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Disassembly of *in vitro* formed lamin head-to-tail polymers by CDC2 kinase

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The nuclear lamina is an intermediate filament-type network underlying the inner nuclear membrane. At the onset of mitosis it depolymerizes, presumably in response to phosphorylation of the lamin proteins. Recently, cdc2 kinase, a major regulator of the eukaryotic cell cycle, was shown to induce lamina depolymerization when incubated with isolated nuclei. Here, we have analysed the structural consequences of lamin phosphorylation by cdc2 kinase using lamin head-to-tail polymers reconstituted *in vitro* from bacterially expressed chicken lamin B_2 protein as a substrate. The effects of phosphorylation were monitored by both a pelleting assay and electron microscopy. We show that lamin B_2 head-to-tail polymers disassemble in response to phosphorylation of specific sites that are phosphorylated also during mitosis *in vivo*. These sites are located within SP/TP motifs N- and C-terminal to the central α-helical rod domain of lamin proteins. Subsequent dephosphorylation of these sites by purified phosphatase 1 allows reformation of lamin head-to-tail polymers. The relative importance of N- and C-terminal phosphorylation sites for controlling the assembly state of nuclear lamins was assessed by mutational analysis. Polymers formed of lamin proteins carrying mutations in the C-terminal phosphoacceptor motif could still be disassembled by cdc2 kinase. In contrast, a single point mutation in the N-terminal site (Ser16 → Ala) rendered head-to-tail polymers resistant to disassembly. These results emphasize the importance of the N-terminal end domain for lamin head-to-tail polymerization *in vitro*, and they demonstrate that phosphorylation-dephosphorylation is sufficient to control the longitudinal assembly of lamin B_2 dimers.

Key words: cdc2 kinase/expression in bacteria/intermediate filament/nuclear lamins/phosphorylation

Introduction

The nuclear lamina is a fibrillar protein network underlying the inner nuclear membrane (e.g. Aebi *et al.*, 1986). During interphase of the cell cycle the lamina is believed to stabilize the nuclear envelope, and to play a role in organizing the distribution of chromatin. During mitosis, it is transiently depolymerized, most probably in response to hyperphosphorylation, and lamina disassembly is thought to represent a prerequisite for nuclear envelope breakdown (for reviews see Newport and Forbes, 1987; Gerace and Burke, 1988; Nigg, 1988, 1989; Burke, 1990). The major components of the nuclear lamina, the lamins, are members of the intermediate filament protein family (Aebi *et al.*, 1986; Fisher *et al.*, 1986; McKeon *et al.*, 1986; Franke, 1987). Prominent features distinguishing nuclear lamins from cytoplasmic intermediate filament proteins include a nuclear localization signal (Loewinger and McKeon, 1988); and the presence (in most lamins) of a C-terminal motif (the so-called CaaX-box) which is subject to a complex series of post-translational modifications, including isoprenylation, proteolytic trimming and carboxyl methylation (Chelsky *et al.*, 1987, 1989; Beck *et al.*, 1988, 1990; Wolda and Glomset, 1988; Farnsworth *et al.*, 1989; Vorburger *et al.*, 1989a; Kitten and Nigg, 1991). These processing events are important for targeting newly synthesized lamins to the nuclear envelope (Holtz *et al.*, 1989; Krohne *et al.*, 1989; Kitten and Nigg, 1991).

Based on biochemical properties and structural criteria, most lamins can be classified as either A- or B-type (Gerace and Blobel, 1980; Burke and Gerace, 1986; Krohne *et al.*, 1987; Höger *et al.*, 1988, 1990; Peter *et al.*, 1989; for reviews see Gerace and Burke, 1988; Nigg, 1989). Whereas expression of at least two distinct B-type lamins is characteristic of most vertebrate cell types (Lehner *et al.*, 1986; Vorburger *et al.*, 1989b; Weber *et al.*, 1990. Höger *et al.*, 1990; see also, Kaufmann, 1989), the expression of A-type lamins appears to be restricted to later stages of vertebrate embryonic development (Lehner *et al.*, 1987; Steward and Burke, 1987; Wolin *et al.*, 1987; Röber *et al.*, 1989, 1990). A- and B-type lamins also display profound differences with respect to their distributions in mitotic cells. When the lamina disassembles and the nuclear envelope breaks down at the onset of mitosis, A-type lamins are released in a soluble state, whereas B-type lamins remain associated with remnants of the nuclear membrane (Gerace and Blobel, 1980; Burke and Gerace, 1986; Stick *et al.*, 1988).

With the identification of the cdc2 kinase as a major regulator of the eukaryotic cell cycle, considerable progress has been made toward understanding the control of entry of cells into mitosis (for reviews see Draetta, 1990; Lohka, 1989; Nurse, 1990; Pines and Hunter, 1990). Mitotically activated cdc2 kinase consists of at least two components, the catalytic subunit $p34^{cdc2}$ and a 45–62 kd member of the cyclin protein family (for reviews see Hunt, 1989; Murray and Kirschner, 1989; Norbury and Nurse, 1989). This complex, purified from *Xenopus* or starfish oocytes (Lohka *et al.*, 1988; Labbé *et al.*, 1989b), accounts for the bulk of the histone H1-kinase activity in mitotic or meiotic (M phase) cells (Arion *et al.*, 1988; Labbé *et al.*, 1988, 1989a; Langan *et al.*, 1989; Chambers and Langan, 1990), and it induces

the physiological effects formerly attributed to an activity called MPF (M-phase promoting factor) (Dunphy *et al.*, 1988; Gautier *et al.*, 1988).

A key issue to be addressed is how cdc2 kinase triggers the multiple structural reorganizations that are characteristic of mitotic cells e.g. chromatin condensation, cytoskeletal rearrangements, nuclear envelope breakdown and lamina disassembly. A number of potential physiological substrates of cdc2 kinase were recently identified (for reviews see Moreno and Nurse, 1990; Pines and Hunter, 1990; Nigg, 1991). These include not only proteins that display enzymatic properties and thus might be activated or inhibited by cdc2 kinase (Morgan *et al.*, 1989; Shenoy *et al.*, 1989; McVey *et al.*, 1989; Mulner-Lorillon *et al.*, 1990), but also abundant proteins with a predominantly structural role (Langan *et al.*, 1989; Peter *et al.*, 1990a,b; Belenguer *et al.*, 1990; Chou *et al.*, 1990; Meijer *et al.*, 1990; Yamashiro *et al.*, 1991). Accordingly, it is possible that at least some of the ultrastructural changes typical of mitotic cells might be controlled by direct phosphorylation of structural targets by cdc2 kinase. Support for this possibility stems from the observation that highly purified cdc2 kinase phosphorylated lamins *in vitro* on M phase-specific sites and, concomitantly, caused depolymerization of the nuclear lamina when incubated with isolated nuclei (Peter *et al.*, 1990a). Moreover, a chicken lamin protein expressed in fission yeast *S.pombe* was shown to be phosphorylated by a mitotically activated kinase, and this lamin kinase activity was temperature-sensitive (ts) in a cdc2 ts mutant strain (Enoch *et al.*, 1991). The mitotic phosphorylation sites on nuclear lamins have been mapped to motifs flanking the central α-helical rod domain (Ward and Kirschner, 1990; Peter *et al.*, 1990a), and mutations affecting these phosphorylation sites were shown to interfere with lamina disassembly *in vivo*, providing strong support for their physiological relevance (Heald and McKeon, 1990).

Recently we have expressed chicken lamin B_2 in *E.coli* and studied its assembly properties by electron microscopy, using both glycerol spraying/low-angle rotary metal shadowing and negative staining techniques (Heitlinger *et al.*, 1991; see also Moir *et al.*, 1990). These studies revealed several distinct stages in lamin assembly, namely: (i) formation of coiled-coil 'myosin-like' dimers; (ii) polar longitudinal association of dimers into head-to-tail polymers; (iii) lateral association of head-to-tail polymers to filaments and; (iv) formation of paracrystalline fibers displaying a characteristic 24.5 nm axial repeat. In addition, preliminary evidence was obtained to suggest that lamin assembly could be inhibited by cdc2 kinase *in vitro* (Heitlinger *et al.*, 1991).

To examine the role of phosphorylation in controlling lamina assembly and disassembly at the molecular level, we have now combined this *in vitro* assembly system with a mutational analysis of M phase-specific lamin phosphorylation sites. We demonstrate that phosphorylation of one particular mitotic phosphoacceptor site on lamin B_2 (serine 16) by cdc2 kinase is sufficient to depolymerize *in vitro* assembled lamin head-to-tail polymers. While phosphorylation does not dissociate lamin dimers (nor inhibit their formation), it interferes with longitudinal interactions between lamin dimers. These results identify the N-terminal head domain of lamin proteins as a major determinant involved in longitudinal assembly of lamin dimers, and they suggest a mechanistic interpretation for phosphorylation-induced mitotic disassembly of the nuclear lamina.

Results

Expression of wild-type and mutant lamin B_2 in E.coli

To study the relative contributions of N- and C-terminal phosphorylation sites to the control of lamina dynamics *in vitro*, phosphoacceptor site mutants of lamin B_2 proteins were constructed by site-directed mutagenesis (Figure 1). In a first mutant (named 'LSPTR') the major mitotic N-terminal phosphoacceptor site (serine 16; Peter *et al.*, 1990a) was replaced by a non-phosphorylatable residue (alanine). In a second mutant (named 'SPSP'), four closely clustered serines (residues 384, 386, 388 and 389) in the C-terminal end domain were mutated to alanines; at least two of these residues (serines 384 and 386) were found to be phosphorylated *in vivo* (K.Weber, M.Peter and E.A.Nigg, unpublished results; see also Ward and Kirschner, 1990). In a third mutant (named 'dmut' for *d*ouble *mut*ant), the two sets of mutations were introduced together into the same cDNA (see Figure 1). The T7-polymerase expression system (Studier and Moffat, 1986) was then used to express wild-type and mutant versions of chicken lamin B_2 in *E.coli*. In all cases, initiation of translation started at the original initiator ATG codon of lamin B_2, resulting in expression of full-length proteins (Heitlinger *et al.*, 1991).

Expression of wild-type lamin B_2 in *E.coli* could readily be visualized after resolving the total cellular proteins by SDS–PAGE, either by Coomassie blue staining (Figure 2A; arrowhead) or by immunoblotting with specific monoclonal antibodies (MAbs; Lehner *et al.*, 1986) (Figure 2B). Qualitatively identical results were obtained when analysing the expression of the various mutant lamin B_2 cDNAs (not shown). However, when compared to authentic lamin B_2 expressed in chicken DU249 cells (Figure 2B, lane 3; arrow), the bacterially expressed proteins migrated with a slightly reduced electrophoretic mobility (Figure 2B, lane 4; arrowhead). This observation indicates that lamin B_2 is neither isoprenylated nor carboxyl methylated by the prokaryotic host. We have in fact shown previously that conversion of a slower migrating precursor to mature lamin B_2 in eukaryotic cells or reticulocyte lysates is due to isoprenylation (Vorburger *et al.*, 1989a, Kitten and Nigg, 1991). In turn, carboxyl methylation does not occur in the absence of prior isoprenylation and proteolytic removal of three amino acids from the CaaX box (Kitten and Nigg, 1991).

The *in vitro* assembly properties of bacterially expressed wild-type lamin B_2 have recently been characterized in detail (Heitlinger *et al.*, 1991). For the purpose of the present study, it was important to ascertain that the various serine to alanine mutations described above did not interfere with the assembly characteristics of wild-type lamin B_2. Therefore, each mutant lamin was expressed in *E.coli* and purified exactly as described previously for the wild-type protein (Heitlinger *et al.*, 1991). Then, the assembly properties of the individual proteins were examined by electron microscopy, using glycerol spraying/low-angle rotary metal shadowing as well as negative staining for sample preparation. None of the mutations shown in Figure 1 was found to significantly interfere with lamin B_2 assembly (data not shown).

In vitro phosphorylation of lamin B_2 by cdc2 kinase

After purification (Heitlinger *et al.*, 1991) or immunoprecipitation from bacterial extracts, wild-type lamin B_2

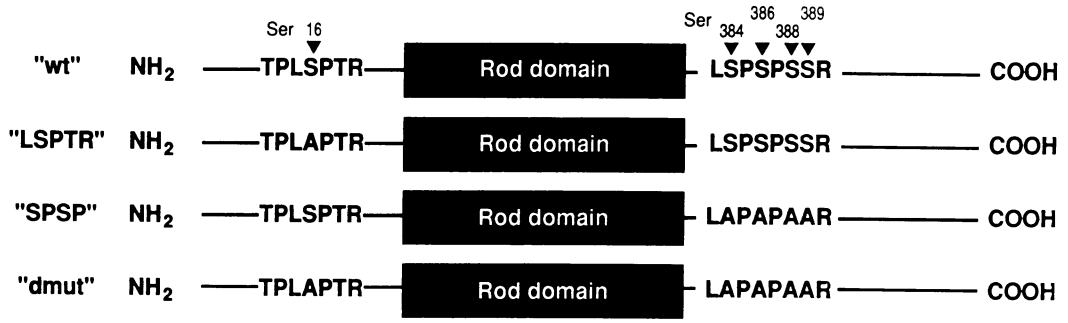

Fig. 1. Schematic representation of wild-type and mutant forms of the chicken lamin B_2 protein. Serine (S) to alanine (A) mutations were introduced into the lamin B_2 cDNA by site-directed mutagenesis (see Materials and methods). The numbers indicate the positions of the corresponding amino acids within the chicken lamin B_2 sequence (Vorburger *et al.*, 1989b). Note that the major mitotic phosphorylation sites flank the central rod domain of the lamin molecule (Ward and Kirschner, 1990; Peter *et al.*, 1990a).

could readily be phosphorylated by highly purified cdc2 kinase (Figure 2C). Mutant lamin B_2 proteins could also be phosphorylated, but, as expected, phosphate incorporation was significantly reduced for the LSPTR and the SPSP proteins, and very low (at most 10–20% of the wild-type level) for the dmut protein (data not shown, see Figure 3). These results confirm that lamin B_2 is a good *in vitro* substrate for cdc2 kinase. More importantly, they rigorously rule out that a co-precipitating kinase might have been responsible for the previously reported phosphorylation of lamin B_2 in immune-complexes prepared from avian cells (Peter *et al.*, 1990a).

To ascertain that phosphorylation of bacterially expressed lamin B_2 occurred on physiological sites, tryptic phosphopeptide maps were analyzed (Figure 3). Tryptic digestion of wild-type lamin B_2 phosphorylated by cdc2 kinase generated two major tryptic phosphopeptides (Figure 3A, panel a, marked by arrowhead and arrow, respectively). These correspond to major phosphopeptides present in maps prepared from *in vivo* labelled M phase lamin B_2 (not shown, see Peter *et al.*, 1990a). A third phosphopeptide could also be detected (Figure 3A, panel a), but this peptide has no counterpart in tryptic phosphopeptide maps of *in vivo* labelled lamins (Peter *et al.*, 1990a), and phosphorylation of the corresponding site(s) is unlikely to play a role in lamina disassembly (see below). Tryptic phosphopeptide maps established from lamin B_2 after phosphorylation with M phase extract were virtually identical to that shown in Figure 3A, panel a (not shown).

Based on our earlier studies (Peter *et al.*, 1990a), the two major phosphopeptides visible in Figure 3A, panel a, can be attributed, respectively, to phosphorylation of serine 16 (arrowhead) and serine 384 and/or 386 (arrow). This assignment is supported by the analysis of tryptic phosphopeptide maps generated after phosphorylating the LSPTR (Figure 3A, panel b), SPSP (Figure 3A, panel c), and dmut proteins (Figure 3A, panel d). It is further confirmed by the finding that phosphopeptide maps resembling the wild-type pattern could be restored by mixing tryptic phosphopeptides generated from the mutant proteins with *in vitro* phosphorylated peptides that had been synthesized to mimic the wild-type versions of the mutated phosphoacceptor sites (Figure 3A, panels e and f; for detailed explanation see Figure legend).

While mutations of the C-terminal residues (serines 384, 386, 388 and 389) specifically and completely eliminated the expected phosphopeptide from the corresponding tryptic

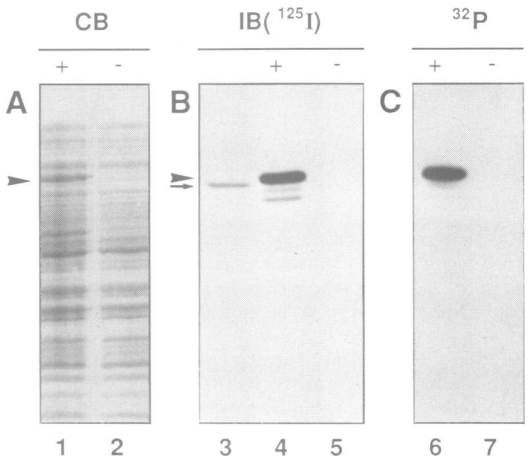

Fig. 2. Expression of full-length lamin B_2 in *E.coli* and phosphorylation by highly purified cdc2 kinase. Full-length chicken lamin B_2 was expressed in *E.coli* using the T7-polymerase system. Expression was driven by phase λ CE6 infection, which provides the T7-polymerase (Studier and Moffat, 1986). Induction is indicated by (+), no induction by (−). **A**: Expression of recombinant lamin B_2 monitored by Coomassie brilliant blue (CB) staining of an SDS gel. Lane 1: total proteins of induced bacteria. Lane 2: total protein of uninduced bacteria. The position of expressed lamin B_2 is indicated by an arrowhead. **B**: Demonstration of lamin B_2 expression by immunoblotting (IB), using a monoclonal antibody (E-3) specific for lamin B_2 (Lehner *et al.*, 1986). Lane 3: total protein prepared from chicken DU249 cells. Lane 4: total protein from induced bacteria. Lane 5: total protein from uninduced bacteria. Note that lamin B_2 expressed in *E.coli* (arrowhead) migrates with a slightly reduced mobility on SDS gels when compared to lamin B_2 from chicken cells (arrow); this result indicates that the bacterially expressed protein is neither isoprenylated nor carboxyl methylated (Vorburger *et al.*, 1989a; Kitten and Nigg, 1991). **C**: Autoradiogram demonstrating *in vitro* phosphorylation of bacterially expressed lamin B_2 by highly purified cdc2 kinase (Labbé *et al.*, 1989b). Lamin B_2 was immunoprecipitated from induced (lane 6) or uninduced (lane 7) bacteria and phosphorylated in the presence of [γ^{32}P]ATP; then, samples were subjected to SDS–PAGE and autoradiography.

maps (Figure 3A, panels c and d, arrow), we note that mutation of serine 16 did not completely abolish phosphate incorporation into the corresponding tryptic peptide (see the faint spots marked by the arrowhead in Figure 3A, panels b and d). Although this result may appear surprising at first, it is readily explained by the data shown in Figure 3B. Phosphoamino acid analysis of wild-type *in vitro* phosphorylated lamin B_2 revealed predominantly phosphoserine, but also a minor amount of phosphothreonine

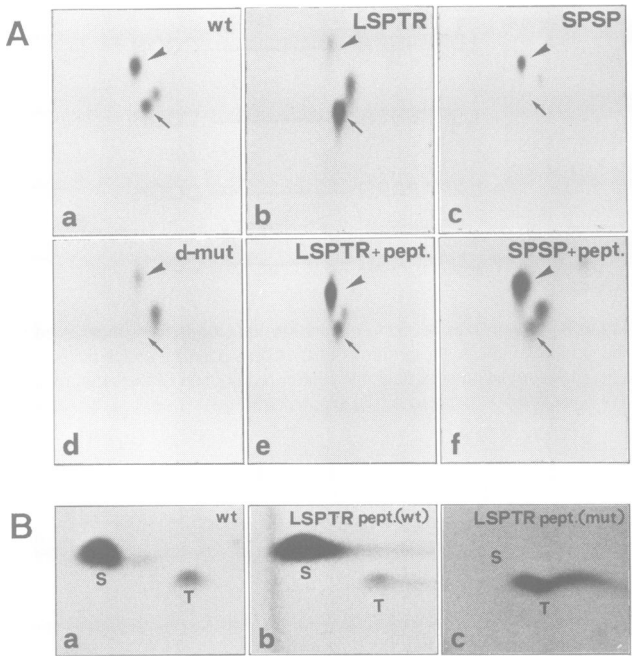

Fig. 3. Phosphorylation of bacterially expressed lamin B_2 occurs on mitotic sites. Lamin B_2 was immunoprecipitated from bacterial extracts expressing either mutant or wild-type protein, and phosphorylated *in vitro* with purified cdc2 kinase in the presence of [γ^{32}P]ATP. The phosphorylated proteins were eluted from SDS gels and either digested with trypsin for phosphopeptide mapping (A) or hydrolysed in 6 M HCl for phosphoamino acid analysis (B). **A**: Phosphopeptide maps obtained from the following lamin B_2 proteins: a: wild-type; b: LSPTR mutant; c: SPSP mutant; d: dmut mutant. In panels e and f, tryptic peptides of either the LSPTR (e) or the SPSP (f) mutants were mixed, prior to thin layer chromatography, with appropriate tryptic digests of *in vitro* phosphorylated synthetic peptides. These were synthesized to span the regions corresponding to the mutated motifs within the lamin B_2 sequence (GTPGGTPLSPTRITRLQ and LKLSPSPSSRVTV, respectively), phosphorylated *in vitro* with cdc2 kinase, and digested with trypsin. Confirming the assignment of the various phosphopeptides, the *in vitro* phosphorylated synthetic peptides run exactly to the positions where tryptic peptides were lost from lamin B_2 due to mutations in the corresponding phosphorylation sites (compare panels e and f with panels b and c). **B**: Phosphorylated wild-type lamin B_2 protein (a) or individual tryptic phosphopeptides (b and c) were subjected to phosphoamino acid analysis. a: wild-type lamin B_2; b: the tryptic LSPTR peptide isolated from the wild-type protein (corresponding to the spot marked by an arrowhead in A, panel a); c: the tryptic LSPTR peptide isolated from the LSPTR mutant protein (corresponding to the spot marked by an arrowhead in A, panel b). Note that the wild-type tryptic LSPTR peptide contains both phosphoserine and phosphothreonine (b), but the corresponding mutant peptide only contains phosphothreonine (c). The trail in the autoradiographic signal visible in c was seen also in the ninhydrin stained phosphothreonine standard (not shown).

(Figure 3B, panel a), consistent with the results of *in vivo* studies (Peter *et al.*, 1990a). The same two phosphoamino acids were recovered also from the isolated tryptic phosphopeptide known to contain serine 16 (Figure 3B, panel b). Thus, the tryptic peptide containing serine 16 must also contain a threonine phosphoacceptor site for cdc2 kinase. Whereas a doubly phosphorylated version of this tryptic peptide would be expected to contain equimolar amounts of phosphoserine and phosphothreonine (and to migrate to a more acidic position on thin layer cellulose plates), the singly phosphorylated peptides are predicted to comigrate. Thus, following mutation of serine 16 to alanine, phosphorylation

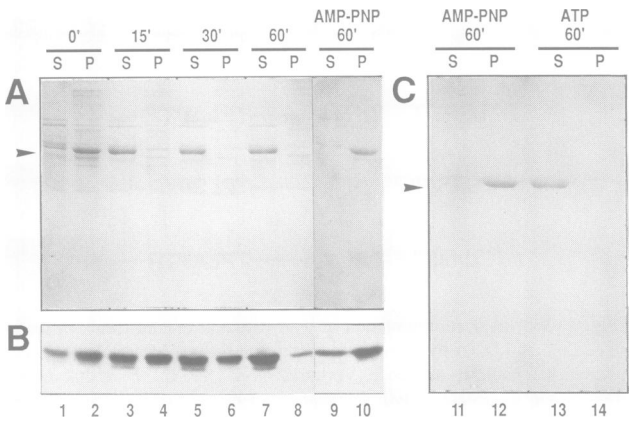

Fig. 4. Biochemical evidence for disassembly of lamin B_2 polymers by either cdc2 kinase or M phase extract. Lamin B_2 head-to-tail polymers were incubated in the presence of ATP with cdc2 kinase (**A** and **C**), or whole cell M phase extract (Nakagawa *et al.*, 1989; **B**) The cdc2 kinase utilized in these experiments was purified either by multiple chromatographic separations (Labbé *et al.*, 1989a; A) or by affinity chromatography on p13-beads (Labbé *et al.*, 1989b; C). At the times indicated, aliquots were removed from the incubations and subjected to centrifugation; the partitioning of lamin B_2 between supernatant (S) and pellet (P) fractions was then analysed by SDS–PAGE, followed by Coomassie brilliant blue staining (A and C) or immunoblotting with anti-lamin B_2 antibodies (B). For control, incubations were carried out in the presence of the non-hydrolysable ATP analogue AMP-PNP instead of ATP (lanes 9–12).

of the corresponding peptide should occur entirely on threonine. As shown in Figure 3B, panel c, this prediction is met. At present we do not know with certainty which threonine is phosphorylated by cdc2 kinase. One prime candidate is residue 13 immediately upstream of serine 16 (see Figure 1), but we cannot exclude a contribution by threonine 9 (Vorburger *et al.*, 1989b). Both of these residues are followed by prolines and, by this criterion, would qualify as potential targets for cdc2 kinase (Shenoy *et al.*, 1989; Peter *et al.*, 1990b).

In vitro disassembly of wild-type lamin B_2 head-to-tail polymers

To determine whether or not M phase extracts and/or cdc2 kinase could disassemble lamin head-to-tail polymers formed *in vitro* from bacterially expressed lamin B_2, such polymers were produced by appropriate dialysis of purified wild-type protein (Heitlinger *et al.*, 1991). The effects of phosphorylation were then examined by a pelleting assay and electron microscopy. Figure 4 illustrates the effects of incubating preformed lamin head-to-tail polymers for various time periods with either one of two types of cdc2 kinase preparations (Figure 4A and C) or with M phase extract (Figure 4B). Following centrifugation, lamin disassembly was monitored by determining the partitioning of lamin B_2 between supernatant (S) and pellet (P) fractions; lamins were detected using either Coomassie blue staining (Figures 4A and C), or, in the case of incubations with whole cell M phase extracts, immunoblotting (Figure 4B). Immediately after addition of cdc2 kinase or M phase extract, the bulk of lamin B_2 was recovered from the pellet fraction, consistent with the presence of extensive polymers (Figures 4A and B, lanes 1 and 2). However, in response to incubation

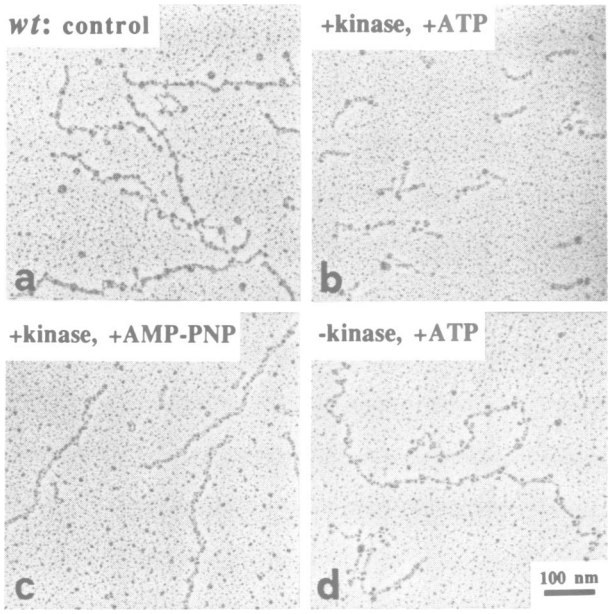

Fig. 5. Electron microscopic evidence for disassembly of wild-type lamin B_2 polymers by cdc2 kinase. Preformed lamin B_2 head-to-tail polymers (**a**) were incubated with purified cdc2 kinase (Labbé *et al.*, 1989b) in the presence of either ATP (**b**) or the non-hydrolysable ATP analogue AMP-PNP (**c**). For further control, lamin B_2 polymers were incubated also with ATP but without cdc2 kinase (**d**). Samples were examined by electron microscopy, using glycerol spraying/low angle rotary metal shadowing. Note that only the addition of both ATP and cdc2 kinase resulted in disassembly of the lamin polymers.

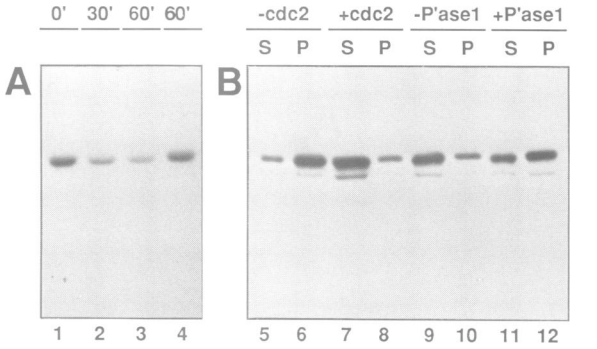

Fig. 6. Purified phosphatase 1 dephosphorylates lamin B_2 and allows reformation of polymers. **A**: After *in vitro* phosphorylation of lamin B_2 by cdc2 kinase in the presence of [γ-^{32}P]ATP, aliquots of the phosphorylated protein were incubated for the times indicated in the presence (lanes 1–3) or absence (lane 4) of purified phosphatase 1. Dephosphorylation was monitored by SDS–PAGE and autoradiography. **B**: Preformed lamin B_2 head-to-tail polymers were incubated in the presence of ATP with (+cdc2) or without (−cdc2) cdc2 kinase. Then, aliquots of the samples were subjected to centrifugation and the polymerization state of lamin B_2 was monitored by immunoblotting of supernatant (S) and pellet (P) fractions (lanes 5–8). Another aliquot of the cdc2 kinase treated sample (i.e. the one containing phosphorylated, depolymerized lamin B_2; lanes 7 and 8) was dialysed against phosphatase buffer, divided into halves and further incubated for 60 min with or without adding purified phosphatase 1. Then, both samples were dialysed against polymerization buffer (Heitlinger *et al.*, 1991), and the partitioning of lamin B_2 between supernatant (S) and pellet (P) fractions was analysed by centrifugation and immunoblotting (lanes 9–12).

with cdc2 kinase or M phase extract, lamin B_2 was progressively solubilized, the time course of solubilization being much more rapid with purified cdc2 kinase than with M phase extract (Figures 4A and B, lanes 3–8). In either case, solubilization was strictly dependent on ATP hydrolysis. No disassembly occurred when Mg^{2+} was omitted (not shown) or when ATP was replaced by the non-hydrolysable analogue AMP-PNP (Figures 4A and B, lanes 9 and 10). The above experiments were carried out with a preparation of cdc2 kinase that had been purified from starfish oocytes by multiple chromatographic separations (Labbé *et al.*, 1989a); as shown in Figure 4C, identical results were obtained when using a highly purified cdc2 kinase prepared by an alternative procedure based on affinity chromatography (Labbé *et al.*, 1989b).

ATP-dependent disassembly of wild-type lamin B_2 polymers by cdc2 kinase could be demonstrated also by electron microscopy (Figure 5). When lamin B_2 head-to-tail polymers (Figure 5a) were incubated with cdc2 kinase for 60 min in the presence of ATP, they disassembled predominantly into dimeric molecules (Figure 5b). As expected from the results presented above (Figure 4), head-to-tail polymers persisted when ATP was replaced by AMP-PNP (Figure 5c) or when cdc2 kinase was omitted from the incubation (Figure 5d).

Phosphatase 1 can reverse cdc2 kinase effects

The results shown above strongly argue that ATP-dependent disassembly of lamin B_2 polymers by cdc2 kinase is mediated by phosphorylation. Thus, one would predict that treating the phosphorylated lamins with an appropriate phosphatase should reverse the effect of cdc2 kinase. In preliminary experiments we used both phosphatase 2A (Stone *et al.*, 1987) and phosphatase 1 (Cohen, 1989) for dephosphorylation of lamin B_2. The latter enzyme was found to be more efficient in dephosphorylating the residues previously phosphorylated by cdc2 kinase (not shown), and was therefore used for further studies. In a first experiment, bacterially expressed wild-type lamin B_2 was phosphorylated by cdc2 kinase in the presence of [γ^{32}P]ATP, and then used as substrate for phosphatase 1. As shown in Figure 6A, lamin B_2 was readily dephosphorylated in the presence (lanes 1–3) but not in the absence of phosphatase 1 (lane 4). Dephosphorylation was approximately linear with time (Figure 6A, lanes 1–3), suggesting that phosphatase 1 could dephosphorylate both N- and C-terminal mitotic sites within lamin B_2 with comparable efficiency. In support of this notion, phosphorylated LSPTR- and SPSP-mutant lamins could both be dephosphorylated by phosphatase 1 (not shown). To assess the effect of phosphatase treatment on lamin–lamin interactions, preformed head-to-tail polymers were treated with (Figure 6B, lanes 7 and 8) or without (Figure 6B, lanes 5 and 6) cdc2 kinase, and aliquots of disassembled (phosphorylated) lamins were incubated *in vitro* in the presence or absence of purified phosphatase 1. When these samples were dialysed against buffer inducing head-to-tail polymer formation (Heitlinger *et al.*, 1991), lamin proteins incubated in the absence of phosphatase 1 remained soluble (Figure 6B, lanes 9 and 10), whereas those incubated in the presence of phosphatase 1 were predominantly recovered in the pellet fraction (Figure 6B, lanes 11 and 12), indicating reformation of head-to-tail polymers.

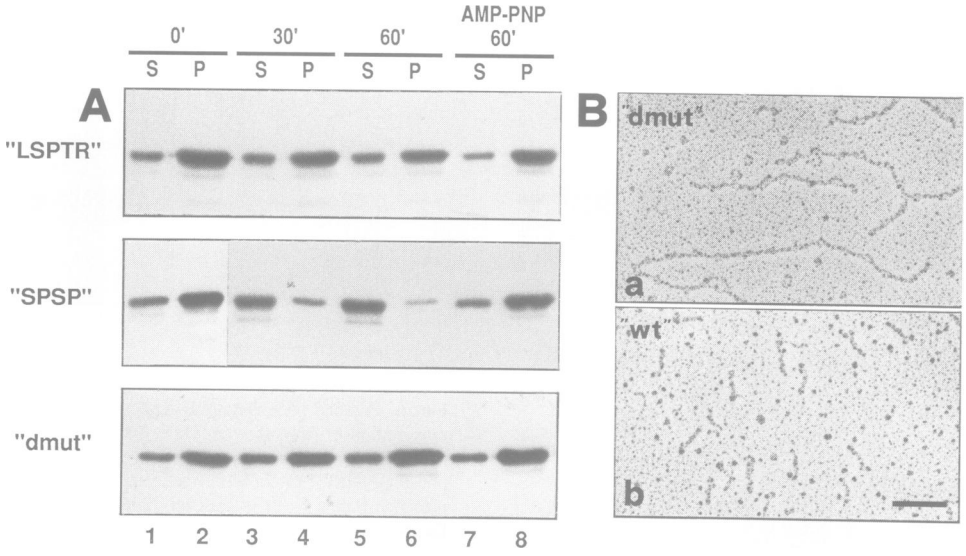

Fig. 7. Disassembly of mutant lamin B_2 head-to-tail polymers with cdc2 kinase. Head-to-tail polymers formed by the various lamin B_2 mutants were incubated with cdc2 kinase in the presence of ATP (A, lanes 1–6 and B, a and b) or the non-hydrolysable ATP analogue AMP-PNP (A, lanes 7 and 8). Aliquots were removed after the times indicated and the effect of phosphorylation was monitored by a pelleting assay (A) and electron microscopy (B). **A**: The polymerization state of lamin B_2 proteins was assessed by centrifugation and immunoblotting of supernatant (S) and pellet (P) fractions, as described in the legend to Figure 4. **B**: Preformed head-to-tail polymers of the dmut protein (a) and wild-type lamin B_2 (b) were incubated for 60 min in the presence of ATP with purified cdc2 kinase. Then, samples were examined by electron microscopy, using glycerol spraying/low angle rotary metal shadowing. Bar = 100 nm.

Mutation of serine 16 to alanine interferes with head-to-tail polymer disassembly in vitro

To assess the relative importance of the N- and C-terminal phosphoacceptor sites for controlling lamina dynamics *in vitro*, we next tested whether or not mutations of mitotic phosphorylation sites would interfere with the disassembly of preformed head-to-tail polymers. For this purpose, polymers were formed using each of the lamin B_2 mutant proteins shown in Figure 1, and incubated for various time periods with either cdc2 kinase (Figure 7) or M phase extract (Figure 8). Incubations were carried out in the presence of ATP, or for control, the non-hydrolysable ATP analogue AMP-PNP. Mutation of serine 16 in the N-terminal head domain of lamin B_2 strongly interfered with disassembly (Figures 7A and 8, see panels labelled 'LSPTR'). Taking into account the fraction of soluble lamin B_2 normally present in the control samples (see Figure 7A, lanes 7 and 8 and Figure 8, lanes 5 and 6), quantification of these results by densitometric laser scanning indicated that $< 10\%$ of the mutant lamin B_2 protein was specifically solubilized. Very similar results were obtained also when studying the disassembly of polymers formed by the dmut protein (Figures 7A and 8, see panels labelled 'dmut'). In contrast, head-to-tail polymers formed by the SPSP mutant protein could readily be disassembled by either cdc2 kinase or M phase extracts (Figures 7A and 8, see panels labelled 'SPSP'), and the kinetics of this disassembly closely resembled those seen with wild-type lamin B_2 (compare Figures 7A and 4). It is worth emphasizing that the LSPTR mutant resisted disassembly not only when using purified cdc2 kinase (Figure 7A) but also when using whole M phase extracts (Figure 8). While this result does not rigorously exclude the existence in M phase extracts of other kinases capable of disassembling wild-type head-to-tail polymers, it demonstrates that such (hypothetical) kinases would have to act through the LSPTR cdc2 phosphoacceptor site.

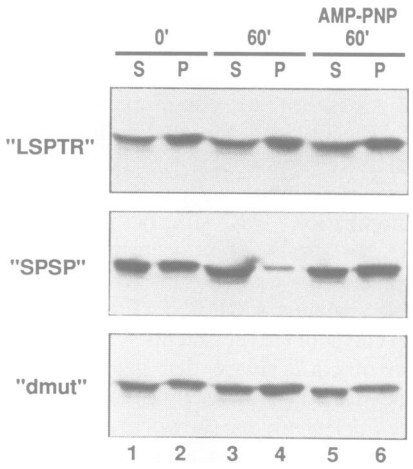

Fig. 8. Disassembly of mutant lamin B_2 polymers by M phase extract. Head-to-tail polymers of the various lamin B_2 mutants were incubated with M phase extract (Nakagawa *et al.*, 1989), in the presence of either ATP (lanes 1–4) or the non-hydrolysable ATP analogue AMP-PNP (lanes 5 and 6). Aliquots were removed at the times indicated, and the partitioning of the lamin B_2 mutant proteins between supernatant (S) and pellet (P) fractions was analysed by centrifugation and immunoblotting.

The above results were confirmed and extended by electron microscopic analysis (Figure 7B). Head-to-tail polymers were formed starting with both wild-type and dmut lamin B_2 proteins (not shown). These polymers were then incubated in parallel with cdc2 kinase, in the presence of ATP. As shown in Figure 7B, dmut polymers resisted disassembly (Figure 7B, panel a), whereas the wild-type lamin B_2 control samples disassembled as expected (Figure 7B, panel b). This result virtually eliminates the caveat that addition of cdc2 kinase to dmut polymers might have induced

non-specific aggregation of the mutant lamin proteins. Instead, it demonstrates that the observed lack of disassembly of dmut polymers (Figures 7A and 8) results from the inability of cdc2 kinase to phosphorylate the mutated M phase specific phosphoacceptor sites.

Discussion

Using polymers reconstituted *in vitro* from bacterially expressed wild-type and mutant chicken lamin B_2 proteins, we have studied the role of cdc2 kinase in controlling the assembly state of nuclear lamins. We show that phosphorylation of mitotic phosphoacceptor sites in lamin B_2 causes disassembly of longitudinal head-to-tail polymers into dimers, but does not disrupt dimers. The effect of phosphorylation by cdc2 kinase is reversible in that subsequent dephosphorylation of lamin dimers by purified phosphatase 1 allows reformation of polymers. By combining the *in vitro* disassembly assay with site-directed mutagenesis of mitotic phosphorylation sites in lamin B_2, we further show that phosphorylation of serine 16, a residue located in the N-terminal end domain of lamin B_2, plays a crucial role in controlling lamin depolymerization. In contrast, phosphorylation of only the SPSP motif C-terminal to the α-helical rod domain does not induce disassembly of lamin B_2 head-to-tail polymers.

Mitotic disassembly of the nuclear lamina network is likely to require disruption of multiple types of protein interactions. These may include both longitudinal and lateral associations between lamin proteins, as well as interactions between lamins and components of the nuclear membrane or peripheral chromatin. It is not possible, at present, to mimic all of these interactions in an *in vitro* system. Our present results provide strong evidence for a role of cdc2 kinase in controlling the disassembly of longitudinal head-to-tail polymers into lamin dimers, but we have no definitive information on the effects of phosphorylation on lateral interactions betwen such polymers *in vitro*. Although highly purified cdc2 kinase could efficiently trigger lamina disassembly in a detergent solubilization assay using whole nuclei (Peter *et al.*, 1990a), it remains possible that mechanisms other than phosphorylation by cdc2 kinase may contribute to control the mitotic disassembly of the nuclear lamina *in vivo*. In particular, Ward and Kirschner (1990) have proposed a participation of other kinases, while Chelsky *et al.* (1987) have attributed an important role to transient demethylation of B-type lamins. With respect to these proposals, it is worth noting that no disassembly of LSPTR mutant lamin polymers occurred in whole cell M phase extracts (Figure 8). Thus, these extracts contain no kinases capable of disassembling lamin head-to-tail polymers through phosphorylation of sites other than serine 16. Also, our results indicate that, at least *in vitro*, lamin assembly-disassembly processes do not require C-terminal modification (i.e. isoprenylation and carboxyl methylation) of lamin B_2 (see also Heitlinger *et al.*, 1991). It would appear more likely, therefore, that cell cycle regulated carboxyl methylation of lamin proteins relates to modulation of their membrane association rather than their polymerization state (Kitten and Nigg, 1991).

Interestingly, *in vitro* phosphorylation of lamin proteins by cdc2 kinase did not affect preformed dimers nor inhibit their formation (see also Heitlinger *et al.*, 1991). This result falls in line with the observation that mitotically disassembled lamin proteins exist predominantly as dimers and/or tetramers *in vivo* (Gerace and Blobel, 1980; Dessev *et al.*, 1990). We also note that the mitotic sites phosphorylated by cdc2 kinase could be dephosphorylated by phosphatase 1, allowing *in vitro* disassembled lamin dimers to reassemble into polymers. Whether or not a type 1 phosphatase plays a similar role *in vivo* remains to be determined, but it is intriguing that genetic evidence indicates a requirement for phosphatase 1 for exit from mitosis (Doonan and Morris, 1989; Ohkura *et al.*, 1989; Axton *et al.*, 1990). Thus, a type 1 phosphatase is active *in vivo* at a time when lamins are dephosphorylated and the nuclear envelope reassembles.

In addition to serine 16, we have detected a second cdc2 phosphoacceptor site within the N-terminal end domain of lamin B_2. This site most probably corresponds to threonine 13, and we note that phosphorylation of the corresponding residue was detectable also in mitotic cells *in vivo* (Peter *et al.*, 1990a, M.Peter and E.A.Nigg, unpublished results). Considering that both serines 384 and 386 within the C-terminal SPSP motif are phosphorylated *in vivo* (K.Weber, M.Peter and E.A.Nigg, unpublished results; see also Ward and Kirschner, 1990), it would seem that the central α-helical rod domain of lamin B_2 is flanked, on either side, by *two* closely spaced phosphorylation sites. However, only the residues corresponding to the major phosphorylation sites (i.e. serine 16 and serine 384 or 386 in the chicken lamin B_2 sequence) were conserved during evolution in all lamin proteins sequenced to date (Höger *et al.*, 1988, 1990; Peter *et al.*, 1989; Vorburger *et al.*, 1989b).

Using a transient transfection assay, Heald and McKeon (1990) found that mutation of the N-terminal serine 22 in human lamin A (i.e. the residue corresponding to serine 16 in chicken lamin B_2) efficiently prevented mitotic lamina disassembly. Mutations of only C-terminal phosphoacceptor sites did not render lamin A resistant to disassembly, but they enhanced the effects of N-terminal mutations (Heald and McKeon, 1990). Interestingly, small deletions removing either the major N- or C-terminal phosphoacceptor site produced significantly greater effects than mere point mutations creating non-phosphorylatable residues. These findings may be rationalized by our observation that two closely-spaced phosphoacceptor sites exist on either side of the central rod domain. Whereas the reported point mutations destroyed only one of these sites on either side, the deletions would be expected to abolish both sites.

To the extent that they emphasize the central role of the N-terminal serine 16 of lamin B_2 in controlling lamina disassembly, our present results are in excellent agreement with those of Heald and McKeon (1990). However, our studies did not reveal a major contribution of C-terminal phosphoacceptor sites to lamin disassembly. Conceivably, phosphorylation of these latter sites might be related predominantly to the control of lateral interactions between head-to-tail polymers, and effects of mutations of these sites might have escaped detection in our assay system. Alternatively, however, it is possible that *in vivo* phosphorylation of sites located C-terminal to the central rod domain might predominantly influence interactions between lamins and other proteins e.g. components of the nuclear membrane or chromatin-associated proteins.

The N-terminal end domain has previously been implicated in lamin–lamin interactions (Georgatos *et al.*, 1988) as well

as in the formation of cytoplasmic intermediate filaments (e.g. Traub and Vorgias, 1983; Geisler and Weber, 1988; Chou *et al.*, 1989). Most interestingly, a recent study indicates that mitotic disassembly of vimentin filaments is likely to result from phosphorylation of the vimentin N-terminus by cdc2 kinase (Chou *et al.*, 1990). The precise physiological phosphoacceptor site(s) on vimentin have not yet been reported, but they are known to lie within an N-terminal 12 kd fragment (Chou *et al.*, 1990). Within this region vimentin does contain a potential phosphoacceptor site for cdc2 kinase (Wood *et al.*, 1989), but it is remarkable that this site does not correspond in position to those present in lamin proteins, suggesting independent evolution. Other members of the cytoplasmic intermediate filament protein family (e.g. keratins) do not contain obvious cdc2 kinase sites (Conway and Parry, 1988), and it is an interesting question how mitotic reorganization of such filament systems is controlled. *In vitro* studies on desmin and vimentin filaments have shown that phosphorylation of N-terminal end domains by either protein kinase A or C could cause filament disassembly or interfere with filament formation, but a physiological role of these kinases during mitosis appears unlikely (Inagaki *et al.*, 1987; Evans, 1988; Geisler and Weber, 1988). In this context, we emphasize that lamin B_2 polymers could not be disassembled by protein kinases A or C, supporting the notion that disassembly of lamin polymers is a specific consequence of phosphorylation at mitotic phosphorylation sites (Peter *et al.*, 1990a, and unpublished results).

Multiple lines of evidence now indicate that cdc2 kinase could control mitotic lamina disassembly by directly phosphorylating lamin proteins. First, highly purified cdc2 kinase induces lamina disassembly when incubated either with isolated nuclei (Peter *et al.*, 1990a; Dessev *et al.*, 1991) or with head-to-tail polymers reconstituted *in vitro* from bacterially expressed chicken lamin B_2 (this study). Second, mutation of the major mitotic phosphoacceptor sites of cdc2 kinase confers resistance to lamina disassembly, both *in vivo* (Heald and McKeon, 1990) and *in vitro* (this study). Third, genetic evidence obtained from a fission yeast strain expressing chicken lamin B_2 strongly suggests a direct interaction between cdc2 kinase and lamin proteins (Enoch *et al.*, 1991). While it is premature to exclude the possibility that kinases other than cdc2 might also act on the mitotic phosphorylation site corresponding to Ser16 in lamin B_2, we emphasize that such hypothetical kinases would have to be indistinguishable from cdc2 with respect to both cell cycle activation and substrate site specificity.

Materials and methods

Site-directed mutagenesis and plasmid constructions

Mutations of the major mitotic phosphorylation sites were introduced into a lamin B_2 cDNA which contained an *NdeI* restriction site at the ATG start codon (Vorburger *et al.*, 1989b; Heitlinger *et al.*, 1991). Mutations were constructed by oligonucleotide directed mutagenesis (Kunkel *et al.*, 1987), using the Muta-gene kit provided by BioRad, and checked by sequencing. The mutated cDNAs were subcloned into the prokaryotic expression vector pAR3038 (Rosenberg *et al.*, 1987) and plasmids transformed into *E.coli* JM109. Recombinant DNA procedures were performed by standard techniques (Sambrook *et al.*, 1989).

Expression and purification of chicken lamin B_2

Expression and purification of wild-type and mutant chicken lamin B_2 proteins were done exactly as described previously (Heitlinger *et al.*, 1991). All mutant proteins were expressed at similar levels and behaved like wild-type lamin B_2 through all purification steps.

Immunochemical techniques and in vitro phosphorylation assays

Immunoprecipitations using the monoclonal antibody E-3 (Lehner *et al.*, 1986) were performed as described previously (Nakagawa *et al.*, 1989), except that, prior to the addition of primary antibodies, extracts were pretreated for 30 min with goat anti-mouse IgG coupled to agarose (Sigma), to remove proteins binding non-specifically. Immunoblotting was carried out as described by Borer *et al.* (1989).

Recombinant lamin B_2 to be used for *in vitro* phosphorylation reactions was immunoprecipitated from bacterial lysates. These were prepared by sonication of *E.coli* in phosphate-buffered RIPA (1% Triton X-100, 1% deoxycholate, 0.1% SDS, 20 mM sodium phosphate pH 7.2, 100 mM NaCl, 20 mM NaF, 0.02% NaN_3, 0.1 mM PMSF, 1% Trasylol). After immunoprecipitation, samples were washed 4 times in RIPA buffer, twice in appropriate kinase assay buffers, and then subdivided into aliquots. One aliquot was routinely used for controlling the efficiency of immunoprecipitations by immunoblotting.

The conditions for phosphorylating lamin proteins with cdc2 kinase were described previously (Peter *et al.*, 1990b). In the case of immunoprecipitated lamins, reactions were carried out at 30°C in the presence of 10 μCi[γ-^{32}P]ATP in final volumes of 50 μl. After 15 min the immunoprecipitates were washed three times with ice-cold RIPA buffer, once with 50 mM Tris–HCl pH 7.5, 1 M NaCl, 0.5% Triton X-100, and twice with PBS. Then, phosphorylated proteins were eluted from the immunobeads with 50 μl of gel sample buffer, boiled for 3 min and analysed by SDS–PAGE and autoradiography.

For phosphorylation of purified lamin B_2 proteins, these were dialysed into appropriate kinase buffer (Peter *et al.*, 1990b). After incubation of ~2 μg substrate with 0.5 μl cdc2 kinase (100 pmol/min/μl) for 15 min at 30°C, reactions were stopped by addition of gel sample buffer. Samples were boiled for 3 min and analysed by SDS–PAGE and autoradiography. Peptides used for *in vitro* phosphorylation reactions were prepared by solid phase synthesis and phosphorylated as described (Peter *et al.*, 1990a).

Tryptic phosphopeptide mapping and phosphoamino acid analysis

Two-dimensional analyses of tryptic phosphopeptides and phosphoamino acid analysis were carried out as described previously (Peter *et al.*, 1990a). Individual phosphopeptides were eluted from TCL-plates as described by Krek and Nigg (1991).

Lamin assembly–disassembly assays

Purified lamin B_2 proteins were assembled into head-to-tail polymers as described in detail elsewhere (Heitlinger *et al.*, 1991). For each reaction, 50 μl of purified lamin B_2 (~0.5 mg/ml) were dialysed for 3 h against equilibration buffer (25 mM Tris pH 8.5, 150 mM NaCl, 1 mM EGTA, 1 mM DTT), followed by dialysis for 1 h against filament buffer (25 mM MES pH 6.5, 150 mM NaCl, 1 mM EGTA, 1 mM DTT). Then, the $MgCl_2$ concentration was adjusted to 10 mM and ATP (or AMP-PNP) was added to 1 mM final concentration. Disassembly reactions were started by addition of purified cdc2 kinases or 20 μl M phase extract (Nakagawa *et al.*, 1989), and incubations were carried out at 30°C. After various time periods aliquots were removed and placed on ice, reactions were stopped by adding EDTA to 10 mM final concentration, and samples were either centrifuged for 1 h (4°C, 100 000 *g*, Beckman TL-100.2 rotor) for sedimentation analyses, or prepared for electron microscopy (see below). Supernatant and pellet fractions were taken up in gel sample buffer and the partitioning of lamin B_2 was analysed by SDS–PAGE, followed by either Coomassie blue staining or immunoblotting.

Phosphatase treatments

To determine activity of phosphatase 1 for a lamin substrate, purified wild-type or mutant lamin B_2 proteins were phosphorylated with cdc2 kinase in the presence of 10 μCi[γ-^{32}P]ATP. The samples were then diluted with 50 vols of phosphatase buffer A (50 mM Tris–HCl pH 7.5, 1 mM EDTA, 0.5% β-mercaptoethanol). 2 μl of purified phosphatase 1 were diluted with 9 vols of substrate buffer (50 mM Tris pH 7.5, 1 mM EDTA, 0.1% β-mercaptoethanol) containing 1 mg/ml BSA. Then 20 μl of substrate buffer containing 3 mM $MnCl_2$ were added and the phosphatase was preincubated for 2 min at 30°C. The dephosphorylation reaction was started by adding 20 μl of phosphorylated lamin B_2 substrate to the pre-treated phosphatase, and incubation was carried out at 30°C. After various time periods aliquots were removed, mixed with gel sample buffer, boiled for 5 min and analysed by SDS–PAGE and autoradiography.

To study the effect of phosphatase 1 on disassembled lamins, lamin B_2 head-to-tail polymers were depolymerized by phosphorylation with cdc2 kinase, as described above, and the depolymerized sample was dialysed for 1 h against phosphatase buffer A. $MnCl_2$ was then added to a final concentration of 1 mM and aliquots of the sample were incubated in equal volumes of phosphatase A buffer, in the presence or absence of phosphatase 1. Both samples were incubated for 1 h at 30°C, then dialysed for 1 h against polymerization buffer (Heitlinger *et al.*, 1991) and centrifuged as described above. Pellet and supernatant fractions were taken up in gel sample buffer and the partitioning of the lamin B_2 protein analyzed by immunoblotting.

Electron microscopy

Glycerol spraying/low-angle rotary metal shadowing was performed as described by Heitlinger *et al.* (1991). Even though no micrographs of negatively stained material are shown in this paper, negatively stained specimens were prepared and used for controls [e.g. to check the assembly properties of the different phosphoacceptor mutants (filaments, paracrystals etc.)]. Specimens were examined in a Hitachi H-7000 transmission electron microscope operated at 100 kV. Electron micrographs were recorded on Kodak SO-163 electron image film at nominal magnifications of either 20 000× or 50 000×. Magnification calibration was performed according to Wrigley (1968) using negatively stained catalase crystals.

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