Sites of Phosphorylation by Protein Kinase A in CDC25Mm/GRF1, a Guanine Nucleotide Exchange Factor for Ras*

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Activation of the neuronal Ras GDP/GTP exchange factor (GEF) CDC25Mm/GRF1 is known to be associated with phosphorylation of serine/threonine. To increase our knowledge of the mechanism involved, we have analyzed the ability of several serine/threonine kinases to analyze the activity of cAMP-dependent protein kinase (PKA) in the phosphorylation of CDC25Mm in fibroblasts overexpressing this RasGEF as well as in mouse brain synaptosomal membranes. In vitro, PKA was found to phosphorylate multiple sites on purified CDC25Mm, in contrast to protein kinase C, calmodulin kinase II, and casein kinase II, which were virtually inactive. Eight phosphorylated serines and one threonine were identified by mass spectrometry and Edman degradation. Most of them were clustered around the Ras exchanger motif/PEST motifs situated in the C-terminal moiety (residues 631–978) preceding the catalytic domain. Ser745 and Ser822 were the most heavily phosphorylated residues and the only ones coinciding with PKA consensus sequences. Substitutions S745D and S822D showed that the latter mutation strongly inhibited the exchange activity of CDC25Mm on Ha-Ras. The multiple PKA-dependent phosphorylation sites on CDC25Mm suggest a complex regulatory picture of this RasGEF. The results are discussed in the light of structural and/or functional similarities with other members of this RasGEF family.

Ras proteins are molecular switches cycling between the active GTP-bound and the inactive GDP-bound forms (1). They control a series of physiological and pathological events associated with growth and differentiation of the cell. Their activity is regulated by GTPase-activating proteins (GAPs)1 that enhance the intrinsic GTPase activity and by guanine nucleotide exchange factors (GEFs) that accelerate the very slow intrinsic GDP/GTP exchange, favoring thus the regeneration of the active form of Ras. The nucleotide exchange mechanism catalyzed by GEFs has been intensively studied and characterized at a three-dimensional structural level (for review, see Ref. 2). Diverse RasGEFs have been identified: SOS1 and SOS2, Ras-GRP, CDC25Mm/GRF1, and GRF2. SOS1 and SOS2 are ubiquititary GEFs and their activation depends on signals from tyrosine kinase receptors (for review, see Ref. 3). RasGRP is a neuronal RasGEF involved in diacylglycerol and calcium regulation of Ras (4, 5). CDC25Mm/GRF1 has been shown to be activated by heterotrimeric G protein coupled receptors, such as muscarinic receptors (6, 7); it is predominantly expressed in the neuronal cell and is involved in calcium-associated signals (8, 9). The highly homologous GRF2 is present in cells from different organs (10) and shows in vivo functional differences from GRF1 (11). In brain extracts, the GRF1/GRF2 hetero-oligomer formation was observed (12). These GEFs are modular proteins containing specific domains. CDC25Mm, a protein of 1262 amino acid residues, shows the following sequence of domains/motifs: pleckstrin homology 1 (PH1), coiled-coil (CC), Ras exchanger motif (REM), and PEST. The RasGEF domain, represented by the conserved catalytic CDC25 domain, is situated in the C-terminal region.

Results in vitro with CDC25Mm (13) and in vivo with SOS (14) indicated that the N-terminal portion of GEF down-regulates the activity of the catalytic domain. Several reports have shown that activation of CDC25Mm is coupled with Ca2+ influx, as well as with formation of a complex with calmodulin and phosphorylation (8, 15). The latter modification was found to concern serine/threonine (6, 16) and very recently also tyrosine residues (7). The nonreceptor tyrosine kinase Src can induce on CDC25Mm a Rac1-GEF activity (18), showing that this protein is an important element for cross-regulation of Ras and Rac-dependent pathways (19), as has been reported for SOS (20) and GRF2 (21). Taken together, these results illus-

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1 The abbreviations used are: GAP, GTPase-activating protein; GEF, GDP/GTP exchange factors; ME, 2-mercaptoethanol; PRA, protein kinase A; PKC, protein kinase C; CaMKII, calmodulin kinase II; CKII, casein kinase II; MBP, maltose-binding protein; 8-Br-cAMP, 8-bromo-cAMP; 8-Br-adenosine-3’,5’-cyclic monophosphate; PDBu, phorbol dibutyrate; PDBu, phorbol dibutyrate; PAGE, polyacrylamide gel electrophoresis; RIPA, radioimmune precipitation assay; PCR, polymerase chain reaction; MALDI, matrix-assisted laser desorption-ionization; GRF, guanine nucleotide releasing factor; REM, Ras exchanger motif; IQ, ilimaquinone; DH, Dbl homology; PH, pleckstrin homology; CC, coiled-coil; HPLC, high performance liquid chromatography.

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trate the complexity of the upstream activation of CDC25Mm, as well as the many aspects that remain to be clarified.

In order to deepen our information on the specific action(s) of kinases on CDC25Mm, in this work we have carried out a series of experiments analyzing the phosphorylation of this GEF by several serine/threonine kinases. PKA was found to be a very efficient phosphotransfer enzyme for CDC25Mm in vivo and in vitro, leading to the phosphorylation of multiple residues that were identified by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry and Edman degradation. Functional modifications induced by phosphorylation of full-length CDC25Mm and by substitutions of single residue sites are also reported.

**EXPERIMENTAL PROCEDURES**

**Materials**—p[γ-32P]ATP (111 Tbjg/mmol) was from PerkinElmer Life Sciences; the PKA catalytic subunit, PKC, and casein kinase II were from Roche Molecular Biochemicals; and calmodulin kinase II was from Calbiochem.

**CDC25Mm Mutagenesis**—Mutants CDC25Mm(574-545) and (745-745) were constructed using a megaprimer PCR method, Pwo polymerase (Roche Molecular Biochemicals) and the gene fragment encoding the C-terminal half-molecule (pGEX-CDC25Mm[631–1262], as template. The first step used oligonucleotides CGTCCGGAAGGTGCCTTCAACATCCC (S745D) or CGTCGGGAAGGTGCCTTCAACATCCC (S745D) as a mutagenic primer and CTTGCGAGCTTCTGACATACAA, which was used as a second primer hybridizing 200 bases downstream on the CDC25Mm open reading frame. The resulting products together with 5’GGGTATGACGCTCTTGATGTTG were cloned in pGEX-CDC25Mm-(631–1262), of which the obtained fragment carrying the substitution was amplified by pGEX-CDC25Mm-(631–1262), and CDC25Mm-(239–591) (DH-PH2), CDC25Mm-(239–480) (DH), CDC25Mm-(978–1262), 50 μM Tris-HCl, pH 7.5, 50 μM MgCl₂, 1 mM dithiothreitol, 2 μM ATP, 370 KBq of [γ-32P]ATP, and 25 units of the catalytic subunit of yeast PKA (a kind gift of Paolo Tortora, University of Milano-Bicocca, Milan, Italy) for 20 min at 30 °C. The samples, washed three times in RIPA buffer, were analyzed by SDS-PAGE and quantified for autoradiography by densitometric scanning.

The synaptosomal membranes were prepared from mouse brain according to Ref. 24 as reported previously (Ref. 25 (see in particular Fig. 2, fraction LP1)). The washed synaptosomal fraction was hypotonically lysed by resuspension in lysis buffer (10 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol) containing protease inhibitors. After 30 min in ice, the suspension was centrifuged for 20 min at 40,000 rpm in a Beckman 50 Ti rotor and the supernatant discarded. The pellet was resuspended and centrifuged for 10 min at 14,000 g. The final synaptosomal membrane fraction was resuspended in phosphorylation buffer (25 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM EGTA) to a final protein concentration of 2 mg/ml. Aliquots (200 μg) were incubated for 5 min at 30 °C (final volume, 200 μl) in phosphorylation buffer in the presence of 10 mM MgCl₂ and 10 μM ATP, 370 KBq of [γ-32P]ATP plus 10 mM 8-bromo-cAMP, 1.5 mM CaCl₂, or 1.5 mM CaCl₂ with 200 nM PDBu. The incubation was stopped by addition of 10% trichloroacetic acid (TCA) and the samples were solubilized in sodium dodecyl sulfate (SDS)-PAGE buffer and electrophoresed on a 10% SDS-PAGE gel. The gel was dried, cut, and transferred to scintillation vials, and the radioactivity was counted. Purification of Phosphoproteins—The phosphorylation mixture (500 μl) containing 250 milligrams of PIA, 1000 pmol of CDC25Mm-(631–1262), 50 μM Tris-HCl, pH 7.5, 5 mM MgCl₂, 1 mM diethiothreitol, and 100 μM PDBu was incubated at 30 °C for 1 h and dialyzed against 50 mM ammonium acetate (pH 8.0). The 32P-labeled wild-type and mutant proteins. Purified proteins were concentrated with Aquacide II (Calbiochem), except for full-length CDC25Mm to avoid aggregation phenomena, and dialyzed against 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 50% glycerol, and 7 mM ME. When stored at −20 °C in this buffer, the recombinant proteins were stable for at least several months. The CDC25Mm fragments (1–1262) protein preparations contained approximately 50% full-length product, the remaining proteins consisting of N-terminal shorter fragments of CDC25Mm (13). The various isolated N-terminal CDC25Mm fragments were more than 90% pure. The catalytic domain CDC25Mm(978–1262) was isolated and purified to homogeneity as described (22).
phosphopeptides were recorded on a matrix-assisted laser desorption-absorbance at 215 nm as well as by radioactivity measurements in a eluted with linear gradients of acetonitrile in 0.1% (v/v) trifluoroacetic acid. Peptides were saturated solution of a nitrocellulose binding assay. The labeled Ha-Ras washed twice with 3 ml of ice-cold buffer C (50 mM Tris-HCl, pH 7.5, 10 mM MgCl2) was examined after incubation with PKA, PKC, and PKD. The CDC25Mm was digested for 5 min at 30 °C in a final volume of 30 μl of appropriate buffer. The specific activity of the different kinases was 1 mmol of phosphate transferred/million/min to peptide or protein substrates. Control experiments (C) contained buffer instead of kinase. Each sample (10 μl) was analyzed on a 10% (w/v) acrylamide SDS-PAGE followed by autoradiography.

Mass Spectrometry Analysis of Phosphopeptides—Mass spectra of phosphopeptides were recorded on a matrix-assisted laser desorption-ionization time-of-flight mass spectrometer. Samples analyzed by MALDI mass spectrometry were prepared by mixing 1.5 μl of matrix (saturated solution of 9-ε-cyano-4-hydroxycinnamic acid (Sigma) in 40% acetonitrile, 0.1% trifluoroacetic acid) with 1 μl of peptide (5–10 pmol). This mixture was then loaded on a stainless steel sample holder and dried in a vacuum. A VG Analytical Tofspec mass spectrometer equipped with a 337-nm laser was used for sample analysis, with a 25-kV acceleration voltage. From 50 to 150 shots were accumulated for each spectrum acquisition in the positive and negative ion mode. Calibration with external standards was obtained with a mixture of peptide LWRF (Tebu) and insulin (Sigma) in a 1/3 concentration ratio (10 pmol total sample load) with the same matrix.

Edman Degradation—Peptides were applied to precycled Polybrene-treated glass fiber discs. Automated sequencing was done on a four-cartridge Procise 494A pulsed-liquid phase sequencer (Applied Biosystems).

Assay for GEF Activity—The dissociation rates of the Ha-Ras-[3H]GDP complexes were measured kinetically at 30 °C by the nitrocellulose binding assay. The labeled Ha-Ras-[3H]GDP complex was prepared by incubation of 2 μM Ha-Ras and 6 μM [3H]GDP (350 Bq/nmol) for 5 min at 30 °C in 100 μl of buffer B (50 mM Tris-HCl, pH 7.5, 1 mM MgCl2, 100 mM NH4Cl, and 0.5 mM 32P bovine serum albumin) containing 3 mM EDTA. Then, 3 mM MgCl2 were added. The reaction mixture (50–200 μl) for the dissociation experiments contained, in buffer B, 0.1–0.2 μM Ha-Ras-[3H]GDP complex and full-length MBP-CDC25Mm, either wild-type or mutant, as indicated in legends to figures. Dissociation kinetics were started by the addition of a 500-fold excess of unlabeled nucleotide. The concentration of glycerol carried over from the CDC25Mm storage buffer was much lower, about 0.5–1% that of PKA in the case of CaMKII and even less with PKC and CKII.

RESULTS

In Vitro Phosphorylation of CDC25Mm by PKA—In order to understand the mechanism by which phosphorylation of GEF could influence the activity of the downstream pathway(s), we have characterized the ability of various serine/threonine kinases to phosphorylate purified full-length CDC25Mm in vitro, an aspect that has yet to be investigated. Phosphorylation of CDC25Mm was examined after incubation with PKA, PKC, CKII, or CaMKII in the presence of γ-32P/ATP. After SDS-PAGE electrophoresis and autoradiography, the labeled band (Fig. 1) corresponding to full-length CDC25Mm was identified by comparison with Coomassie Blue-stained gel. In the experimental conditions chosen, each kinase was added in an amount capable of phosphorylating 300–500-fold more protein substrate than the amount of CDC25Mm present in the incubation mixtures. A pronounced phosphorylation was obtained with PKA, whereas the effects of the other three kinases was much lower, about 0.5–1% that of PKA in the case of CaMKII and even less with PKC and CKII.

These results led us to quantify the extent of PKA-dependent phosphorylation. Noteworthy, all of the CDC25Mm preparations used were purified in native conditions and showed a well characterized RasGEF activity. Fig. 2 reports the kinetics of the CDC25Mm phosphorylation (A) and the phosphate incorporation as a function of PKA (B) and ATP (C) concentrations. CDC25Mm phosphorylation reached a plateau value after 20 min of incubation in the presence of 10 milliunits of PKA and 100 μM ATP. Phosphorylation stoichiometry measurements were performed at a CDC25Mm concentration of 70 nM. Higher CDC25Mm concentrations led to a decrease of the phosphorylation stoichiometry. For example, under the same conditions (10 milliunits of PKA), the use of a 10-fold higher concentration of CDC25Mm led to a 6 times lower stoichiometry. For each labeling, special care was taken to control the specific incorporation of 32P into CDC25Mm. As detailed under “Experimental Procedures,” two methods were used for phosphorylation stoichiometry determination. These are, quantification of the trichloroacetic acid-insoluble radioactivity of the samples from the phosphorylating mixture, on one hand, and measurement of the radioactivity of excised CDC25Mm band from SDS-PAGE, on the other hand. The stoichiometry of phosphorylation of CDC25Mm, as determined with these two methods, represented about 4 mol of phosphate covalently incorporated/mol of CDC25Mm. For comparison, the molar stoichiometry obtained with CaMKII was 0.01–0.02.

PKA Phosphorylates CDC25Mm in Vivo—We then investigated whether PKA phosphorylation of CDC25Mm also occurs in intact cells. NIH 3T3 fibroblasts overexpressing CDC25Mm (clone NIH-RG7) were treated with forskolin in order to increase cAMP level and thus activate PKA. Total cell extracts were then analyzed by Western blotting with CDC25Mm antibodies. Fig. 3A shows that the treatment with forskolin rapidly causes a reduction in the electrophoretic mobility of the protein, persisting at least up to 30 min. This shift in mobility is due to phosphorylation, since treatment of immunoprecipitated CDC25Mm with acidic potato phosphatase restored the higher mobility of the protein (data not shown). A back phosphorylation experiment confirmed that PKA phosphorylates in vivo CDC25Mm. CDC25Mm was isolated from control cells and from cells pretreated with forskolin for
and 45 min, and then subjected to in vitro phosphorylation by the catalytic subunit of PKA. As shown in Fig. 3B, the amount of [32P]phosphate incorporated into CDC25Mm isolated from treated cells was much lower (40–50%) than that incorporated in CDC25Mm from untreated cells. Lowering the stoichiometry of phosphate incorporation might reflect in vivo phosphorylation of CDC25Mm by PKA.

We previously showed by subcellular fractionation of mouse brain that CDC25Mm is associated with synaptosomal membranes and is enriched in postsynaptic densities (25). Synaptosomal membranes contain PKA, PKC, and CaMKII (24, 25), and, under conditions allowing the activity of the different kinases, it is possible to identify endogenous physiological substrates (24). To investigate whether CDC25Mm is phosphorylated by PKA in its physiological environment, mouse brain synaptosomal membranes were isolated and incubated in a kinase assay in the presence of [γ-32P]ATP without or with 8-Br-cAMP. Additionally, calcium alone or calcium and PDBu (an activator of PKC) were tested. Membranes were solubilized, and CDC25Mm was immunoprecipitated and analyzed by SDS-PAGE and autoradiography. As shown in Fig. 3C, 8-Br-cAMP markedly stimulated the phosphorylation of CDC25Mm in the synaptosomal membrane system, while only a very modest incorporation of [32P]phosphate was observed in the presence of calcium and calcium plus PDBu. This experiment indicates that PKA can heavily phosphorylate CDC25Mm in the physiological environment.
neuronal tissue, whereas other kinases (calcium/calmodulin-dependent and protein kinase C) are much less active toward this protein.

Definition of the Phosphoserine/Phosphothreonine-containing Regions—In order to characterize the PKA phosphorylation sites with respect to the various domains of CDC25Mm, different fragments of CDC25Mm (corresponding to known functional domains, such as the catalytic C-terminal domain (Ras-GEF domain), the Rac-GEF domain (DH), and various motifs, like PH and IQ) were constructed, isolated, and examined for their PKA-dependent phosphorylation. The $^{32}$P-labeled proteins were visualized by autoradiography and quantified after gel band extraction. In Fig. 4, we compare the phosphorylation of the full-length molecule with that of the isolated domains. The $^{32}$P incorporation in the N-terminal moiety and its shorter fragments was not significant. In addition, as revealed by autoradiography, $^{32}$P incorporation in the full-length molecule was comparable to that of the C-terminal half-moiety, whereas the isolated C-terminal Ras-GEF domain was not a PKA substrate. These results were confirmed by quantitative measurements of the phosphorylation content (data not shown). Accordingly, the region encompassing residues 631–978 contains the major sites of phosphorylation by PKA. Thus, the phosphorylation sites were clustered in a poorly characterized region of the protein containing the REM and PEST motifs.

Identification of PKA-dependent Phosphorylation Sites on CDC25Mm—To identify the modified residues, CDC25Mm-(631–1262) phosphorylated in vitro by PKA was digested by trypsin. The resulting phosphopeptides were first subjected to two-dimensional fingerprinting, of which a representative map is shown in Fig. 5. Several radioactive spots were visible. The nine main spots eluted represented 28%, 20%, 15%, 10%, 8%, 6%, 4%, 3%, and 3% of the total radioactivity recovered from the thin layer plate. Each of the nine recovered spots were further purified by reverse phase HPLC on a C18 column. Between 80% and 90% of the radioactive peptides were eluted from the column. Radioactive fractions were analyzed by MALDI mass spectrometry. Labeled peptides showed a mass increase of 80 Da, which is characteristic for peptides with single phosphorylation sites.

The molecular masses of phosphorylated peptides are listed in Table I. Seven phosphopeptides corresponding to seven discrete phosphorylation sites on CDC25Mm-(631–1262) were identified (Table I). Since the rules governing peptides desorption in MALDI mass spectrometry are not yet fully elucidated, one could not exclude that some peptides did not desorb from the matrix. Therefore, automated Edman degradation was also performed. The results are shown in Table II. The combination of MALDI and Edman degradation revealed nine phosphopeptides corresponding to serines 643, 691, 745, 793/794, 822, 849, 1123/1124, and 1146, and threonine 798.
Serine 643

FIG. 6.

Serine 691

Serine 798

Serine 849

Serine 1123/1124

Serine 1146

As already mentioned, most of phosphorylated residues are clustered around the REM/PEST region, with the exception of two weakly radioabeled sites located in the RasGEF domain (Fig. 6). It should be noted that the latter two sites were not previously identified by using the isolated RasGEF domain as substrate for the PKA experiments. The other seven sites were located between residues 643 and 849. Five PKA consensus sites are present in the primary structure of CDC25Mm (residues 83, 726, 745, 822, and 916) of which only Ser745 and Ser822 were found phosphorylated. Moreover, determination of the phosphorylation extent after thin layer fingerprinting and HPLC separation indicated the peptides containing Ser745 and Ser822 as the two major phosphorylated ones.

Effect of Phosphorylation on the RasGEF Activity and Site-directed Mutagenesis of Ser745 and Ser822—In order to determine the functional effect of phosphorylation on the full-length CDC25Mm, we have measured the dissociation kinetics of Ha-Ras-p21GDP in the presence of purified CDC25Mm preincubated with PKA. These experiments showed an inhibitory effect of CDC25Mm phosphorylation on the Ras nucleotide exchange but only to a modest extent (about 15% of the control values, data not shown). One should, however, stress that due to technical reasons these measurements required CDC25Mm phosphorylated in vivo. In contrast, the GEF activity of CDC25Mm(S822D) was also close to that of the wild-type factor. In the contrast, the GEF activity of CDC25Mm(S822D) was much lower, corresponding to less than one third of that of the wild-type protein (Fig. 7, A and B). These results propose Ser822, a residue situated in the PEST region, as a candidate for a physiological function.

Protein phosphorylation is one of the most important mechanisms of regulation of signal transmission in the cell. It has been object of intensive investigations in the many GEFs of the Rho family, and it has recently evoked a renewed interest in the RasGEF of the CDC25Mm/GRF1 type. Even if the phosphorylation of this neuronal RasGEF and its association with the activation have been known for several years (8, 6), our knowledge of the phosphorylation sites and the biochemical mechanisms involved therein is as yet uncertain. However, since treatment of the isolated exchange factor with protein phosphatase I, specific for serine/threonine residues, abolished the ligand-induced increase in exchange activity (6), serine/threonine kinases can be expected to participate, at least in part, in the regulation of the CDC25Mm activity. This problem has been approached in this work by analyzing various serine/threonine kinases. Observations are reported, demonstrating in vivo and in vitro the selective participation of PKA in the phosphorylation of CDC25Mm and the in vitro identification of the PKA-dependent phosphorylation sites located in the C-terminal moiety.

A possible involvement of CDC25Mm in a PKA-dependent Ras activation had been proposed previously on the basis of the observation that in cortical neurons the level of active Ras (Ras-GTP) was strongly enhanced in response to treatment with forskolin (26). Pretreatment with H89, a specific inhibitor of PKA, reduced this activation. In this regard, it is worth mentioning that Mattingly (16) has recently reported from in vivo and in vitro experiments that phosphorylation of serine 916 was in part dependent on PKA. Phosphorylation of CDC25Mm by PKA has been shown in our work to take place in vivo.

PKA Phosphorylation of CDC25Mm/GRF1

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Our in vivo data satisfactorily agree with those obtained in vivo.
FIG. 7. RasGEF activity of CDC25Mm mutated in position Ser<sup>745</sup> and Ser<sup>822</sup>. The GEF activity of purified full-length mutated CDC25Mm proteins was determined as stimulation of the dissociation rate of the Ha-Ras-p21-GDP complexes (see "Experimental Procedures"). Several CDC25Mm preparations of either wild-type or mutants and several CDC25Mm concentrations were used to compare their specific activities. Panel A represents the relative GEF activities of CDC25Mm(S745A), CDC25Mm(S745D), CDC25Mm(S822A), and CDC25Mm(S822D), as percentage of the GEF activity of the wild-type product. Panel B shows a typical experiment determining the GEF activity of the CDC25Mm products. The dissociation rates of Ha-Ras-p21<sup>3H</sup>GDP complexes were measured kinetically at 30 °C using the nitrocellulose binding assay, in the absence (×) or in the presence of 200 nM CDC25Mm-wt (white circle) or 200 nM CDC25Mm(S822D) (black circle).

vitro using well characterized purified components, which indicate that several serine and threonine residues are the targets for CDC25Mm phosphorylation by PKA. The plateau value of phosphate incorporation in CDC25Mm in the presence of PKA corresponded to a stoichiometry of 4 mol of phosphate groups covalently attached/mol of full-length CDC25Mm. In buffer conditions that are optimal for the activity of PKC, CaMKII, and CKII, these kinases failed to significantly phosphorylate the full-length CDC25Mm. The catalytic C-terminal domain (285 amino acids) of CDC25Mm as well as isolated domains of the N-terminal region did not appear to contain phosphorylation sites. Accordingly, the region encompassing residues 631–978 was shown to bear most of the PKA phosphorylation sites on the exchange factor. These results were confirmed by the structural analysis of the isolated phosphopeptides by MALDI mass spectrometry and Edman degradation, which identified nine phosphorylated sites corresponding to serines 643, 691, 745, 793/794, 822, 849, 1123/1124, and 1146 and threonine 798 in the primary structure of CDC25Mm. Most of the Ser/Thr residues were clustered in the C-terminal half of the protein, in a region containing the REM (fragment 639–686) and the PEST (fragment 789–853) motifs. This region, preceding the RasGEF catalytic domain with the conserved CDC25 homology, is not well characterized as yet.

Of the nine PKA-specific phosphorylation sites identified in the present work, only two (Ser<sup>745</sup> and Ser<sup>822</sup>) coincided with the PKA consensus sites that we have deduced from the analysis of the CDC25Mm primary structure. The fact that these two residues were the most strongly phosphorylated made them priority candidates for site-directed mutagenesis (see below). The two weakly labeled Ser<sup>1123</sup>/Ser<sup>1124</sup> and Ser<sup>1146</sup>, identified in the catalytic region of CDC25Mm, were not found phosphorylated in the isolated C-terminal catalytic domain, suggesting that their accessibility to PKA can be influenced by the presence of the other domains. Ser<sup>1146</sup> is not conserved in human GRF1, whereas both Ser<sup>1123</sup> and Ser<sup>1124</sup> are conserved in murine, rat, and human GRF1. In CDC25Mm, substitution of Ser<sup>1124</sup> has been reported to influence the GEF activity (27). The adjacent Ser<sup>1123</sup> is homologous to SOS Ala<sup>877</sup>, which in the three-dimensional model hydrogen-bonds Gln<sup>70</sup> of Ha-Ras (28), a residue situated on α-helix 2 of the switch 2 region that is essential for the exchange reaction (29, 30). A phosphorylated residue, Ser<sup>642</sup>, was identified in the REM sequence, a conserved stretch essential for the function and structural integrity of the RasGEF family. Ser<sup>642</sup> is homologous to SOS residue Leu<sup>609</sup>, which, in the three-dimensional model, interacts with the RasGEF domain of SOS (28). The other phosphorylated serines (residues 691, 745, 793/794, 798, 822, and 849) are clustered around the PEST motifs in the most divergent region of GRF1 and GRF2, in which the weak homology is associated with a variable extension of the PEST motifs. Of these residues, serine 798 is not conserved whereas residues 793/794 and 822 are conserved in all known GRF1 but not in GRF2, like Ser<sup>216</sup>. It is possible that they have a regulatory function specific for GRF1. Worth mentioning, GRF1 and GRF2 are very similar in the primary structure, but different in tissue specificity. Even though residues 691, 745, and 849 are located in a very diverging region, they are serines or threonines in CDC25/GRF1Mm, GRF1rat, GRF1Hs, GRF2Mm, and GRF2Hs. This observation makes it likely that these residues play a functional role such as the interaction with as yet unidentified ligands.

PEST motifs are known in a number of cases to constitute targets of signals for proteolytic events (31). In the case of CDC25Mm, its in vitro treatment with the Ca<sup>2+</sup>-dependent protease calpain was found to lead to the production of C-terminal fragments cleaved in the region of the REM/PEST motifs (13). However, so far, no data have been reported about the half-life of CDC25Mm in the neuronal cell. In this regard, we would like to mention that a newly discovered subclass of evolutionary conserved Ras-like protein, kB-Ras, has recently been shown to interact specifically with a PEST region of the protein ligand Ikβ, decreasing its proteolytic degradation (32). Noteworthy is also the observation that S. cerevisiae GEFs Cdc25p and Sdc25p display short half-lives in the yeast cell (33).

Phosphorylation of Ser<sup>916</sup>, which is in part dependent on the action of PKA (16), has not been identified in our study. The PKA-dependent phosphorylation of this residue was reported not to induce any relevant effect on the RasGEF activity, whereas the carbachol-dependent phosphorylation of the same residue by an as yet unidentified kinase was necessary for full activation of the exchange factor in vitro. It is probable that the minor importance of the PKA action together with differences in the in vitro approach (in the case of Mattingly (Ref. 16), a short GRF1 fragment of 83 amino acid residues was used) justifies the discrepancy.

An important point, that remains to be assessed, is the effect of the PKA-dependent phosphorylation of the RasGEF activity of CDC25Mm. From the results in vitro reported by Mattingly and Macara (16), we were expecting a stimulation of the GEF activity of CDC25Mm. However, we could only observe that in vitro phosphorylation of CDC25Mm induces a slight inhibition of the GDP dissociation rate from the Ha-Ras-GDP complex. As already mentioned, this result could be, at least in part, the consequence of the lower phosphorylation efficiency obtained
under the conditions required for the functional test. More impressive was the 70% inhibition that we detected by substituting Ser\textsuperscript{822} with aspartate, a mutation mimicking the negative charge of the phosphate. In contrast, no effect was obtained by replacing Ser\textsuperscript{745} with aspartate. One should here stress that under the conditions required for the functional test, it is possible that Ser\textsuperscript{745} is involved in other functions not identified as yet. We would like to mention that CDC25Mm has been involved in the mechanisms of long term memory (34, 35) and has been reported to be an important postnatal regulator of the synthesis and release of growth hormone (36). The detailed analysis of all the combinations of residues susceptible of phosphorylation in vitro would doubtless contribute to a better comprehension of their functions.

Recently, Kiyono et al. (18) have shown that GRF1 can also be phosphorylated by the nonreceptor kinase Src. This modification slightly enhanced the exchange activity on Rac1, a member of the Rho family of GTPases. In contrast, no effect on the RasGEF activity of GRF1 was observed. Altogether, these observations suggest a very complex situation for the regulation of CDC25Mm/RasGEF activity(ies) by phosphorylating events, in which more than one kind of kinases and several pathways are likely to be involved. The fine tuning of switch molecules like Ras proteins, which are central elements of pathway networks, makes reasonable the existence of several regulatory mechanisms, in which GEF, the factor responsible for the regeneration of the active form of Ras, can be expected to be one of the most important component involved.

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