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Design and Synthesis of a Peptide Derived from Positions 195–244 of Human cdc25C Phosphatase

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Abstract: We have designed, synthesized and purified a 51 amino acid peptide derived from an essential domain of human cdc25C phosphatase. *In vivo*, differential phosphorylation of this domain regulates either the induction of mitotic processes, or the checkpoint arrest of eukaryotic cells in response to DNA damage. Peptide synthesis was achieved using the stepwise Fmoc strategy and resulted in an important yield of highly pure peptide. The final peptide was identified by amino acid analysis, electrospray mass spectrometry and nuclear magnetic resonance, which revealed that one of the two methionines within the peptide was oxidized into its sulphoxide derivative We investigated whether this 51 amino acid peptide folded into secondary structures in solution by circular dichroism and observed the formation of alpha helices in TFE. Finally, we verified that this peptide could bind to its biologically relevant 14-3-3 partner *in vitro* by fluorescence spectroscopy. Copyright © 1999 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: human cdc25C; 51 a.a. peptide; SPPS synthesis; Fmoc strategy; mass analysis

INTRODUCTION

Identification and understanding of the functional role of specific sequences present in large proteins is still, for a large part, dependent on the study of small peptides issued from a delimited amino acid sequence within a protein. However, in some cases, whether for structural or functional studies, such an approach may require high yields of long peptides of 50 to 100 residues. Although methods used for the synthesis of peptides have been seriously improved in the last decades, large-scale synthesis (>20 mg) of long peptides with a high degree of purity (>95%) still constitutes a challenge [1].

Cdc25C is a protein phosphatase which induces entry into mitosis through dephosphorylation and activation of cdc2-cyclinB at the G2/M transition [2–6], and which is involved in the checkpoint control of DNA damage [7–10]. Regulation of cdc25C activity is dependent upon its reversible phosphorylation *in vivo*. Activation of the mitotic function of cdc25C at the G2/M transition occurs concomitantly with its phosphorylation at several sites: Thr 48, 67 and 130 and Ser 122 and 214 in human cdc25C [11–16]. In contrast, following DNA damage in synchronized mammalian cells, cdc25C is predominantly phosphorylated on serine 216 [10,17]. Whereas phosphorylation of the mitotic sites of cdc25C has been proposed to be controlled by cdc2-cyclin B in a positive amplification loop [11–14], phosphorylation of serine 216 in asynchronously

Abbreviations: Boc, *tert*-butyloxycarbonyl; *t*Bu, *tert*-butyl; DCM, dichloromethane; DMF, *N*,*N*-dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, 1-hydrobenzotriazole; NEM, 4-ethylmorpholine; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulphonyl; TBTU, *O*-(1H-benzotriazol-1-yl)-*N*,*N*,*N′*,*N′*-tetramethyluronium tetrafluoroborate; TCEP, Tris(2-carboxyethyl) phosphine; TFA, trifluoroacetic acid; Trt, triphenylmethyl; TFE, trifluoroethanol; PEG-PS, polyethylene glycol graft polystyrene.

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HeLa cells was shown to be achieved by a protein kinase of 36–38 kDa, which bound to a region of human cdc25C located between positions 200 and 256 [17]. This region contains a putative bipartite nuclear localization sequence (NLS) close to Ser216, which is conserved in all members of the cdc25 family, except in mouse and hamster, suggesting that reversible phosphorylation of Ser216 might regulate the nuclear localization of cdc25C proteins [17]. Phosphorylation of Ser216 following DNA damage is achieved by chk 1, a protein kinase required for DNA damage checkpoint arrest in fission yeast [8–10].

This phosphorylation occurs within an RSXSXP sequence (X is any amino acid), Ser216 being the second serine, and thus creates a potential 14-3-3 binding site [10]. *In vivo*, 14-3-3 has indeed been shown to bind to human cdc25C, phosphorylated on Ser216 in G1, S and G2 phases, and in response to DNA damage, upon activation of the checkpoint control mechanism [10]. From these data, several authors have proposed that binding to 14-3-3 would sequester human cdc25C, thus preventing it from functionally interacting with and activating cdc2 [9,10].

In order to investigate the structural and biological characteristics as well as the regulation of the domain in human cdc25C encompassing phosphorylation sites Ser214 and Ser216, the site of interaction with 14-3-3, and the putative NLS, we devised a strategy to synthesize and purify large amounts of a 51-amino acid peptide derived from positions 195 to 244 of human cdc25C (called MP51), to which we added a C-terminal reactive cysteine group. Here, we describe the synthesis by Fmoc strategy and the purification of this peptide, together with its identification by amino acid analysis, electrospray mass spectrometry and nuclear magnetic resonance. We also present preliminary data concerning its ability to form secondary structures in solution, and show that this peptide can interact with recombinant 14-3-3 *in vitro*.

MATERIALS AND METHODS

Chemicals

Purex grade DMF stored over 4 Å molecular sieves, HPLC grade acetonitrile, peptide synthesis grade piperidine and pure TFA were purchased from SDS (Peypin, France). TBTU, HOBT and Fmoc-Arp(Pbf)-OH were obtained from Senn Chemicals (Gentilly, France). Fmoc-L-Cys(Trt)-PEG-PS resin and other Fmoc-amino acids were obtained from PerSeptive Biosystems (Voisin-le-Bretonneux, France). The following protecting groups were used for side-chain protection: *t*Bu (Ser, Tyr, Thr, Asp, and Glu), Boc (Lys), Trt (Cys, Asn and Gln) and Pbf (Arg). Microgranular carboxymethyl cellulose (GM52) was purchased from Whatman Biosystems (Maldstone, UK)

Analytical Procedures

Analytica1 HPLC was performed on a Waters system (Milford, MA) with an Aquapore RP 300 column, C8, 7 μm, 220 × 4.6 mm (Brownlee Lab., Applied Biosystems, San José, CA) and semipreparative HPLC was performed with a Nucleosil 300, C8, 5 μm column, 200 × 20 mm, SFCC (Neuilly-Plaisance, France). The amino acid content of peptide hydrolysates was determined with a High Performance Analyzer (Model 7300, Beckman Instruments, Fullerton, CA). Electrospray mass spectrometry analyses were earned out in the positive ion mode using a Trio 2000 VG Biotech Mass Spectrometer (Altringham, UK) and a Platform II Micromass Spectrometer (Manchester, UK).

Peptide Synthesis

Peptide synthesis was performed on 0.7 g of Fmoc-L-Cys(Trt)-PEG-PS resin (0.12 mmole of Cys/g) with a 9050 Pepsynthetizer Milligen (PerSeptive Biosystems) and according to the Fmoc/*t*Bu system as described by the manufacturer. Fmoc deprotection was achieved by percolating 20% piperidine in DMF through the reaction column (1.5 ml/min; 8 min for residues 50–27, 9 min for residues 26–17, 10 min for residues 16–10 and 11 min for the remainder of the sequence). Fmoc amino acids (six equivalents) were activated by the addition of equimolar amounts of TBTU and HOBT diluted to 0.27 M with 6.86% NEM in DMF. Coupling was obtained by recycling this solution through the reaction column for a standard 60 min. Double coupling (60 min) was performed according to a prediction program for coupling difficulty (J. Méry, unpublished) at Ile_{43}, Thr_{42}, Lys_{33}, Arg_{31}, Pro_{30}, Arg_{29}, Asn_{28}, Arg_{19}, Tyr_{18}, Arg_{14} and Ser_{13}. For all residues, after the last coupling reaction, unreacted sites were capped for 6 min with 0.3 M *N*-acetylimidazole in DMF.

Side-chain Deprotection

After deprotection of the terminal amino group, the side-chain protected peptidylresin was transferred

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into a 100 ml glass reactor (provided with two polyethylene sinters and connected to a manual apparatus), washed four times with DMF, ethanol and DCM and dried under a nitrogen stream for 30 min.

Deprotection was achieved with 10 ml of TFA/ethanedithiol/thioanisolel/water/triisopropylsilane/phenol (85:5:5:2:2:7.5). After 2 h, 5 ml of TFA/ethanedithiol/thioanisole (90:5:5) were added and stirring was continued for 2 h. The reactor content and a 5 ml TFA wash were dropwise filtered into 200 ml of cold diethylether and the crude peptide was collected by filtration, washed three times with cold diethylether and dried under vacuum for 30 min (400 mg).

Preliminary Purification by Ion Exchange on CM52 Cellulose

The crude peptide was diluted in 10 ml of 0.01 M sodium acetate pH 5 (buffer A), loaded onto a CM52 cellulose column (1.8×10 cm) and eluted with a linear salt gradient (0–0.2 M NaCl in buffer A at a flow rate of 50 ml/h, detection at 276 nm).

Semi-preparative HPLC Purification

The fractions retained after the ion exchange chromatography were concentrated to about 10 ml and purified by two successive semi-preparative HPLC runs with a linear elution gradient of 20–22% acetonitrile/TFA 0.05% (buffer B) in TFA/water 0.1% (buffer C) for 90 min at a flow rate of 9 ml/min. Fractions corresponding to peak 3 were pooled and freeze-dried.

Peptide Analysis

Amino acid analysis was performed by hydrolysing 3 to 4 nmol peptide with 0.2 ml of 5.7 N HCl containing 0.9% (w/v) of phenol *in vacuo* at 110°C in sealed glass tubes for 20 and 76 h. The content of serine, threonine and methionine was calculated by extrapolation at time 0 of the values obtained at 20 and 76 h. For determination of the cysteine content (as cysteic acid), performic oxidation was conducted as described by Stewart and Young [18] prior to a conventional 20 h hydrolysis.

Circular Dichroism Spectra

CD spectra were recorded on a Mark V dichrograph (Jobin–Yvon, Paris) using 1 mm thick quartz cells, and peptide concentration in the 0.05–0.1 mg/ml range.

NMR Measurements

NMR data were acquired on a Bruker AMX 600 spectrometer. The peptide solution was prepared by dissolving 5 mg of peptide in 0.5 ml of a mixture H_2O/TFE-d_3 (2/1). Chemical shifts were referred to internal TSP-d_4. COSY, TOCSY and NOESY were used for proton assignments and HSQC for carbon identification.

Fluorescence Binding Experiments

Binding experiments were performed by trytophan fluoresence spectroscopy essentially as described previously by Heitz *et al.* [19], using recombinant pmal-human 14-3-3ζ (kindly provided by Dr D. Fisher, CRBM-CNRS, Montpellier, France) and the 51-amino acid cdc25-derived peptide in potassium phosphate buffer (150 mM potassium phosphate, pH 7.2, 1 mM EDTA, 5% glycerol). Fluorescence measurements were performed at 25°C with a Spex II Jobin–Yvon fluorolog spectrofluorometer, with spectral bandpasses of 4 nm for both excitation and emission. Intrinsic tryptophan fluorescence was excited at 290 nm and emission spectra were recorded between 310 and 400 nm. Measurements were corrected as previously described by Divita *et al.* [20], plotted and fitted according to a standard quadratic equation using the Grafit software (Erathicus Software Ltd) with the following equation:

$$F = F_{\text{ini}} - \{(DF)[(E_t + L + K_d) - (E_t + L + K_d)^2 - 4E_tL]^{1/2}\}/2E_t$$

in which F is the relative fluorescence intensity, F_{ini} is the relative fluorescence intensity at the beginning of the titration, DF is the variation of fluorescence intensity between the initial value and that obtained at saturating concentrations of substrate (L), i.e. in this case the peptide, E_t is the total concentration of 14-3-3 and K_d is the dissociation of the enzyme–substrate complex.

RESULTS AND DISCUSSION

Peptide Design

With the aim of studying the structural and functional characteristics and regulation of the domain within human cdc25C, we set upon designing and synthesizing a peptide derived from the sequence of human cdc25C, including the putative bipartite NLS [17] which would encompass these different functional sites. The sequence of the corresponding

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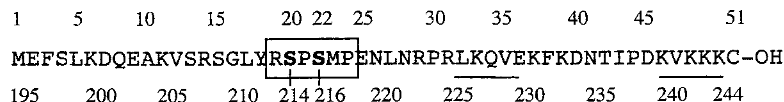

Figure 1 The sequence of MP51: the 51 amino acid peptide derived from residues 195–244 of human cdc25C, with an additional C-terminal reactive cysteine group is shown. Positions of residues are numbered from the N-terminus to the C-terminus, starting from the first methionine (above the sequence), and with respect to positions in full-length human cdc25C (below the sequence). Phosphorylation sites Ser 214 and Ser 216 are in bold. The 14-3-3 consensus binding site is boxed. The putative bipartite NLS is underlined.

peptide, termed MP51 together with the different functional sites mentioned is presented in Figure 1.

Synthesis and Purification

The desired deprotected peptide was obtained by the standard Fmoc procedure from an initial 0.7 g of Fmoc-L-Cys(Trt)-PEG-PS resin. As shown in Figure 2, HPLC analysis of the crude peptide obtained revealed the presence of four major peaks (designated as 1, 2, 3 and 4), eluting at the expected elution range, together with several minor ones. Given that the synthesis procedure included a capping step after introduction of each amino acid, these minor peaks most likely corresponded to truncated peptides; they were therefore discarded. Aliquot fractions of the four major peaks were analysed by amino acid analysis following acidic hydrolysis, as well as by electrospray mass spectrometry. Peaks 2, 3 and 4 revealed molecular weights close to the theoretical one, but peak 3 yielded a better amino acid analysis. Subsequent purification steps were consequently devoted to the isolation of peak 3. Given their unsatisfactory amino acid analysis, the products of the other major peaks were not identified in detail. In addition, these peptides proved to be definitely different from that of peak 3, as after HPLC purification and coinjection with peak 3 no comigration could be detected.

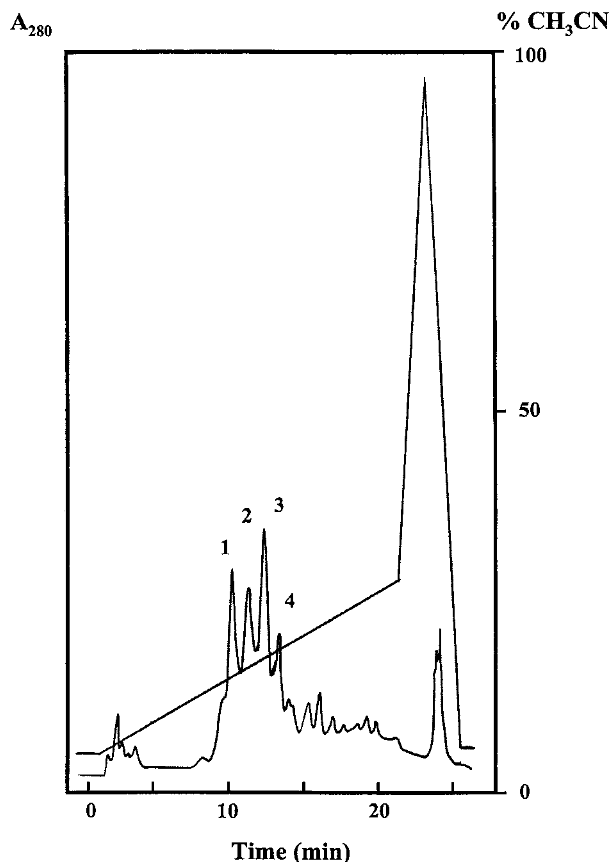

Figure 2 Analytical HPLC of the crude peptide. Reversed-phase HPLC analysis of crude peptide was performed on an Aquapore RP300 column, C8, 7 μm (0.46 × 20 cm) with a 25 min linear elution gradient of CH_3CN/TFA 0.05% (buffer B) in TFA/H_2O = 0.1% (buffer A). The flow rate was 1.5 ml/min and CH_3CN percentage is represented by a dotted line. Detection was measured at 276 nm, AUFS: 0.1.

Semi-preparative purification of the peptide product corresponding to peak 3 yielded 25.4 mg peptide, which proved to be homogenous by analytical HPLC (Figure 3) and electrophoresis on a 20% SDS-polyacrylamide gel. Moreover, as shown in Table 1, amino acid analysis of this purified peptide was in very good agreement with that expected from the amino acid composition. Further identification of the purified peptide was undertaken by electrospray mass spectrometry. As shown in Figure 4, the corresponding spectra yielded molecular peaks at 6004 ± 1.7 (Trio 2000) or 6010 ± 6.9 (Platform II) (expected 5996). In addition to the peak at 6010, peaks at M + 114 and M + 228 could be observed, corresponding to M + 1 TFA and M + 2 TFA.

Although the data above allowed us to attribute peak 3 unambiguously to MP51, a discrepancy remained between the expected and experimental mass of the peptide. The excess mass being of 14

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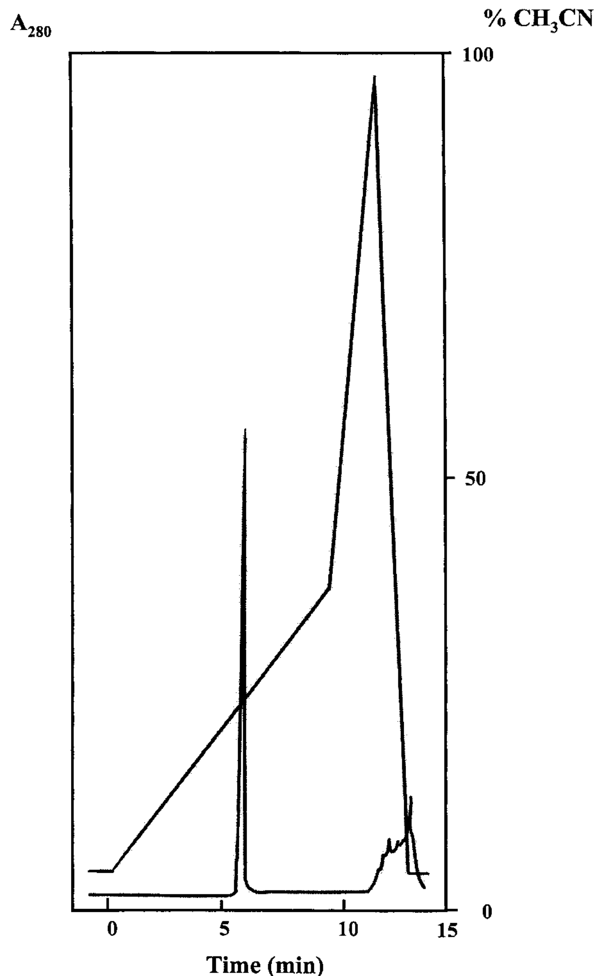

Figure 3 Analytical HPLC of the purified peptide. Same conditions as in Figure 2, except a 15 instead of a 25 min linear elution gradient was used.

Da and the peptide containing two methionine residues, which are known to be very sensitive to oxidation, we hypothesized that one or both of these residues might be oxidized into a sulphoxide derivative. If so, however, oxidation would result in a slightly different amino acid analysis compared to that obtained. One explanation could be that hydrolysis prior to amino acid analysis is known to induce reduction of sulphoxide derivatives into methionine [21]. Final confirmation of methionine oxidation was provided by NMR analysis. Respective ^{1}H and ^{13}C resonances of the two methionine residues were identified unambiguously using COSY, TOCSY, NOESY and HSQC experiments. While protons and carbons of Met 1 displayed expected chemical shift values, the γ and ε proton and carbon resonances observed for Met 23 showed large downfield shifts compared to Met 1 (Table 2). Both the proton and carbon chemical shifts of Met 23 resonances are indicative of the presence of a sulphoxide derivative. In addition, that only one spin system should be detected for each methionine reveals that only one methionine, Met 23, is fully oxidized into its sulphoxide form, whereas Met 1 is absolutely not affected by the oxidation process. The identification of only one oxidized methionine in MP51, versus both or both in part is rather unexpected when considering an oxidation process inherent to the synthesis procedure. We have indeed previously synthesized several other peptides con-

Table 1 Amino Acid Analysis of the Purified Peptide from Peak 3 of the First Analytical HPLC (Figure 2)

Type of amino acid	Number of residues	
	Theoretical	Experimental
Asx	6	6 × 0.99
Ser[a]	5	5 × 0.96
Pro	4	4 × 0.99
Ala	1	1 × 1.05
Val	3	3 × 1.01
Ile	1	1 × 0.95
Tyr	1	1 × 1.03
Lys	9	9 × 1.00
Thr[a]	1	1 × 0.93
Glx	6	6 × 0.99
Gly	1	1 × 1.04
Cys[b]	1	1 × 1.11
Met[a]	2	2 × 0.90
Leu	4	4 × 1.00
Phe	2	2 × 0.97
Arg	4	4 × 1.03

[a] Values extrapolated at time 0 using the value at 20 and 76 h hydrolysis.
[b] Determination as cysteic acid obtained after performic oxidation prior to a 20 h conventional hydrolysis.

Table 2 NMR Analysis of Methionine Residues 1 and 23 in the Peptide

		δ ^{1}H (ppm)	δ ^{13}C (ppm)
Met 1	γCH_2	2.61–2.56	30.6
	εCH_3	2.12	16.1
Met 23	γCH_2	2.99	50.9
	εCH_3	2.73	38.9

^{1}H and ^{13}C chemical shifts of the γCH_2 and εCH_3 side chain groups of the two methionines of the peptide.

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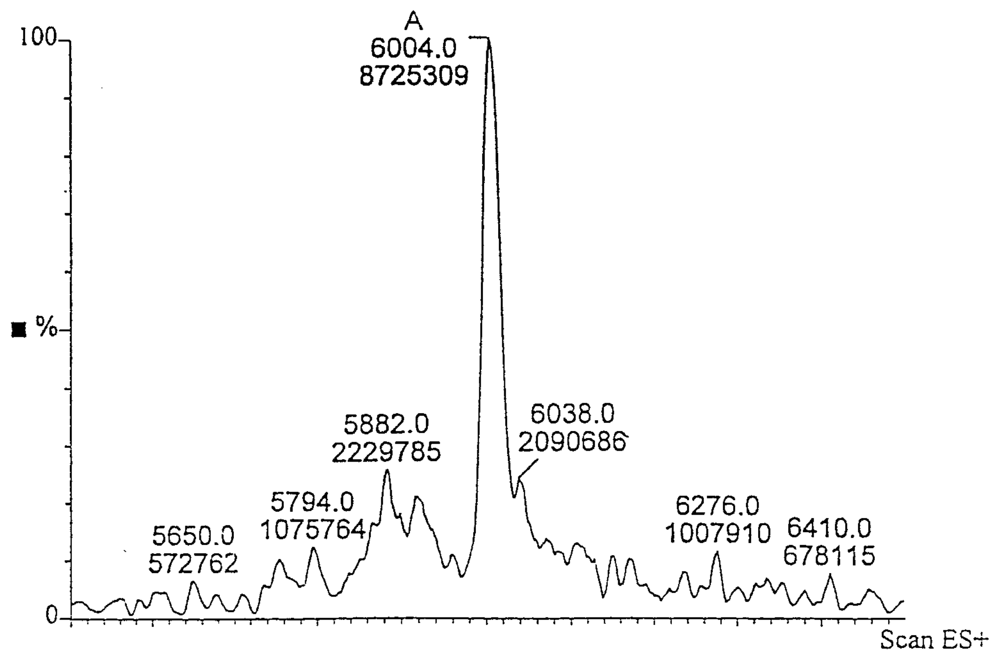

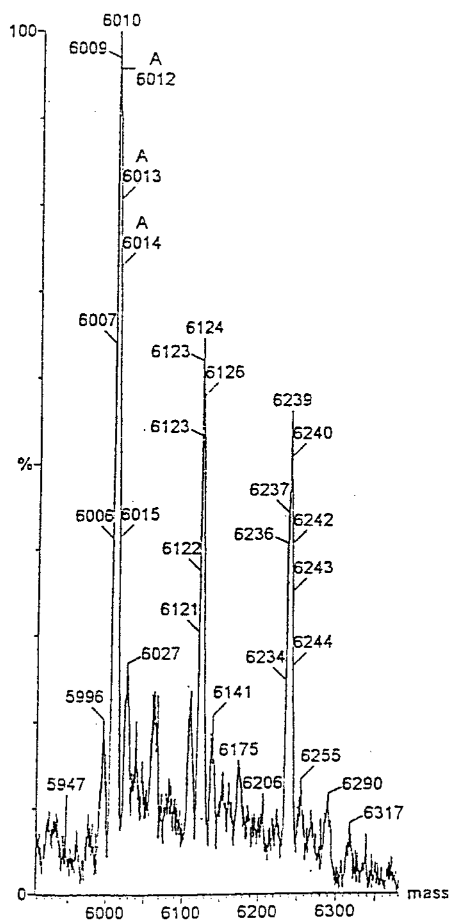

Figure 4 Electrospray mass spectra. Electrospray mass spectra of peptide were carried out in the positive ion mode using a Trio 2000 VG Biotech mass spectrometer (upper part) and a Platform II Micromass spectrometer (lower part).

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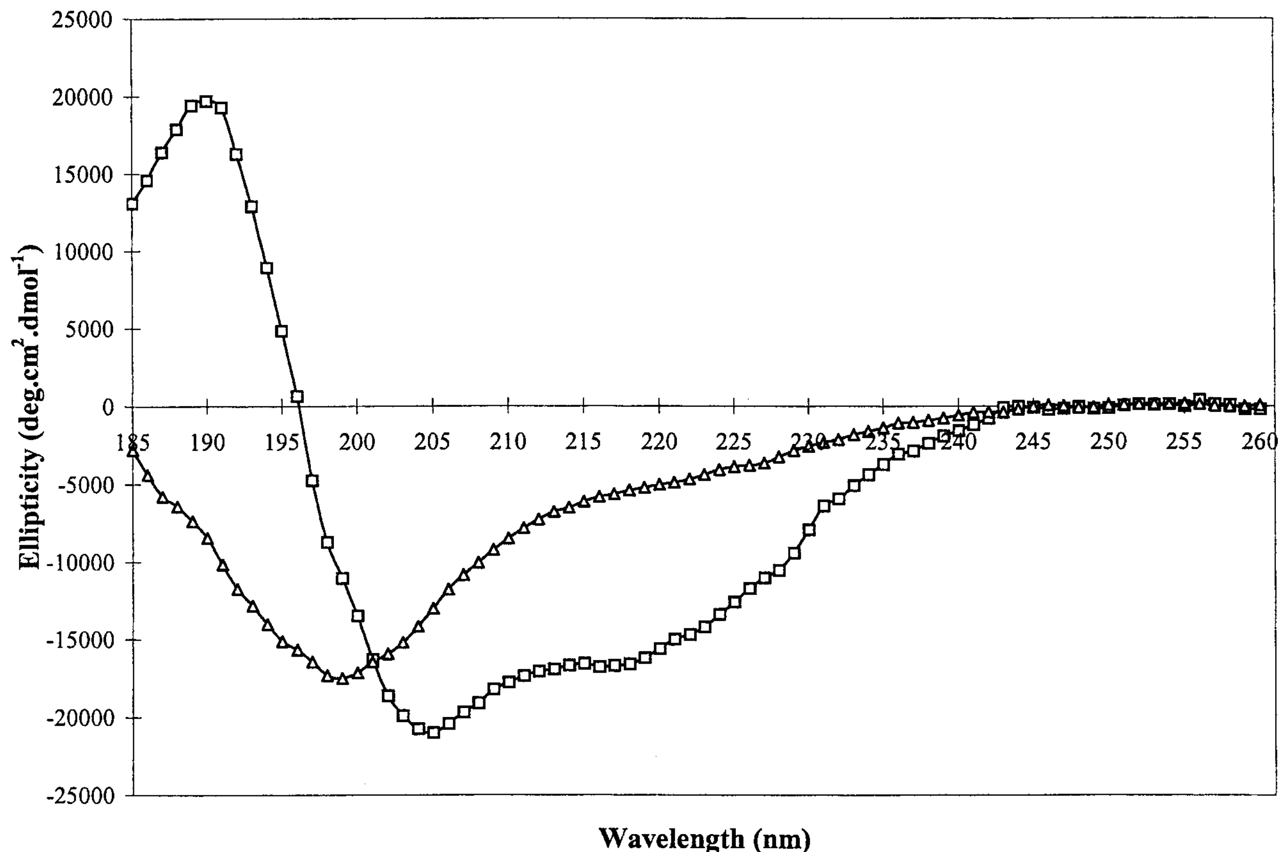

Figure 5 CD analysis of MP51 in different media. Spectra recorded at 0.1 mg/ml peptide in H_2O (△) and in 33% TFE (□).

taining a methionine residue within their sequence in our laboratory using the same Fmoc strategy, which implies the use of very mild conditions for the removal of the protecting groups, and have never so far detected the presence of methionines in an oxidized form [22]. This observation suggests that the nature of the residues surrounding a methionine residue may influence the oxidation process during peptide synthesis. Attempts to reduce the oxidized methionine under mild conditions [23] which avoid cleavages of the peptide chain, failed. Only very small amounts of the reduced peptide could be recovered, the remainder corresponding to the initial oxidized peptide.

Preliminary Conformational Analysis

In order to determine whether purified MP51 could adopt secondary structures in solution, we analysed its structural characteristics by CD in different media. The peptide was initially diluted to a final concentration of 0.1 mg/ml in water and any structural variations due to changes of the medium were measured as a function of changes of the initial CD spectrum. As shown in Figure 5, in water the spectrum showed a single minimum at 198 nm, indicative of a nonordered structure. We tested different pH conditions by addition of HCl or NaOH and any possible influence of buffering (20 mM phosphate buffer; pH 6.8) but could not detect any changes (data not shown). Only addition of TFE (*ca.* 33%) induced the formation of an α-helical structure, characterized by two minima at 207 and 217 nm associated with an α-helical structure, characterized by two minima at 207 and 217 nm associated with a maximum at 193 nm (Figure 5). Identification of the domains involved in helix formation is currently under way.

Binding Experiments

Several authors have shown that cdc25 proteins can interact with 14-3-3 isoforms both *in vitro* and *in vivo*. Human 14-3-3 isoforms, β and ε were found to interact with both human cdc25A and cdc25B both *in vitro* and *in vivo* [24]. Similarly, human

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cdc25C was shown to co-immunoprecipitate with 14-3-3 partners during interphase *in vivo* [10]. Human cdc25C is thought to interact with 14-3-3 via the RSXSXP sequence (X is any amino acid), the two serines corresponding to positions 214 and 216, respectively, a domain central to MP51 [10]. However, *in vivo* this interaction appears to be dependent on phosphorylation of human cdc25C on serine 216 [10].

We investigated whether MP51 synthesized and purified in this study could effectively interact with a biologically relevant partner, 14-3-3, in the absence of phosphorylation, similarly to recombinant cdc25A and cdc25B [24], or whether this interaction could not take place *in vitro*, in the absence of phosphorylation on serine 216, and measured its affinity for recombinant human 14-3-3ζ by tryptophan fluorescence spectroscopy. Briefly, 100 nM human 14-3-3ζ protein was incubated in potassium phosphate buffer and changes in its intrinsic tryptophan fluorescence were measured upon addition of increasing concentrations of MP51. As shown in Figure 6, addition of the peptide quenched the intrinsic fluorescence of 14-3-3 to 40% its initial value with saturating concentrations of peptide (1000 nM). Fitting of the data yielded a K_d value of 122 ± 24 nM. These results confirm that MP51 can interact with human 14-3-3ζ *in vitro* in the absence of phosphorylation on serine 216. This interaction takes place with high affinity and induces conformational changes in the environment of the tryptophan groups of 14-3-3. Further investigations will involve phosphorylation of serines 214 and 216, respectively, so as to determine whether phosphorylation modulates the affinity of this peptidic domain for 14-3-3 partners.

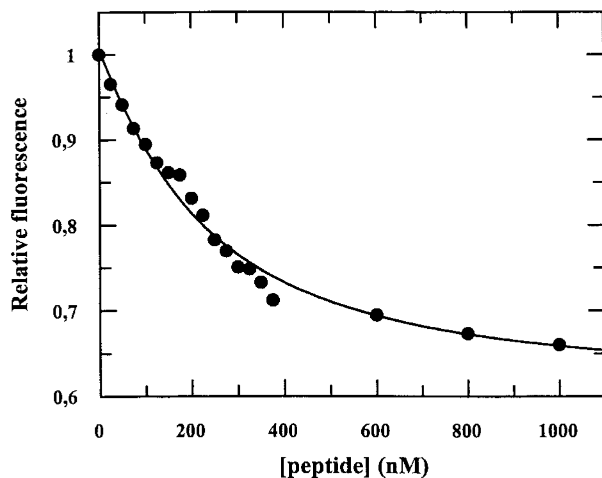

Figure 6 Interaction between MP51 and human 14-3-3ζ. 100 nM human pmal-14-3-3ζ protein was incubated at 25°C in potassium phosphate buffer (150 mM potassium phosphate, pH 7.2, 1 mM EDTA, 5% glycerol) and changes in its intrinsic tryptophan fluorescence were measured upon addition of increasing concentrations of MP51 from 10 to 1000 nM. Values measured for different concentrations of peptide are shown, together with their best fitting curve.

CONCLUSION

In this paper we have described the Fmoc SPPS and purification of a 51-amino acid peptide derived from an essential phosphorylation-site domain of human cdc25C phosphatase, with an additional C-terminal cysteine. Although one methionine within the peptide sequence was quantitatively oxidized into its sulphoxide derivative and despite the challenging length of this peptide, we were able to obtain important yields of highly pure peptide, sufficient for preliminary conformational studies. Although the peptide does not exhibit any secondary structures in water, we have shown that it can adopt an α-helical structure in solution in the presence of 33% TFE. Finally, given the functional importance of the domain reproduced by this peptide, we have investigated its ability to interact with a biologically relevant partner. Our results show that this peptide can interact with 14-3-3ζ with high affinity *in vitro*, in the absence of phosphorylation. Given the important yield of pure peptide obtained, we intend to pursue a more extensive structural characterization, as well as further investigations concerning, in particular, the role and effect of phosphorylation.

Acknowledgements

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