Volume 292, number 1,2, 205–209 FEBS 10362 November 1991

ADONIS 001457939101065M

Identification of phosphorylation sites on murine nuclear lamin C by RP-HPLC and microsequencing

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Received 20 September 1991

Isolated interphase lamin C, obtained from Ehrlich ascites tumor cells, was digested by Lys-C endoproteinase, the resulting peptides separated by reversed-phase HPLC and subjected to microsequencing in order to identify phosphorylation sites in interphase and following phosphorylation in vitro by cdc2-kinase, protein kinase C (PKC) and protein kinase A (PKA), respectively. Nuclear lamin C showed partial phosphorylation of Ser^{392} and Ser^{409}, and possibly Ser^{407} in interphase. Phosphorylation was increased in response to cdc2-kinase at Ser^{390} and Ser^{392} and to PKC at Ser^{572}. The N-terminal peptide (aa 1–32) containing consensus sequences for the 3 kinases was phosphorylated by cdc2-kinase, PKC and PKA. The sequence data suggests that multiple molecular switches via lamina modification control the dynamic behaviour of the nucleoskeleton during the cell cycle.

Nuclear lamin; Phosphorylation; cdc2-Kinase; PKA; PKC; RP-HPLC; Microsequencing

1. INTRODUCTION

The nuclear lamina is a fibrillar meshwork lining the nucleoplasmic surface of the nuclear membrane. It is believed to be important for nuclear envelope integrity and the organization of chromatin (for review see [1–3]. In mammalian cells the lamins A, B and C, members of the intermediate filament protein family [4,5], are the major molecular components of the nuclear lamina [6,7]. The hyperphosphorylation of the nuclear lamins is thought to be important for nuclear envelope breakdown during mitosis [4,5,7,8,10,11] (for review see [1–3]. Nuclear envelope breakdown is controlled by a kinase activity referred to as M-phase promoting factor (MPF) [11–14]. The catalytic subunit of MPF, a serine/threonine specific protein kinase, is homologous to the 34 kDa product of the *Schizosaccharomyces pombe* cdc2 gene [15,16]. Recent results identified MPF/cdc2-kinase as one of the kinases that act on the lamins [9–11]. In addition, other kinases are discussed to be involved in the lamina dynamics [10,11]. In this work we determined phosphorylation sites of murine lamin C, isolated from interphase nuclei and following phosphorylation with cdc2-kinase, PKC and PKA, respectively, using Lys-C digestion, RP-HPLC and microsequencing.

Abbreviations: RP-HPLC, reversed phase high-performance liquid chromatography; TFA, trifluoroacetic acid; aa, amino acid; PTH-amino acid, phenyl-thiohydantoin amino acid; PTH-DTT, phenyl-thiohydantoin-dithiotreitol; cdc2-kinase, cell division cycle 2 kinase; PKC, protein kinase C; PKA, protein kinase A, catalytic subunit; Lys-C lysine-C endoproteinase.

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2. MATERIALS AND METHODS

2.1. *Materials*

Endoproteinase Lys-C was from Boehringer, Mannheim, Germany; [γ-^{32}P]ATP from Amersham, Braunschweig, Germany; HPLC-columns for peptide separation and solvents for automatic protein sequencing were from Applied Biosystems, Weiterstadt, Germany. A Sykam HPLC was used for preparative peptide separation, Applied Biosystems HPLC A 130 for radioactive samples. Protein kinase A was from Sigma, München, Germany. A protein mixture, containing all known enzymes of PKC, was purified in a modified procedure according to [17,18] and kindly provided by Dr W. Schiebler, Hoechst AG, Frankfurt/Main, Germany. The cdc2-kinase purified according to [19] was kindly provided by Dr G. Draetta, EMBL, Heidelberg, Germany. Murine lamins were isolated as described previously [20].

2.2. *In vitro phosphorylation assays*

Purified A-type lamins were precipitated [21] and sonicated in the respective kinase buffers. Protein kinase assays were carried out under the following buffer conditions: cdc2-kinase: 50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 2 mM DTT, 1 mM ATP, 0.2 μl purified cdc2-kinase preparation (~0.02 μM); PKC: 30 mM Tris, pH 7.4, 8 mM $MgCl_2$, 1 mM EGTA, 2 mM DTT, 1 mM ATP, 1.1 mM $CaCl_2$, 50 μg/ml phosphatidylserine, 25 μg/ml diacylglycerol, 0.1 μM PKC; PKA: 30 mM Tris, pH 7.4, 8 mM $MgCl_2$, 7 mM DTT, 1 mM ATP, 1 mM EDTA, 0.05 μM PKA. Reactions were carried out in a final volume of 50 μl in the presence of 20 μCi [γ-^{32}P]ATP (3000 Ci/mM), at 4°C for 10 min for PKA and PKC, and 30°C for 45 min for cdc2-kinase. The phosphorylated lamins were separated by SDS-PAGE.

2.3. *Protease cleavage*

The protein bands were excised from the polyacrylamide gels, washed with 10 mM EDTA, dried, re-swollen and incubated with 1.2

μg Lys-C per band (10 μg) in cleavage buffer (0.2 *N,N*-methylmorpholine, pH 8.0, 1 M urea) for 22 h at 37°C.

2.4. *Peptide elution*

The peptides were eluted by 2 centrifugation steps, firstly in 500 μl cleavage buffer (14 000 × *g*, 2.5 h, 4°C), secondly in 500 μl 1 M urea, 0.1% TFA (18 000 × *g*, 30 min, 20°C). The mixed eluates were applied to RP-HPLC.

2.5. *Peptide purification and sequencing*

The peptides were separated by RP-HPLC using a 7 μm C8 column (2 × 250 mm) applying a gradient of 10% acetonitrile/0.1% TFA, to 90% acetonitrile/0.1% TFA, at a flow rate of 0.2 ml/min. Peptide fractions were collected and radioactivity was measured by Cerenkov counting. Sequence analysis of RP-HPLC separated peptides was carried out by automatic sequencing (477A, Applied Biosystems).

3. RESULTS

3.1. *HPLC-analysis of Lys-C digested lamin C prior to in vitro phosphorylation*

On average 20 μg of lamin C were digested for 22 h using Lys-C and separated by RP-HPLC (Fig. 1). The separation resulted in a number of peptides allowing for the characterization of an almost complete protein sequence (Fig. 1, Table I). Twenty-three peaks were analyzed by sequence analysis. The sequenced peptides could be assigned to the complete protein sequence derived from a murine lamin C-DNA sequence ([22], Table I). Polymorphisms were observed in 7 peaks. One cleavage product, aa 379–417/18/20, was detected in four peaks (No. 5,7,8,9), indicating a post-translational modification and/or alternative cleavage on its KKRKC-terminal end by Lys-C. In 2 peaks (No. 5 and 8, Fig. 1) containing these cleavage products, certain serine residues were not detectable by sequence analysis. These steps in Edman degradation, position 392 in cleavage product 379–417/18/20 (peak No. 5), positions 407 and 409 in cleavage product 379–417/18/20 (peak No. 8) showed a constant or increased signal for PTH-DTT-dehydroalanine. The presence of the PTH-DTT-dehydroalanine adduct concomitant with the absence of PTH-serine indicates that a phosphorylated serine was at this position as previously described for other phosphoproteins [23]. For lamin C this indicates that Ser^{392}, Ser^{409}, and possibly Ser^{407} are partially phosphorylated in interphase. Alkaline phosphatase treatment of the combined peaks 3–9 including the cleavage products with the aa-sequence 379–417/18/20 resulted in the alteration of retention times in the RP-HPLC profile and in the appearance of Ser^{392} in sequence analysis in all the peaks containing the respective cleavage product (results not shown), providing further evidence for partial in vivo phosphorylation of these serines in interphase lamin C.

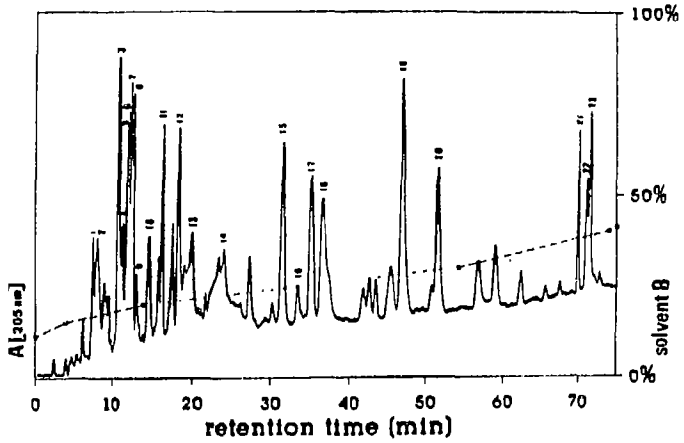

Fig. 1. Profile of RP-HPLC separated interphase lamin C peptides. Lamin C was digested by Lys-C, the resulting peptides were separated by RP-HPLC using a 5–90% acetonitrile gradient, 0.1% TFA.

Table I

Sequence analysis of RP-HPLC separated peptides following Lys-C digestion of lamin C

Peak No.	Amino acid sequence	Residues
1	RRHETRLV...	220–233
2	NIYSEELRETK	209–219
	EAALSTALSEK	145–155
3	LESSESRS...	421–450
	RKLESSES...	419–450
4	RKLESSESRSSFSQHART...	419–450
5	KEGDLLAAQARLK	123–135
	LLEGEEERLRLSPS#PTSQRS...	379–417/18/20
6	ERARLQLELSK	98–108
7	LLEGEEERLRLSPSPTSQ...	379–417/18/20
	EGDLLAAQARLK	124–135
	ERARLQLELSK	98–108
8	LLEGEEERLRLSPSPTSQRSRG-PASS-SS•QS#Q--G-V...	379–417/18/20
	EGDLLAAQARLK	124–135
9	LLEGEEERLRLSPSPTSQ...	379–417/18/20
10	DLEALLNSK	136–144
11	LALDMEIHAYRK	367–378
	LVRSLTMVEDNEDDDEDGEE...	543–574
12	LRDLEDSLARERDTSRRLLAEK	320–341
13	DLEALLNSKEAA...	136–155
14	SNEDQSM...	458–486
	AQNT-G...	516–542
15	AGQVVTIWASGAGATHSPPTDLV-K	491–515
16	QREFES#LADALQELRAQHEDQ-VE◇...	234–260
17	QREFESRLADALQELRAQ-HEDQV...	234–260
18	QLQDEMLRRVDAENRLQTLK	182–201
19	LDNARQSAERNSNLVGAAHE...	271–311
20	EREMAEMRARMQQQLDEYQE...	342–366
21	EDLQELNDRLAVYIDRVRSL...	33–78
22	◇	0–32
23	◇	0–32

Sequences of RP-HPLC separated peptides were assigned to the sequence of the whole lamin C protein deduced from cDNA analysis [22]. S#, no serine but increase of PTH-DTT-dehydroalanine detected in this position compared to previous step of Edman degradation. S•, no serine detected; level of PTH-DTT-dehydroalanine remained constant, compared to the previous step of Edman degradation. ◇, N-terminus of the peptide blocked. The peptide was identified following additional trypsin digestion and sequencing. ..., peptide not completely sequenced; –, aa not determined.

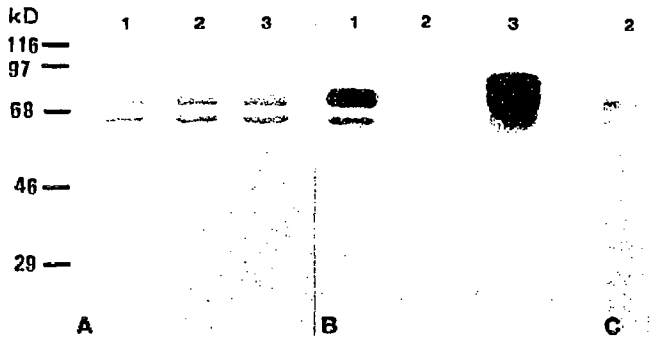

Fig. 2. SDS-PAGE and autoradiography of the purified A-type lamins. Lamins A/C phosphorylated by cdc2-kinase (lane 1), PKA (lane 2) and PKC (lane 3), (A) Coomassie stain, (B) autoradiography, (C) autoradiography 5 × exposure time of lamin A/C phosphorylated by PKA (lane 2).

3.2. *SDS-PAGE of in vitro phosphorylated murine lamin C*

Purified A-type lamins were phosphorylated by cdc2-kinase, PKC and PKA, respectively, and separated by SDS-PAGE. Fig. 2 shows that both A-type lamins, lamin A and C, can be phosphorylated by purified cdc2-kinase, PKC and, less efficiently, by PKA. We analyzed the phosphorylation of lamin C in detail. The bound phosphate, expressed in mol phosphate per mol of lamin C was: 0.09 mol for cdc2-kinase, 0.11 mol for PKC, and 0.02 mol for PKA, respectively.

3.3. *HPLC-analysis of Lys-C digested lamin C phosphorylated in vitro*

A-type lamins were phosphorylated with cdc2-kinase, PKC, and PKA in the presence of [γ-^{32}P]ATP, digested with Lys-C and applied to RP-HPLC. Figure 3 shows the resulting chromatograms and the corresponding Cerenkov counts. Cdc2-kinase phosphorylates cleavage products in 4 peaks (Fig. 3A, Table II). Phosphopeaks No. 1–3 contained the cleavage products encompassing aa 379–417/18/20 whereas peak 4 was identified as the N-terminal peptide of lamin C. These cleavage products include the motif S/TPX^{R}/K/^{S}/T for cdc2-kinase. Our sequence data showed an increase of PTH-DTT-dehydroalanine for position 390 and less prominently for position 392. However, in similar experiments, phosphoserine at position 392 was clearly detected as measured by the increase of PTH-DTT-dehydroalanine. ^{32}P was incorporated in 8 radioactive peaks following phosphorylation of lamin C with PKC (Fig. 3B, Table II). In radioactive peak No. 6, containing peptide aa 543–574, the conserved serine 572 is apparently phosphorylated. Additionally, the N-terminal peptide (peak 8) and the peptides encompassing aa 379–417/18/20 solely present in peaks 2–4 are phosphorylated. By analogy, radioactivity detected in the polymorphic peaks 1 and 5 may be incorporated in this peptide. However, for these peptides the phosphorylated residues could not be detected. A weak phosphorylation of the N-terminal peptide was observed following phosphorylation by PKA (aa 1–32, Fig. 3C, Table II).

Table II

Sequences of RP-HPLC separated peptides following Lys-C digestion of in vitro phosphorylated lamin C

Peak No.	Amino acid sequence	Residues
A 1	ERARLQLELSK	98–108
	LLEGEEERLRLS$^{\#}$PS•PTSQ...	379–417/18/20
	RKLESSESRSSFSQHART...	419–450
2	LLEGEEERLRLSPSPTS...	379–417/18/20
	ERARLQLELSK	98–108
3	LLEGEEERLRLSPSPTS...	379–417/18/20
	DLEALLNSK	136–144
4	◇	1–32
B 1	RKLESSESRSSFSQHARTSG-L...	419–450
	LLEGEEERLRLSPSPTSQRSRG...	379–417/18/20
	LESSESRSSFSQHARTSG-LAV...	421–450
2	LLEGEEERLRLSPSP...	379–417/18/20
3	LLEGEEERLRLSPSPTSQ...	379–417/18/20
4	LLEGEEERLRLSPSPTSQR...	379-417/18/20
5	LLEGEEERLRLSPSPTSQ...	379–417/18/20
	SLTMVEDNEDDDEDGEEL...	545–574
6	LVRSLTNVEDNEDDDEDGE-ELLHHHRVSGS•...	543–574
	DLEALLNSK	136–144
	LALDNEIHAYRK	367–378
7	SNEDQSNGN...	458–486
	AQNT-G-GSS...	516–542
8	◇	1–32
C 1	◇	1–32

Sequence analysis of RP-HPLC separated radioactive peaks, following lamin C phosphorylation with cdc2-kinase (A), PKC (B) and PKA (C). The peptides were assigned to the sequence of the whole lamin C protein deduced from cDNA analysis [23]. S$^{\#}$ no serine but increase of PTH-DTT-dehodroalanine detected in this position, compared to previous step of Edman degradation. S$^{\bullet}$, no serine detected; level of PTH-DTT-dehydroalanine remained constant, compared to the previous step of Edman-degradation. ◇, N-terminus of the peptide blocked; identification of the peptide following additional trypsin/ digestion and sequencing. ..., peptide not completely sequenced; –, aa not determined.

4. DISCUSSION

We determined phosphorylation sites of lamin C by a combination of different methods, Lys-C digestion, RP-HPLC separation and sequencing of the resulting peptides. The sequence information was used to identify putative phosphorylation sites in interphase lamin C and following in vitro phosphorylation. Alterations in PTH-serine/PTH-DTT-dehydroalanine ratios were determined to identify phosphoserine residues as previously described [23]. Sufficient quantities of purified lamins allowed us to recover on average 150 pmol of lamin C peptides for direct sequence analysis.

It could be shown that in interphase Ser^{392} and Ser^{409} are partially phosphorylated. The sequence surrounding the conserved Ser^{392} matches the consensus sequence

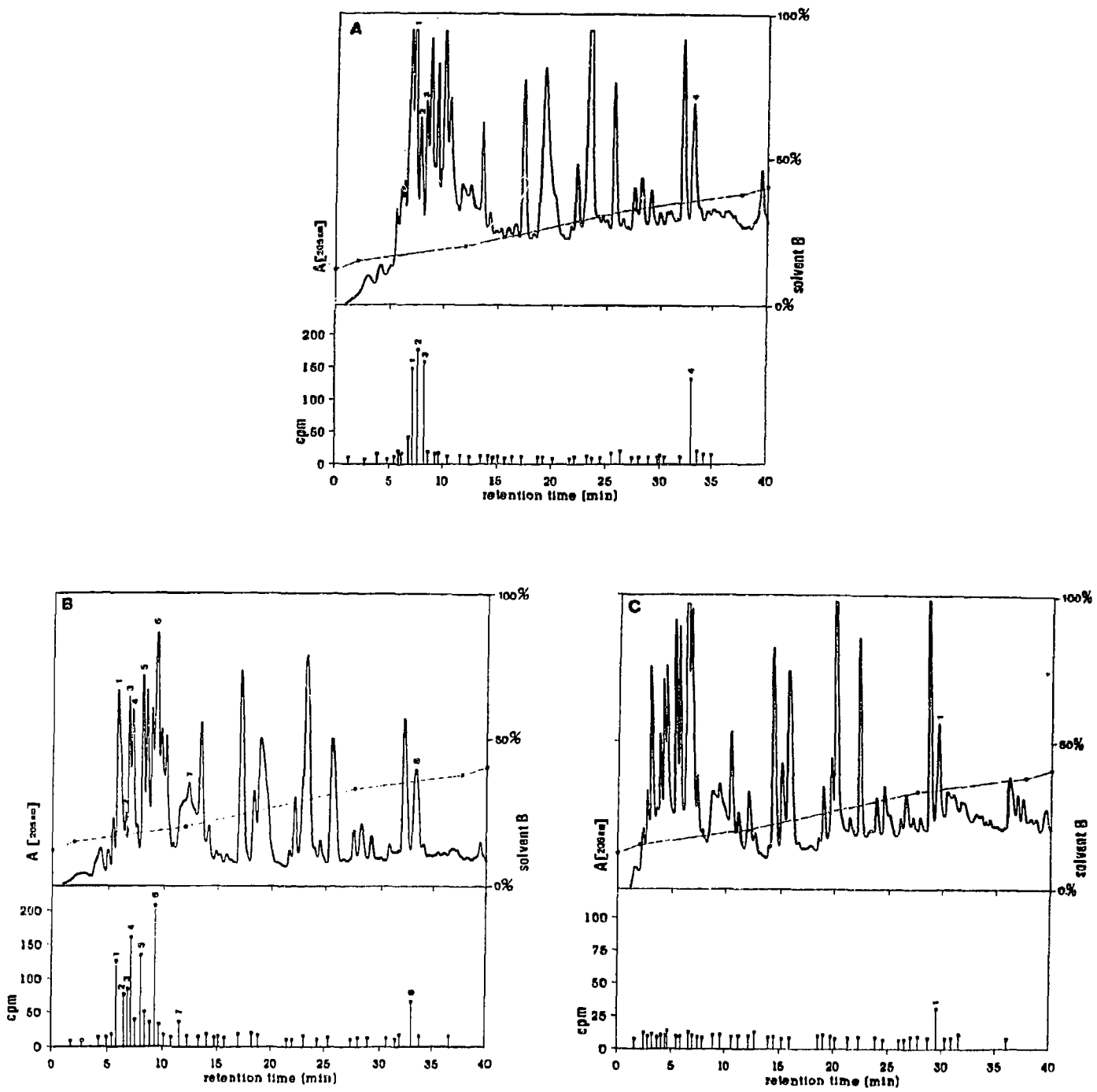

Fig. 3. Profile of RP-HPLC separated lamin C peptides following in vitro phosphorylation with cdc2-kinase (A), PKC (B), and PKA (C), respectively. The in vitro phosphorylated lamin C was digested by Lys-C, the resulting peptides were separated by RP-HPLC using a 10–90% acetonitrile gradient, 0.1% TFA.

for the cdc2-kinase [24]. Ser^{409} is not included in a known kinase recognition motif, but is conserved as S/T amino acid in man, mouse, and *Drosophila*. We also obtained some evidence for phosphorylation of Ser^{407} in interphase which has been suggested to be phosphorylated by S6-kinase II [11]. Partial phosphorylation of these residues, especially of Ser^{392} a mitosis-specific phosphorylation site in vivo [11], may be explained by an equilibrium of phosphorylated to unphosphorylated sites in interphase as a result of limited kinase and/or phosphatase action. Thus, lamina disassembly/reassembly during the cell cycle may only require partial modification of respective phosphorylation sites.

We have identified cdc2-kinase, PKC, and PKA as

potential lamin kinases. Cdc2 phosphorylated Ser^{390} and Ser^{392} and a non-identified aa in the N-terminal peptide. The 3 phosphorylation sites described here exist outside the end of the rod domain. As yet Ser^{390} has not been described as a site for cdc2-kinase. It is highly conserved but it does not match the known cdc2-kinase consensus sequence. It has been suggested that the consensus sequence assigned to cdc2-kinase requires modification [11] and may include the R L S P S P motif. The overall level of lamin phosphorylation with cdc2-kinase at Ser^{392} was 1:5 compared to 1:9 of that found in interphase. This suggests that the kinase operated at its mitosis-specific motif. The efficiency of the enzyme under in vitro conditions can only partially account for the substantially higher ratios of overall phosphate incorporated into mitotic lamins [8], reflecting a much lower efficiency of the enzyme under in vitro conditions and/or conformational alterations in isolated lamins.

The reported modification of Ser^{22} [9,10] was confirmed indirectly by the observed incorporation of radioactivity into the N-terminal peptide (aa 1–32). A determination of the position by sequence analysis is currently under investigation.

PKC may represent a physiologically relevant lamin kinase [25]. Our results show phosphorylation of the C-terminal peptide at Ser^{572} as well as the peptides encompassing aa 379–417/18/20 and the N-terminal peptide outside the rod domain. The peak No. 6 which had incorporated the highest amount of ^{32}P-phosphate consisted of 2 peptides, aa 543–573 and aa 367–378, the latter containing no Ser and Thr residues. Thus, the C-terminal S/TXR/K motif for PKC is only present once in the C-terminal peptide and the serine included in this motif may be the site of phosphorylation. However, the phosphoserine residue could not be clearly detected by the ratios of PTH-serine to PTH-DTT-dehydroalanine. The peptide encompassing aa 379–417/18/20 is clearly phosphorylated as shown in Table II, peaks 2–4. The peptide contains two PKC sites, Ser^{395} and Thr^{416}. The first site has previously been shown to be phosphorylated by PKC [11]. The N-terminal peptide was also phosphorylated by PKC. Again, the exact position, most likely within the conserved PKC consensus sequence of this peptide, remains to be determined.

PKA which is also thought to be a physiologically relevant lamin kinase yields in a weak phosphorylation of the N-terminal peptide. A candidate for phosphorylation is the partially conserved RRXS/T motif with a threonine residue in man and mouse and a serine residue in *Xenopus*.

In summary, the sequence analysis of interphase as well as in vitro phosphorylated lamin C shed new light on the complex and possibly cooperative action of several kinases regulating the dynamic behaviour of lamins in the cell cycle.

Acknowledgements: The authors thank Dr G. Draetta for kindly providing us with purified cdc2-kinase; K. Lepper and K. Jouvenal for skillful technical assistance and S. Lenz for preparing the manuscript. The work contains parts of the PhD thesis of Martin Eggert and was financially supported by a grant from the Deutsche Forschungsgemeinschaft (SFB-272) to E. Jost.

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