Phosphorylation of Histone H1 through the Cell Cycle of Physarum polycephalum

24 SITES OF PHOSPHORYLATION AT METAPHASE*

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H1 phosphorylation has been studied through the precise nuclear division cycle of Physarum polycephalum. The number of sites of phosphorylation of Physarum H1 is very much larger than the number of sites reported for mammalian H1 molecules which is consistent with the larger molecular weight of Physarum H1. At metaphase all of the Physarum H1 molecules contain 20–24 phosphates. Immediately following metaphase, these metaphase-phosphorylated H1 molecules undergo rapid dephosphorylation to give an intermediate S phase set of phosphorylated H1 molecules containing 9–16 phosphates. Progressing into S phase newly synthesized H1 is phosphorylated and eventually merges with the old dephosphorylated H1 to give a ladder of bands 1–20. By the end of S phase or early G2 phase, there is a ladder of bands 1–16 all of which undergo phosphate turnover. Further into G2 phase the bands move to higher states of phosphorylation, and by prophase all of the H1 molecules contain 15–24 phosphates which increases to 20–24 phosphates at metaphase. These results support the proposals that H1 phosphorylation is an important factor in the process of chromosome condensation through G2 phase, prophase to metaphase.

In the current model for the nucleosome the very lysine-rich histone H1 is located on the periphery of the disc-shaped structure sealing off 2 turns of DNA which are coiled around a core of the histone octamer (1, 2). We have shown that H1 (3–5), H5 (6, 7), and H1* (8) are conformationally homologous and in solution contain 3 well-defined structural domains: (i) a flexible basic N-terminal region; (ii) an apolar central globular region; and (iii) a flexible basic C-terminal half of the molecule. Their central apolar globular regions are all about 80 residues, and this constancy of size, but not of sequence, has been related to their binding site in the nucleosome (8–10) which is probably at the DNA entry and exit points to the nucleosome (9, 10). The flexible N- and C-terminal regions are probably involved in stabilizing the 34-nm supercoil of nucleosomes and generating higher-order chromatin structure, though how they function is not understood even in outline. These regions, particularly the C-terminal domain, are subjected to extensive reversible phosphorylations which must modulate their interactions in chromatin. Using the multinuclear macrolasmodium of Physarum polycephalum which exhibits a very high natural synchrony of nuclear division (2 to 3 min) in a 9-h cycle we have shown that hyperphosphorylation of H1 occurs through G2 phase/metaphase (11, 12). These observations led to the proposal that this G2-phase/metaphase H1 hyperphosphorylation initiated and controlled chromosome condensation though other later events were thought to be involved in the final packaging of the metaphase chromosomes. Similar behaviors of the H1 phosphorylations have also been observed for synchronized CHO cells (13, 14), rat hepatoma cells (15), and HeLa cells (16). Histone H3 also undergoes a late metaphase phosphorylation which has been correlated with chromosome condensation (17). Recently the identification of temperature-sensitive G2 phase cell mutants ts85 and FT210 have added considerable support for an involvement of H1 and H3 phosphorylation in chromosome condensation (18–21); FT210 most probably has a thermolabile defect in H1 kinase at the nonpermissive temperature. Another type of temperature-sensitive mutant tsBN2 exhibits premature chromosome condensation at the nonpermissive temperature. Another type of temperature-sensitive mutant tsBN2 exhibits premature chromosome condensation exactly paralleled by hyperphosphorylation of H1 and phosphorylation of H3 at the mitosis-related sites of phosphorylation (21). Although these recent studies add support to the proposed roles of H1 and H3 phosphorylation in the process of chromosomal condensation, a direct causal relationship remains to be demonstrated.

In a subsequent study of H1 phosphorylation during the P. polycephalum cell cycle (22) where H1 hyperphosphorylation prior to mitosis was observed, it was reported, contrary to all previous studies of a range of cell types, that H1 was not dephosphorylated after mitosis. In earlier studies it has been assumed that the number of sites of mitosis-related phosphorylation of Physarum H1 was similar to the 5 or 6 sites of phosphorylation found for mammalian cells (see Ref. 15). However, a recent characterization of P. polycephalum histones (23) showed that the molecular weight of the H1 was 30,700, some 50% larger than found for H1 molecules from higher eukaryotes. Chymotrypsin digestion of Physarum H1 shows that whereas its N-terminal and central domains are similar in size to those of mammalian H1 the Physarum C-terminal peptide was very much larger and co-migrated with intact calf thymus H1 (24). Because many metaphase-related sites of H1 phosphorylation are in the C-terminal domain (15) these observations raise the distinct possibility that the

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1 H. Yasuda and M. Yamada, unpublished data.
number of sites of phosphorylation of *Physarum* H1 is much larger than found for mammalian H1 molecules. We show here that the number of phosphates and their distribution during the cell cycle are as follows: S phase, 1-20; G2 phase, 1-16; prophase, 15-24; and metaphase, 20-24.

**MATERIALS AND METHODS**

*Growth of Physarum Macroplasmodium and 32P Labeling—Microplasmodia were grown in semi-defined medium (25) with a hematin concentration of 2.5 μg/ml. Exponentially growing microplasmodia were washed twice with water, and 2 ml of settled microplasmodia were transferred onto Whatman No. 50 filter paper, supported by No. 4 filter paper and a metal grid in a Petri dish. Medium was added after 1 h of fusion in the dark. Mitosis was detected by phase-contrast microscopy of smears fixed in ethanol (26). After mitosis 2 or at mitosis 3 the macroplasmodia were frozen in liquid nitrogen. For 32P pulse label, the microplasmadia were grown for 24 h in medium which omitted KH2PO4. After transferring the microplasmodia onto filter paper and fusion, the KH2PO4-depleted medium was added and mitosis 2 was determined. At 6.5 h after mitosis 2, the plasmodia were placed into fresh KH2PO4-depleted medium containing 1 μCi of 32P. After 30 min of label, the plasmodium was shock frozen (“G2 phase”). When early prophase of mitosis 3 was detected (metaphase = 30 min), the plasmodium was labeled for 30 min (“metaphase”); the “S phase” plasmodia were obtained by pulse-labeling plasmodia for 30 min at metaphase plus 30 min. For continuous labeling, microplasmodia were grown in KH2PO4-depleted medium for 24 h. After fusion on the filter paper, KH2PO4-depleted medium as well as 1 μCi of 32P was added. At 6.5 h after mitosis 2 (= G2 phase), metaphase, and at mitosis 3 plus 1 h (= S phase) the synchronized plasmodium were harvested.

*Isolation of Histone H1*—The frozen macroplasmodia were scraped off the filter paper into a 40% guanidine HCl solution (40% guanidine HCl, 50 mM KH2PO4, 50 mM K2HPO4, pH 6.8). Total basic proteins were extracted as described (23), except that a 5% guanidine HCl solution was used to bind the protein to Bio-Rex 70 (cation exchange resin). After elution of the proteins from Bio-Rex 70 with 0.1 M phosphate (Escherichia coli, Worthington, acid-urea-gel sample buffer and the samples loaded on the gel. p1 acid-urea slab gel with 6 M urea (0.75-1.15 vol g). The supernatant was saved and the pellet was re-2.

*RESULTS*

*Physarum* macroplasmodia were harvested in G2 phase, at metaphase, and in S phase. Total basic proteins were extracted. Perochlic acid-extracted histone H1, isolated and characterized as described by Mende et al. (23), was electrophoresed on AU-polyacrylamide gels and silver stained (Fig. 1). The gels (lanes 1-3) show a ladder of bands and up to 24 or 25 bands can be counted over the three lanes. For H1 extracted in G2 phase (M + 6.5 h) there were 15 or 16 bands in a ladder running from band 1; at metaphase, bands 20-24 or 25 were very strong with weak bands 1-3 and 15; at S phase (M + 1 h) a ladder of bands from 1 to 20 was observed with bands 1-3 being more intense than the upper bands. Possibly the weak bands 1-3 in the metaphase H1 (lane 2) resulted from a very small S phase component; either the harvest of metaphase was displaced very slightly into S phase or preparation for S phase is already occurring in the *Physarum* macronucleus at metaphase because *Physarum* has no G1 phase. To identify possible sources of protein contamination, basic proteins were perchoric acid extracted from microplasmodia (lane 4) and total proteins insoluble in 5% perchloric acid but soluble in 0.6 M H2SO4 were extracted in G2 phase (lane 5), at metaphase (lane 6), and in S phase (lane 7) from macroplasmodia. For the microplasmodial H1 a ladder of bands 1-15 or 16 is observed with bands 7-9 being more intense which may be a contaminant of the residual proteins. To identify sources of protein phosphorylation, other than histone H1, autoradiographic patterns were obtained from 32P pulse-labeled acid-extracted core proteins at G2 phase (lane 8), metaphase, (lane 9), and S phase (lane 10). Just one of these bands, under the conditions of the pulse-labeled experiment, shows a cell cycle specific phosphorylation which migrates to a position corresponding to H1 bands 17 and 18.

Two questions arise from the above data. The first question concerns whether all of the changes in the gel patterns result from protein phosphorylation and the second raises the possibility that some of the observed complexity may be due to H1 primary sequence variants. Fig. 2A shows acid-urea gels of H1 isolated from macroplasmodia arrested at G2 phase (lane 2), early prophase (lane 3), metaphase (lane 4), and S phase (lane 5). Lanes 3P and 4P show the effect of alkaline phosphatase on early prophase H1 and metaphase H1, respectively. This treatment reduces the early prophase H1 bands 15-24 to a single band, and the same effect is observed for the metaphase 18-24 bands. In the gels of Fig. 2, equal volumes of the different phosphorylated states of H1 (lanes 2, 3, and 4) and of phosphorylated H1s plus alkaline phosphatase (lanes 3P and 4P) were applied. These corresponded to 100 μg of H1 for lanes 2, 3, and 4 and 50 μg of H1 for lanes 3P and 4P. For lane 3, the integrated area corresponding to 100 μg of H1 was 8.74 mm2, whereas for lane 3P the integrated area corresponding to 50 μg of H1 was 4.41 mm2. Normalizing to 100 μg of protein these areas are almost identical. For lane 4 the integrated area was 6.90 mm2 (100 μg of H1) whereas for lane 4P the integrated area was 3.33 mm2 (50 μg of H1). Again normalizing to 100 μg of protein the areas are found to be identical to within 3 to 4%. For both lanes 3P and 4P the same amount of H1 was recovered following alkaline phosphatase treatment. Thus there are no primary sequence variants of *Physarum* H1, and its cell cycle complexity shown in Fig. 1 can be attributed to H1 phosphorylation.

Fig. 2B shows the AU region of a 7.5% polyacrylamide gel containing SDS (28) of H1 samples isolated from G2 phase (lane 2), early prophase (lane 3), metaphase (lane 4), and S phase (lane 5). Although in the SDS gels the behaviors of H1 are less pronounced, in the SDS gels they exactly parallel...
HI Phosphorylation through the Physarum Cell Cycle

**FIG. 1.** Separation of Physarum H1-HMG proteins and core proteins on AU gels. Lanes 1–4, perchloric acid-extracted silver-stained H1-HMG proteins from macroplasmodia at G2 phase (lane 1), metaphase (lane 2), S phase (lane 3), and from microplasmodia (lane 4). Lanes 5–7, nuclear proteins extracted with 0.6 N H2SO4 after perchloric acid extraction of H1-HMG proteins and silver-stained (lanes 8–10) autoradiographs of these gels following 32P pulse label. Lanes 5 and 8, G2 phase; lanes 6 and 9, metaphase; and lanes 7 and 10, S phase.

**FIG. 2.** Separation of perchloric acid-extracted Physarum proteins on AU gels (A) and on SDS gels (B and C). A, silver-stained proteins in the H1 region of the AU gels are shown; microplasmodal H1 (lane 1), G2-phase (lane 2), early prophase (lane 3) and early prophase after alkaline phosphatase treatment (lane 3P), metaphase (lane 4) and metaphase after alkaline phosphatase treatment (lane 4P), S phase (lane 5). B, Coomassie Brilliant Blue-stained proteins in an SDS gel. 2, G2 phase; 3, early prophase; 4, metaphase; 5, S phase. Only the H1 region is shown. C, Coomassie Brilliant Blue-stained Physarum H1 and its chymotrypsin digestion products, H1 COOH and H1 NH2.

those of the same H1 samples on the long acid-urea gels; the higher the phosphate content the lower the mobility and the more complex the behavior the broader the band. It can be seen in Fig. 2B (lane 5) that although the S phase H1 band is broad reflecting the complexity of S phase H1 phosphorylation there is no component in this lane which co-migrates with the metaphase H1 phosphorylation. This is also seen in the acid-urea gels of Fig. 2A (lane 5). From this data it seems clear that there is not a metaphase-phosphorylated H1 component resistant to dephosphorylation in S phase as was previously reported (22).

Chymotryptic digestion of bovine H1 results in an initial cleavage of the peptide bond C-terminal of phenylalanine 106 in the globular domain of the molecule (24). The N-terminal peptide 1–106 and the C-terminal peptide 107–216 have been identified. Chymotryptic digestion of Physarum H1 also results initially in 2 peptides, one of which co-migrates with the bovine N-terminal peptide and has been identified as the N-terminal region of Physarum H1; the other peptide co-migrates close to the intact bovine H1 and has been identified as the C-terminal peptide of Physarum H1 (24). These data show that the N-terminal and central globular regions of Physarum H1 are very similar in size to those of bovine H1. The 50% increased molecular weight of Physarum H1 is accommodated by an increase in the size of the C-terminal domain such that it is at least double the size of the bovine C-terminal domain. Most of the mitosis-related phosphorylation of mammalian H1 is located in the C-terminal domain of the molecule (15). In gels of the chymotryptic fragments of Physarum H1 (Fig. 2C) the cell cycle complexity in H1 phosphorylation resides largely in its C-terminal domain. Presumably the large increase in the number of sites of Physarum H1 phosphorylation is required by the large increase in the size of the C-terminal domain.

Fig. 3 gives densitometer scans of Physarum H1 S phase, early prophase, metaphase, and G2 phase. These scans show both the high levels and complexity of S phase H1 phosphorylations. Each inflection and small peak in these scans is a
distinct band visible in the gels. In S phase the highest levels of phosphorylations are in the lower bands and give a peak at band 7; a second group of bands appears to give a peak at band 13. As the macroplasmodium progresses into G2 phase the lower states of H1 phosphorylation must be further phosphorylated to give a group of bands of phosphorylated H1 with a peak at band 13. In early prophase (e.g., 25 min before metaphase) histone H1 moves to higher states of phosphorylation to give a group of bands between 15 and 24 phosphorylations with a peak of °P label at 22 phosphates. At metaphase the overall levels of phosphorylation increase from this to give a group of bands between 20 and 24 with a peak at 23 phosphates. In this metaphase scan and possibly in the early prophase scan there is also a small component of S phase H1 phosphorylation. In the S phase scan there is no component of the metaphase H1 phosphorylation pattern.

°P pulses were applied to macroplasmodia 30 min prior to harvest at M + 6.5 h (G2 phase), M, and M + 1 h (S phase). The silver stained gels are shown in Fig. 4: lane 1 (G2 phase), lane 2 (M), and lane 3 (S phase) with the corresponding °P autoradiographs in lanes 4, 5, and 6. By comparison with the early prophase H1 (M - 30 min) (Fig. 2, lane 3) and the other metaphase H1 (Fig. 2, lane 4) it can be concluded that the metaphase H1 of Fig. 4, lane 2, has been harvested late and is probably a few minutes into S phase. This lane shows the presence of an S phase H1 component, bands 9-16, as well as the metaphase bands 20-24. This is fortunate because the °P autoradiograph (lane 5) shows that °P turnover in the 30 min prior to metaphase is located in bands 17-24. Thus bands 9-16 in lane 2 must contain cold phosphate and result from the dephosphorylation of previously phosphorylated H1, i.e., old H1. It follows further either that the °P label incorporated in H1 during the 30 min prior to metaphase labels specific metaphase sites and is the first to be removed in early S phase or that H1 phosphorylated early, i.e., before pulse labeling, is dephosphorylated early. Either interpretation implies nonrandom patterns of H1 phosphorylation. The pattern of S phase H1 bands, lane 3, is very similar to that shown for another S phase H1 (Fig. 1, lane 3) with bands 1-3 and bands 9-17 staining strongly. The corresponding °P label autoradiograph (lane 6), however, shows that the lower bands 1-3 are very strongly labeled whereas the upper bands are only weakly labeled. Further, the weak °P label does not correspond directly with the stained bands (lane 3) but appears to be displaced to higher band numbers; stained bands 9-13 contain lower levels of °P label than bands 14-22. There appears to be three patterns of behavior: (i) rapid °P turnover in the lowest bands 1-3 which are the newly synthesized H1 being phosphorylated; (ii) an intermediate group of bands 9-16 which result from the dephosphorylation of old H1 containing cold phosphate; and (iii) this intermediate group of bands overlaps with another group of bands 14-22 which result from old H1 containing higher levels of °P. H1 isolated in G2 phase (M + 6.5 h) gives stained bands 1-16 (lane 1), and the corresponding autoradiograph (lane 4) gives °P-labeled bands 1-16 with the higher levels of label in bands 4-13, particularly bands 4-7. These patterns appear to be the overlap of phosphorylated new H1 and dephosphorylated old H1.

**DISCUSSION**

A summary of the different patterns of H1 phosphorylation through the cell cycle is given in Fig. 5. At metaphase, 20-24 states of H1 phosphorylation are observed, and phosphate turnover occurs in these states. A late metaphase or early S phase harvest shows metaphase bands 19-24 in which phos-
phosphate is turning over, bands 9–16 which contain cold phosphate derived from old H1, and indications of band 1 very weakly labeled. One hour into S phase there are two groups of bands, bands 1–3 strongly labeled and attributed to the phosphorylation of new H1 to low states and a group of bands 9–22 which contain very weak labeling in bands 13–22. Further into S phase there is a set of bands 1-19 which is very similar to the patterns of bands in early G2 phase 5 h after metaphase. One hour further into G2 phase at M + 6.5 h the pattern of bands is beginning to move to higher states of phosphorylation, which then further increases to bands 15–24 in early prophase and to bands 20–24 at metaphase. The high states of Physarum H1 phosphorylation clearly "amplify" patterns of behaviors observed for mammalian cells. The molecular weight of Physarum H1 has been estimated to be 30,700 (23), i.e. about 300 residues. Its amino acid composition contains 6.70 mol % of threonine and 8.71 mol % of serine which corresponds to about 20 threonines and 26 serines, substantially more than is required to accommodate the 24 sites of phosphorylation. The cycle of H1 phosphorylation events appears to be as follows; as the nuclei go from metaphase into S phase the 20–24 phosphorylation states of metaphase H1 are dephosphorylated to intermediate states of S phase H1 containing 9–20 sites of phosphorylation. S phase sites 9–16 contain cold phosphate, and it follows that the incorporation during the 30-min metaphase pulse label must be rapidly removed after metaphase which clearly indicates the presence of a group of metaphase-related phosphorylation sites. One hour into S phase the dephosphorylation of these metaphase sites is largely complete giving "old" S phase H1 with 9–20 phosphates, and newly synthesized H1 with 1–3 phosphates has appeared. This new H1 is further phosphorylated and overlaps with the "old" S phase H1 to give the pattern of bands 1–16 by early G2 phase. Through G2 phase all the H1 molecules, new and old, are increasingly phosphorylated to give the early prophase states of phosphorylation containing 16–23 phosphates and finally the metaphase H1 states of 20–24 sites of phosphorylation.

The behaviors described above provide an explanation for the conclusions of Fischer and Laemmli (22) that metaphase H1 was not dephosphorylated following nuclear division and through S phase and G2 phase newly synthesized H1 was increasingly phosphorylated until it reached the highest levels of phosphorylation at metaphase. These studies were carried out at much lower resolution using SDS gels, and from Fig. 4 (lane 3) it is easy to see that the two bands would be observed corresponding to phosphorylated new H1 and old S phase H1 with 9–16 phosphates. Progressing through the cell cycle the new H1 would be increasingly phosphorylated and merge with the old S phase H1 to give the observed lower resolution behaviors (22). At these lower resolutions it would not be possible to separate the high states of metaphase H1 phosphorylation from the intermediate states.

The role of intermediate S phase phosphorylation is not known though it has been associated with S phase functions. Physarum H1 is very much larger than mammalian H1 and probably requires higher levels of S phase and phosphorylation for its functions, e.g. the redistribution of old H1 and the deposition of new H1 during chromatin replication. It has been proposed that the G2 phase/metaphase H1 hyperphosphorylation initiates and controls chromosome condensation although other later events in G2 phase or prophase may be required to complete final stages of condensation to the metaphase chromosome (11, 12). It can be seen in Figs. 1–4 that all of the H1 molecules are phosphorylated to 20–24 states of phosphorylation at metaphase, and it follows that H1 phosphorylation is a general factor affecting all chromatin associated with H1, i.e. probably inactive chromatin. In the Physarum cell cycle H1 kinase activity increases 17-fold on going from S phase to late prophase; this increase precedes and presumably drives H1 phosphorylation (12). This increase in activity is a kinase activation process (30). As has been pointed out a wide range of cell cycle advancements obtained by the fusions of pairs of Physarum macromplasmodia in different stages of the cell cycle can be predicted by averaging the individual levels of H1 kinase (12), and it has been shown that the addition of heterologous H1 kinase activity advances mitosis by up to 1 h when added 3 h prior to metaphase (31, 32). Thus although a direct causal relationship between H1 phosphorylation and chromatin condensation is still lacking there is an impressive body of evidence supporting these proposals.

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REFERENCES
H1 Phosphorylation through the Physarum Cell Cycle

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