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Protein myristoylation in protein–lipid and protein–protein interactions

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Abstract

Various proteins in signal transduction pathways are myristoylated. Although this modification is often essential for the proper functioning of the modified protein, the mechanism by which the modification exerts its effects is still largely unknown. Here we discuss the roles played by protein myristoylation, in both protein–lipid and protein–protein interactions. Myristoylation is involved in the membrane interactions of various proteins, such as MARCKS and endothelial NO synthase. The intermediate hydrophobic nature of the modification plays an important role in the reversible membrane anchoring of these proteins. The anchoring is strengthened by a basic amphiphilic domain that works as a switch for the reversible binding. Protein myristoylation is also involved in protein–protein interactions, which are regulated by the interplay between protein phosphorylation, calmodulin binding, and membrane phospholipids.

Keywords: Protein modification; Myristoylation; Protein–lipid interaction; Protein–protein interaction; Phosphorylation

1. Introduction

Since the discovery of protein myristoylation in the catalytic subunit of cAMP-dependent protein kinase and in the regulatory subunit (subunit B) of calcineurin (protein phosphatase 2B) [1,2], various proteins involved in signal transduction as well as those of viral origin have been shown to be fatty-acylated [3]. The modification is often essential for the proper functioning of these proteins. For example, the transforming activity of $p60^{src}$ from Rous Sarcoma Virus is dependent on its myristoylation [4]. A non-myristoylated MARCKS (myristoylated alanine-rich C kinase sub-

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strate) can only partially rescue the abnormalities found in the MARCKS knockout mice [5]. However, the mechanism by which the modification exerts its effects is still largely unknown [3,6].

It is generally assumed that hydrophobic acyl groups, such as myristoyl and palmitoyl groups, are involved in protein–membrane interactions. Due to its intermediate hydrophobicity, myristoylation has been implicated in the reversible membrane association [7,8]. Studies from our own and other laboratories have established that such a mechanism, in fact, is operative in the phosphorylation-dependent translocation of MARCKS between the cytosolic and membrane fractions [9,10]. In contrast, palmitoylation is considered to provide a more stable membrane anchoring. A specific palmitoyl thioesterase exists to liberate the palmitoylated protein from the membrane [11]. Although myristoylation has been considered to be static, we have demonstrated the presence of demyristoylase activity in the brain [12–14].

Not all acylated proteins are membrane bound. The first protein that was found to be myristoylated, cAMP-dependent protein kinase, is not a membrane protein. In the case of calcineurin, the second myristoylated protein found, the myristoylation does not seem to affect its association with membranes, and both myristoylated and non-myristoylated forms are present in membrane and soluble fractions [15,16]. Interestingly, the modification has been shown to affect the stability of both proteins [15,17]. This suggests that the myristoyl moiety interacts with the protein. In fact, the myristoyl group of recoverin is hidden inside a hydrophobic pocket, and there seems to be a specific interaction between the acyl group and the protein. Upon Ca^{2+} binding, a drastic conformational change of the protein occurs, which results in the protrusion of the acyl moiety from the protein and allows the protein to bind to membranes [18].

The involvement of the modification in protein–protein interactions has also been the subject of intensive studies [15,19–21]. Recently, we have shown that the modification is directly involved in the interaction of a brain-specific protein kinase C substrate, NAP-22, with calmodulin [22]. The acyl chain interacts specifically with the hydrophobic pocket of calmodulin. Since the basic domain adjacent to the myristoyl group was found to be important for the NAP-22–calmodulin interaction, the interplay of the myristoyl group and the basic domain seems to function in a manner analogous to that found in the myristoylation-mediated protein–membrane interaction [23,24]. In the present article, the roles of protein myristoylation in both protein–membrane and protein–protein interactions and the regulation of these interactions by protein phosphorylation, calmodulin binding, and membrane phospholipids will be discussed.

2. Myristoylation in protein–membrane interactions

2.1. Phosphorylation-dependent membrane interaction of MARCKS: a prototype

MARCKS is a major in vivo substrate of protein kinase C (PKC) in various cells and tissues [25]. Although the phosphorylation of MARCKS has been used as a specific marker for PKC activation in previous studies, our recent work has established that the MARCKS protein is a substrate of various kinases belonging to the MAP kinase family [26–28]. Since MARCKS also binds Ca^{2+}-calmodulin, the protein functions as one of the crosstalk points of a variety of signal transduction pathways. Recent gene-targeting studies suggest that MARCKS is essential during development [29]. Its physiological function, however, has yet to be determined.

The most striking feature of MARCKS is its stimulation-dependent translocation between the membrane and soluble fractions [30]. PKC, which is usually found in the cytosol, goes to the membrane upon stimulation (Fig. 1). In contrast, MARCKS is found in the membrane fraction in resting cells, but phosphorylated MARCKS moves out of the membranes to the cytosol. Since MARCKS is myristoylated at its N-terminus and lacks hydrophobic amino acids, it has been generally assumed that the acyl moiety is directly involved in the membrane anchoring of the protein. However, the myristoyl moiety has only 14 carbons,

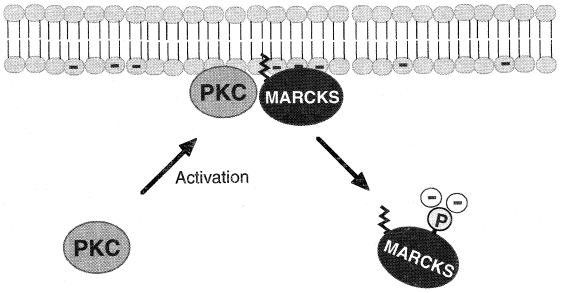

Fig. 1. Stimulation-dependent translocation of protein kinase C and its major substrate protein, MARCKS. PKC, found in the cytosol in resting cells, goes to the plasma membrane and phosphorylates MARCKS. Phosphorylated MARCKS dissociates from the membrane.

and its intermediate hydrophobicity is not sufficient to stably anchor an otherwise hydrophilic protein to the membrane [8]. Furthermore, how the phosphorylation at the PKC phosphorylation domain, which is found in the middle of the molecule (Fig. 2), affects the interaction of the myristoyl group with membrane lipids remains unknown. A vague image of a phosphorylation-dependent 'conformational change' comes to mind, but the MARCKS protein does not assume a fixed conformation, but shows a rather random structure [31]. MARCKS belongs to the class of hydrophilic proteins, such as tau protein, that assume a set of different conformations [32].

To elucidate the mechanism of the phosphorylation-dependent translocation of MARCKS, we first tested its ability to bind to membrane phospholipids in a reconstituted system [9]. The MARCKS protein, purified from membrane fractions of bovine brain, was mixed with various phospholipids, and the interaction between MARCKS and lipid vesicles was examined by sucrose density gradient centrifugation [9]. We demonstrated that the purified MARCKS protein has the intrinsic ability to bind to phospholipid membranes. We then examined whether the PKC-dependent phosphorylation affects the binding. Interestingly, MARCKS showed phosphorylation-dependent translocation only when the vesicles contained acidic phospholipids. We further demonstrated that the PKC phosphorylation domain, with a basic amphiphilic nature, binds only to acidic phospholipids [9].

These results can be explained by assuming two membrane binding sites, as illustrated in Fig. 3. The N-terminal myristoyl moiety together with the 10-amino acid N-terminal domain, with a basic amphiphilic nature, binds to neutral (phosphatidylcholine) as well as acidic (phosphatidylserine) phospholipids, mainly through a hydrophobic interaction. The second binding site, the PKC phosphorylation domain with a basic amphiphilic nature, binds only to acidic phospholipids. When MARCKS is mixed with vesicles containing only neutral phospholipids, it binds to the membranes through only the N-terminus [9,10]. The phosphorylation of the PKC phosphorylation domain does not affect the binding. In contrast, MARCKS binds to vesicles containing acidic phospholipids through two binding sites: the myristoyl N-terminal domain and the phos-

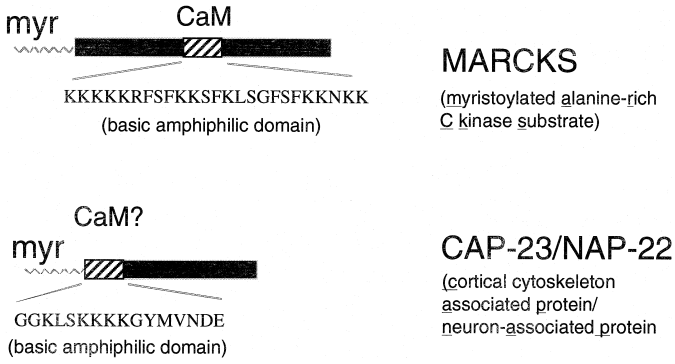

Fig. 2. Structures of two members of the MARCKS family of acidic hydrophilic PKC substrate proteins. Both proteins are myristoylated at the N-terminus and have a basic amphiphilic domain as the PKC-phosphorylation and calmodulin-binding domain.

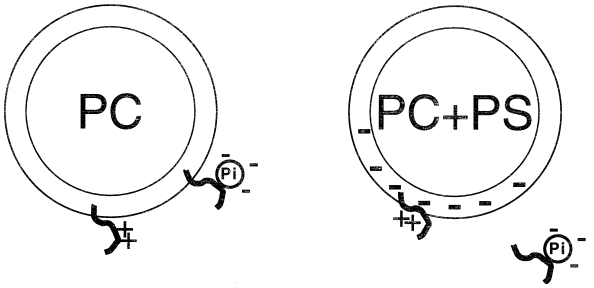

Fig. 3. MARCKS binds to phospholipid membranes through two binding sites. The myristoylated head domain can interact with both neutral (phosphatidylcholine, PC) and acidic (phosphatidylserine, PS) membranes, mainly through hydrophobic interactions. The basic amphiphilic domain interacts only with acidic phospholipids. Phosphorylation of the latter dissociates the MARCKS protein from acidic membranes, but not from neutral membranes.

phorylation domain. Phosphorylation of the latter not only diminishes the affinity of the domain, but also changes the overall charge of the molecule. The incorporation of three to four phosphoryl groups increases the acidity of the protein, and the charge repulsion between the protein and the acidic membranes is strong enough to 'pull out' the myristoylated hydrophobic head from the membrane [9].

Work from this and other laboratories not only confirmed our conclusion but also demonstrated that the same mechanism functions in other myristoylated proteins, such as src kinase [33]. One interesting result in these studies is the observation that the binding of calmodulin to the phosphorylation domain of MARCKS also liberates the MARCKS protein from the acidic membranes [34]. Since calmodulin is highly acidic, the binding of calmodulin has the effect of charge reversal, similar to that of phosphorylation. The phosphorylation domain with the basic amphiphilic nature, therefore, works as a phosphorylation- and calmodulin-dependent reversible membrane-binding domain in conjunction with protein myristoylation.

2.2. Interaction of the basic amphiphilic domain with membrane phospholipids

MARCKS is not the only protein that shows stimulation-dependent translocation between the membrane and soluble fractions. The endothelial isozyme of nitric oxide synthase (eNOS) has been shown to behave similarly [35]. Interestingly, the eNOS protein is myristoylated and has a calmodulin-binding domain with a basic amphiphilic nature [36,37]. This prompted us to examine the ability of eNOS to bind to membrane phospholipids through its calmodulin-binding domain [38]. Peptides derived from the calmodulin-binding domains of three isozymes of NOS were found to bind specifically to acidic phospholipids. Since the nitric oxide synthase activity of NOS is dependent on the binding of calmodulin to the NOS proteins, these results raise the interesting possibility that the NOS enzymatic activity is regulated by the binding to acidic phospholipids. Furthermore, the calmodulin-binding domain of eNOS was found to be a good substrate of PKC. Phosphorylation of one specific threonine residue by PKC leads to the dissociation of the domain from phospholipid membranes [38]. Phosphorylation of the domain also affects the calmodulin-binding to the NOS proteins, resulting in the inactivation of the enzymatic activity (Matsubara et al., in preparation). Thus, it is easily conceivable that the calmodulin-binding domain of the eNOS isozyme functions together with the N-terminal myristoyl moiety in the reversible translocation of the protein, in a manner similar to that found in MARCKS (Fig. 4). In addition, the dynamic palmitoylation-depalmitoylation cycle of eNOS may play an important role in the specific localization of the protein to caveolae.

GAP-43 (*g*rowth-*a*ssociated *p*rotein of apparent molecular mass of 43 kDa) is one of the major proteins found in the neuronal growth cone [39]. It is a major PKC substrate protein, and shows sequence homology to MARCKS. GAP-43 has a calmodulin-binding domain that also functions as the PKC phosphorylation domain. Although its physiological function is not known, GAP-43 has been suggested to be involved in neuronal plasticity [40,41]. Since GAP-43 binds to membrane phospholipids [42], we have analyzed the ability of the calmodulin-binding domain of GAP-43 to bind to phospholipids [43]. We demonstrated that the calmodulin-binding domain of GAP-43 binds

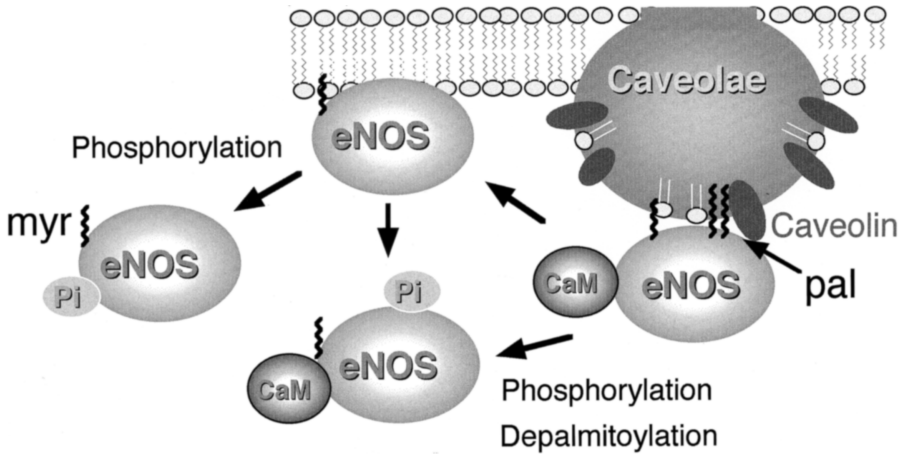

Fig. 4. Interaction of endothelial nitric oxide synthase (eNOS) with membrane microdomains. Both protein–protein and protein–lipid interactions play roles in the eNOS–membrane interaction and the translocation of the protein. Palmitoylation may be responsible for the localization of eNOS in the caveolae. Note that most of the modifications involved are reversible, and that the eNOS–membrane interaction is dynamic.

specifically to acidic phospholipids. Although the synthetic peptide derived from the calmodulin-binding domain (QASFRGHITRKKLKGEK) is shorter and contains fewer basic amino acids than the peptide derived from MARCKS, the quantitative analysis of the binding to phospholipids by titration calorimetry showed that the GAP-43 peptide has an affinity comparable to those of other membrane-binding peptides [10,43]. Furthermore, calmodulin binding as well as PKC-dependent phosphorylation reverses the binding of the peptide to phospholipid membranes. Therefore, a regulatory mechanism similar to that found with MARCKS functions in the GAP-43-membrane interaction. Although GAP-43 shares some overall structural features with MARCKS, such as the lack of hydrophobic amino acids and the richness in acidic and hydrophilic amino acids, it is not myristoylated, but instead is palmitoylated at two Cys residues near its N-terminus. Interestingly, the GAP-43 protein isolated from the membrane fractions of bovine brain used in our study was not palmitoylated [26]. The two Cys residues were oxidized and formed a disulfide bridge between them [43]. This not only suggests that the calmodulin-binding domain suffices for the tight binding of GAP-43 to the membranes, but also implies that the regulation of GAP-43 is effected in a complex manner, in which dynamic palmitoylation and oxidation through nitric oxide, as well as protein phosphorylation and calmodulin binding, work either independently or cooperatively. Therefore, a mechanism similar to the reversible translocation of eNOS may also operate in this case (Fig. 4).

The elucidation of the calmodulin-binding domain with a basic amphiphilic nature as a phosphorylation- and calmodulin-dependent membrane-interacting domain in MARCKS and GAP-43 led to the finding of similar domains in other proteins. Adducin, a membrane-cytoskeletal protein, contains a MARCKS-like calmodulin-binding domain that serves as a PKC phosphorylation domain. MAP1B, a microtubule-associated protein, binds to acidic membrane phospholipids as well, and the interaction of the protein with microtubules is controlled by lipid binding [44]. The basic amphiphilic domains, therefore, function as membrane-targeting domains in various proteins, and the interaction is often reversible, and is regulated by various factors, such as protein phosphorylation.

2.3. Conformation of the basic amphiphilic domain in solution and in the membrane

How does the calmodulin-binding domain with the basic amphiphilic nature interact with phos-

pholipids? To address this question, we studied the conformations of synthetic peptides derived from the calmodulin-binding domains of GAP-43 and eNOS. The CD spectra of these peptides show random structures in solution, but the peptides assume α-helical structures upon binding to phospholipid membranes [31,38,43,45]. The α-helical content of the peptides depends on the phospholipids used and on the sequences of the peptides. Therefore, specific structural interactions exists, rather than mere ionic interactions between the basic amphiphilic domain and the phospholipids. The phospholipid head groups seem to be 'recognized' by these proteins. In some cases, acidic phospholipids of special interest, such as phosphatidylinositol phosphates, show high affinities, suggesting that a possible regulatory mechanism may exist for the interaction of these proteins with membranes and with other signal transducing components.

From the structural viewpoint, it is interesting to note that the calmodulin-binding domains of these proteins show an amphiphilic nature when they assume an α-helical structure [46]. As clearly seen in the helical wheel projection of the eNOS peptide (Fig. 5a), the hydrophobic and basic hydrophilic amino acids segregate on the opposite sides of the helix. It is therefore reasonable to assume that the basic hydrophilic surface interacts with the acidic polar head groups of the phospholipids, while the hydrophobic surface is slightly embedded in the apolar part of the lipid bilayers (Fig. 5b). Interestingly, a similar calmodulin-binding domain in MARCKS was demonstrated to assume an extended structure in the membrane-bound state [47]. This may be due to the presence of two long hydrophilic basic stretches surrounding the central basic amphiphilic domain (Fig. 2), which is characteristic of the MARCKS calmodulin-binding domain. We demonstrated that the same domain binds to calmodulin in an extended form, which is quite unusual for calmodulin-binding domains (see below). We also found that the conformation of the MARCKS calmodulin-binding domain changes, depending on the ionic strength. This suggests that the MARCKS domain can assume two conformations in the lipid-bound state; one is extended and the other is α-helical. Thus, the interaction mode of the MARCKS domain with membrane phospholipids seems to be more complicated than that found in the calmodulin-binding proteins, such as the NOS isozymes and GAP-43. More detailed studies on the membrane-bound structures by other biophysical techniques, such as solid-state NMR, are clearly necessary.

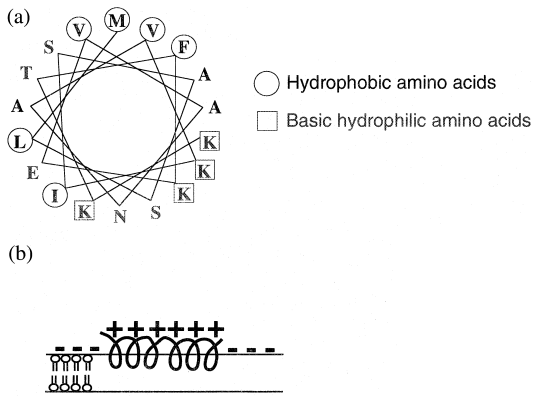

Fig. 5. (a) Helical wheel presentation of the calmodulin-binding domain of eNOS. Hydrophilic and hydrophobic amino acids segregate on either side of the helix, forming an amphiphilic helix. (b) Schematic representation of the interaction between an amphiphilic α-helix and acidic membranes. Hydrophobic residues interact with the apolar inner phase of the membrane, while basic residues interact with the polar heads.

2.4. Conformation of basic amphiphilic domains in calmodulin complexes

Since the calmodulin-binding domain of MARCKS binds to phospholipid membranes in an elongated structure, it is interesting to determine the structure of the domain in the calmodulin complex. The entire MARCKS protein has been shown to have an elongated, rod-shaped structure by rotary shadowing [48]. Our circular dichroism (CD) spectroscopic study showed that MARCKS does not have a distinct structure in solution [31]. The presence of myristoylation does not have a significant effect on the structure. When the MARCKS protein or a peptide derived from the calmodulin-binding domain was complexed with calmodulin, no significant change in the secondary structure was observed. In contrast, peptides with a canonical calmodulin-binding

motif derived from various calmodulin target proteins have been shown to bind to calmodulin in α-helical conformations [49]. A search in the protein database revealed other calmodulin-binding proteins with characteristics similar to that of MARCKS. In contrast to the conventional calmodulin-binding sequence, they are characterized by the presence of many basic amino acids (often more than 50%) and by the lack of large hydrophobic amino acids. Some of these proteins, such as F52 and the Ras-related GTP binding protein (Ral-A), have been shown to bind to calmodulin in non-helical conformations [50,51].

3. Myristoylation in protein–protein interactions

3.1. Ca^{2+}- and myristoylation-dependent binding of NAP-22 to calmodulin

As discussed above, the roles of protein myristoylation in protein–lipid interactions have been well established. However, the involvement of acylation in protein–protein interactions has been the subject of much speculation, but no clear experimental evidence has been presented. The CAP-23/NAP-22 protein has been characterized as a cortical cytoskeleton-associated protein of 23 kDa in chicken brain [52], and as a neuron-specific acidic protein of 22 kDa in the rat [53]. It shows clear homology to GAP-43 and MARCKS, although the homology between GAP-43 and MARCKS is less evident. The protein shares the overall characteristics of MARCKS and GAP-43; acidic, very hydrophilic, and without any large hydrophobic amino acids. It is a good substrate of PKC and binds calmodulin as well. These three proteins, therefore, constitute a family of acidic heat-stable PKC substrate proteins that are proposed to be involved in neuronal functions [41]. Unlike other family members, however, CAP-23/NAP-22 lacks a canonical calmodulin-binding domain (Fig. 2). The protein has been shown to bind to calmodulin in a Ca^{2+}-dependent manner, and deletion studies indicated that the N-terminal domain is essential for binding [54]. Using synthetic myristoylated peptides, as well as myristoylated and non-myristoylated recombinant proteins, we narrowed down the calmodulin-binding domain of CAP-23/NAP-22 to the myristoylated N-terminal domain of nine amino acids [22]. Interestingly, the binding of the protein to calmodulin is myristoylation dependent; only the myristoylated recombinant protein binds to calmodulin. It was found that the myristoyl moiety and a few basic amino acids together with a hydrophobic amino acid, a leucine, seem to be important for the binding (Fig. 6). This is reminiscent of the canonical calmodulin-binding motif identified in various calmodulin target proteins. In the typical calmodulin-binding motif, two critical hydrophobic residues surround a 12-amino-acid basic amphiphilic central domain [49]. The two hydrophobic amino acids interact with each of the two calmodulin half domains. We concluded that the two hydrophobic groups in the N-terminal domain of CAP-23/NAP-22, namely, the myristoyl moiety and the sole Leu residue, interact with the calmodulin half domains [22]. To our knowledge, this is the first clear-cut demonstration of the direct involvement of protein myristoylation in a protein–protein interaction. Since the same myristoylated N-terminal basic domain binds to both acidic membrane phospholipids and calmodulin, there seems to be a complex interplay between the various signal transduction pathways. Phosphorylation of the N-terminal domain by PKC leads to the dissociation of CAP-23/NAP-22, from both the membranes and calmodulin. Furthermore, phosphorylation of the protein by PKC was also found to be myris-

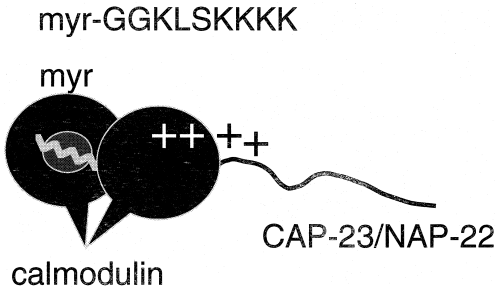

Fig. 6. Interaction of myristoyl peptides with calmodulin. One hydrophobic residue, leucine, and a few basic residues, leucine, and a few basic residues, lysines, seem to be important for the interaction.

toylation-dependent [22]. Thus, protein myristoylation seems to play important roles in various protein–protein interactions.

4. Dynamic regulation of protein myristoylation

4.1. Enzymatic demyristoylation of MARCKS

In contrast to protein palmitoylation, which is regulated by dynamic enzymatic palmitoylation and depalmitoylation, myristoylation has been considered to occur co-translationally and to be static. During our study on the MARCKS proteins isolated from the cytosolic and membrane fractions, we noticed the presence of a MARCKS fraction that showed weak affinity to the calmodulin affinity column [12]. A detailed study on the molecular structure of the fraction by mass spectrometry revealed that the fraction contains a non-myristoylated form of MARCKS. The non-myristoylated form is enriched in the cytoplasmic fraction and amounts to 20–25% of the MARCKS in the fraction. These results suggest either that the co-translational myristoylation is not tightly coupled or that the myristoylated MARCKS protein can be demyristoylated.

To pursue the latter possibility, we tested various fractions from bovine brain for MARCKS de-myristoylation activity. Myristoylated MARCKS, purified from bovine brain, was incubated with subcellular fractions, and the decrease in the apparent molecular weight in SDS gel electrophoresis, due to demyristoylation, was monitored [13]. It was found that the cytoplasmic fraction of brain synaptosomes contains MARCKS demyristoylation activity. Since the same fraction has already been shown to contain myristoyl transferase activity, these results suggest that MARCKS undergoes dynamic myristoylation and demyristoylation in brain synaptosomes. It is interesting to note that the demyristoylation reaction was found to be calmodulin-dependent; calmodulin binding to the phosphorylation domain in the middle of the MARCKS molecule inhibits the demyristoylation activity [13]. Since the demyristoylase activity takes place in the N-terminal myristoylation domain, this may suggest that the two domains, one in the N-terminus and the other in the middle of the molecule, are in close proximity in the calmodulin-bound form. This is understandable if one considers the necessity of the myristoyl group in the MARCKS–calmodulin interaction [12]. A similar mode of intramolecular regulation between the myristoyl moiety and other domains of a protein has been demonstrated in the case of recoverin [18,55]. More detailed structural studies on the MARCKS–calmodulin complex should elucidate the role of the acylation in the protein–protein interaction.

5. Conclusions

The roles played by protein myristoylation in protein–lipid interactions have become fairly clear. In most cases, a basic amphiphilic domain or a simple basic domain containing more than a few basic amino acids work together with the myristoyl moiety. The binding of an acidic protein, such as calmodulin, or protein phosphorylation of the basic domain diminishes the affinity of the domain to the acidic phospholipid membrane, which leads to the dissociation of the entire protein from the membrane. Interestingly, the same combination of a myristoyl moiety and a basic domain is involved in the binding of a myristoylated protein (CAP-23/NAP-22) to calmodulin. The reversible translocation of various signal transducing proteins, therefore, is controlled by the interplay between distinct factors, including protein phosphorylation, calmodulin binding, and specific acidic phospholipid metabolites.

Acknowledgements

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