Phosphorylation of Linker Histones by a Protein Kinase A-like Activity in Mitotic Nuclei

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Micronuclear linker histones of the ciliated protozoan, Tetrahymena thermophila, are extensively phosphorylated in vivo. Each of these polypeptides, α, β, γ, and δ, contains sites for phosphorylation by cyclic-AMP dependent protein kinase (PKA) but not Cdc2 kinase, and some data have been presented implicating PKA kinase in their phosphorylation in vitro and in vivo (Sweet, M. T., and Allis, C. D. (1993) Chromosoma 102, 637–647; Sweet, M. T., Jones, K., and Allis, C. D. (1996) J. Cell Biol., in press). In this report we have extended these analyses by showing that Cdc2 and PKA kinase are not evenly distributed between micro- and macronuclei. Macronuclei, but not micronuclei, contain a 36-kDa polypeptide that is immunoreactive with p34cdc2 antibodies. In contrast, a 40-kDa polypeptide is detected with PKA antibodies in micronuclei, that is not detected in macronuclei. In support, extracts from micronuclei, but not macronuclei, contain a kinase activity that resembles some, but not all, characteristics of PKA from other sources. Immunodepletion experiments using anti-PKA antibodies show that a 40-kDa polypeptide can be specifically removed from these extracts with a concomitant loss in kinase activity. Microsequence analyses of δ demonstrate that this linker histone is phosphorylated in vivo on two PKA consensus sequences located in its carboxyl-terminal domain, an optimum PKA consensus sequence, Arg-Lys-Asn-Ser, and a less optimal PKA sequence, Lys-Ser-Ser-Val. Collectively, these results suggest that PKA or a PKA-like kinase is responsible for the phosphorylation of linker histone in mitotically dividing micronuclei. In contrast, macronuclei, which divide amitotically, phosphorylate linker histone H1 using a distinct, Cdc2-like kinase.

A variety of kinases and phosphatases have been shown to play an implicit role in the regulation of fundamental cell cycle processes. For example, a family of closely related cyclin-dependent protein kinases is thought to catalyze a series of phosphorylation events associated with cell-cycle progression (for reviews see Refs. 3–6). During mitosis, several proteins such as linker histones (7, 8), lamins (9), and cytoskeletal proteins (10, 11) undergo stage-specific, reversible phosphorylation. The biological consequences of these phosphorylation events are largely unclear.

It has long been suggested that hyperphosphorylation of the linker histone H1 during mitosis is causally linked to mitotic chromosome condensation (reviewed in Ref. 8), although this relationship remains unproven and controversial (discussed in Refs. 12 and 13). It has also been proposed that phosphorylation of linker histone may act as a first-step mechanism to promote transient decondensation of the chromatin fiber, allowing access of specific factors (such as the SMC family of nonhistone proteins; reviewed in Refs. 13 and 14) in a variety of cell cycle-regulated processes including chromosome condensation (15). Mounting evidence has shown that chromosome condensation can occur in the absence of H1 or H1 phosphorylation in vitro (16–18) and in vivo (12, 19).

The ciliated protozoan, Tetrahymena thermophila, provides an ideal model for unraveling complex relationships between H1 phosphorylation, gene expression, and mitotic chromosome condensation. Each vegetative cell contains two types of nuclei, a somatic macronucleus that divides amitotically and a germ-line micronucleus that divides mitotically. Both nuclei contain linker-associated polypeptides that differ dramatically. Macronuclei, for example, contain a H1 that resembles vertebrate H1s in several properties including growth or division-associated phosphorylation by a Cdc2-like kinase (20). In contrast, micronuclei contains four distinct polypeptides (α, β, γ, and δ) that are also phosphorylated in growing or dividing cells (21). If Cdc2 kinase is solely responsible for linker histone phosphorylation and mitotic chromosome condensation in Tetrahymena, α, β, γ, and δ would be expected to be phosphorylated by this enzyme. However, α, β, γ, and δ do not contain any obvious recognition sequence for Cdc2 kinase (22), and none of these polypeptides are phosphorylated by this kinase in vivo under conditions where macronuclear H1 is extensively phosphorylated (1). Interestingly, all four of these polypeptides contain at least one canonical phosphorylation site for cAMP-dependent protein kinase (PKA) (22), although none of the in vivo sites of phosphorylation have been identified.

In this study, we have continued to explore the relationship between PKA and linker histone phosphorylation in mitotic micronuclei in Tetrahymena with emphasis on δ. Immunoblotting data support the notion that PKA (or a PKA-like activity) exists in micronuclei during most stages of the life of the cell, and antibodies against PKA can immuno-deplete δ kinase activity from micronuclear extracts. Microsequence analyses confirm that δ is phosphorylated in vivo on two serine residues.

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1 The abbreviations used are: PKA, cAMP-dependent protein kinase; HPLC, high performance liquid chromatography; RP-HPLC, reverse phase-HPLC; MOPS, 3-(N-morpholino)propanesulfonic acid; NBS, N-bromosuccinimide; PKI, PKA inhibitor.
embedded in the carboxyl terminus; both sites conform to PKA recognition sites. Collectively, these results suggest that a PKA or a PKA-like kinase is responsible for phosphorylation of δ in micronuclei and support the general hypothesis that PKA kinase(s) play an important, and previously unsuspected, role in mitosis.

**Materials and Methods**

**Cell Culture and Labeling Conditions**

*T. thermophila* strains CU428 (ChxChx-cy-SI/VII) and CU427 (Mpr/Mpr [6 mp-s VI]) were grown in 1% enriched protease peptone under standard conditions as described previously (23). Previous data had shown that δ phosphorylation was maximal in early mating cell cultures, 2–4 h after initiating mating (2). Therefore, 2.5-h mating cultures were utilized in this study. Conjugation was induced according to Bruns and Brussard (24) with modifications described by Allis and Dennison (25). Mating cultures were phosphorylated in vitro by starving cells of each mating type separately at approximately 1–2 × 10⁷ cells/ml in 10 mM Tris, pH 7.4, in the presence of [32P]orthophosphate (12.5 μCi/ml) for 2–3 h before adding 2 × growth medium and growing cultures for at least one generation (2–5 × 10⁶ cells/ml). Labeled cells were then starved and mated as described above.

**Preparation of Nuclei and Nuclear Proteins**

 Macronuclear and micronuclear proteins were isolated as described by Gorovsky et al. (25), except that the nuclei isolation buffer contained 1% 1-iodoacetamide, 1% phenylmethylsulfonyl fluoride, and 10 μM sodium butyrate, but not spermidine. Where indicated, purified preparations of micro- and macronuclear were isolated following sedimentation at unit gravity according to Allis and Dennison (25). In order to better preserve labile phosphorylation modifications, chromemuric-phosphorylsulfonic acid (final concentration 0.1 mM) was added to the cell homogenization buffer. δ was partially purified from sulfuric acid extracts of micronuclei by reverse phase (RP-HPLC using a C8 column (Brownlee)) with a linear gradient of 5–90% acetonitrile containing 0.1% trifluoroaceticacid (changing at a rate of 0.45%/min) with a flow rate of 1 ml/min.

**Preparation of Nuclear Extracts**

 Macroc- and micronuclear extracts from log-phase growing cells were used to prepare active kinase extracts immediately following isolation. Nuclei were washed once in isolation buffer and then resuspended (at 5 × 10⁹ macronuclei/ml and 1 × 10⁹ micronuclei/ml) in 10 × phosphorylation buffer (1 M NaCl, 250 mM MOPS, pH 7.4, 100 mM MgCl₂, 10 mM dithiothreitol, and 0.1% Nonidet P-40). Nuclei were lysed in this buffer for 10 min before a 9-fold volume of cold water was added; the lysate was then vortexed well and then microcentrifuged for 15 min. The clarified supernatant was removed and used immediately or frozen at −20 °C. Extracts prepared in this fashion remained active for kinase activity for up to 1 week at −20 °C.

**In Vitro Phosphorylation**

Enzymes, HeLa Cdc2 kinase, bovine or Paramecium PKA kinase, or crude Tetrahymena micronuclear extracts (extract from approximately 1 × 10⁶ micronuclei/ml) were added to each reaction immediately prior to the addition of [γ-32P]ATP (100–150 units; 1 unit transfers 1 pmol of P/min), as described previously (1). Incubations proceeded at 30 °C for 15 min, and duplicate samples from each reaction were removed, applied to P81 filter paper (Whatman International, Maidstone, United Kingdom), and processed for liquid scintillation counting (26).

**Immunoprecipitation**

Ten microliters of micronuclear extract was incubated with 5 μl of either preimmune serum or immune serum for 2 h with gentle shaking at 4 °C. As a negative control, immune serum was added to buffer without nuclear extract. Protein A-Sepharose (15 μl) was added to each mixture and the incubation continued at 4 °C for 1 h more. Following centrifugation, the unbound supernatant was removed from each incubation mixture, and 5 μl was analyzed in *in vitro* kinase reactions with and without δ as substrate. Immunoblotting analysis was done on an aliquot of the immunoprecipitation supernatant. Protein bound to the Sepharose beads was also analyzed in this fashion after solubilization with Laemmli sample buffer.

**Chemical and Enzymatic Cleavages**

N-Bromosuccinimide—RP-HPLC-purified δ dissolved in 180 ml of 5% acetic acid was cleaved by adding 20 ml of 20 mM N-bromosuccinimide (NBS) freshly dissolved in 5% acetic acid and incubating 2 h at room temperature in the dark (27). The resultant peptides were purified by RP-HPLC using a C18 column with a 0–90% acetonitrile linear gradient as described above. The single *in vivo* phosphorylated NBS fragment from δ, which eluted at approximately 8% acetonitrile, was identified by scintillation counting and gel analysis on 50% acrylamide gels (see below).

Endoprotease Lys-C—In *in vivo* phosphorylated NBS peptide from δ, isolated as described above, or *in vitro* phosphorylated δ or a synthetic δ peptide was cleaved by mixing endoprotease Lys-C (Boehringer Mannheim) with peptide at 1:100 (w/w) in 0.1 M ammonium bicarbonate buffer, pH 9.0, and incubating at 37 °C for 1–2 h. A second equal aliquot of enzyme was then added to the reaction mixture and incubation continued overnight (28). Where indicated, peptides were purified by cation-exchange HPLC using a polyCAT A column (PolyLC Inc.) with a 0–0.3 M NaClO₄ linear gradient (in a 10 mM phosphate buffer, pH 6.5) increasing at a rate of 0.3 M NaClO₄/min. Peptides were also purified by elution with water from acrylamide 50% polyacrylamide gels (29) as described previously (30). Due to the small size and positive charge of several of the δ peptides analyzed in this study, carboxymethylcellulose membrane was chosen for best retention of peptides (31). Peptides were eluted from carboxymethylcellulose membranes for sequencing, as described previously (31). Small peptides were also analyzed by thin layer chromatography (TLC) using a phospho-chromatography buffer (57% 1-butanol, 25% pyridine, and 7% glacial acetic acid) as described by Boyle et al. (32).

**Preparation of Synthetic Peptides and Sequencing**

Peptides used in this study (see Fig. 2 for details) were synthesized by the solid phase procedure on a peptide synthesizer (model 430A; Applied Biosystems Inc., Foster City, CA). Peptides were cleaved from the resin and analyzed by RP-HPLC, amino acid analysis (Pico Tag system; Waters Associates, Milford, MA), and mass spectroscopy analysis (model 477A protein sequencing system; Applied Biosystems Inc.) was used to confirm the sequence of peptides and to identify sites of serine phosphorylation on purified peptides.

**Electrophoresis and Immunoblots**

One-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis systems have been described previously (33, 34). Acrylamide gel electrophoresis of small peptides has also been described previously (29). Immunoblot analyses of SDS-polyacrylamide gels were done as described previously (35). Balanced protein loads of samples were ensured by staining parallel gels of equivalently loaded samples and by staining immunoblots directly with Ponceau red stain. Immunoreactivity was detected by alkaline phosphatase-conjugated secondary antibodies or by chemiluminescence as indicated. Polyclonal antiserum against the catalytic subunit of PKA was prepared as described (36). Antibodies against yeast p34cdc2 and macronuclear cdc2 were a generous gift from the Beach laboratory.

**Results**

Unexpected Partitioning of Cdc2 and PKA between Micro- and Macronuclei—The unique partitioning of mitosis-related events to micronuclei along with a specialized set of linker-associated polypeptides, α, β, γ, and δ, raises the question which kinase is responsible for their phosphorylation *in vivo*. If, as suggested by our earlier studies (1), PKA or a PKA-like activity is responsible for micronuclear linker histone phosphorylation, we reasoned that the catalytic subunit of this kinase should be present in micronuclei. When an antibody raised against the catalytic subunit of PKA from Paramecium is used to probe a blot of total nuclear protein from extensively purified micro- and macronuclei, a single, strongly immunoreactive band with an apparent molecular mass of 40 kDa is observed in lanes containing micronuclear protein (Fig. 1A). Unexpectedly, at all stages of the life cycle examined (growing, starved, and 2 h mating), macronuclei contain little or no detectable PKA catalytic subunit.

Opposite results are obtained when identical samples are probed with antibodies raised against yeast recombinant p34cdc2.
Incorporation of [32P]phosphate is observed when the synthetic bovine catalytic PKA are shown in Fig. 3. Significant phosphorylation with macro- and micronuclear extracts and purified bovine PKA subunit. In contrast, the synthetic H1 peptide is not a good substrate for either of these kinase reactions, although this peptide is an excellent substrate with purified preparations of human Cdc2 kinase (data not shown). This result is consistent with our inability to detect p34^cdk2 in micronuclei (Fig. 1B). In agreement with our previous studies (20), a 36-kDa polypeptide is detected in micronuclear samples when probed with an antibody-generated yeast p34^cdk2. Surprisingly, this polypeptide is not detected in micronuclear isolated from the same cells. Thus, macronuclei and micronuclei are distinguished not only by distinct, non-overlapping linker histones, but also by the kinases that might be responsible for phosphorylating them.

**Micronuclear Extracts Phosphorylate Synthetic δ Peptide in Vitro**—Previous experiments suggested that δ, the smallest of the micronuclear linker polypeptides, was phosphorylated in vivo on at least one serine located in the carboxy-terminal third of the protein (1). It was suggested that PKA or a PKA-like kinase was responsible for this phosphorylation since the catalytic subunit of bovine PKA also phosphorylated the same CNBr-generated peptide in vitro. As shown in Fig. 2A, three putative sites for PKA phosphorylation are contained in the carboxy terminus of δ. Two serines matching the most stringent PKA consensus sequence (Arg-Arg/Lys-Xxx-Ser, underlined) are shown as black boxes; one serine contained within a less stringent consensus sequence (Arg-Xxx-Xxx-Ser) is indicated as an open box. These two classes of PKA consensus motifs are utilized in the majority of the PKA target sequences (37).

In order to determine whether macro- or micronuclei contain kinase activities capable of phosphorylating PKA or Cdc2 substrates, a salt extract was prepared from each type of nuclei and tested with either of two contrasting peptides. One peptide, containing the three putative PKA sites described above, was synthesized for use as a model PKA substrate (δ peptide, Fig. 2A); a second peptide, containing two consensus sequences for Cdc2 kinase from macronuclear H1 (H1 peptide, Fig. 2B), was synthesized for use as a model Cdc2 substrate. In vitro phosphorylation with macro- and micronuclear extracts and purified bovine catalytic PKA are shown in Fig. 3. Significant incorporation of [32P]phosphate is observed when the synthetic δ peptide is used as a substrate with either crude micronuclear extract or purified bovine catalytic PKA. In contrast, the synthetic H1 peptide is not a good substrate for either of these kinase reactions, although this peptide is an excellent substrate with purified preparations of human Cdc2 kinase (data not shown). This result is consistent with our inability to detect p34^cdk2 in micronuclei (Fig. 1B).

To determine if the PKA activities assayed in Fig. 3 display properties characteristic of PKA in higher organisms, parallel studies were repeated in the presence of the well known, competitive inhibitor of mammalian PKAs (PKI; Ref. 38). Unlike bovine PKA, which is clearly inhibited by this peptide, neither purified Paramecium (39) nor crude Tetrahymena PKA activity is inhibited by 5 mM PKI (Fig. 3). These data suggest that ciliate PKAs as well as PKAs from other sources (40) differ significantly from PKA in higher organisms. Another hallmark of PKA is its dependence on cAMP. While cAMP reproducibly increases the kinase activity of the crude micronuclear extract...
Gardles of the source of PKA, micronuclear extracts or purified acrylamide gel and identified by autoradiography (Fig. 5). Re-identical conditions. Phosphopeptides resulting from these di-
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PKA from Paramecium (data not shown) or bovine heart, the phosphopeptide maps produced are essentially identical when assayed in this fashion. In addition, these kinases produce identical phosphopeptide maps when either synthetic δ peptide (Fig. 5, lanes 2 and 3) or intact δ (data not shown) are used as in vitro substrates.

A comparison of in vivo versus in vitro phosphorylated peptides was then performed to determine whether in vitro phosphorylation of δ peptide produces a similar map as δ phosphorylated in vivo. δ, labeled in vivo with [32P]orthophosphate, was recovered by RP-HPLC and cleaved with Lys-C endopepti-
dase. Digestion products were then analyzed as above. These results demonstrate that the in vivo phosphopeptide map of δ (Fig. 5, lane 1) is comparable with, but not identical to, that of δ phosphorylated in vitro. Interestingly, not all of the peptides are phosphorylated to the same extent. In all cases tested, in vitro and in vivo, peptide c is labeled to the greatest extent. Peptide a (variable in different digests, see below) is phosphorylated to a lesser extent, while peptide(s) b, which resolve to varying extents, is typically poorly phosphorylated. To confirm that each of the above peptides with similar acid-urea poly-
acylamide gel mobilities are indeed identical peptides, the bands were excised from the gel and analyzed by thin layer chromatography. In all cases phosphopeptides with the same electrophoretic mobility on the acid-urea gel had the same mobility on the thin layer chromatography plates, and those with different electrophoretic mobilities also differed in chro-
matographic mobility (data not shown).

PKA Site Utilization on δ Is Ordered in Vivo—Phosphory-
lated isoforms of linker histones migrate with a reduced mobility on SDS gels (for example, see Ref. 41). Previous experiments suggested that, like macronuclear H1, the mobility of δ on an

SDS gel is reduced by phosphorylation and that δ is maximally phosphorylated during early stages of the sexual pathway, conjugation (2). As shown in Fig. 6A, three distinct bands of δ are well resolved when HPLC-purified δ from 2.5-h mating cells

FIG. 4. Immunoprecipitation of PKA and immunodepletion of kinase activity from micronuclear extracts. A, micronuclear extracts were incubated with preimmune serum (lane 1) or antibodies against the catalytic subunit of Paramecium (lane 2). Extracts prior to immunoprecipitation (Extr.) and a reaction in which extract was not added (lane 3) were also analyzed for the presence of the 40-kDa immuno-reactive protein. Immunoblots of corresponding supernatants and immunoprecipitates are shown. B, in vitro analysis of kinase activity remaining in the supernatant after above immunoprecipitations. by about 20%, it does not show a significant requirement for cAMP. The significance of this result is not clear, but may be due to the high salt extraction conditions used to prepare the extract.

Immunodepletion of Kinase Activity from Micronuclear Extract—Antibodies against the Paramecium catalytic subunit were used to precipitate PKA from crude micronuclear extracts (Fig. 4A). A 40-kDa polypeptide is precipitated with the anti-

PKA (lane 2), but this peptide is not precipitated with preim-
mune serum (lane 1) or when extract is not added to the immune reaction (lane 3). Supernatants from these precipita-
tion were then assayed for kinase activity as described above. Preimmune sera has little effect on the kinase activity of the supernatant, while the supernatant from the immune reaction shows an 80% decrease in kinase activity (Fig. 4B). These immunodepletion data are consistent with the suggestion that PKA or a PKA-like kinase is responsible for δ phosphorylation in micronuclei.

Mapping of Phosphorylation Sites in δ—The amino acid se-
quence of the carboxyl terminus of δ suggests that there are three potential phosphorylation sites that could be utilized by PKA. Shown in Fig. 5 (top) are the expected peptides resulting from a limit digestion of synthetic δ peptide with lysine-specific (Lys-C) endopepti-

nase. If δ is phosphorylated by PKA using optimal PKA recognition motifs, we predicted that two [32P]-labeled peptides would be produced following Lys-C endopepti-
dase digestion, one with a single labeled serine, Asn-Ser-

Thr-Ser-Lys, and a second with two potential phosphoserines, Arg-Arg-Ser-Ser-Ser-Lys (see the serines in black and white boxes in Fig. 5).

To test this prediction, synthetic δ peptide was phosphoryl-
ated in vitro by bovine PKA catalytic subunit or the crude Tetrahymena micronuclear extract and subjected to a limit digestion with Lys-C endopeptinase. In addition, RP-HPLC-purified δ was phosphorylated in vitro and digested under identical conditions. Phosphopeptides resulting from these di-
gestion were then resolved on a long (30 cm) 50% acid-urea acrylamide gel and identified by autoradiography (Fig. 5). Regard-

less of the source of PKA, micronuclear extracts or purified

FIG. 5. Phosphopeptide maps of δ labeled in vivo or synthetic δ peptide labeled in vitro by PKA or micronuclear extracts. Top, amino acid sequence and predicted fragments produced from a limit digestion of synthetic δ peptide with Lys-C endopeptinase (indicated by the diagonal lines). Putative PKA consensus motifs and phosphorylation sites are indicated as in Fig. 2. Bottom, 32P autoradiogram of phosphopeptides resolved in a long (30 cm) 50% acid-urea gel. Synthetic δ peptide was used as a substrate and labeled in vitro with either micronuclear extracts (lane 2) or bovine PKA (lane 3). For comparison, δ labeled in vivo is shown in lane 1. Those peptides in common between in vivo and in vitro labeled substrates are indicated with arrows (a, c) or a bracket (b). Bracket denotes two phosphopeptides that were not always well resolved; peptide a radiolabel intensity varied from one digestion to the next.
In vivo labeled δ was electrophoresed in a 30-cm-long 10% SDS-polyacrylamide gel and resolved into three bands denoted slow (S), medium (M), and fast (F). The identity of each of the three bands as δ was confirmed by immobility using a general anti-δ antibody (data not shown). The corresponding 32P autoradiograph of this gel is shown immediately to the right. Densitometric analysis of the stained gel and autoradiograph suggested that the relative specific activity of the slow isoform was twice that of the medium isoform; no 32P label is associated with the fastest migrating isoform even when the autoradiograph is intentionally overexposed. The two bands labeled S and M were then excised from the gel and cleaved with Lys-C endoproteinase, and digestion products were resolved on an 10 cm acid-urea gel. An autoradiograph of this gel is shown in B. The relative mobility of Lys-C endoproteinase peptides labeled b and c (with arrow) are equivalent to those in Fig. 5 but appear different due to the smaller size of the gel used. In this experiment, no radiolabel was observed at the position of peptide a.

Densitometric analysis of the stained bands and corresponding autoradiograph indicate that the relative specific activity of the slowest migrating band (labeled S) was approximately twice that of the intermediate mobility band (M) (data not shown), suggesting that δ is phosphorylated at two distinct sites during this stage of the life cycle (see below). In contrast, the fastest band (F) is not phosphorylated and most likely represents the dephosphorylated isoform of δ.

In order to determine if phosphorylation of δ occurs in a random or ordered fashion, the labeled bands resolved in Fig. 6A were excised and the individual isoforms extracted. Each band was then separately cleaved with Lys-C endoproteinase, and the digestion products were resolved in a high resolution acid-urea gel. The resulting phosphopeptide map clearly documents a non-random pattern of phosphorylation site utilization in the δ isoforms resolved by SDS-gel electrophoresis. Consistent with the idea that the band labeled M is a monophosphorylated isoform of δ, a single labeled phosphopeptide is observed after Lys-C digestion of isoform M (Fig. 6B, peptide c). In contrast, the slowest migrating species (band S), a putative phosphorylated isoform of δ, produces two labeled peptides (b and c) with similar relative mobility to the peptides labeled b and c in Fig. 5. The identification of these bands was confirmed by in vivo phosphorylation sites in δ—To rigorously determine the phosphorylation sites utilized in δ, labeled in vivo with [32P]orthophosphate, was cleaved with NBS (see Fig. 7A) and the resultant single phosphopeptide was purified by RP-HPLC. Direct microsequence analysis verified that this peptide was indeed the indicated carboxyl-terminal portion of δ (the length of the arrow in Fig. 7A corresponds to the amino acid sequence obtained). During each cycle of microsequencing, an aliquot of each cleaved phenylthiohydantoin-derivative was collected and the presence of 32P was directly determined by scintillation counting. As anticipated, the first indicated serine, contained within the optimal PKA consensus sequence Arg-Lys-Asn-Ser-Val-Ser-Lys (see Fig. 7A, arrow c), is phosphorylated (Fig. 7). Since this peptide was phosphorylated in vivo and in vitro with a wide range of PKAs, including bovine, Paramecium (data not shown), and Tetrahymena (Fig. 5), our data suggest that serine 215, two amino acids removed from a single lysine, can be phosphorylated by a wide range of authentic PKAs. Interestingly, peptide b, the slowest migrating of these peptides, is detected only in band S (Fig. 6B). Collectively, these results suggest that PKA first phosphorylates δ at serine 201 contained in the optimal consensus sequence Arg-Lys-Asn-Ser and then becomes a maximally phosphorylated, dephosphorylated isoform utilizing serine 215 contained in the less optimal PKA consensus sequence Lys-Ser-Ser-Val.

Interestingly, the second "expected" optimal consensus sequence Arg-Arg-Ser-Ser-Ser is not utilized for phosphorylation in vivo or in vitro with any of the substrates that we have tested. However, we found it is possible to phosphorylate this
optimal PKA consensus sequence when the neighboring sequence of the δ peptide is altered. Omission of a single serine (serine 205, see Fig. 7A; a mistake made during the original synthesis of the δ peptide) that lies between the two optimal PKA consensus sites changes the Lys-C phosphopeptide map of δ significantly. Using this “mutated” δ peptide, phosphopeptides a and c are not observed and a new, faster migrating phosphopeptide with the sequence Ser-Arg-Arg-Ser-Ser-Ser-Lys is obtained (data not shown). As expected for a PKA optimal consensus motif, microsequence analysis shows that the second serine following the second arginine is phosphorylated (data not shown). The above second serine is phosphorylated when micronuclear extract, Paramecium PKA, or bovine PKA is used as a source of kinase with the altered peptide substrate, and thus as expected, the second “expected” optimal PKA consensus motif is a good in vitro substrate for most PKAs. However, unexpectedly, the Arg-Arg-Ser-Ser-Ser-Ser-209 peptide (Ser-209 underlined) motif is not utilized when Ser-205 is present in the δ model peptide substrate. These results suggest that conformation of δ plays an important role in determining which sites of phosphorylation are utilized in δ in vitro and in vivo.

**DISCUSSION**

One of the more unexpected results to emerge from this study is the clear and non-overlapping partitioning of PKA and Cdc2 kinase catalytic subunits between micro- and macronuclei of *Tetrahymena*, respectively. Several lines of evidence suggest that PKA or a PKA-like kinase, but not Cdc2, is responsible for phosphorylation of δ (and likely other linker histones) in mitotic micronuclei. First, micronuclei, but not macronuclei, contain a 40-kDa polypeptide that is immunoreactive with antibodies raised against the catalytic subunit of Paramecium PKA. Given the clear evidence documenting the regulation of transcription by phosphorylation (42) and, in particular, the involvement of PKA in a subset of these phosphorylation events (43, 44), the complete absence of PKA catalytic subunit in transcriptionally active macronuclei is striking and unexpected. Equally remarkable is the absence of p34<sup>cdc2</sup> in micronuclei, given the suspected role of this kinase in a variety of mitosis-associated events (3).

Second, micronuclear extracts and more purified PKAs from Paramecium and bovine heart produced essentially identical in vitro phosphopeptide maps with two in vitro substrates, δ or a synthetic δ peptide spanning all known potential PKA phosphorylation sites in δ (1). Importantly, phosphopeptide maps obtained with all three sources of PKA are comparable with that obtained with in vivo phosphorylated δ. Third, antibodies against the PKA catalytic subunit immunoprecipitated a 40-kDa protein from micronuclear extracts, and this immune serum, but not preimmune serum, depleted kinase activity from crude micronuclear extracts. Finally, two in vivo sites of phos-

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**FIG. 7. Identification of in vivo phosphorylation sites in δ.** A, the amino acid sequence of the carboxyl-terminal peptide resulting from the NBS cleavage of δ. The PKA consensus sequence phosphorylation sites are shown, optimal (black boxes) and less optimal (open boxes), as in Figs. 2 and 5. Two serines utilized in vivo (see B) are indicated with P; asterisk denotes the position of the serine residue that was accidentally omitted during the synthesis of the δ peptide (see text for details). Arrows correspond to results obtained by microsequence analyses in B such that the length of each arrow corresponds directly to the appropriate amino acids confirmed by microsequencing. The method used for generating each of the phosphopeptides used in the microsequence analyses is as follows: NBS peptide, reverse phase-purified NBS cleavage fragment of in vivo phosphorylated δ; peptide a, Lys-C endoproteinase-generated peptide of in vivo phosphorylated δ; peptide b, Lys-C endoproteinase-generated peptide of in vivo phosphorylated δ that was purified by acid-urea gel electrophoresis, excised and eluted; peptide c, Lys-C endoproteinase-generated peptide of in vitro phosphorylated δ peptide that was purified by acid-urea gel electrophoresis as well as in vitro phosphorylated δ peptide that was purified by cation-exchange chromatography; peptide c, Lys-C endoproteinase-generated peptide of in vitro phosphorylated δ peptide that was purified by elution from an acid-urea gel. B, histograms of direct measurements of [32P] in the phenylthiohydantoin-derivatives during microsequencing. NBS and Lys-C endoproteinase peptides were generated as described above. As indicated by the length of the arrow in A above, essentially the entire in vivo labeled NBS peptide from δ was sequenced (from Gly-188 to Ser-217). However, only [32P] counts/min data between Thr-196 and Arg-206 are plotted; no other counts/min above background levels were detected.
Phosphorylation in δ have been identified. Both sites conform with recognition sites for PKA (37), although other kinases with similar sequence requirements have been reported (45). Collectively, these results lend strong support to the idea that PKA or a PKA-like kinase is present in micronuclei and that this activity is at least partially responsible for phosphorylation of δ in vivo.

Our data suggest, however, that several differences exist between ciliate and mammalian PKAs. Unlike bovine PKA, neither Paramecium nor crude Tetrahymena PKA activity is inhibited by PKI. These results are in agreement with the findings that Paramecium and Dictyostelium PKAs, respectively, are poorly inhibited by this peptide (39, 40). Although, the catalytic core of the PKA is highly conserved between most species (Ref. 46; reviewed in Ref. 47), sequence differences in the recognition site between isomers of the PKA subunit in yeast and mammalian cGMP-dependent protein kinase reduce these enzymes’ affinities for the Kemptide substrate and PKI (48, 49).

Our data strongly suggest that phosphorylation of δ by PKA is influenced by both protein sequence and conformation. Studies using model substrates with mammalian PKAs have suggested that substrates containing a pair of basic residues (usually arginines) one to three amino acids removed from the site of phosphorylation are generally favored over substrates with a single basic residue (37). Based upon this general rule, two putative sites of phosphorylation by PKA were predicted from the sequence of the carboxyl terminus of δ (1).

Our data demonstrate, however, that the above prediction is upheld for only one of these sites. Serine 201, contained within the optimal consensus sequence Arg-Lys-Asn-Ser, is the exclusive site of monophosphorylation within δ in vivo and is a highly preferred site in vitro. Unexpectedly, a second serine 209 contained within an optimal PKA recognition motif, Arg-Arg-Ser-Ser-Ser, is not utilized in vivo or in vitro. However, PKA, regardless of source, did utilize serine 209 when a synthetic peptide spanning this region, but missing serine 205, was used a substrate. Since this sequence is not utilized in vivo or in vitro when the correct δ peptide is used as substrate, we favor the interpretation that some aspect of δ’s conformation, possibly altered by the absence of serine 205, lowers the efficiency that this site is utilized. Surprisingly, a second serine 215, embedded within the sequence Lys-Ser-Ser-Val is phosphorylated when δ is phosphorylated in vitro or in vivo (site b, Fig. 6A). This less optimal PKA phosphorylation site contains a hydrophobic valine one amino acid after the phosphorylation site, a requirement shown to contribute to efficient substrate binding with mammalian PKA (50). These data support our contention that the overall structure of δ plays an important role in site utilization by PKA in vivo.

Previous studies have indicated that micronuclear linker histones are extensively phosphorylated in growing and mating cells, but not in starved cells (21). Our results (Fig. 1) demonstrate that PKA catalytic subunit is detected in micronuclei from growing, starved, and young mating cells, and at first glance, it appears that the amount of the 40-kDa subunit does not change appreciably with the physiological state of the cell. However, the crystal structure of PKA (50) demonstrated the importance of post-translational phosphorylation (at Thr-197) and suggested possible roles of phosphorylation in promoting PKA activation (reviewed in Ref. 47). Whether Tetrahymena PKA undergoes physiologically regulated modification during its life cycle is unknown.

Recent data suggest that an increasingly important mechanism in the role of phosphorylation events is the subcellular location of kinase or phosphatase subunits (reviewed in Ref. 51). For example, activation of PKA also requires a well known, cyclic AMP-dependent dissociation of catalytic subunit from an inhibitory regulatory subunit. Studies of fluorescence-tagged PKA injected into mammalian cells found that upon dissociation the free catalytic subunit moved from the cytoplasm to the nucleus while the regulatory subunit remained in the cytoplasm (52, 53). These data suggest the possibility that the activity of the catalytic subunit in the micronucleus may be modulated by a regulatory subunit that is selectively targeted to micronuclei during select stages of the life cycle. Along this line, the R subunit of PKA has been detected in Dictyostelium nuclei, and, interestingly, its amount in nuclei increases upon differentiation (54).

Although the biological function of linker histone phosphorylation is presently unknown, it has been proposed that it may facilitate chromatin decondensation facilitating factor access to DNA (15). Condensed chromatin, in contrast, is thought to be stabilized by dephosphorylated linker histone isoforms. Consistent with this model is the recent finding that phosphorylation of δ in micronuclei is linked (temporally and spatially) to a transient period of chromatin decondensation and transcription activation during meiotic prophase (2). We hypothesize that phosphorylation of the two carboxyl-terminal PKA sites identified in δ in this study acts to destabilize or decondense micronuclear chromatin, thereby facilitating the binding of factors required for activation including unique histone variants such as hv1 (55). Moreover, Lamb and co-workers (56) have demonstrated that inhibition of PKA in living mammalian cells results in rapid chromatin condensation at all phases of the cell cycle. Based on these results, we speculate that phosphorylation of PKA sites in mammalian H1s may play a previously unsuspected role in chromatin decondensation.

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