Phosphorylation of the myristoylated protein kinase C substrate MARCKS by the cyclin E–cyclin-dependent kinase 2 complex in vitro

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The myristoylated alanine-rich C-kinase substrate (MARCKS) purified from brain was recently characterized as a proline-directed kinase(s) substrate in vitro [Taniguchi, Manenti, Suzuki and Titani (1994) J. Biol. Chem. 269, 18299–18302]. Here we have investigated the phosphorylation of MARCKS by various cyclin-dependent kinases (Cdks) in vitro. We established that Cdk2, Cdk4 and, to a smaller extent, Cdk1 that have been immunoprecipitated from cellular extracts phosphorylate MARCKS. Comparison of MARCKS phosphorylation by protein kinase C (PKC) and by the purified cyclin E–Cdk2 complex suggested that two residues were phosphorylated by Cdk2 under these conditions. To identify these sites, Cdk2-phosphorylated MARCKS was digested with lysyl endoprotease and analysed by electrospray MS. Comparison with the digests obtained from the unphosphorylated protein demonstrated that two peptides, Gly138–Lys140 and Ala138–Lys142, were phosphorylated by cyclin E-Cdk2. The identity of these peptides was confirmed by automatic Edman degradation. On the basis of the consensus phosphorylation sequence described for Cdk2, and on MS/MS analysis of the Ala138-Lys142 peptide, we concluded that Ser137, one of the phosphorylation sites identified in vitro, and Thr140 were the Cdk2 targets in vitro. None of the other sites described in vitro were phosphorylated in these conditions. Interestingly, a preliminary phosphorylation of MARCKS by PKC improved the initial rate of phosphorylation by Cdk2 without modifying the number of sites concerned. In contrast, phosphorylation of MARCKS by Cdk2 did not significantly affect further phosphorylation by PKC.

Key words: cell cycle, cyclin-dependent kinases, mass spectrometry, phosphoprotein.

INTRODUCTION

The myristoylated alanine-rich C-kinase substrate (MARCKS) has been used for many years as a marker for protein kinase C (PKC) activation in various cell systems. The PKC phosphorylation sites lie in the ‘phosphorylation site domain’, a 25-residue basic sequence highly conserved between various species. Four serine residues are the targets for PKC in vitro but only three of them have been described as physiological phosphorylation sites (reviewed in [1,2]). The cellular phosphorylation of MARCKS by PKC triggers the translocation of the substrate from the plasma membrane to the cytoplasmic and/or lysosomal compartments [3–5]. The molecular mechanism of this translocation was elucidated through experiments in vitro with reconstituted membrane models [6,7] and by the ectopic cellular expression and localization of various mutants [8,9]. This process was called the ‘myristoyl electrostatic switch’ because hydrophobic interactions of the N-terminal myristoyl moiety with the membrane were involved, as well as electrostatic interactions of the phosphorylation site domain with the acidic lipids of the bilayer. In vitro, this phosphorylation also negatively regulates the interaction of MARCKS with calmodulin [10,11] and F-actin [12], although there has been no absolute evidence for the cellular occurrence of this process until now.

PKC phosphorylation seems to be a key mechanism of MARCKS regulation, but other modifications of the protein have been described in vitro and in vivo that might influence its cellular function. In particular, six new phosphorylation sites located in the N-terminal half of the sequence have been identified in vivo on the bovine protein purified from brain [13]. Five of them are composed of the Ser-Pro motif, which is the minimal consensus sequence for phosphorylation by cyclin-dependent kinases (Cdks), and one is the Pro-Xaa-Ser-Pro sequence characteristic of MAP kinase phosphorylation. A seventh phosphorylated serine residue was recently identified in the C-terminal sequence of the protein [14]. After the publication of these results, phosphorylation of MARCKS in vitro by MAP kinase and some Cdks was investigated. MAP kinase was found to phosphorylate MARCKS on the expected Pro-Xaa-Ser-Pro site but the authors could not detect any evidence of an equivalent process in the cell [15]. In another study, phosphorylation of MARCKS by Cdc2 kinase was reported but the corresponding phosphorylation sites were not identified [16]. Finally, the PKC-related PRK1 kinase, one of the possible cellular targets of the small G-proteins of the Rho family, was found to phosphorylate MARCKS on the same sites as did PKC [17].

In the present study we investigated the phosphorylation of MARCKS by various Cdks in vitro. We focussed on the cyclin E–Cdk2 complex and identified two sites specifically phosphorylated by this kinase. The possible biological relevance and meaning of these results are discussed.

MATERIALS AND METHODS

Cell cultures

Tumour-derived choroidal melanoma cells (OCM-1) were cultured in RPMI medium with 5% (v/v) fetal calf serum as

Abbreviations used: Cdk, cyclin-dependent kinase; MARCKS, myristoylated alanine-rich C-kinase substrate; PKC, protein kinase C.

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described previously [18]. For serum deprivation, cells were maintained for 48 h in the same medium in the absence of serum. To block the cells at the G1/S transition of the cell cycle, 1 µg/ml aphidicolin was added to the medium for 15 h. Cells were recovered by treatment with trypsin and further processed for immunoprecipitation experiments.

**Immunoprecipitations and kinase activity measurements**

For immunoprecipitations of Cdk1, Cdk2 and Cdk4, cells were lysed with the radioimmunoprecipitation assay buffer containing 50 mM Heps, pH 7.3, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM dithiothreitol, 1 mM NaF, 0.1 mM sodium vanadate, 10 mM β-glycerophosphate, 10% (v/v) glycerol, 2 µg/ml aprotinin, 2 µg/ml leupeptin, 2 µg/ml pepstatin, 1 mM PMSF and 0.1% (v/v) Tween 20. The lysates were cleared by centrifugation at 4°C for 5 min at 12700 g. The Cdk1, Cdk2 and Cdk4 kinases were immunoprecipitated with Protein A-Sepharose CL-4B beads precoated with 5 µg of the respective antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). The kinase activities of the resulting immunoprecipitates were measured as follows: 1 µg of each substrate [recombinant human MARCKS protein, histone H1 (Sigma) and recombinant pRb (QED Biosciences)] was incubated with the immunoprecipitate in the presence of 25 mM Heps buffer (pH 7.4)/10 mM MgCl2/1 mM dithiothreitol/100 µM unlabelled ATP/3 µCi of [γ-32P]ATP (Amersham) for the indicated durations at 30°C in a total reaction mixture of 30 µl. The reaction was stopped with 10 µl of 4× Laemmli sample buffer; the corresponding fractions were subjected to SDS/PAGE separation by the method of Laemmli [19]. The corresponding gels were dried and 32P incorporation into the substrate was analysed by autoradiography with X-ray Hyperfilm MP (Amersham). To quantify 32P incorporation, the Coomassie Blue-stained substrate was excised from the gel and counted for radioactivity in 10 ml of water.

**Expression and purification of cyclin E–Cdk2**

S9 cells were co-infected with baculovirus encoding His-tagged cyclin E (multiplicity of infection 10) and Cdk2 (multiplicity of infection 100). Infected cells were collected after 3 days and centrifuged at 400 g for 10 min at room temperature. The pellet was resuspended in lysis buffer [10 mM Tris/HCl (pH 7.5)/25 mM NaCl] at 5×106 cells/ml and broken with a Dounce homogenizer on ice; 1 vol. of the resulting lysate was diluted with 4× volume of solubilization buffer [40% (v/v) glycerol/100 mM NaH2PO4/1 M NaCl/0.08% (v/v) Tween 20/0.2% 2-mercaptoethanol (pH 7.5)], kept on ice for 15 min and then centrifuged at 100000 g for 1 h at 4°C. The resulting supernatant was adjusted to 500 mM NaCl and subjected to gel-filtration chromatography on a Superdex 75 HR 26/60 column in HBHS buffer [20 mM Heps/500 mM NaCl/0.02% (v/v) Tween 20] at a flow rate of 2 ml/min. The fractions were tested for the presence of Cdk2 and cyclin E by Coomassie Blue staining of SDS gels, by Western blots with antibodies against Cdk2 and cyclin E, and by H1 kinase activity. The total protein amount was measured with the Lowry test from Bio-Rad. The cyclin E–Cdk2 complex was eluted with an apparent molecular mass of 85 kDa. The corresponding eluted fractions were pooled and immediately subjected to cobalt affinity chromatography on a 500 µl column of Talon beads (Clontech) equilibrated in HBHS buffer, pH 7.8. The sample was loaded on the column at a maximal flow rate of 0.5 ml/min, followed by sequential washes with HBHS buffer and phosphate buffer (50 mM, pH 6.8). The elution was performed with a 0–250 mM imidazole gradient in the elution buffer [20 mM Tris/HCl (pH 7.6)/100 mM NaCl/0.005% (v/v) Tween 20] at 1 ml/min over 3 h. The cyclin E–Cdk2 complex, detected as described previously, was eluted in the 10–50 mM imidazole fractions. To remove the imidazole, these fractions were applied to a PD10 desalting column in kinase activity buffer [20 mM Tris/HCl (pH 7.5)/100 mM NaCl/1 mM EDTA/1 mM dithiothreitol/0.005% (v/v) Tween 20/10% (v/v) glycerol] and kept at –70°C until used.

**Phosphorylation of MARCKS by purified kinases and degradation by lysyl endopeptidase**

Recombinant MARCKS (500 ng) was incubated with 1 ng of PKM (the catalytic fragment of PKC) (Biomol Research Laboratories) or 50 ng of the cyclin E–Cdk2 complex at 30°C in the phosphorylation mixture described above. This apparently large amount of purified cyclin E–Cdk2 complex in the reaction was required because only 10–20% of the complex was active, as deduced from activation experiments performed with the Cdk2-activating kinase AKT (results not shown). For MS analysis, the phosphorylation was performed for 30 min, a time corresponding to maximal 32P incorporation by both Cdk2 and PKM. The reaction mixture was then heated at 90°C for 3 min to inactivate the enzymes. The digestion with lysyl endopeptidase (Sigma) was then performed on the heat-stable resulting fraction as described previously and the reaction was stopped with 0.1% (v/v) trifluoroacetic acid [13]. The product of the reaction was used for MS analysis.

**MS analysis and Edman degradation sequencing**

HPLC–MS analysis was performed by coupling a model Alliance HPLC system (Waters, Milford, MA, U.S.A.) to the electrospray mass spectrometer. The separation performed on the Macherey Nagel Nucleosil 300-5 C18, narrow-bore column (2 mm × 125 mm) was flow-split by a stainless steel Valco tee (Supelco, Saint-Germain-en-Laye, France), with approx. 1/15 of the column effluent being directed towards the mass spectrometer by means of a fused silica capillary. Absorbance was monitored at 214 nm. The separation of the peptide fragments was obtained at 250 µl/min with a gradient of 0–60% (v/v) buffer B in buffer A over 90 min [buffer A, 0.1% (v/v) trifluoroacetic acid in water; buffer B, 0.08% (v/v) trifluoroacetic acid in acetonitrile]. The electro-spray mass spectra were obtained on a VG BioQ triple quadrupole mass spectrometer (m/z range 4000) upgraded by the manufacturer so that it gained Quattro II performance (Micromass Ltd., Altrincham, Greater Manchester, U.K.). Scanning was performed in the m/z range 300–2000 in 10 s. The resulting cone voltage was 50 V. The mass spectrometer was calibrated externally by using horse heart myoglobin multiply-charged ions. Fractions containing the peptides of interest were collected from the HPLC separation and sequenced on a 473A sequencer (Perkin-Elmer, Applied Biosystems Division, Foster City, CA, U.S.A.). For the MS/MS analysis, the peptide mixture was desalted on a pulled glass capillary microcolumn packed with 2 µl of Poros oligoR3 resin (PerSeptive Biosystems, Framingham, MA, U.S.A.). After the column had been washed with 5% (v/v) formic acid, the peptides were eluted with 50% (v/v) acetonitrile/5% (v/v) formic acid directly into a spraying capillary. Nano-electrospray mass spectra were acquired on an API III triple quadrupole mass spectrometer (PE Sciex, Ontario, Canada) equipped with a nanospray ion source. The mass spectrometer was operated in MS/MS mode with Q1 set to transmit a mass window of 2 Da.
RESULTS

Phosphorylation of MARCKS by Cdk1, Cdk2 and Cdk4 in vitro

Cdk1, Cdk2 and Cdk4 were immunoprecipitated from cell extracts. The corresponding kinase activities were measured with histone H1 (Cdk1) or pRb (Cdk2 and Cdk4) and with purified human recombinant MARCKS protein as substrates. Phosphorylations for 10, 30 and 45 min were performed for each reaction. The corresponding autoradiograms are shown in Figure 1(A). All three kinases were active towards their physiological substrates (H1 and pRb) and towards purified MARCKS. The kinetics of MARCKS phosphorylation by Cdk2 and Cdk4 was similar to that observed with pRb (in each case the maximum was reached after 30 min). In contrast, the kinetics of MARCKS phosphorylation by Cdk1 was low by comparison with the phosphorylation of histone H1. Normalization of MARCKS phosphorylation to that of pRb (for Cdk2 and Cdk4) and of histone H1 (for Cdk1) confirmed that Cdk2 and Cdk4 phosphorylate MARCKS and pRb with similar kinetics, although the phosphorylation of MARCKS was slightly slower than that of pRb. In contrast, the phosphorylation of MARCKS by Cdk1 was slow in comparison with that of histone (Figure 1B), suggesting that MARCKS is a poor substrate for this kinase.

To verify that these activities were not due to non-specific contaminating kinases in the immunoprecipitates, a first immunoprecipitation with antibodies against Cdk1, Cdk2 or Cdk4 was performed and the corresponding immunodepleted supernatants were used for a second immunoprecipitation. The kinase activities present in the two pellets were compared. The outcome (results not shown) demonstrated that preliminary immunodepletion drastically decreased the kinase activity in the second immunoprecipitate, suggesting that all three kinase activities detected with MARCKS as the substrate were actually due to Cdk1, Cdk2 and Cdk4 respectively.

To confirm that Cdk2 phosphorylates MARCKS under these conditions we tested the effect of olomoucine, a specific inhibitor of Cdk2 [20]. As shown in Figure 1(C), this compound strongly decreased the phosphorylation of MARCKS by the Cdk2 immunoprecipitate. Finally, Cdk2 immunoprecipitates from serum-starved (G0–G1) and aphidicolin-blocked (G1–S) cell fractions were tested for their ability to phosphorylate MARCKS. Cdk2 activity is usually low in serum-starved cells and high in G1–S-blocked cells. We found almost no phosphorylation of MARCKS with the immunoprecipitate from quiescent cells, whereas a strong incorporation of 32P was detected when the aphidicolin-treated cell extract was used (Figure 1C).

Taken together, these results demonstrate that Cdk2, Cdk4 and, to a smaller extent, Cdk1 phosphorylate MARCKS in vitro. In the following experiments we focused our study on the phosphorylation of MARCKS by the cyclin E–Cdk2 complex.

Phosphorylation of MARCKS and pRb by purified cyclin E–Cdk2 complex

Cdk2 and cyclin E were overexpressed in Sf9 insect cells, and the complex was purified as described in the Materials and methods section. A Coomassie Blue-stained SDS gel of the purified complex is shown in Figure 2(A). This complex was tested for its ability to phosphorylate MARCKS and pRb. As expected both MARCKS and pRb were substrates for cyclin E–Cdk2 (Figure 2B). The relatively low level of 32P incorporated into MARCKS in comparison with pRb probably reflected the different numbers of phosphorylation sites in the two proteins. Because the pRb protein contains seven or eight sites of phosphorylation by Cdk2 in vitro [21], the results suggest that this kinase could phosphorylate only some of the seven sites described in vitro in the MARCKS sequence. As a control, we also used purified brain neuromodulin as a Cdk2 substrate. This protein has some biochemical and sequence similarities to MARCKS, and was also reported as a proline-directed kinase substrate in vitro [22]. As shown in Figure 2(B), no significant phosphorylation of neuromodulin was detected under the conditions used for the phosphorylation of MARCKS and pRb. These results confirm that MARCKS is a Cdk2 substrate in vitro.

Comparison of the phosphorylation of MARCKS by PKM and Cdk2

Because MARCKS has been extensively described as a physiological PKC substrate, we compared the time course of the
Figure 2  Phosphorylation of MARCKS, pRb and neuromodulin by purified cyclin E–Cdk2

(A) Cdk2 and cyclin E were overexpressed into Sf9 insect cells and purified as described in the Materials and methods section; 8.5 μg of the resulting fraction was separated by SDS/PAGE and stained with Coomassie Blue. The positions of molecular mass markers (lane M) are indicated (in kDa) at the right. (B) MARCKS (M, lane 1), pRb (Rb, lane 2) or neuromodulin (Nm, lane 5) (500 ng of each) were phosphorylated with 50 ng of the purified Sf9 cyclin E–Cdk2 complex for 30 min in the presence of [γ-32P]ATP. The reaction was stopped with Laemmli sample buffer and the fractions were separated by SDS/PAGE. The 80 kDa polypeptide phosphorylated in the pRb fraction is probably a degradation product of this protein. Controls in the absence of substrate (lane 3) or in the absence of Cdk2 in the presence of MARCKS (lane 4) are also shown.

phosphorylation of MARCKS by PKM (which phosphorylates MARCKS in the same way as PKC does in vitro) and by Cdk2. The corresponding autoradiograms (Figure 3A) and quantifications (Figure 3B) are shown. Similar, but not identical, kinetics of phosphorylation were observed with the two enzymes, with a maximal 32P incorporation occurring after 20 min. We found that 2 mol of phosphate per mol of MARCKS protein were incorporated on phosphorylation by Cdk2. In comparison, phosphorylation by PKM led to the incorporation of 4 mol of phosphate per mol of protein, in good accordance with the previous descriptions of the phosphorylation of MARCKS by PKC in vitro (see [1,2]). These results demonstrate that PKC and Cdk2 phosphorylate MARCKS with similar kinetics in vitro and suggest the existence of two sites that are phosphorylated by Cdk2 under these conditions.

Identification of the phosphorylated sites by electrospray MS analysis

To identify the sites of phosphorylation by Cdk2, we proceeded as follows: 5 μg of MARCKS was phosphorylated by Cdk2 and the reaction mixture was subjected to digestion with lysyl endoprotease. As a control, the same amount of MARCKS was incubated in the phosphorylation mixture without Cdk2. The product of the degradation was subjected to HPLC–MS analysis as described in the Materials and methods section. Because the transfer of phosphate from ATP to Ser or Thr residues increases the mass of the corresponding peptide by 80 Da, we investigated the existence of peptides with a difference of 80 Da between the control and the phosphorylated fractions. Most of the peptide masses measured were the same in both the phosphorylated and the control fractions, except for two. A peptide with a mass of 1500 Da was detected in the control but not in the phosphorylated fraction, where in contrast a 1580 Da peptide was found that was absent from the control fraction (Figure 4). This molecular mass matched well with peptide 10 (residues 138–152; see Figure 6) from the human MARCKS sequence (theoretical mass 1500 Da). Edman degradation sequencing of this fraction confirmed the identity of the peptide (results not shown). Similarly, peptide 3

(residues 12–30), which contains one of the sites identified in vivo (Ser37), was phosphorylated by Cdk2 (1785 Da in the control and 1865 Da after phosphorylation by Cdk2). All the other peptides
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Figure 4  MS analysis of phosphorylation of MARCKS by cyclin E–Cdk2

MARCKS was phosphorylated (phospho MARCKS) or not (control) by Cdk2 and digested with lysyl endoprotease as described in the Materials and methods section. The digests were analysed by HPLC–MS and the presence of peptides with an increased mass of 80 Da in the phosphorylated fraction was investigated by comparison with the control fraction. This occurred in peptides Gly12–Lys30 and Ala138–Lys152, which had respective molecular masses of 1785 and 1500 Da and were both identified as doubly charged ions (m/z 893 and 751 respectively for the non-phosphorylated forms, and m/z 933 and 791 after phosphorylation). These experimental values fitted perfectly with those expected from the sequence of the protein. The phosphorylated and non-phosphorylated forms of Gly12–Lys30 were eluted from the HPLC column with retention times of 37 and 38.5 min respectively. The phosphorylated and non-phosphorylated forms of Ala138–Lys152 were eluted at 33.2 and 35.5 min respectively. The non-phosphorylated forms of both peptides were absent from the phosphorylated fraction, suggesting strongly that phosphorylation of these sites was complete. All the other peptides from the N-terminal half of MARCKS were found unchanged in the two fractions (results not shown).

from the N-terminal half of the protein were identified and their masses were identical in the two fractions (results not shown). We failed to identify the C-terminal peptide, which also contains a phosphorylated site in vivo. However, because the number of sites identified by MS and by 32P incorporation were in good agreement, we concluded that only peptides 3 and 10 contained the phosphorylation sites for Cdk2.

Because there is only one serine residue in the correct configuration for Cdk2 phosphorylation peptide, which also contains a phosphorylated site in vivo. However, because the number of sites identified by MS and by 32P incorporation were in good agreement, we concluded that only peptides 3 and 10 contained the phosphorylation sites for Cdk2.

The identification of the phosphorylated residue in peptide 10 was less evident because two threonine and two serine residues were present in the sequence. To identify the phosphorylated residue, nanospray MS/MS analysis of the peptide was performed (Figure 5). As shown, the difference between $y_2$ and $y_3$ corresponds to a phosphothreonine residue. Furthermore, the position of the y series ($y_1$, $y_2$, $y_3$–$y_{15}$) indicates that all the C-terminal fragments except $y_1$ and $y_5$ contained a phospho group. Because the unphosphorylated $y_1$ ion that corresponded to the C-terminal fragment starting from the second proline residue in the peptide was not seen, we conclude that Thr136 is the sole phosphorylation site in the peptide. In good agreement with these results, Thr136 is in the optimal configuration (Thr-Pro-Lys-Lys) for phosphorylation by Cdk2, whereas the other residues are not. Taken together, these results demonstrate that Ser27 and Thr136 in the human MARCKS sequence are phosphorylated by cyclin E–Cdk2 in vitro. A summary of these results is shown in Figure 6.

Phosphorylation of MARCKS by PKC affects phosphorylation by Cdk2

The phosphorylation of MARCKS by PKC is a key mechanism for the regulation of this protein, and probably triggers conformational modifications in solution. For this reason we investigated whether this phosphorylation of MARCKS modified further phosphorylation by Cdk2. MARCKS was first subjected to maximal phosphorylation by PKM. As a control, the same incubation was performed in the absence of the enzyme. The incubation mixture was then heated for 3 min at 90 °C, in order to inactivate the kinase completely. The incubation mixture was then heated for 3 min at 90 °C, in order to inactivate the kinase completely. As a heat-stable protein, MARCKS remains in solution in these conditions and we verified that this treatment did not modify the parameters of
MARCKS phosphorylated by cyclin E–Cdk2 was digested with lysyl endoprotease; the resulting peptide mixture was subjected to MS/MS analysis as described in the Materials and methods section. The doubly charged precursor ion (m/z 792) was selected and subjected to collision-induced dissociation. yₙ₊₁ ions are larger than yₙ ions of the unphosphorylated peptide by 80 Da; yₙ₊₁ ions were formed by the loss of H₃PO₄ from the yₙ ions (loss of 98 Da).

Figure 6 Summary of the sites of phosphorylation by Cdk2 on the N-terminal half of MARCKS

The N-terminal sequence of MARCKS is shown. The PKC phosphorylation site domain (PSD) is indicated by the box at the end of the sequence. The theoretical lysyl endoproteinase-generated peptides are indicated by the arrows above the sequence and numbered from 1 to 10 from the N-terminus to the C-terminus. Peptides 3 and 10 correspond to Gly₁₂–Lys₃₀ and Ala₁₃₈–Lys₁₅₂ respectively and were phosphorylated. The phosphorylated residues Ser₂₇ and Thr₁₅₀ are underlined, in bold characters and indicated with an asterisk.

its phosphorylation by either PKM or Cdk2 (results not shown). Phosphorylation by Cdk2 was then performed in the presence of [γ-³²P]ATP for different durations on these two fractions. The corresponding autoradiogram is shown in Figure 7. Preliminary phosphorylation by PKM clearly improved the initial rate of phosphorylation by Cdk2. However, the total amount of ³²P incorporated remained the same in the two fractions, as shown in Figure 7(B). These results probably reflect some PKC-induced conformational modification that might facilitate the access of Cdk2 to its phosphorylation sites but does not open additional sites. This also confirmed that the sites of phosphorylation by PKC and Cdk2 were different. We also tested whether Cdk2 phosphorylation conversely modified further phosphorylation by PKC, but we did not observe a significant difference between the two fractions (results not shown).

DISCUSSION

The MARCKS protein has been previously identified as a substrate for proline-directed kinase(s) in vivo [13]. Here we demonstrate that the cell cycle regulators Cdk2, Cdk4 and, to a smaller extent, Cdk1 phosphorylate MARCKS in vitro. The phosphorylation of MARCKS by Cdk₁ in vitro has already been reported [16], but this is the first description of the phosphorylation of MARCKS by Cdk2 and Cdk4. Although obtained in vitro, our results point to the specificity of this phosphorylation in two ways. First, the heat-stable protein neuromodulin, which also contains proline-directed phosphorylation sites in vivo [22], was not phosphorylated by Cdk2. Secondly, only two residues were phosphorylated by Cdk2 in vitro, although the primary sequence of MARCKS contains more than ten serine or threonine residues in the minimum described consensus sequence for this kinase (serine–proline or threonine–proline).

We also demonstrate in this work that Cdk4 phosphorylates MARCKS in vitro. Interestingly, maximal phosphorylation by Cdk4 was higher than by Cdk2 (see Figure 1), suggesting that different and/or additional sites were concerned. Although some identical phosphorylation sites were described for Cdk2 and Cdk4 on pRb, other sites were specific for one or other of these
two kinases [21]. When the sequence specificities of Cdk2 and Cdk4 kinases were investigated [23], the Ser/Thr-Pro-Xaa-Yaa (where Yaa is a basic residue) sequence was found to optimal for Cdk2 but not for Cdk4. Further experiments will be needed to establish whether the sites of phosphorylation of MARCKS by Cdk4 are the same as for those by Cdk2.

The experiments performed with the purified cyclin E-Cdk2 complex yielded additional information on the phosphorylation of MARCKS by this kinase. We established the stoichiometry of this phosphorylation in different ways. It was already known that MARCKS contains four sites of phosphorylation by PKC

\textit{in vitro}. On the basis of these results, and on the comparison of maximal 32P incorporation by PKM and Cdk2, we concluded that there are two sites of phosphorylation by Cdk2. Confirmation of these results and identification of the corresponding modified residues were achieved by electrospray MS analysis. Interestingly, of the seven phosphorylation sites identified \textit{in vivo}, only Ser118 was modified by Cdk2. This raises the possibility, but does not demonstrate, that this kinase could be responsible for this phosphorylation \textit{in vivo}. It is important to note that the sequence surrounding this residue correlates perfectly with the consensus Ser/Thr-Pro-Xaa-Lys/Arg sequence described previously for Cdk2. The second residue phosphorylated by Cdk2 was Thr196, which has not been described as a phosphorylation site \textit{in vitro}. Interestingly, this residue is adjacent to the PKC phosphorylation domain, previously described as a key regulating region of the protein. In the present study we observed that a preliminary phosphorylation of MARCKS by PKM increased the initial rate of phosphorylation by Cdk2. By taking these results together, it was tempting to speculate that the well-established phosphorylation of Ser118, Ser143, Ser157 and Ser170 by PKM positively modified the access of Cdk2 to Thr196. Further experiments are needed to confirm this hypothesis.

The phosphorylation of MARCKS \textit{in vitro} by Cdk1 (Cdc2) and Cdk5 has already been described. These phosphorylations were found to modify the biochemical properties of the protein in some way, in particular its interaction with calmodulin [16]. Although the residues phosphorylated by Cdk1 and Cdk5 were not identified, phosphopeptide analysis suggested the existence of common, as well as distinct, sites, including both serine and threonine residues. The identification of these sites and their comparison with those described \textit{in vivo} are now needed for a better understanding of the possible regulation of MARCKS by these modifications.

The p42 MAP kinase also phosphorylates MARCKS \textit{in vitro} on Ser118 of the human sequence [15]. This residue was also found phosphorylated \textit{in vivo}, and is in the correct consensus sequence (Pro-Xaa-Ser-Pro) for phosphorylation by MAP kinase. In their study, the authors did not detect the phosphorylation of MARCKS by MAP kinase in cells stimulated by platelet-derived growth factor. However, they addressed the question of this phosphorylation after a short period (10 min) of stimulation by platelet-derived growth factor and of subsequent activation of MAP kinase. The eventual phosphorylation of MARCKS after long-term activation of MAP kinase remains to be investigated.

The phosphorylation of MARCKS \textit{in vitro} by kinases other than PKC is poorly documented. The major information comes from the MS analysis performed on the purified brain protein, on which seven serine residues were phosphorylated by unidentified proline-directed kinases(s) [13]. Given the tissue origin of which seven serine residues were phosphorylated by unidentified kinases from the MS analysis performed on the purified brain protein, on established phosphorylation of Ser62 by Cdk2. By taking these phosphorylation domain, previously described as a key regulating in previously for Cdk2. The second residue phosphorylated by Cdk2 does not demonstrate, that this kinase could be responsible for only Ser27 and Thr136. For example, the consensus sequence of phosphorylation by Cdk1 is the same as that for Cdk2, and Cdk1 has been shown to phosphorylate cytoskeletal components (intermediate filaments and microtubule-associated proteins) involved in the G1-M transition [27]. Experiments are now in progress to establish the phosphorylation states of MARCKS during the cell cycle.

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