Evidence for a non-myristoylated pool of the 80 kDa protein kinase C substrate of rat brain

R. A. Jeffrey McILHINNEY and Kate McGLONE

M.R.C. Anatomical Neuropharmacology Unit, Department of Pharmacology, University of Oxford, South Parks Road, Oxford OX1 3QT, U.K.

A protein of 80 kDa apparent molecular mass was found to be specifically myristoylated in rat brain cytosols derived from either whole brain or synaptosomes. The attachment of the fatty acid took place in the absence of protein synthesis, since the cytosols did not incorporate [^{14}C]lysine into protein, nor did cycloheximide affect the incorporation of the myristic acid into the protein. The fatty acid was incorporated into the protein via an acid-labile/alkali-resistant band, and Pronase digestion of the labelled protein showed that the lipid was covalently linked to a glycine residue. Together, these data suggested that the myristic acid was amide-linked to the *N*-terminal residue of the protein. The protein was identified as one of the major protein kinase C substrates, the MARCKS (myristoylated alanine-rich C kinase substrate) protein, by showing that Ca^{2+} stimulated its phosphorylation, by its heat stability and by immune precipitation (using an antiserum to the MARCKS protein). Incorporation of myristic acid into intact protein continued for up to 12 h, despite the fact that over this period some degradation of the protein could be demonstrated. In pulse-chase experiments, the pattern of loss of the incorporated fatty acid was similar to that of the protein itself, and therefore the loss of radioactivity probably reflects protein degradation rather than specific de-acylation of the protein. Together, these results suggest that there is a pool of unacylated MARCKS protein in the rat brain.

INTRODUCTION

The processing of proteins by the covalent attachment of saturated fatty acids, termed acylation, is now a widely recognized phenomenon (for reviews see Towler *et al.*, 1988a; Grand, 1989; Schmidt, 1989). Interest in this modification of proteins has been stimulated by the large number of receptor, regulatory, oncogene, viral and coupling proteins that have been found to be acylated. For example, acylated proteins in the central nervous system include myelin proteolipid protein (Folch & Lees, 1951), myelin DM 20 (Agrawal *et al.*, 1982), the α -subunit of the Na^+ channel (Schmidt & Caterall, 1987), calcineurin B (Aitken *et al.*, 1982), the α_0 and α_1 subunits of guanine-nucleotide-binding protein (G-protein) (Schulz *et al.*, 1987; Buss *et al.*, 1987) and the MARCKS (myristoylated alanine-rich C kinase substrate) protein (Stumpo *et al.*, 1989). The first three of these are examples of proteins modified by the covalent attachment of palmitate, whereas the last three proteins are modified by the amide linkage of myristic acid to an *N*-terminal glycine. The myristoylation of proteins is thought to be a co-translational process which takes place as the protein is synthesized and is susceptible to protein synthesis inhibitors such as cycloheximide (Magee & Courtneidge, 1985; McIlhinney *et al.*, 1985; Wilcox *et al.*, 1987).

The enzyme responsible for the attachment of the lipid, *N*-myristoyl transferase (NMT), has been shown to be present at high levels in rat brain (Glover *et al.*, 1988; McIlhinney & McGlone, 1990) and not to be uniformly distributed within the central nervous system (McIlhinney & McGlone, 1990). In the course of these studies and while developing an assay for NMT, it was observed that there were endogenous protein acceptors for fatty acid acyltransferases in rat brain cytosols and that one of these, a protein of 80 kDa apparent molecular mass, was specifically myristoylated (McIlhinney & McGlone, 1989).

In the present paper we show that the acylation of this protein *in vitro* does not depend on continued protein synthesis, and that the bound fatty acid is attached via an amide bond to the *N*-

terminal glycine of the protein. In addition, we have identified the protein as the recently named MARCKS protein (Stumpo *et al.*, 1989), one of the major protein kinase C substrates in rat brain. The implications of these results are discussed.

MATERIALS AND METHODS

Materials

[^3H]Myristic acid (22.2 Ci/mmol), [^3H]palmitic acid (30 Ci/mmol) and [^{14}C]lysine (300 mCi/mmol) were purchased from Amersham International. [^3H]Stearic acid was produced by Amersham International by reduction of vaccenic acid with ^3H and was purified as previously described (McIlhinney & McGlone, 1989). Myristoyl chloride and palmitoyl chloride were obtained from Aldrich Chemical Co. Myristic acid, palmitic acid, lithium CoA, fatty acid CoA synthetase and ATP were all purchased from Sigma. All other chemicals were obtained from FSA Ltd., and were of the highest quality available. Reverse-phase f.p.l.c. columns (Pep RPC HR 5/5) were obtained from LKB Pharmacia Ltd. H.p.l.c.-grade acetonitrile and trifluoroacetic acid were purchased from Rathburn Chemicals.

Tissue preparation

Male Wistar rats were stunned and decapitated, and the brains were removed and quickly placed in ice-cold 10 mM-Tris/HCl, pH 7.4, containing 0.32 M-sucrose. All subsequent procedures were performed at 4 °C. The brains were washed free of Tris/sucrose and homogenization was performed with a Dounce homogenizer in 5 vol. of cold extraction buffer: 20 mM-Tris/HCl, pH 7.4, containing 1 mM-EDTA, 1 mM-dithiothreitol, pepstatin (8 $\mu\text{g}/\text{ml}$), leupeptin (20 $\mu\text{g}/\text{ml}$) and aprotinin (20 $\mu\text{g}/\text{ml}$). The homogenates were centrifuged (100 000 g_{av} ; 1 h) and the supernatants were either assayed fresh or stored at -70 °C until needed. Synaptosomes were prepared as described by Gray & Whittaker (1962) and lysed by freeze-thawing in extraction

buffer followed by centrifugation ($100\,000\ g_{av.}$; 1 h), and the resulting supernatants were frozen in portions at $-70\ ^\circ\text{C}$.

Labelling

Portions ($400\ \mu\text{g}$) of the cytosols or synaptosomes were incubated with the appropriate fatty acid ($25\ \mu\text{Ci}$) or [^{14}C]lysine ($5\ \mu\text{Ci}$) in $400\ \mu\text{l}$ of a buffer containing ATP ($5\ \text{mM}$), lithium CoA ($1\ \text{mM}$), MgCl_2 ($5\ \text{mM}$), EGTA ($0.1\ \text{mM}$), Tris/HCl ($10\ \text{mM}$) and Hepes ($15\ \text{mM}$), pH 7.2. These conditions were chosen to mimic those used in the NMT assay, which were optimal for the myristoylation of peptides (McIlhinney & McGlone, 1989, 1990). In those incubations in which cycloheximide was used to prevent protein synthesis, it was added to give a final concentration of $5\ \mu\text{g}/\text{ml}$. Labelling with [^{32}P]ATP in the presence and absence of calcium was performed as described by Albert *et al.* (1986).

Immunoprecipitation and SDS/PAGE

Immunoprecipitation was carried out by incubating $5\ \mu\text{l}$ of a rabbit anti-MARCKS serum (kindly provided by Dr. P. Greengard, The Rockefeller University, New York, NY, U.S.A.) with portions ($100\ \mu\text{l}$) of labelled cytosol overnight at $4\ ^\circ\text{C}$. The bound antigen was then precipitated by rotating these incubations with $25\ \mu\text{l}$ of Protein A-Sepharose for 2 h at $4\ ^\circ\text{C}$. Then the beads were washed three times with $50\ \text{mM}$ -Tris/HCl, pH 7.5, containing 0.05% Nonidet P-40, and the bound material was eluted with $50\ \mu\text{l}$ of SDS/PAGE sample buffer (Laemmli, 1970). Proteins were separated by SDS/PAGE on 10% gels using the discontinuous buffer system of Laemmli (1970), and the positions of the proteins were determined by autoradiography of the gels following impregnation with either 2,5-diphenyloxazole (PPO) (Bonner & Laskey, 1974) or sodium salicylate (Chamberlain, 1979).

Fatty acid analysis

Fatty acid analysis was performed on gel bands excised from salicylate-impregnated gels, using the autoradiogram as a guide to the position of the proteins. The fatty acids incorporated into the proteins were released from the gel slices by hydrolysis with KOH followed by HCl (McIlhinney *et al.*, 1985) and extracted into hexane. In order to examine the amino acid linkage of the myristic acid, the $80\ \text{kDa}$ band was cut from the gels and exhaustively digested with Pronase (McIlhinney & McGlone, 1990), and the released radioactivity was extracted into chloroform/methanol ($1:1$, v/v). Analysis of the released materials was performed by reverse-phase chromatography using a Pep RPC HR 5/5 column as previously described (McIlhinney & McGlone, 1990), using ^{14}C -labelled fatty acid and synthetic [^3H]myristoylglycine as standards.

Protein estimations

Protein was determined using the Bio-Rad dye-binding assay, with BSA as the standard.

Western blotting

Cytosols prepared from synaptosomes were incubated as described above, except that unlabelled myristic acid ($1\ \mu\text{g}$) replaced the radioactive fatty acid. At the times indicated, the incubations were terminated by heating at $100\ ^\circ\text{C}$ for 10 min followed by centrifugation ($10\ \text{min}$; $10\,000\ g_{av.}$). The proteins remaining in the supernatant were recovered by precipitation with 5 vol. of cold acetone and incubation for 16 h at $-20\ ^\circ\text{C}$ followed by centrifugation. The precipitated proteins, which included the MARCKS protein, were separated by SDS/PAGE and transferred to nitrocellulose using the LKB Multiphor dry blotter and the discontinuous buffer system recommended by the manufacturers. Following transfer, the nitrocellulose was

blocked by incubation in phosphate-buffered saline ($10\ \text{mM}$ -sodium phosphate (pH 7.2)/ $0.14\ \text{M}$ -NaCl) containing BSA (1% , w/v) and Tween 20 (0.05% , v/v) for 16 h. The blocking buffer was also used throughout for the other incubations. The MARCKS protein was detected by first incubating the blot with a rabbit anti-MARCKS serum (as described above) at a dilution of $1:300$ for 2 h at room temperature. The first serum was removed by washing and the blot was incubated for a further 2 h with a swine anti-(rabbit immunoglobulin) serum coupled to horseradish peroxidase (Dako) at a dilution of $1:500$. After washing with phosphate-buffered saline, the position of the immunopositive bands was revealed by incubating the blot with 4-chloro-1-naphthol ($0.5\ \text{mg}/\text{ml}$) in Tris/HCl ($0.05\ \text{M}$; pH 7.5) containing NaCl ($0.2\ \text{M}$) and hydrogen peroxide (0.003% , v/v) for 15 min. Control incubations were performed by omitting the first antibody.

RESULTS

Incubation of rat brain synaptosomes indicated that there was an $80\ \text{kDa}$ protein that was specifically labelled with myristic acid but not with stearic or palmitic acid (Fig. 1). Given that under the conditions of these incubations we would not expect to find protein synthesis, this was somewhat surprising, since myristoylation is normally co-translational (Wilcox *et al.*, 1987). To test whether in fact protein synthesis was contributing to the myristoylation of the $80\ \text{kDa}$ band, we incubated rat brain cytosols with myristic acid in the presence of a concentration of cycloheximide which would prevent protein synthesis. The results were unequivocal in that cycloheximide had no effect upon the incorporation of myristic acid (Fig. 1) and, furthermore, there was no incorporation of [^{14}C]lysine into protein in lysates, which supported the myristoylation of the $80\ \text{kDa}$ band (Fig. 1). Consequently the myristoylation of this protein could not be linked to protein synthesis in this 'in vitro' system.

It was therefore important to determine how the fatty acid was being attached to the protein, and to show that it was being

Fig. 1. Myristoylation of the $80\ \text{kDa}$ protein does not depend on protein synthesis

Rat brain cytosols were incubated for 1 h with myristic (Myr), palmitic (Pal) or stearic (St) acid, and the labelled proteins ($100\ \mu\text{g}$) were analysed by SDS/PAGE. In separate experiments cytosols were incubated with myristate in the presence (+C) or absence (-C) of cycloheximide at a concentration ($5\ \mu\text{g}/\text{ml}$) known to fully inhibit protein synthesis. Under the incubation conditions used in these experiments, there is no apparent protein synthesis in the cytosols, as judged by their failure to incorporate [^{14}C]lysine (^{14}C) into protein under conditions which do permit the acylation of the $80\ \text{kDa}$ protein (Myr). In this and all subsequent gels, the arrows show the positions of the marker proteins fructose-6-phosphate kinase ($84\ \text{kDa}$) and pyruvate kinase ($58\ \text{kDa}$).

Fig. 2. Myristic acid is incorporated into the protein intact and is amide-linked to a glycine residue

The 80 kDa protein was excised from the gels and digested exhaustively with Pronase. This released the majority of the radioactivity found in this band, and more than 80% of this (a) co-chromatographed with a myristoylglycine standard (b).

incorporated intact. Slices containing the labelled 80 kDa and 50 kDa proteins were excised from gels impregnated with salicylate, using the autoradiographs as a guide. The slices were then subjected to sequential alkaline and acid hydrolysis, and the released fatty acid was analysed by h.p.l.c. In ten experiments, $80 \pm 7\%$ of the radioactivity in the 50 kDa band was released by the alkaline treatment, suggesting that the lipid was ester-linked to this protein. The 80 kDa protein, however, required acid hydrolysis for the release of the radioactivity bound to it ($75 \pm 13\%$), suggesting that the fatty acid was not ester-linked. The fatty acid released from both proteins was identified as intact myristic acid (results not shown). In order to analyse further the nature of this linkage, gel slices were subjected to exhaustive Pronase digestion and the fragments released were analysed by h.p.l.c. The results showed that 80% of the released radioactivity co-chromatographed with a synthetic *N*-myristoylglycine standard (Fig. 2), and the remainder with myristic acid. This suggests that the myristate was indeed linked to the *N*-terminal glycine of the protein by an amide bond, as found for most myristoylated proteins.

Since we had previously shown that rat brain synaptosomes contained NMT (McIlhinney & McGlone, 1990), the ability of cytosols prepared from these to myristoylate the 80 kDa protein was examined. The results showed that these cytosols do indeed show a time-dependent incorporation of myristic acid into the 80 kDa protein, similar to that of the total brain cytosols (Fig. 3). Interestingly, prolonged incubations of up to 8 h demonstrated

Fig. 3. Cytosols prepared from rat brain synaptosomes can also *N*-myristoylate the 80 kDa protein in a time-dependent manner

Cytosols were prepared from rat brain synaptosomes as described in the Materials and methods section and incubated with [3 H]myristic acid for 2, 4 or 6 h. Analysis of the labelled proteins (100 μ g) indicated that the incorporation of the label increased with time. Even after prolonged incubations of 8 h, the acylation of this protein was predominantly with myristic acid (see 8-Myr and 8-Pal).

that the 80 kDa protein remained the only protein specifically myristoylated. The small degree of incorporation of palmitate into the 80 kDa protein with these prolonged incubations might be expected, since NMT is not absolutely specific for fatty acid chain length (McIlhinney & McGlone, 1989). It should be noted that no additional myristoylated protein bands appeared in these long-term incubations, even after 12 h of incubation (see Fig. 5).

The identity of the 80 kDa protein was established by immunoprecipitating the protein, using a rabbit antiserum against the rat brain MARCKS protein, from cytosols prepared from rat brain labelled with [3 H]myristate for 1 h. The results (Fig. 4a) showed that the serum specifically precipitated the 80 kDa protein. Its identity was confirmed by showing that the immune-precipitated myristoylated protein co-migrated on SDS/polyacrylamide gels with the phosphorylated form of the MARCKS protein obtained by calcium stimulation of protein kinase C (Fig. 4b). The identity of the myristoylated protein in the cytosols prepared from rat brain synaptosomes was also confirmed as the MARCKS protein by immune precipitation, and again there was time-dependent acylation (Fig. 4c). The protein was heat-stable, remaining in solution when the cytosols were boiled at 100 $^{\circ}$ C for 10 min (results not shown). This is also a property of the MARCKS protein (Graff *et al.*, 1989a) and provides additional evidence for the identity of the myristoylated protein as the MARCKS protein.

Since the myristoylation of the 80 kDa protein could be due to either the acylation of a pool of non-myristoylated protein, or the de-acylation and re-acylation of the protein, experiments were performed to see if the attached fatty acid did turn over in the brain cytosols. Cytosols prepared from synaptosomes were labelled for 4 h with [3 H]myristate and then unlabelled myristate (10 μ g) was added. At the time points indicated, samples of the incubation were removed and equal amounts were analysed on SDS/polyacrylamide gels. The results show that under these conditions there was significant loss of radioactivity from the 80 kDa band (Fig. 5a). Nevertheless, in the absence of unlabelled myristic acid, the incorporation of radioactive myristate into the protein continued for up to 12 h (Fig. 5a). In order to determine whether this loss of labelled protein was due to the release of

Fig. 4. Identification of the 80 kDa protein as the major brain protein kinase C substrate or MARCKS protein

Rat brain cytosol was labelled with myristic acid for 1 h and the cytosols were immunoprecipitated with a rabbit antiserum to the MARCKS protein (*a*; Ser). The precipitation was controlled by using Protein A-Sepharose alone in the second step of the reaction (*a*; Con). The results showed that the antiserum (Ser) specifically immunoprecipitated the 80 kDa myristoylated band. Its identity was confirmed by showing that it was phosphorylated in the presence of calcium (*b*; +Ca) and that the myristoylated protein (*b*; Myr) co-migrated with the ^{32}P -labelled protein. This immunoprecipitated band was also acylated in a time-dependent manner after 2, 4 and 6 h of labelling with ^3H myristate (*c*) in cytosols prepared from rat brain synaptosomes.

Fig. 5. Pulse-chase and immunoblotting of the MARCKS protein at different times of incubation

Cytosols from rat brain synaptosomes were labelled for 4 h with ^3H myristic acid, after which $10\text{ }\mu\text{g}$ of unlabelled myristic acid was added and the incubation was continued for a further 8 h. At the times indicated after the addition of the unlabelled myristate, samples ($100\text{ }\mu\text{g}$) of the incubation were analysed by PAGE (*a*). As a control for degradation of the protein, a 12 h incubation was run in parallel. A similar incubation was performed using unlabelled myristic acid alone and samples of this were transferred on to nitrocellulose paper, and the MARCKS protein was detected by immunoblotting with the anti-MARCKS serum (*b*). The double and triple arrows represent the top and bottom of the gel respectively.

attached fatty acid, or to degradation of the protein, the amounts of MARCKS protein in such incubations was determined by Western blotting. The results (Fig. 5*b*) indicated that the loss of MARCKS protein was discernible by 4 h and was accompanied by the appearance of a 70 kDa immunopositive band which was apparent by 4 h, and readily so at 6 and 8 h. Both the radioactively labelled protein and the immunoreactive protein showed a similar pattern of degradation, with the loss of activity becoming apparent at 4 h and being more discernible thereafter. Therefore the loss of radioactivity appeared to be due to the breakdown of the protein rather than selective loss of the fatty acid. It should be noted, however, that the 70 kDa degradation fragment of the MARCKS protein did not appear to be radioactively labelled.

DISCUSSION

The results presented here establish that the 80 kDa protein which is specifically myristoylated *in vitro* in cytosols prepared from rat brain is the protein kinase C substrate termed MARCKS protein by Stumpo *et al.* (1989). Furthermore, they provide the first direct demonstration that this protein is *N*-terminally acylated at a glycine residue. This had previously been suggested by both its *N*-terminal sequence and the fact that a synthetic *N*-terminal peptide sequence of this protein could act as a substrate for NMT (R. A. J. McIlhinney & K. McGlone, unpublished work; Graff *et al.*, 1989*b*). The fact that the myristoylation of the protein is occurring normally in a system in which there is no protein synthesis and that it is unaffected by protein synthesis inhibitors was somewhat surprising.

Normally the *N*-terminal myristoylation of cell proteins takes place as the proteins are being synthesized and is susceptible to protein synthesis inhibitors (McIlhinney *et al.*, 1985; Magee & Courtneidge, 1985), as is the myristoylation of the MARCKS protein itself (Aderem *et al.*, 1988; James & Olson, 1989). Indeed, nascent peptides which are already myristoylated can be isolated from polyribosomes, and therefore myristoylation appears to be an early event in protein synthesis (Wilcox *et al.*, 1987). The observations described here therefore require some explanation, and three hypotheses seem plausible.

Firstly, the phenomenon could be an artefact of the preparation of the lysates. This could occur by release of the unacylated protein from polyribosomes during the homogenate preparation. Since the protein is intact, and as discussed above myristoylation is an early event in protein synthesis, this explanation seems unlikely, since it would result in the release of peptides smaller than the intact protein. Furthermore, such a process would also affect other proteins, and a range of specifically myristoylated proteins would be expected in the lysates. This is not what is observed, with the most striking feature of our observations being that the only protein myristoylated specifically is the MARCKS protein. Therefore we consider this explanation to be improbable, a conclusion which is supported by our observation that the myristoylation of the MARCKS protein takes place in cytosols prepared from synaptosomes, in which little protein synthesis takes place.

An alternative explanation is that the protein is undergoing de-acylation and re-acylation in the lysates. However, the half-life of the attached myristate is generally similar to that of the protein to which it is attached (Buss *et al.*, 1984), and these proteins have fairly long half-lives (Buss *et al.*, 1984; McIlhinney *et al.*, 1985). If de-acylation and re-acylation were to account for the myristoylation of the MARCKS protein in the cytosols, then it would have to be specific for this protein, since other myristoylated proteins are not apparent in our experiments. Also, in a recent paper on the synthesis of the MARCKS protein in BC3H1 myocytes, pulse-labelling of the protein with myristate

revealed no evidence for alterations in the levels of myristoylation of the protein in response to phorbol ester stimulation (James & Olson, 1989). Finally, our results suggest that the loss of radioactive myristic acid from the MARCKS protein described above is due to the degradation of the protein itself rather than to a selective loss of the fatty acid. However, it should be noted that the 70 kDa immunopositive fragment of the MARCKS protein which appears with the breakdown of the protein is not myristoylated. This could imply that the loss of the *N*-terminal region of the protein is an early event in the degradation of the protein, and it is tempting to speculate that the de-acylation of the protein may render it more susceptible to degradation, as has been shown for gastric mucins (Slomiany *et al.*, 1984, 1988). It is perhaps surprising that, despite the obvious loss of the immunopositive MARCKS protein by 8 h of incubation, there is a continued increase in the incorporation of myristic acid into the protein. The simplest explanation for this apparent paradox would be if the rate of degradation were less than the rate of acylation.

The third explanation for the results reported here is that there is a pool of unacylated MARCKS protein in both the rat brain and synaptosomal cytosols which can be acylated when these are provided with the appropriate conditions. Given the normally tight coupling of myristoylation to protein synthesis this might seem implausible, since it would seem to necessitate a sequestration of some of the MARCKS protein into a synthetic pathway where it was either not acylated or had the myristic acid removed after synthesis. However, a number of reports suggest that such a mechanism might indeed exist. Firstly, a 52 kDa mitochondrial protein has been described which is synthesized in the cytoplasm, transported to the mitochondrion and there myristoylated (Vijayasarathy *et al.*, 1989). Secondly, the coupling protein transducin has an *N*-terminal sequence that is consistent with the protein being myristoylated, and indeed a synthetic peptide with this sequence will act as a substrate for NMT (Towler *et al.*, 1988b), but the protein itself is not acylated (Buss *et al.*, 1987). Both of these proteins therefore provide evidence for a mechanism for their synthesis without obligatory myristoylation, and consequently similar mechanisms may be operating to produce a pool of unacylated MARCKS protein. In this context, it is interesting to note that during the purification of the MARCKS protein from bovine brain it was noted that 30% of the protein exhibited different hydrodynamic properties from the major peak of material which the authors fully characterized (Albert *et al.*, 1987). Otherwise the chromatographic characteristics of the two fractions were similar. Therefore the authors suggested that the minor component could represent a structurally different subpopulation of the protein. One plausible explanation for these observations is that this 30% of the MARCKS protein could represent the unacylated pool found in the experiments described in this paper. The significance of such a pool of unacylated MARCKS protein remains unclear.

When macrophages were exposed to a priming stimulus, it was found that the MARCKS protein was myristoylated rapidly in response to the stimulus. In order to explain this, Aderem *et al.* (1988) postulated that there might be a pool of unacylated MARCKS protein present in these cells. A pool of unacylated MARCKS protein such as that observed here would be consistent with this hypothesis and provide a means for the rapid acylation of this protein in response to a physiological stimulus. In this regard, it is interesting to note the presence of a pool of unacylated MARCKS protein in cytosols derived from synaptosomes. Unfortunately, due to the degradation of the protein and the non-equilibrium conditions of labelling used in our experiments, we were unable to estimate the size of this pool.

In conclusion, the results presented here demonstrate that, in cytosols prepared from whole rat brain or synaptosomes, the MARCKS protein can be myristoylated in the absence of protein synthesis. This myristoylation takes place on the *N*-terminal glycine of the protein, and the fatty acid is attached by an amide bond. Although our results do not preclude the possibility of an acylation/de-acylation cycle causing this myristoylation of the MARCKS protein *in vitro*, the simplest explanation consistent with our data and with what is known about the general myristoylation of proteins is that it is due to the presence of a pool of unacylated MARCKS protein present in the cytosols. These results are intriguing in that they suggest that, for this protein at least, myristoylation may be a more complex phenomenon than a simple 'once and for all' co-translational modification of proteins.

We thank Dr. Paul Greengard and Dr. Angus Nairn for their generous donation of the rabbit anti-MARCKS serum.

REFERENCES

- Aderem, A. A., Albert, K. A., Keum, M. M., Wang, J. K. T., Greengard, P. & Cohn, Z. A. (1988) *Nature* (London) **332**, 362–364
- Agrawal, H. C., Randle, C. L. & Agrawal, D. (1982) *J. Biol. Chem.* **257**, 4588–4592
- Aitken, A., Cohen, P., Santikarn, S., Williams, D. H., Calder, A. G., Smith, A. & Klee, C. B. (1982) *FEBS Lett.* **150**, 314–318
- Albert, K. A., Walaas, S. I., Wang, J. K. & Greengard, P. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 2822–2826
- Albert, K. A., Nairn, A. C. & Greengard, P. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7046–7050
- Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 83–88
- Buss, J. E., Kamps, M. P. & Sefton, B. (1984) *Mol. Cell. Biol.* **4**, 2697–2704
- Buss, J. E., Mumby, S. M., Casey, P. J., Gilman, A. G. & Sefton, B. M. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7493–7497
- Chamberlain, J. P. (1979) *Anal. Biochem.* **98**, 132–135
- Folch, J. & Lees, M. B. (1951) *J. Biol. Chem.* **191**, 807–817
- Glover, C. J., Goddard, C. & Felsted, R. L. (1988) *Biochem. J.* **250**, 485–491
- Graff, J. M., Stumpo, D. J. & Blackshear, P. J. (1989a) *J. Biol. Chem.* **264**, 11912–11919
- Graff, J. M., Gordon, J. I. & Blackshear, P. J. (1989b) *Science* **246**, 503–506
- Grand, R. J. A. (1989) *Biochem. J.* **258**, 625–635
- Gray, E. G. & Whittaker, V. P. (1962) *J. Anat.* **96**, 79–88
- James, G. & Olson, E. N. (1989) *J. Biol. Chem.* **264**, 20928–20933
- Laemmli, U. K. (1970) *Nature* (London) **227**, 680–685
- Magee, A. M. & Courtneidge, S. A. (1985) *EMBO J.* **4**, 1137–1144
- McIlhinney, R. A. J. & McGlone, K. (1989) *Biochem. J.* **263**, 387–391
- McIlhinney, R. A. J. & McGlone, K. (1990) *J. Neurochem.* **54**, 110–117
- McIlhinney, R. A. J., Pelly, S. J., Chadwick, J. K. & Cowley, G. P. (1985) *EMBO J.* **4**, 1145–1152
- Schmidt, J. W. & Caterall, W. A. (1987) *J. Biol. Chem.* **262**, 13713–13723
- Schmidt, M. F. G. (1989) *Biochim. Biophys. Acta* **988**, 411–426
- Schulz, A. M., Tsai, S.-C., Kung, H.-F., Oroszlan, S., Moss, J. & Vaughn, M. (1987) *Biochem. Biophys. Res. Commun.* **146**, 1234–1239
- Slomiany, A., Jozwiak, Z., Takagi, A. & Slomiany, B. L. (1984) *Arch. Biochem. Biophys.* **229**, 560–567
- Slomiany, B. L., Murty, V. L. N., Sarosiek, J., Piotrowski, J. & Slomiany, A. (1988) *Biochem. Biophys. Res. Commun.* **151**, 1046–1053
- Stumpo, D. J., Graff, J. M., Albert, K. A., Greengard, P. & Blackshear, P. J. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 4012–4016
- Towler, D. A., Gordon, J. I., Adams, S. P. & Glaser, L. (1988a) *Annu. Rev. Biochem.* **57**, 69–99
- Towler, D. A., Adams, S. P., Eubanks, S. R., Towery, D. S., Jackson-Machelski, E., Glaser, L. & Gordon, J. I. (1988b) *J. Biol. Chem.* **263**, 1784–1790
- Vijayasarathy, C., Bhat, N. R. & Avadhani, N. G. (1989) *J. Biol. Chem.* **264**, 772–775
- Wilcox, C., Hu, J. S. & Olson, E. N. (1987) *Science* **238**, 1275–1278