A Ca$^{2+}$-calmodulin dependent histone 3 kinase was partially purified from a low salt (150 mM NaCl) nuclear extract of mouse leukemia cells by calmodulin-Sepharose affinity chromatography. In vitro, the kinase activity transferred $\gamma$-phosphate from ATP to histone 3 to form an acid-labile and alkaline-stable linkage. Under the assay conditions 1.8 mol of phosphate are incorporated per mol of histone 3. Upon modification of arginine residues with phenylglyoxal prior to phosphorylation, a considerable decrease in the amount of phosphate transferred to histone 3 was observed. Amino acid analysis revealed that H3 was phosphorylated on arginine residues. To identify the phosphorylated peptide(s), histone 3 was cleaved with cyanogen bromide prior to phosphorylation. The phosphorylated mixture was then separated by gel filtration high-performance liquid chromatography under denaturing conditions. Fragments I (N-terminal 10.3-kDa peptide) and III (C-terminal 1.7-kDa peptide) were both phosphorylated. Amino acid sequencing further revealed that the molar yields of 3 of the 4 arginines present in the phosphorylated cyanogen bromide fragment III were reduced by a factor of about 10 compared with the corresponding arginines from the unphosphorylated fragment. In the case of fragment I, 25 cycles of Edman degradation revealed that the recovery of only arginine 2 was reduced by a factor of 20. The putative phosphorylation sites are arginines 2, 128, 129, and 131. The sequence information offered an indirect evidence that these arginines were the sites of phosphorylation. The kinase described in this report represents a first member of a potentially important new class of kinases which are Ca$^{2+}$-calmodulin dependent and which phosphorylate arginine.

Protein phosphorylation plays an important role in a number of cellular activities, including initiation of mitosis (reviewed by Nurse, 1990) and regulation of transcription (reviewed by Bohmann, 1990). Among the proteins phosphorylated during these processes are transcription factors (Jackson et al., 1990), protein kinase C (Bohmann et al., 1990), and histones 1 and 3 (Gurley et al., 1974). Phosphorylation of H3 is a mitotic event (Gurley et al., 1978; Marks et al., 1973; Gurley et al., 1974) and has been suggested to be closely associated with chromatid condensation (Shibata et al., 1990). Stimulation of quiescent cells by growth factors, phorbol esters, okadaic acid, and protein synthesis inhibitors also leads to rapid phosphorylation of H3 (Mahadevan et al., 1991). Within minutes of stimulation, the proto-oncogenes c-fos and c-jun were transcriptionally activated and structural changes in chromatin was observed (Mahadevan et al., 1991). Mahadevan et al. (1991) have suggested that the rapid phosphorylation of H3 modulates nucleosomal characteristics and potentially regulates early gene expression at the structural level.

Our present knowledge of the kinases that phosphorylate H3 is limited to an H3 kinase identified as a component of HeLa nucleosomes (Simpson, 1978), a 38-kDa chromatin-bound H3 kinase from calf thymus (Shoemaker and Chalkley, 1978, 1980), and the catalytic subunit of cAMP-dependent kinase used by Shibata et al. (1990). Early reports indicated that the phosphorylation of H3 is regulated by physiological concentrations of Ca$^{2+}$ in butyrate-treated HeLa cells (Whitlock et al., 1980, 1983). Recent reports demonstrate that the level of intracellular Ca$^{2+}$ is related to such events as nuclear envelope breakdown and chromatin condensation (Keith et al., 1985; Rao et al., 1990). In addition, calmodulin (CaM) (1) (primary mediator of Ca$^{2+}$-dependent signaling in eucaryotic non-muscle cells) together with Ca$^{2+}$ was shown to play an important role in regulating cell cycle-related events, including G2/M transition (Lu et al., 1993 and for review see Means et al., 1991). In a previous report from our laboratory, a Ca$^{2+}$-CaM-activated H3 kinase was identified as a component of calf thymus chromatin (Wakim et al., 1990). In this report a similar and probably identical kinase complex was partially purified from mouse leukemia nuclear extracts and used as the source of H3 kinase in the identification of the phosphorylated amino acids and their sites in H3. The data presented indicate that H3 is phosphorylated in a Ca$^{2+}$-CaM-dependent manner on arginine residues at potentially four different sites, three of which are in the C-terminal tail of the protein.

Phosphorylation of basic amino acids including histidine, lysine, and arginine lead to the formation of acid labile phosphoramidate linkages as summarized by the following reaction.

\[
\text{Protein-NH} + \text{ATP} \rightarrow \text{protein-N-PO}_2 + \text{ADP} \quad \text{(Reaction 1)}
\]

The formation of phosphoramidate linkages has been reviewed by Matthews and Huebner (1984) and Huebner and Matthews (1985). The existence of N-linked phosphate in proteins in vivo has been reported in a variety of cell types and subcellular compartments (Huang et al., 1991). In vitro, histone 4 has been shown to be a good substrate for a histidine

\footnote{The abbreviations used are: CaM, calmodulin; TEA, triethylamine; TEAA, triethylammonium acetate; HFLC, high-performance liquid chromatography.}
kinase identified in Physarum polycephalum nuclear extracts and purified from Saccharomyces cerevisiae whole cell extracts (Huang et al., 1991). Phosphorylase was found in H1 and phosphorylase in myelin basic protein (Chen et al., 1977; Steiner et al., 1980). An arginine-specific protein kinase has been isolated from the pellet of a 450 mM NaCl extract of rat liver chromatin and phosphorylated an 11-kDa protein (Levy-Favatier et al., 1987).

Kinases that generate P-N linkages have received much less attention than those generating P-O linkages primarily because of the technical difficulties related to the identification of the phosphorylated proteins and the phosphorylated amino acids. For example, acid precipitation of phosphoproteins with trichloroacetic acid, fixation of polyacrylamide gels with acetic acid, or HPLC chromatography in the presence of trifluoroacetic acid or any other method utilizing acidic conditions, commonly used in the case of acid stable phosphoproteins, must be excluded because they lead to a rapid reversal of the phosphorylation reaction (Hultquist, 1968; Wei and Matthews, 1990a, 1990b). In this report we demonstrate the existence of a kinase in the nucleus of mouse leukemia cells which phosphorylated arginine residues primarily in the C-terminal tail of H3.

**EXPERIMENTAL PROCEDURES**

**Materials—**Calf thymus histones were purified according to Bonner et al. (1968). Calmodulin was purified by the method of Gopalakrishna and Anderson (1982). CaM-Sepharose was prepared from purified CaM and calmodine bromide-activated Sepharose 4B (Pharmacia LKB Biotechnology Inc.) according to the manufacturer’s instructions. Phosphoarginine was purchased from Sigma. [γ-32P]ATP was purchased from Amersham Corp.

**Purification of H3—**H3 was purified in a single step by reversed phase HPLC using a Vydac C-18 column and an acetonitrile gradient in the presence of 0.1% trifluoroacetic acid. Calf thymus histones (200 μg) were dissolved in 100 μL of 6 mM guanidine hydrochloride containing 10% β-mercaptoethanol, heated at 55 °C for 20 min, and applied to the column. The column was eluted at 1 mL/min with an increasing acetonitrile gradient.

**Mouse Leukemia Cell Culture—**Murine L1210 cells were grown in 1530 media supplemented with 10% heat-inactivated bovine calf serum (HyClone, Inc.) and maintained in logarithmic state by serial passage of the media. Cells were harvested by centrifugation at 1,000 × g and stored at −70 °C.

**Isolation of the Ca2+-CaM-dependent Histone 3 Kinase Activity—**The mouse leukemia L1210 cell pellet was thawed, suspended in 50 volumes of homogenization buffer (10 mM potassium phosphate, 320 mM sodium sucrose, and 10 mM NaF, pH 6.9) and extracted while stirring for 30 min. The mixture was centrifuged at 10,000 × g for 30 min. The supernatant (nuclear extract) was applied to a CaM-Sepharose affinity column in the presence of Ca2+ and eluted with a buffer containing EGTA instead of Ca2+. The EGTA eluate contained Ca2+-CaM-binding proteins or protein complex. Sucrose density gradient centrifugation showed that the EGTA eluate consisted of a complex but did not add to the purity of the kinase (data not shown). Consequently, we chose to use the EGTA eluate as the source of the phosphorylating activity. Equal amounts (1 μg of protein) of enzyme were used per assay. The nuclear extract and the EGTA eluate were assayed for possible endogenous phosphorylating activities in the absence of histone substrates. In the case of the nuclear extract, three proteins with apparent molecular masses of 105, 65, and 41 kDa were phosphorylated in a Ca2+-CaM independent manner (Fig. 1, lanes 1 and 2). The EGTA eluate contained an endogenous protein with an apparent molecular weight of 55 kDa that was phosphorylated in a Ca2+-CaM-dependent manner (Fig. 1, lanes 3 and 4). This either represented autophosphorylation of the kinase(s) of interest or one of its subunits or simply a protein that co-migrated with the EGTA eluate and which happened to be phosphorylated by that kinase in a Ca2+-CaM-dependent manner. It also seems possible that the 55-kDa protein could be the CaM-kinase II (α subunit). Phosphorylation was also performed in the presence of a mixture of all five calf thymus histones. H1 and H2B were the preferred substrates for the nuclear extract, and their phosphorylation was not activated in the presence of Ca2+-CaM (Fig. 1, lanes 5 and 6). The EGTA eluate, on the other hand, contained a kinase that specifically phosphorylated H3 in a Ca2+-CaM-dependent manner (Fig. 1, lanes 7 and 8).
Phosphorylated H3 was 1.8 mol of phosphate/mol of H3. The samples were then neutralized and H3 repurified as above. It purified histone fractions.

Phosphorylation of Purified H3 was then desalted by ultrafiltration using a Centricon 3 microconcentrator. The maximum stoichiometry of phosphorylation in the presence of Ca2+ and calmodulin was observed (Fig. 2). In fact, by this method all five histones can be purified in a whole histone mixture by reversed phase HPLC using a Vydac C-4, calf thymus histones. Histone 3 is the predominant protein phosphorylated by the EGTA eluate in the presence of Ca2+ and calmodulin.

Identification of Phosphoarginine in Phosphorylated H3—Phosphorylated H3 was base-hydrolyzed and the resulting amino acids derivatized and analyzed as described under “Experimental Procedures.” As can be observed from Fig. 5A, phosphoarginine can be separated from phosphohistidine, all of which were elute isocratically under the analysis condition. Fig. 5B represent the isocratic part of the amino acid analysis of phosphorylated H3. The 32P counts eluted at identically the same retention time as phosphoarginine. This strongly indicated that arginine is the likely phosphorylated amino acid.

Cyanogen bromide cleavage of H3 was performed prior to phosphorylation of the peptide mixture. CB I, representing the N-terminal 10.3-kDa peptide, and CB III, representing the C-terminal peptide were both phosphorylated (Fig. 6). CB III contained about three times the amount of the bound 32P compared to CB I. CB III lacked any serine, threonine, or tyrosine. Thus these amino acids could be excluded from being the phosphorylated residues at least in this peptide. For the purpose of microsequencing, fragment 1 was desalted by repeated washes through a Centricon 3 and fragment II by reversed phase HPLC at pH 6.9.

In this report we demonstrate that nuclear extracts from mouse leukemia cells contain a Ca2+-CaM-dependent kinase that is capable of phosphorylating histone 3 (H3) on arginine residues. Based on the amino acid sequence information obtained using a cyanogen bromide digest of H3 prepared prior to phosphorylation, we suggest that 3 out of the 4 arginine residues present in the 15 amino acid C terminus of H3 and arginine 2 at its N terminus are the likely phosphorylation sites. This is the first such activity to be identified which is Ca2+-CaM-dependent and which phosphorylates the basic amino acid arginine. A similar kinase was identified from rat

**DISCUSSION**

In this report we demonstrate that nuclear extracts from mouse leukemia cells contain a Ca2+-CaM-dependent kinase that is capable of phosphorylating histone 3 (H3) on arginine residues. Based on the amino acid sequence information obtained using a cyanogen bromide digest of H3 prepared prior to phosphorylation, we suggest that 3 out of the 4 arginine residues present in the 15 amino acid C terminus of H3 and arginine 2 at its N terminus are the likely phosphorylation sites. This is the first such activity to be identified which is Ca2+-CaM-dependent and which phosphorylates the basic amino acid arginine. A similar kinase was identified from rat.
Chromatin-bound Histone 3 Arginine Kinase

**Fig. 3.** Purification of phosphorylated H3 by gel filtration HPLC on a tandem of Bio-Sil TSK 250 and 125 columns (60 x 0.46 cm). Phosphorylated H3 was purified from the assay mixture by gel filtration HPLC in the presence of 6 M guanidine hydrochloride and 10 mM potassium phosphate at pH 6.9 at 1 ml/min (A). Purified phospho-H3 was either treated with acid or base as described under “Experimental Procedures” and repurified by gel filtration as above. B and C, represent the repurification of phospho-H3 after acid treatment (B) and base treatment (C). As can be observed from B, acid treatment led to loss of the radioactivity from co-eluting with the protein peak. Base treatment led to no loss of radioactivity (C).

**Fig. 4.** Autoradiogram of phenylglyoxal-treated phosphorylated H3. H3 was treated with phenylglyoxal prior to phosphorylation in the presence of CaM followed by SDS-polyacrylamide gel electrophoresis and autoradiography. Equal amounts of protein were loaded in each lane. Lane 1 represents the phosphorylation of untreated H3. Lanes 2 and 3 represent the phosphorylation of H3 that have been treated with 0.5 and 5 mM phenylglyoxal, respectively, prior to phosphorylation.

Liver chromatin which also phosphorylated arginine (Levy-Favatier et al., 1987). The kinase described by Levy-Favatier et al. (1987) consists of a single subunit of an apparent molecular mass of 34 kDa capable of autophosphorylation and phosphorylates a single chromosomal protein of 11 kDa. The kinase presented in this paper phosphorylated H3 preferentially and did not show any phosphorylated band at 34 kDa. Also the rat liver kinase was isolated from the pellet of a 0.46 M NaCl chromatin extraction. The kinase presented in this paper is isolated from a low salt (150 mM NaCl) extract of chromatin. We believe that the two kinases are different.

The maximum stoichiometry of phosphorylation of H3 was found to be 1.8 moles phosphate per mole H3. This was less than the expected 4 moles which could be either due to unequal phosphorylation of the four arginines in question or due to partial loss of the phosphate during the purification of the phosphorylated H3. The fact that more than one mole phosphate per mole H3 was observed, indicates multiplicity of phosphorylation sites which is in agreement with the rest of our data.

Phosphorylation of H3 is believed to be involved with chromatin condensation (Shibata et al., 1990). The mitotic-specific sites of H3 phosphorylation according to these authors were serines 10 and 28 both of which are present in the N terminus of H3. In their experiments they used the catalytic subunit of CAMP-dependent kinase to phosphorylate H3 and precipitated the phosphorylated protein with trichloroacetic acid, thus eliminating any potential phosphoramidate linkages. Our finding that H3 was phosphorylated on arginine residues in a Ca"+-CaM-dependent manner neither contradicts nor supports their observations, since a different enzyme was used, but demonstrate that H3 was phosphorylated on arginine residues primarily present in the C-terminal tail of the protein. Based on earlier reports indicating that the C-terminal short tail of H3 extended from the globular central domain of the protein (Bradbury, 1983), any change in the charge of that region of the protein will be suspected of modifying the binding of H3 to DNA. Certainly, phosphorylation of arginine will change the overall charge of the C-terminal tail and is likely to alter the association of H3 with DNA during nucleosome assembly.

Phosphorylation of H3 is known to be stimulated by Ca"+
Fig. 5. Reversed phase HPLC of base hydrolyzed and phenylisothiocyanate-derivatized amino acids. Over 95% of the radioactivity was eluted within the first 10 min of isocratic separation of the derivatized amino acids. A, His(P), Lys(P) and Arg(P) standards. Tyr(P) was also included in the standard prior to base hydrolysis but showed no peak in the first 10 min of separation. B, elution profile of amino acids from phosphorylated H3 (solid line) and the radioactivity (dotted line).

Fig. 6. Purification of the cyanogen bromide fragments of H3 after phosphorylation of the mixture in the presence of CaM. The phosphorylated cyanogen bromide mixture was chromatographed using gel filtration HPLC on a tandem of Bio-Sil TSK 250 and 125 columns at 1 ml/min in the presence of 6 M guanidine hydrochloride. The effluent was monitored by the absorbance at 226 nm (solid line) and by radioactivity of 32P (dotted line). at physiological concentrations (Whitlock et al., 1980, 1983). Calmodulin, which is the primary mediator of Ca2+-dependent signaling in eucaryotic none muscle cells, has been shown to regulate different stages of the cell cycle, including G2/M transition (Lu et al., 1993). The existence of a Ca2+-CaM-dependent nuclear kinase which specifically phosphorylated H3 is no surprise. Such an activity was first observed to be a component of a complex purified from calf thymus nuclear

Fig. 7. N-terminal sequencing of phosphorylated CB I (A) and CB III (B). The picomole recoveries of the amino acids of the unphosphorylated (solid lines) and phosphorylated (dotted lines) peptides are presented. Amino acids at the different cycles are represented by single letter notations.
material (Wakim et al., 1990). The activity described in the present report was extracted from nuclear material of mouse leukemia cells and is believed to be identical to that from calf thymus but is over 100 times more abundant than in thymus tissue.

It was a surprise to find out that the phosphate transferred to H3 is acid-labile and base-stable and explained the difficulties that we initially encountered in purifying the phosphorylated peptide by standard reversed phase HPLC in the presence of trifluoroacetic acid. Modification of arginine residues using phenylglyoxal prior to phosphorylation led to a considerable decrease in the overall charge of that region and potentially regulating the phosphorylated CB I11 revealed a considerable decrease in the recoveries in the case of the unphosphorylated fragment. This indicated either that CB I11 is phosphorylated at a three time faster rate that CB I. This indicated either that CB III is about three times more phosphorylated than CB I or that CB III is phosphorylated at a three time faster rate than CB I under the assay conditions. Amino acid sequencing of phosphorylated CB III revealed a considerable decrease in the molar recoveries of 3 of the 4 arginines compared with their recoveries in the case of the unphosphorylated fragment. This either means that phosphorylated arginines elute at a position other than arginine, which we were not able to identify, or bind to the polybrene filter in a manner similar to the acid stable phosphoamino acids, including phosphoserine and phosphothreonine. Upon applying equal amounts of either arginine or phosphoarginine to the sequencer, it was observed that phosphoarginine eluted at exactly the same retention time as arginine. However, the recovery of phosphoarginine was about 20 times less than arginine. The amino acid sequence information offers indirect evidence about the sites of H3 phosphorylation. Taken together, the data presented in this paper strongly indicated that the C-terminal tail of H3 was phosphorylated on arginine residues, thus altering the overall charge of that region and potentially regulating the binding of H3 to DNA during nucleosome assembly/disassembly.

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