Stimulation of a Ca\(^{2+}\)-Calmodulin-activated Histone 3 Arginine Kinase in Quiescent Rat Heart Endothelial Cells Compared to Actively Dividing Cells

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A Ca\(^{2+}\)-calmodulin-activated histone 3 kinase was partially purified from nuclear extracts of dividing and quiescent rat heart endothelial cells. The histone 3 phosphorylating activity was 20–100-fold higher in quiescent than in dividing cells. Base hydrolysis followed by amino acid analysis revealed that histone 3 was phosphorylated on arginine. Further investigations were conducted to determine whether phosphorylation of histone 3 also occurred in vivo. Cells were incubated for 3 h in a phosphate-free medium supplemented with \(^{32}\)P-phosphoric acid. It was observed that the nuclear content of arginine-phosphorylated histone 3 was considerably higher in quiescent than in dividing rat heart endothelial cells. The histone 3 arginine kinase is a component of a complex containing a Ca\(^{2+}\)-dependent calmodulin-binding protein of apparent molecular mass of 85 kDa. Using polyclonal antibodies to an 85-kDa protein, also the major Ca\(^{2+}\)-dependent calmodulin-binding component of the histone 3 arginine kinase from calf thymus, an immunoreactive protein of identical apparent molecular mass was found to be present in equal amounts both in dividing and quiescent cells. We propose that the 85-kDa protein is either the histone 3 arginine kinase or one of its subunits and that phosphorylation of histone 3 is involved with cell cycle exit in eukaryotes.

Protein phosphorylation/dephosphorylation plays an important role in a number of cellular activities in eukaryotes (reviewed in Ref. 1). Such activities include initiation of mitosis (reviewed in Ref. 2) and regulation of transcription (reviewed in Ref. 3). Among the proteins phosphorylated are transcription factors (4, 5), topoisomerase II (6), and histones 1 and 3 (7, 8). Phosphorylation of histone 3 (H3\(^3\)) is associated with mitosis (7, 9, 10) and is suggested to be closely associated with chromatin condensation (11). Stimulation of quiescent cells by growth factors, phorbol esters, okadaki acid, and protein synthesis inhibitors also leads to the rapid phosphorylation of H3 (12). Mahadevan et al. (12) suggested that the rapid phosphorylation of H3 modulates nucleosomal characteristics and potentially regulates early gene expression at the structural level. The phosphorylation sites of H3 in all of the above reports were shown to be in the N-terminal region of H3, on serine or threonine residues. Our knowledge of the kinases that phosphorylate H3 is limited to a kinase identified as a component of the HeLa cell (13), a 38-kDa chromatin-bound H3 kinase from calf thymus chromatin (14, 15), the catalytic subunit of cAMP-dependent kinase used by Shibata et al. (11), and a chromatin-bound Ca\(^{2+}\)-Calmodulin-activated kinase from calf thymus (16) and from mouse leukemia cells (17). The kinase from mouse leukemia cells is the first to phosphorylate a basic amino acid (arginine) in a Ca\(^{2+}\)-Calmodulin-dependent manner (17). Interestingly, three of the four phosphorylated arginine residues in H3 are within the 15-amino acid C-terminal tail of H3. Such a modification in the C terminus of the protein will alter the overall charge of that region and potentially alter the binding of H3 to DNA during nucleosome assembly/disassembly. The present report is intended to demonstrate that the Ca\(^{2+}\)-Calmodulin-dependent arginine phosphorylation of H3 is correlated with cell cycle exit in rat heart endothelial (RHE) cells and that the H3 kinase is a regulated kinase, potentially a component of a cascade responsible for cell cycle exit.

EXPERIMENTAL PROCEDURES

Materials—Calf thymus histones were prepared according to Bonner et al. (18). Calmodulin was purified by the method of Gopalakrishna and Anderson (19). CaM-Sepharose was prepared from purified CaM and cyanogen bromide-activated Sepharose 4B (Pharmacia Biotech Inc.) according to the manufacturer’s instructions. Biotinylated calmodulin was purchased from Biomedical Technologies Inc. [\(^{32}\)P]ATP and \([\alpha-^{32}\)P]phosphoric acid were purchased from Amersham Corp.

RHE Growth Conditions—RHE cells were plated at 3 × 10\(^5\) cells (for dividing) and 8 × 10\(^5\) (for nondividing) per 35-mm plate (Falcon) and placed at 37 °C, 5% CO\(_2\) for 48 h in 4% Fetal Clone Serum (Hyclone) in DMEM/Ham’s F-12 medium (Hyclone). Confluent cultures were washed three times with Hanks’ balanced salt solution and placed in serum-free DMEM/Ham’s F-12 medium for 24 h prior to harvest. Five plates of each of dividing and quiescent cells were then placed in serum-free, phosphate-free DMEM plus 50 ng/ml insulin-like growth factor-I and 100 ng/ml basic fibroblast growth factor with 12.5 units/ml heparin plus 100 mCi of [\(^{32}\)P]ATP per plate for 3 h before collection. Nuclei were isolated from companion cultures treated simultaneously, stained with Hoechst dye, and then analyzed by flow cytometry to determine the cell cycle characteristics of the isolated population of cells.

Isolation of the Ca\(^{2+}\)-Calmodulin-dependent Histone 3 Kinase Activity and Histone 3 Kinase Assay—Both the isolation and assay of the kinase were performed as described by Wakim and Aswad (17).

Base Hydrolysis and Identification of Phosphoarginine—Both hydrolysis and amino acid identification were performed as described by Wakim and Aswad (17).

Immunoprecipitations—Immunoprecipitations were performed according to a protocol provided by Oncogene Science. Briefly, 100-mm plates were collected in 9 ml of PBS/TDS, pH 7.25 (10 mM Na\(_2\)HPO\(_4\), 150 mM NaCl), 1% Triton X-100, 10 mM sodium deoxycholate, 0.1% SDS,
30 mM sodium azide, 1 mM NaF). Lysates were then put through three freeze-thaw cycles and clarified by centrifugation at 4°C, 10,000 × g for 5 min. Samples of the supernatants were quantitated (BCA, Pierce). Equal amounts of protein were brought up to 1 ml with PBS/TDS. 15 μl of protein G plus/protein a agarose (Oncogene Science) and 5 μl of α-200 were added to each sample and incubated overnight at 4°C with gentle agitation. Pellets were collected by centrifugation at 4°C, 2,500 rpm, 15 min, and washed four times with 1 ml of PBS/TDS. Final pellets were then resuspended in SDS sample buffer. Samples were separated on 9% SDS-urea gels. Gels were then either silver-stained according to GEClode color silver stain procedure (Pierce) or transferred to PVDF membranes. Blots were treated with rabbit anti-85-kDa primary antibody followed by goat anti-rabbit IgG conjugated to horseradish peroxidase. Blots were finally stained with TMB (Kirkegaard and Perry Laboratories, Inc.).

RESULTS AND DISCUSSION

CaM affinity-purified fractions from RHE cells were assayed for their histone phosphorylating activities (Fig. 1). The reaction mixtures contained 100 μg of total histones and 2 μg of CaM affinity-purified fractions per assay. Equal amounts of histones (15 μg) were loaded per lane. As can be observed from Fig. 1, dividing cells contained a considerably lower level of H3 phosphorylating activity compared to the activity from quiescent cells (Fig. 1, lanes 1 and 3, respectively). The presence of Ca\(^{2+}\)-CaM in the assay did not noticeably activate the H3 kinase from dividing cells (Fig. 1, lane 2), but activated the kinase from quiescent cells by a factor of at least 10 times (Fig. 1, lane 4). The band labeled H3 (deg.) has been identified as H3, lacking its N-terminal 27 amino acids, which is also phosphorylated by the Ca\(^{2+}\)-CaM kinase from quiescent cells. In fact, the presence of H3 (deg.) in the assay mixture helped us exclude the N terminus from being the primary site of H3 phosphorylation. The phosphorylated amino acid in H3 was identified as arginine. Briefly, \(^{32}\)P-labeled H3 was hydrolyzed for 3 h in 3 M KOH at 124°C. Samples were cooled on ice, and equimolar amounts of perchloric acid were added in order to precipitate salts and to leave the amino acids in solution. Samples were then centrifuged, and the supernatants were dried. Amino acids were derivatized with phenylisothiocyanate and analyzed according to Wakim and Aswad (17). It was observed that over 90% of the phosphate-labeled amino acids migrated with identical retention times as that of phosphoarginine (data shown below). This is in agreement with earlier observations from our laboratory showing that H3 is phosphorylated primarily on three arginine residues present in its C-terminal tail (17). Also shown in Fig. 1 is the presence of a Ca\(^{2+}\)-CaM-dependent phosphoprotein with an apparent molecular mass of 50 kDa. The identity of this protein has not been determined. It is clear from Fig. 1 that quiescent cells contain at least 20-100-fold more of the Ca\(^{2+}\)-CaM-dependent H3 phosphorylating activity than do dividing cells.

To further pursue the possibility that arginine phosphorylation of H3 is related to cell quiescence it was important to demonstrate differences in the levels of arginine phosphorylated H3 in vivo between dividing and quiescent cells. RHE cells were collected either while dividing, after reaching confluency followed by a 2-day serum deprivation, or after reaching confluency then replating in the presence of serum to obtain dividing cells. As can be seen from Fig. 2, approximately 25% of the cells were in transit through the S phase (Panels A and C) while only a minimum of 2.6% of the cells grown to confluency and with serum deprivation were in the S phase (Panel B). Under confluent conditions the majority (over 90%) of the cells were arrested within the G\(_{\text{0/1}}\) phase of the cell cycle. In parallel, in vivo incorporation of \(^{32}\)P into H3 was also determined in dividing and confluent cells. As described under “Experimental Procedures,” cells were incubated in the presence of \([\alpha-^{32}\text{P}]\) phosphoric acid for 3 h. Nuclear extracts were prepared, and proteins were separated onto two identical 16% SDS-polyacrylamide gels. Equal amounts of protein were loaded per lane. Both gels were transferred to PVDF membranes. One blot was treated with 10% acetic acid and the other with 0.5 M KOH for 3 h at 50°C. Fig. 3 represents an autoradiogram of the acid (A) and base (B) treated blots. It is clear from this figure that, in the case of quiescent cells, a phosphorylated H3 band was observed in the blot that was treated with KOH (Fig. 3B, lane 2). The same band was not observed in the blot treated with acid (Fig. 3A, lane 2). This indicated that in the case of quiescent RHE cells a basic amino acid was phosphorylated in H3. In the case of dividing cells, the autoradiograph showed that the same phosphorylated band was absent from both acid- and base-treated blots (Fig. 3, A and B, lanes 1 and 3). The phosphorylated amino acid in H3 was further identified to be arginine (Fig. 4). The H3 band from quiescent cells was excised from the blot and base-hydrolyzed followed by identification of the phosphorylated amino acid. As can be observed from Fig. 4, about 90% of the \(^{32}\)P counts migrated with an identical retention time as phosphoarginine. This strongly indicates that the principle phosphorylated amino acid is arginine.

Polyclonal antibodies to the 85-kDa protein from calf thymus were raised in rabbits and used to identify immunoreactive proteins present in RHE cell extracts. It was observed that an immunoreactive protein with 85-kDa apparent molecular mass was present in both dividing and quiescent cells (Fig. 5B, lanes 1 and 2, respectively). Silver staining of CaM affinity-purified fractions from dividing and quiescent cells (Fig. 5A, lanes 1 and 2).
2, respectively) showed very similar protein patterns. The patterns of immunoprecipitated proteins from dividing and quiescent RHE cells were also very similar (Fig. 5A, lanes 3 and 4, respectively). As can be observed from Fig. 5B, lanes 1 and 2, the 85-kDa immunoreactive protein was detected at a nearly equivalent amount by immunoprecipitation of proteins from both dividing and quiescent cells. The intense band below the 85-kDa protein represents the IgG heavy chain. The data presented thus far demonstrate that phosphorylation of arginine in H3 potentially plays a role in cell cycle exit and that the kinase responsible for its phosphorylation, while present in nearly equal amounts in both dividing and quiescent cells, is activated 20–100-fold in quiescent cells.

The H3 arginine phosphorylating activity consisted of a protein-DNA complex and no detectable RNA. The ratio of protein to DNA was 95:5%, w/w. The complex contained a Ca\(^{2+}\)-CaM-binding protein of 85-kDa apparent molecular mass (Fig. 6). Lane 1 represents silver staining of the CaM affinity-purified complex after protein separation on a 12% SDS-polyacrylamide gel. Lane 2 represents the binding of biotinylated CaM to an identical sample after blotting to nitrocellulose. As can be observed from lane 2, a protein with an 85-kDa apparent molecular mass bound CaM in a Ca\(^{2+}\)-dependent manner. The 85-kDa protein from RHE is believed to be either identical or homologous to the 85-kDa CaM-binding protein isolated from calf thymus (16). Both proteins have the same apparent molecular mass, were present in the CaM affinity fraction purified under identical conditions, and bound CaM in a Ca\(^{2+}\)-dependent manner. We propose that the 85-kDa protein is either
H3 arginine kinase or one of its subunits and that phosphorylation of H3 is involved with cell cycle exit in eukaryotes.

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