proteins can be heated to 95 °C for 5 min or acidified to pH 4 without irreversible denaturation, a property that has initially been used to develop purification procedures.

MARCKS proteins therefore belong to the class of 'natively unfolded' proteins [70]. These proteins occur "as a mixture of rapidly equilibrating monomeric conformers, which, on average, contain little secondary structure and no hydrophobic core" [70]. Other proteins in this class, such as neuromodulin [also known as GAP-43 (growth-associated protein of 43 kDa)], CAP-23 (cortical cytoskeletal-associated protein of 23 kDa), neurogranin, NAP-22 (neuronal tissue-enriched acidic protein of 22 kDa) and tau, not only share their physico-chemical properties with MARCKS proteins, but also belong to the same functional class, namely being a target for both CaM and PKC and, presumably, being involved in the regulation of the (actin) cytoskeleton (reviewed in [10,11]). Furthermore, some proteins that overall are folded, globular proteins contain a domain with similarity to the MARCKS ED. These proteins include, for example, AKAP-79 (A-kinase anchoring protein-79) [71] and adducin [72]. The domains similar to MARCKS in these proteins might also mediate cross-talk between PKC and CaM (reviewed in [10]). Similar observations have been summarized in the paradigm that key regulatory checkpoints of several signal transduction pathways are occupied by 'natively unfolded' proteins [73].

This concept of signalling via unfolded proteins is in contrast with the signalling concept realized in the widely studied receptor tyrosine kinase pathways. Proteins participating in these pathways are modular proteins consisting of a series of individually conserved domains which are independent folding units and whose function can be predicted from the primary sequence [74,75]. In these proteins (among them some actin-binding proteins [76]), modules with distinct functions are combined to result in a protein able to interact simultaneously with several different partners, thus allowing signalling and cross-talk. The main characteristics of modular proteins are as follows. First, by gene duplication and subsequent evolution, many related modules can be generated {e.g. pleckstrin homology (PH) domains with different substrate specificity [77]}. Secondly, quick evolution is possible, by assembly of pre-existing modules to different genes by recombination. Thirdly, modular proteins can act as scaffolding or adaptor proteins, assuring spatial closeness of different proteins, e.g. to link a receptor with a downstream effector kinase or to connect proteins from different signalling pathways.

While MARCKS proteins also fulfil the function of cross-talk, all their interaction partners converge on the ED: the different MARCKS ligands compete for the ED. Using small, unfolded proteins as cross-points shows at least three advantages. First, proteins containing a single interaction 'hub' can be smaller and thus more 'economic' than large, modular proteins. Secondly, temporal regulation becomes possible. The information of the status of a first signal transducer can be transferred to a second mediator (see above). Finally, it might be that the ability to bind to structurally diverse ligands such as membrane lipids and CaM requires the structural flexibility that only a 'natively unfolded' protein can offer.

NEW BIOLOGICAL FUNCTIONS OF MARCKS PROTEINS

Besides cross-talk between CaM and PKC, other biological functions of MARCKS proteins have been suggested. One hypothesis identified the control of the actin cytoskeleton as the function of MARCKS proteins [6]. This was based on the finding that MARCKS proteins are localized at the plasma membrane, close to the cortical actin network. Also, changes in the phosphorylation status and/or the subcellular localization of MARCKS proteins are concomitant with cellular events such as phagocytosis and neurosecretion that depend on the actin cytoskeleton. However, evidence for a direct interaction between MARCKS proteins and actin has been scarce. More recently, new data regarding the interaction of the lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) with MARCKS, together with an increased understanding of the contribution of PIP_2 to the regulation of the actin cytoskeleton, suggest an alternative model for the regulation of the actin cytoskeleton by MARCKS proteins. Furthermore, cellular studies have recently identified additional levels of regulation of MARCKS, affecting both the myristoyl moiety and the ED. Finally, studies with MARCKS- or MRP-deficient mice have probed the function of MARCKS proteins in the intact organism.

Is actin downstream of MARCKS?

The report that MARCKS can bind to actin [60] provoked widespread interest. It was shown that MARCKS can bind to and cross-link actin *in vitro*, and that both PKC and CaM inhibit this effect. Thus it was proposed that MARCKS integrates signals from the two pathways into the control of the downstream target and effector molecule, actin [6]. Activation of either of these two pathways would lead to a local release of actin by MARCKS, and a local softening of the actin cytoskeleton with increased plasticity.

Several explanations have been advanced in order to explain the actin cross-linking activity of MARCKS [60], as well as the actin bundling activity of the ED of MARCKS proteins [60, 78,79]: (1) MARCKS might contain a single actin-binding site within the ED and cross-link actin through dimerization [6]; (2) two actin-binding sites might be present within the MARCKS ED [80]; and (3) the ED of MARCKS proteins may bundle actin filaments by diminishing the electrostatic repulsion between the filaments [78,79], by analogy with the DNA condensation observed in the presence of polycations.

Besides bundling, both MARCKS and MRP ED peptides induce rapid polymerization of G-actin. This effect, which can be regulated by PKC and CaM, requires the N-terminal pentalysine sequence [81]. Kinetic analysis concluded that free and ED-bound actin monomers nucleate separately, while combination of free and ED-bound species is allowed during the subsequent nucleation step [79]. However, intact MRP demonstrated much less pronounced effects on the formation and structure of F-actin *in vitro* and, in contrast with the conclusions reached for MARCKS [60], no convincing evidence for a cross-linking

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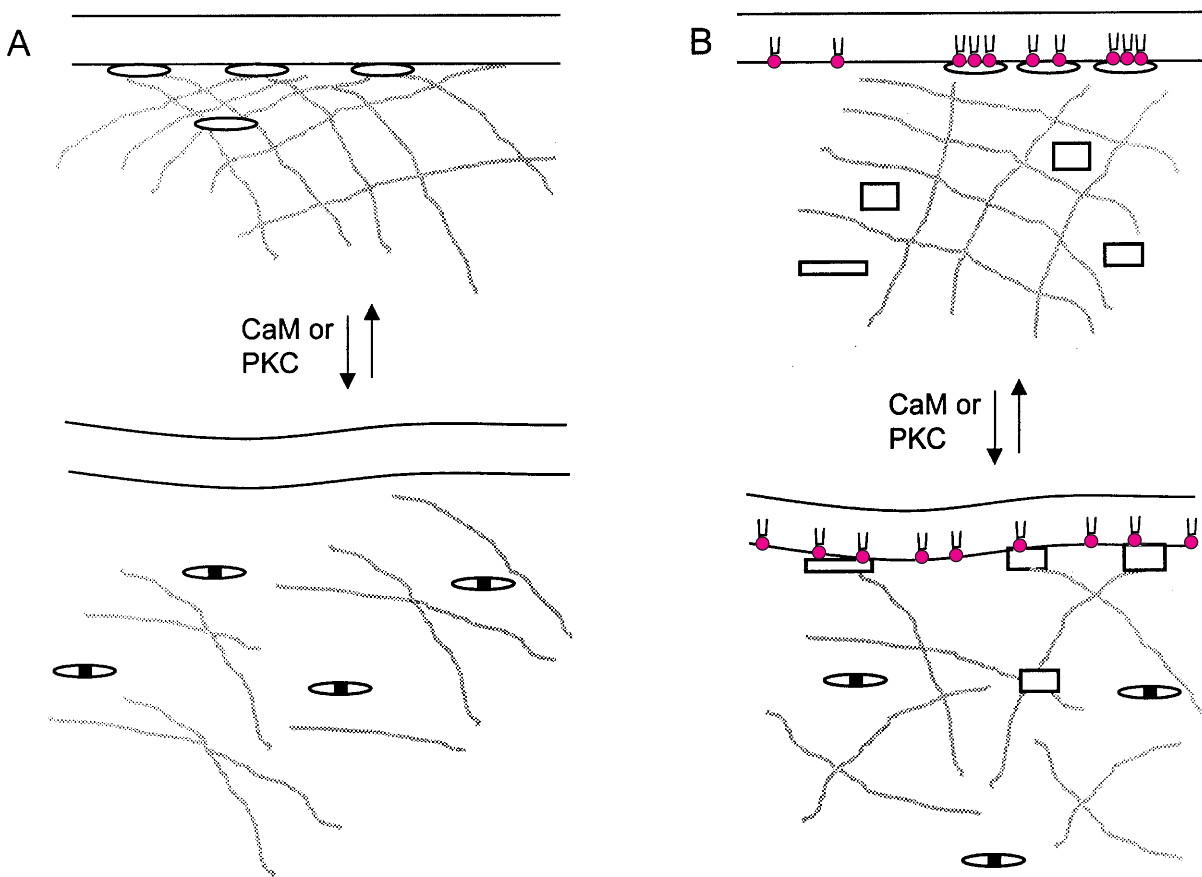

Figure 3 Two proposed modes of regulation of the actin cytoskeleton by MARCKS proteins

(**A**) MARCKS (ovals) binds directly to actin and tethers its to the plasma membrane. Activation of PKC or CaM results in modification and translocation of MARCKS, and release of actin. (**B**) MARCKS sequesters PIP_2 (lipids with head groups depicted red) in an area of the plasma membrane. Activation of PKC or CaM results in release of PIP_2, which becomes available for actin-binding proteins (rectangles). See the text for further details.

activity was found [82]. Further data are necessary in order to fully understand the interactions of MARCKS proteins with actin and their physiological relevance.

Regulation of actin by MARCKS via PIP_2?

It has become clear that polyphosphoinositide lipids play important roles in different signalling pathways [83–85]. PIP_2 is implicated in cell motility and regulation of the actin cytoskeleton [83–88] and, more recently, PIP_2 has been connected to the regulation of exocytosis and endocytosis [89,90], all cellular processes involving MARCKS proteins.

In contrast with other polyphosphoinositides, which are produced enzymically only in activated cells, PIP_2 is continuously present in the plasma membrane of most mammalian cells, comprising 1–5 % of the total lipid. However, recent evidence suggests that PIP_2 can act as signalling lipid. For example, the dynamics of actin are increased drastically if the total pool of PIP_2 is masked by a PH domain or by the PIP_2-binding agent neomycin [87,91]. Also, biophysical methods demonstrated that manipulation of PIP_2 levels by overexpression of the phospholipase C-δ (PLC-δ) PH domain or a phosphatase modulates the force required for pulling the plasma membrane from the cytoskeleton [92]. In order for PIP_2 to regulate different signal transduction pathways, the availability of PIP_2 to cellular components has to be regulated. This might occur by regulated local synthesis and/or hydrolysis, or by a reversible sequestration mechanism.

Recent studies suggest that MARCKS is an ideal candidate to regulate the local availability of PIP_2 [36,37,91]. MARCKS and PIP_2 occur at a similar cellular concentration of approx. 10 μM. MARCKS was found to co-localize with F-actin first around blebs, then in ruffles, and later in the lamellapodia [93]. Recent studies have shown that PIP_2 is also concentrated in ruffles [94,95] and in phagosomes [96], where it could co-localize with MARCKS. Immunofluorescence experiments demonstrated co-localization of MARCKS and PIP_2 in fixed cells [91]. PIP_2 that is sequestered by MARCKS could be released by PKC and/or CaM and become locally available for high-affinity binding to other proteins. This hypothesis is supported by evidence from biophysical, biochemical and cell biological experiments, as discussed below.

MARCKS and the MARCKS ED inhibit PLC-induced PIP_2 hydrolysis in phosphatidylcholine/phosphatidylserine/PIP_2 vesicles [15]. The MARCKS ED peptide also inhibits hydrolysis of PIP_2 by PLC-β_1 and PLC-δ_1 in phosphatidylcholine/phosphatidylserine/PIP_2 monolayers [37]. The simplest explanation for these inhibitory effects is that the ED sequesters PIP_2. Most interestingly, inhibition of the PLC-δ_1-induced hydrolysis of PIP_2 can be reversed by either CaM or PKC, both of which result in

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translocation of the MARCKS ED peptide into solution and in the release of PIP_2 [15,37]. Direct binding measurements demonstrated that 10 nM PIP_2 can bind 50% of the MARCKS ED available [36,37]. For comparison, the PLC-δ_1 PH domain and neomycin bind to PIP_2 with dissociation constants of 1 and 10 μM respectively [97,98]. The strong binding of the MARCKS ED to PIP_2 probably involves formation of an electrically neutral complex of one peptide to three or four lipid molecules [37].

If these observations also hold true *in vivo*, cells should compensate for an increased MARCKS concentration by increasing the concentration of PIP_2. Indeed, it was found that overexpression of wild-type MARCKS, but not of a mutant lacking the ED, in PC12B cells increased the total amount of PIP_2 in the cells [91]. Cells overexpressing wild-type MARCKS showed enhanced neurite outgrowth and an increased amount of actin-based structures (spikes) at the plasma membrane. Similar effects but symmetrical cell spreading were observed when high levels of neomycin or lithium (to inhibit phosphoinositide metabolism) were administered to quiescent cells [91]. Another hint of the potential importance of MARCKS–PIP_2 interactions comes from psychiatric research: chronic exposure of cells to lithium used to treat bipolar illness causes a reduction in MARCKS expression that is reversed by the addition of inositol to the cell medium [99].

In contrast with PIP_2 binding by the PH domain of PLC-δ_1, binding of the MARCKS ED to PIP_2 is a non-specific electrostatic interaction [37]. For example, the MARCKS ED binds equally strongly to the equally strongly charged lipids PIP_2 and phosphatidylinositol 3,4-bisphosphate, whereas the PLC-δ_1 PH domain binds less strongly to phosphatidylinositol 3,4-bisphosphate than to PIP_2 [100]. Also, increasing the salt concentration 5-fold from 100 to 500 mM decreases the binding of PIP_2 to the MARCKS ED 100-fold. Further, as mentioned above, binding of PIP_2 to MARCKS might involve an electrically neutral complex containing several lipid molecules. Finally, Lys_{13} oligomers resembling the total valence of the MARCKS ED bind as strongly to PIP_2 as does the MARCKS ED, peptides with a valence of $+9$ bind approx. 100-fold less strongly than MARCKS, and peptides with valences of less than $+7$ bind only weakly [101].

The two mechanisms discussed above for the regulation of the actin cytoskeleton by MARCKS proteins are depicted in Figure 3. Unphosphorylated myristoylated MARCKS could cross-link actin to the plasma membrane via one of the mechanisms proposed in the previous section. Phosphorylation or CaM complexation of the ED would then result in the release of MARCKS, and thus also actin, from the membrane (Figure 3A). Alternatively, binding of MARCKS to the plasma membrane could locally sequester the PIP_2 pool in resting cells (red full circles in the plasma membrane in Figure 3B). Phosphorylation or CaM complexation of the ED would result in the release of MARCKS from the membrane, and thus free PIP_2 to interact with other proteins, for example with actin-binding proteins known to bind to PIP_2, such as Neural Wiskott–Aldrich Syndrome Protein (N-WASP) [85,88,102]. In this model, no direct binding of MARCKS proteins to actin is required.

De- and re-myristoylation and specific proteolysis

Besides cycles of phosphorylation/dephosphorylation, which happen on a rather short time scale, other post-translational modifications might influence the properties of MARCKS proteins. Recent reports have indicated the possibility of demyristoylation/remyristoylation cycles, as well as of N-terminal cleavage leading to irreversible demyristoylation. Work over the last few years has also established the potential physiological significance of limited proteolysis close to or within the ED.

N-myristoylation is chemically stable, and is generally considered to be an irreversible co-translational event catalysed by the enzyme myristoyl-CoA: protein N-myristoyltransferase. Surprisingly, a significant amount of unmyristoylated MARCKS is found in the cytosolic fraction of cell extracts [103,104], indicating that co-translational myristoylation is not complete and/or that post-translational demyristoylation occurs. Indeed, a demyristoylation activity has been identified in calf brain cytosolic extracts that processes both MARCKS [105] and MRP [31]. MARCKS processed by this calf brain enzymic activity can be remyristoylated *in vitro*, as can MRP demyristoylated by an activity found in macrophage cytosol [106]. In addition, macrophage extracts contain another activity that processes MARCKS, a protease that specifically cleaves myristoylated MARCKS between residues 6 and 7 (lysine and threonine), but does not cleave unmyristoylated MARCKS. MRP is not affected, most probably because the sequence recognized in MARCKS is not found in MRP [106].

The susceptibility of MARCKS proteins to proteolysis has been noticed during the development of purification procedures. However, proteolysis has also been observed *in vivo*. For example, exposure of MARCKS to the cytosol of Ras-transformed fibroblasts results in degradation, most probably by cathepsin L. This effect was decreased after phosphorylation by PKC, indicating a possible role of PKC-mediated phosphorylation in the stability of MARCKS [107]. Similarly, MARCKS of human foreskin fibroblasts was specifically cleaved at the ED, and phosphorylation of MARCKS by PKC prevented cleavage [108]. The protease was later identified as the lysosomal enzyme cathepsin B, with which MARCKS might be able to interact after being targeted to lysosomes through a specific motif found in the N-terminal half of MARCKS [109].

The protozoan parasite *Leishmania* invades host macrophages and interferes with their signal transduction pathways. It was found that *Leishmania* depletes the cells of both MARCKS and MRP [110]. Further studies showed that MRP is cleaved within the ED by the major surface protease of *Leishmania*, leishmanolysin. Again in this case, phosphorylation of MRP by PKC inhibited degradation [111]. Accordingly, a pseudo-phosphorylated MRP ED peptide containing a triple mutation replacing serine residues by aspartic acids was shown to be a highly effective leishmanolysin inhibitor, with potential clinical applications (S. Corradin, personal communication). While these reports have established an interesting mode of irreversible regulation of MARCKS proteins, i.e. specific proteolysis, the significance of these observations is currently not clear.

MARCKS during embryogenesis

The human *MACS* gene is located on chromosome 6q21 [112], while the *MLP* gene is found at 1p34 [113]. Analysis of their promoter sequences has revealed multiple transcription factor binding sites, in agreement with the different levels of regulation observed. As already mentioned, MARCKS proteins show tissue-specific expression patterns, with high levels in several regions of the brain [7,114,115]. Both MARCKS and MRP mRNAs are expressed in the brain and spinal cord from early stages of development [113]. Furthermore, MARCKS proteins are required for embryogenesis, as revealed by several gene knock-out studies.

Mice heterozygous for MARCKS appear normal [116], but exhibit impaired spatial learning [117]. Mice heterozygous for

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MRP also appear normal [118,119]. In contrast, mice lacking both alleles of either MARCKS or MRP show severe phenotypes of brain misformations and perinatal death [116,118,119], indicating that there is no functional redundancy between MARCKS and MRP. Embryos lacking MARCKS show severe abnormalities of the central nervous system, and all die around birth [116]. Whereas in one study all MRP-null mice had exencephaly (lethal failure to close the neural tube) [119], in another study only around 60% of homozygous MRP mutants developed exencephaly [118]. Exencephaly is caused on the cellular level by impaired and/or misguided cellular migration, again implicating MARCKS proteins in migration.

The MARCKS-null mice were used to perform structure–function studies in the intact animal. Expression of non-myristoylatable MARCKS (N-terminal glycine mutated to alanine) in *MACS*$^{-/-}$ mice corrected the severe anatomical defects characteristic of MARCKS-deficient mice, resulting in apparently normal morphology. However, the majority of these animals still died around birth, with the cause of death not being determined. The remaining 25% of these transgenic mice survived the perinatal period, and appeared to be normal and fertile. Around half of the unmyristoylated protein was found to be cytosolic, with the other half being associated with the particulate fraction [120]. Surprisingly, these results indicate that myristoylation of MARCKS is not required for many of the *in vivo* functions of the protein.

OUTLOOK

MARCKS proteins have been studied for two decades and, as the results gathered in this review demonstrate, much has been learned. Figure 4 summarizes our current knowledge of the molecular interactions of MARCKS proteins as described throughout this review. MARCKS is now used as a tool and marker for PKC activity. Many data show that MARCKS proteins can mediate cross-talk between PKC and CaM. Membrane binding of MARCKS proteins is well understood, despite unexplained differences between MARCKS and MRP. Furthermore, the involvement of MARCKS proteins in cellular processes relying on the actin cytoskeleton is very likely, even though the exact molecular mechanisms are still under investigation. MARCKS proteins may regulate actin directly via binding and possibly cross-linking, or, more likely, indirectly via actin-regulating PIP_2-dependent proteins, since MARCKS can bind, sequester and locally release PIP_2. Finally, proteolytic and demyristoylating activities that affect MARCKS proteins have been identified, indicating further possible mechanisms of regulation.

However, recent studies indicate that there is even more to MARCKS proteins. At least seven additional phosphorylation sites were found in bovine MARCKS using electron-spray MS and phospholabelling techniques [121,122]. *In vivo* and *in vitro* studies indicate that MARCKS can be phosphorylated

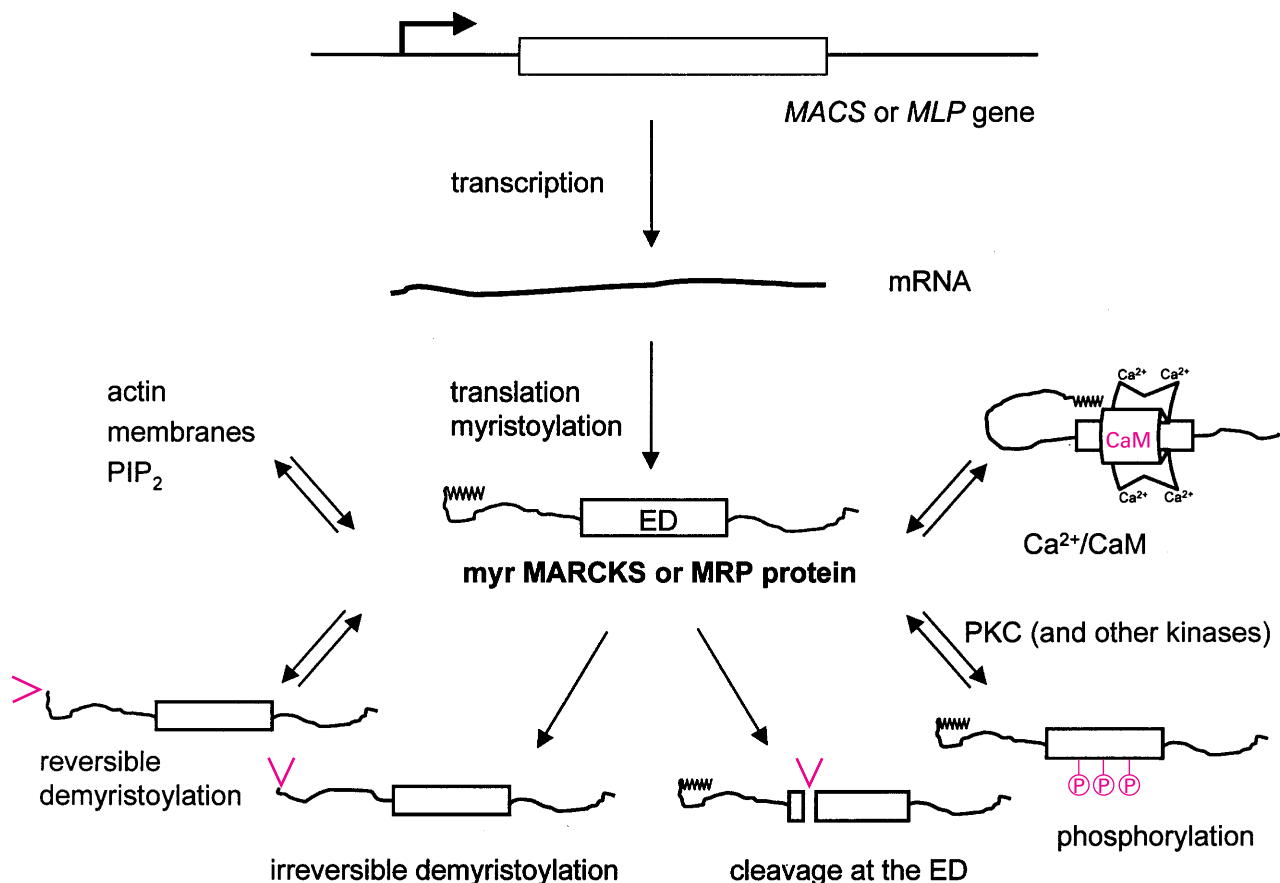

Figure 4 Summary of the lifespan of a MARCKS molecule

Shown are the generation and the possible fates of a MARCKS protein molecule from transcription to proteolysis, including potential interaction partners. Emphasized in red are the post-translational modifications discussed, with open arrowheads indicating the sites of action of modifying enzymes other than kinases.

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by proline-directed kinases, such as mitogen-activated protein kinases and cyclin-dependent kinases [121–123]. While further kinases that phosphorylate MARCKS proteins have also been reported by others, the role of these non-PKC kinases in the biology of MARCKS proteins is currently not clear. The same holds true for another interaction reported recently. Poly(ADP-ribose) is a homopolymer that is produced by poly(ADP-ribose) polymerase and is implicated in the cellular stress response. Poly(ADP-ribose) was shown to bind to the ED of MARCKS proteins, inhibiting binding to CaM and to membranes, phosphorylation by PKC and actin filament polymerization induced by the MRP ED [124]. While these emerging topics indicate that there will be much more to learn about MARCKS proteins, already the currently established results should help to increase our understanding of other signalling pathways and their components.

Because of space limitations we were not able to cite the work of many of our colleagues who have made valuable contributions to this field. Our own work on MARCKS proteins has been funded by grants to G. Schwarz (Biozentrum Basel) and to S. McLaughlin (SUNY Stony Brook), and by a grant from the Ciba-Geigy-Jubiläumsstiftung to G. V. We thank D. Murray (Cornell University, New York, NY, U.S.A.) for providing the peptide model shown in Figure 2, S. McLaughlin for critically reading the manuscript, and the staff of Cold Spring Harbor Laboratory (Cold Spring Harbor, NY, U.S.A.), where parts of this review were written, for their generous support and hospitality. Please note that the opinions expressed in this article are solely the authors', and do not necessarily reflect those of their respective employers.

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