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NUCLEOSIDE TRIPHOSPHATE BINDING AND HYDROLYSIS BY HISTONE H1

Riitta-Maaria Mannermaa and Jouko Oikarinen

Collagen Research Unit, Biocenter and Department of Medical Biochemistry, University of Oulu, Kajaanintie 52A, SF-90220 Oulu, Finland

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We present here further evidence supporting that histone H1 contains a nucleotide binding site interacting e.g. with ADP, ATP, GDP and GTP. The finding is in accordance with the previous observation that nucleotides modulate recognition of DNA by H1. Most interestingly, H1 appears to be capable of hydrolyzing NTPs and incorporating phosphate to exogenous proteins. The mode of nucleotide action on H1 may be considered highly analogous to that of GTPases. Nuclear receptors may thus act through mechanisms similar to those for receptors on the plasma membrane. © 1992 Academic Press, Inc.

The DNA in eukaryotes is packed into nucleosomes in which two turns of DNA are twined around a core structure that consists of pairs of histones H2A, H2B, H3 and H4 (1-4), while H1 binds the nucleosome externally to the hinge/spacer region (4). Aggregation of nucleosomes to higher order structures appears to be a crucial step in the inactivation of the chromatin (5,6), and this process is triggered by H1, which is thought to act as a eukaryotic repressor (7-10).

H1 interacts preferentially with A+T-rich DNA regions, this interaction taking place through SPKK motifs in its tails (11). A method based on DNA affinity chromatography was recently developed for the isolation of H1 in the native form, and experiments with H1 isolated from rat liver (12) and an H1.03-lacZ' fusion protein produced in E. coli have indicated that H1 may display further sequence-specificity in its DNA binding (13). It binds with high affinity to the 5'-TTGGCAnnnTGCCAA-3' motif, the consensus recognition sequence for CTF/NF-I (14). This sequence-specific interaction may thus serve as a target for regulation of the H1 function.

H1 is present in both active and inactive chromatin (15-17), although it is thought to be more loosely bound to the chromatin along actively transcribed genes than in areas not as efficiently expressed (17-19). The impairment has been attributed to the globular domain of H1 and not the tails (19), and we have recently proposed that NTPs may be involved in the

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process (20,21). Alternatively, a mechanism may involve introduction of phosphate to H1 (22,23). The present work was undertaken to characterize further the process leading to the impaired binding, since a region of H1 displays homology with nucleotide binding sites in protein kinases (24).

EXPERIMENTAL

Purification of DNA binding proteins - Acid-purified H1 was prepared from rat liver using PCA extraction (25). Proteins recognizing the 5'-TTGGCAnnnTGCCAA-3' motif on DNA (yielding affinity-purified H1 under the conditions used) were isolated from rat liver principally as described (12). In the present experiments, the affinity matrix was generated by filling in a double-stranded oligonucleotide (25/22-mer) containing the motif with biotinylated nucleotides and subsequently by coupling the labeled DNA to streptavidin-agarose. Affinity-purified H1 displays slightly different mobility in glycerol-SDS-PAGE from acid-purified H1 (Fig. 1). The histone H1-*lacZ'* fusion protein produced in *E. coli* was purified on an anti-ß-galactosidase affinity matrix (13).

Chemical modification of proteins - Lys residues were acetylated with acetic anhydride. The protein fraction (5 to 10 µg) was incubated for 30 min at 37°C in the presence of 5 mM acetic anhydride and precipitated with acetone. The dried pellet was resuspended in a buffer containing 0.1 M NaCl, 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA, and added to various assays or subjected to glycerol-SDS-PAGE. Radioactive labeling with [^{3}H]acetic anhydride (100 mCi/mmol) was performed in a similar manner, replacing acetic anhydride with 1 mCi of the labeled compound.

The various proteins were labeled radioactively at the carboxyl groups with [^{14}C]DCCD (60 mCi/mmol) (see 26), and subjected to glycerol-SDS-PAGE.

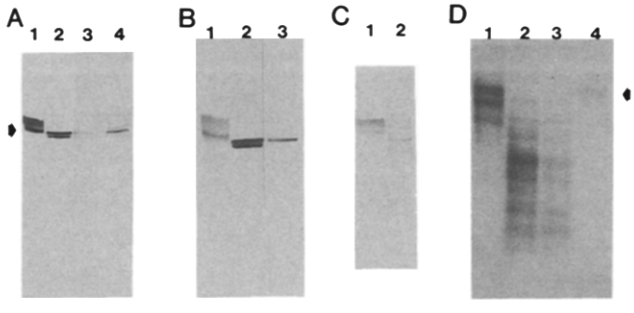

Fig. 1. Comparison of the acid and affinity-purified H1. A) Purities of the Coomassie-stained proteins. Lane 1 contains 3 µg of H1 isolated by PCA extraction and lanes 3 and 4 1 to 2 µg of proteins isolated on the DNA affinity matrices with the NF-I binding site from the α_2(I) collagen promoter or the NF-I consensus motif, respectively. In lane 2 the H1 of lane 1 was acetylated with acetic anhydride prior to electrophoresis. The position of the 31 kDa molecular weight standard is indicated by an arrow. Lanes 1 to 3 in B contain similar samples to those in lanes 1, 2 and 4 of Panel A, respectively, but labeled with [^{14}C]DCCD prior to electrophoresis. C) Western analysis of 10 µg of the acid-purified H1 (lane 1) and 3 µg of the affinity-purified protein isolated on the NF-I consensus matrix (lane 2), using polyclonal antibodies to H1. D) V8-protease maps of the acid-purified H1 (lane 2) and of the affinity-purified protein isolated on the consensus matrix (lane 3). Lanes 1 and 4 contain the corresponding undigested proteins. The proteins (5 µg) were acetylated using [^{3}H]acetic anhydride prior to cleavage.

Labeling with [^{14}C]glucose (>230 mCi/mmol) was carried out as described previously for [^{14}C]acetaldehyde (27). Lys adducts were stabilized using sodiumcyanoborohydride, and the incorporation of radioactivity was determined using glycerol-SDS-PAGE and fluorography.

Determination of NTP hydrolysis activity - Phoshorylation activity in the various preparations was determined by incubation with 1 µCi of [γ-^{32}P]ATP (>3000 Ci/mmol) (28). After the incubation the EDTA concentration was adjusted to 25 mM to terminate incorporation. The incorporation of radioactivity into H1 or dephosphorylated casein (29) was estimated from the pellet after precipitation with acetone by glycerol-SDS-PAGE and autoradiography for 3 h at -70°C.

In order to determine [^{32}P]labeled P-Ser, P-Thr and P-Tyr, the labeled proteins were separated by glycerol-SDS-PAGE, visualized by staining with Coomassie Brilliant Blue, eluted from the gel and hydrolysed in 6 M HCl at 110°C for 18 h or 3 h. The hydrolyzates were dissolved in a buffer containing 1 mg/ml of each of the phosphorylated amino acids unlabeled and subjected to two-dimensional TLC (30). The phosphorylated amino acids were identified by staining with a ninhydrin spray and the presence of [^{32}P] label was detected by autoradiography.

Assays for nucleotide binding - Labeling with [^{14}C]Ac-CoA was performed by incubating acid-purified H1 for 30 min at 37°C in the presence of 100 nCi of the label (50 mCi/mmol) (20). The proteins were precipitated with acetone, and incorporation of the label was determined by glycerol-SDS-PAGE and fluorography.

Covalent labeling of the nucleotide binding site of H1 with a radioactive analog, [^{14}C]FSBA (40 mCi/mmol), was performed virtually as described (31). After the incubation, the protein was precipitated with acetone and subjected to glycerol-SDS-PAGE and fluorography.

Additional methods - Immunoprecipitation experiments were performed on microtiter plates. H1 antibodies (12) were bound to the bottom of the wells, washed with TBS and the remaining binding sites blocked with BSA. Subsequently, the affinity-purified H1 alone or labeled with [^{14}C]FSBA was incubated in the wells for 2 h at room temperature, whereafter the supernatant fraction was subjected to determination of phosphorylation activity or the bound fraction was washed with TBS and dissolved in a buffer containing 1% (w/v) SDS and radioactivity was determined by liquid scintillation counting.

Electrophoresis on glycerol-SDS-polyacrylamide gels was performed as described (32). The gels containing [^{32}P]labeled proteins were subjected to autoradiography and those containing [^{14}C] or [^{3}H]labeled proteins to fluorography (33). Unlabeled proteins were visualized by staining with either Coomassie Brilliant Blue or silver (34). The radioactive label was quantified using a Kontes K495 000 densitometer connected to a Spectra-Physics SP4100 Computing Integrator. V8-protease peptide (35) and Western analyses (36) were carried out as described.

RESULTS AND DISCUSSION

Specific and saturable binding of nucleotides to H1 - H1 can be labeled nonenzymatically in the presence of [^{14}C]Ac-CoA (20,37), and incorporation of radioactivity to H1 can be determined using glycerol-SDS-PAGE (Fig. 2). The binding of [^{14}C]Ac-CoA to H1 is saturable, and a k_D value of 45 µM has previously been determined for the binding (20). Since this labeling does not occur in a time dependent manner, it may be assumed that the binding does not involve hydrolysis of Ac-CoA. In addition, no Mg^{2+} is needed. Effect of various nucleotides on this labeling was studied further in the present work. Addition of an excess of ATP, ADP and to some extent that of

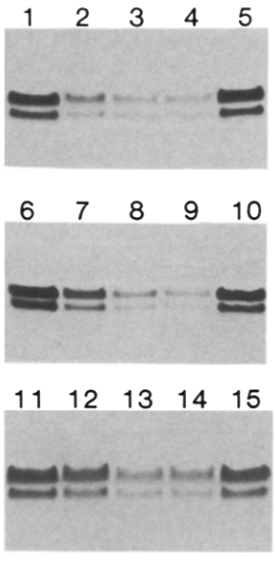

Fig. 2. Specific labeling of H1 with [^{14}C]Ac-CoA. Lanes 1, 5, 6, 10, 11 and 15 contain H1 labeled with [^{14}C]Ac-CoA. Lanes 2, 3 and 4 contain reactions carried out in the presence of 0.2, 0.5 and 1 mM ATP respectively, lanes 7, 8 and 9 those in the presence of 0.5, 1 and 2 mM ADP, and lanes 12, 13 and 14 in the presence of 1, 2 and 4 mM AMP respectively. The competition experiments were carried out in the presence of 5 mM Mg^{2+}.

AMP efficiently counteracted the [^{14}C]Ac-CoA labeling (Fig. 2). A k_D value of 15 µM was calculated from the competition experiments in the presence of Mg^{2+} for ATP, which indicates that the affinity of ATP to H1 is high enough to be regarded as an enzyme-substrate interaction.

In order to confirm that the nucleotides specifically bind to H1, H1 was incubated in the presence of [^{14}C]FSBA, an ATP analog, and an excess of unlabeled FSB. This approach was chosen because it has previously been demonstrated that phosphates and phosphate analogs bind to H1 (38) and interfere with the binding of [^{14}C]Ac-CoA to H1 (20), and it is possible that nucleoside phosphates interact in a nonspecific manner with the Lys-rich C-terminal tail of H1. The binding is more dependent on the base in the case of FSBA. After separation by glycerol-SDS-PAGE and fluorography, the H1 bands were discerned faintly labeled radioactively with [^{14}C]FSBA. An excess of unlabeled ATP, GTP and FSBA (Fig. 3A), and by that of ADP, GDP, AMP and GMP or phosphate analogs counteracted the labeling.

In order to further confirm that the nucleotide binding is specific, H1 was labeled with [^{14}C]DCCD at its carboxyl groups or with [^{14}C]glucose at its Lys ε-amino groups, and effect of nucleotides on the labeling was studied. BSA was included as an internal control. ATP was able to counteract the [^{14}C]DCCD labeling of H1 but not that of BSA (Fig. 3D). On the

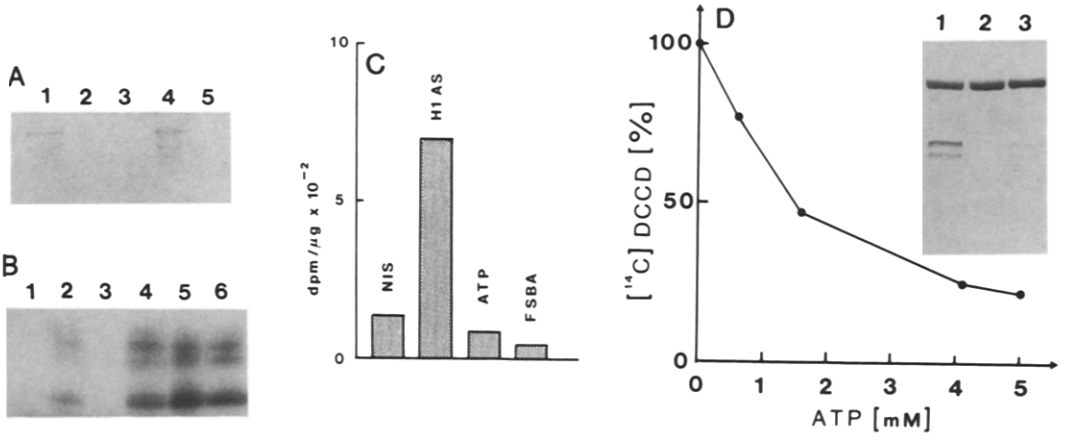

Fig. 3. Specific recognition and processing of ATP by H1. A) Labeling of H1 with [^{14}C]FSBA. Lanes 1 to 4 contain 100 ng of the affinity-purified H1 labeled with [^{14}C]FSBA, and lanes 2 and 3 that labeled in the presence of 100 µM ATP and unlabeled FSBA respectively. Lane 5 does not contain any protein. B) Precipitation of a protein required for phosphate transfer by antibodies to H1. Lanes 2 and 3 contain phosphorylation reactions carried out after pretreatment with an antiserum to H1 and lanes 4 and 5 after pre-treatment with the pre-immune serum. Lane 1 is a negative control with acid-purified H1 and no affinity-purified H1 or serum, and lane 6 a positive control with acid-purified H1 and affinity-purified H1 but no serum. C) Precipitation of the protein that binds [^{14}C]FSBA by the H1 antibodies. NIS represents precipitation with the pre-immune serum and the others with the H1 antiserum. ATP and FSBA represent precipitation of radioactivity with the antiserum after labeling in the presence of 100 µM ATP and FSBA respectively. D) Specific counteraction of the labeling of H1 with [^{14}C]DCCD by ATP. The effect of increasing ATP concentrations on the labeling of the acid-purified H1 is displayed. The proteins labeled were analyzed by glycerol-SDS-PAGE, and the insert demonstrates the differential effect of ATP on the labeling of H1 and BSA. Lane 1 contains 1 µg of both BSA and H1, lane 2 BSA alone and lane 3 both BSA and H1 with 1 mM ATP.

other hand, H1 and BSA were labeled upon incubation for 18 h with [^{14}C]glucose, but this labeling could not be counteracted by an excess of any of the nucleotides or by phosphate analogs (not shown). In the light of these experiments it is evident that nucleotides bind to H1 in a specific manner. H1 does not, however, display a strict specificity for the nucleotide base or sugar.

The NTP is hydrolyzed upon binding to H1 - Previous results had demonstrated that nucleotides modulate interaction of H1 with DNA (20,39). NTPs tend to inhibit H1 DNA binding, NDPs stimulate, while NMPs are ineffective (39). Recent results have indicated that NTPs display a biphasic effect in the presence of Mg^{2+}, while non-hydrolyzable NTP analogs such as GTPγS are clearly inhibitory (20). These results imply that the NTP may be hydrolyzed by H1 in the presence of Mg^{2+}. The inherent capability of H1 to hydrolyze NTPs may enable reversible interaction of H1 with DNA in the presence of nucleotides (see 20,21).

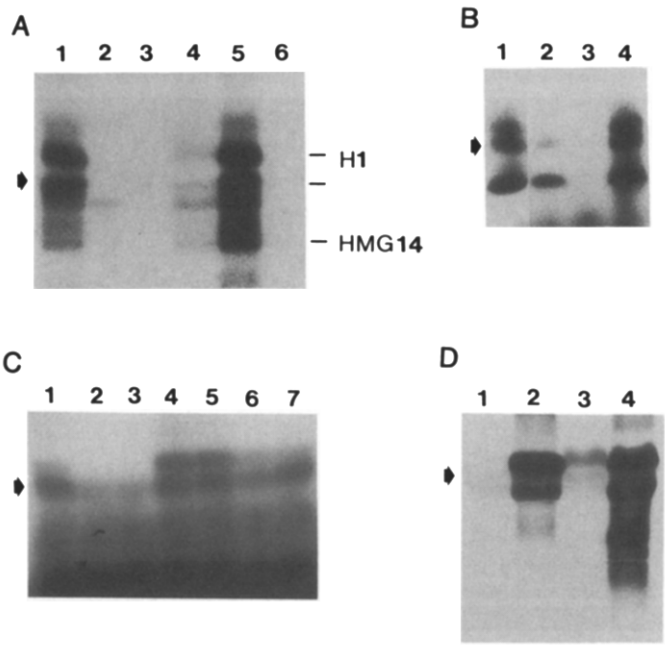

Fig. 4. Phosphorylation of proteins by affinity-purified H1 and its sensitivity to various inhibitors. A) Labeling of H1 with [γ-^{32}P]ATP. Lanes 1 and 3 to 6 contain acid-purified H1, and lanes 1 to 2 and 4 to 6 affinity-purified material. Lane 4 contains 100 µM unlabeled ATP and lane 6 25 mM EDTA. B) Counteraction of the labeling by various inhibitors. All lanes contain both acid and affinity-purified H1. Lanes 2 and 3 contain 100 µM quercetin and FSBA as inhibitors, respectively. C) Counteraction of the labeling by nucleotides. All lanes contain both acid and affinity-purified H1. Lanes 2 and 3, 4 and 5, and 6 and 7 contain in addition 1 mM GTP, NAD and Ac-CoA as a competitor respectively. D) Labeling of H1 and casein with [^{32}P]ATP in the presence of the H1-lacZ' fusion protein. Lanes 1 and 2 contain 1 µg of casein, and lanes 3 and 4 in addition 1 µg of acid-purified H1. Lanes 2 and 4 contain 100 ng of the fusion protein.

Incorporation of radioactivity from [γ-^{32}P]ATP to H1 itself or dephosphorylated casein was used as a measure of the rate of NTP hydrolysis. Acid-purified H1 displayed a negligible rate of phosphate incorporation. Upon incubation in the presence of affinity-purified H1 considerable incorporation of phosphate into H1 or casein could nevertheless be demonstrated (Fig. 4). This incorporation was dependent on Mg^{2+} (Fig. 4A), an optimum of 7 mM being determined, and was readily counteracted by an excess of ADP, GDP, GTP and Ac-CoA (Fig. 4C) or DCCD, quercetin and FSBA (Fig. 4B). A K_m value of 150 µM was determined for ATP and 0.8 µM for casein, and V_{max} with casein was 0.2 µmol/min/mg H1. H1 was needed for incorporation to take place since pretreatment with antibodies to H1 totally prevented the phosphorylation (Fig. 3B and C). For further confirmation of the role of H1 in NTP hydrolysis, the experiments were repeated using an

H1-lacZ' fusion protein produced in E. coli. Phosphorylation of casein and H1 clearly occured upon addition of the fusion protein (Fig. 4D). Subsequently, casein labeled in the presence of [γ-^{32}P]ATP was subjected to acid hydrolysis and determination of P-Ser, P-Thr and P-Tyr. Radioactivity was observed in the positions of P-Ser and P-Thr in TLC.

Homology of H1 with ATP/GTPases - When the structure of ras-p21 (40,41), a GTPase, is compared with that of H1 (42), a conservation of certain functionally crucial amino acid residues is observed. The conserved sequences in ras-p21 include NKCD and SAKTRQG sequences in its C-terminal part which are involved in recognition of guanine (41), and the former of which corresponds to LFNKKD in an analogous position in many other GTPases (40). An FRLNKKPGE sequence may be identified in an analogous position in H1 and is well-conserved in all H1 subtypes (24).

A G-G--G sequence (P loop or equivalent) may be identified in the nucleotide-binding domain of all GTPases (43) and protein kinases (24). No homologous sequence can be identified in an analogous position in H1 but in a position located towards the C-terminus, flanking the FRLNKKPGE sequence (24). This turn in H1 can nevertheless be envisaged as taking part in stabilization of the binding of phosphate in a similar manner to the turn in protein kinases and ras-p21.

Analogy of the nucleosome function to the mode of action of GTPases - H1 binds in the nucleosome to the hinge region externally (4), and would therefore be easily accessible for regulation of its function and may become dissociated from the complex in response to diverse stimuli in a similar way to G_α bound to G_β and G_γ. Consistent with this, previous results from many laboratories have indicated that H1 is more loosely bound to the chromatin in active areas than in inactive ones (7,15,19). Similarly to G_α (40), H1 is subject to (ADP-ribosyl)ation (44), and interaction of both G_α and H1 with regulatory subunits or DNA is affected by AlF_4^- (20,45).

The mode of nucleotide interaction with H1 displays analogy to that identified with GTPases on the plasma membrane (20,40,46). Since the NTP can be hydrolyzed by H1 in the presence of Mg^{2+}, the function of the nucleosome may be considered highly analogous to the plasma membrane GTPases (see 21). Major differences are observable in the phosphate transfer properties and nucleotide specificity of H1 when compared with G_αs, however. The present results suggest thus that nuclear receptors may share a common mode of action with the receptors coupled with GTPases on the plasma membrane, acting by facilitating nucleotide exchange and altering interaction of H1 with the nucleosome (21,46).

It is possible that a protein kinase activity co-purifies with H1 from rat liver. It should be noted, however, that i) the phosphate transfer

activity coelutes with the H1 DNA binding activity when affinity-purified H1 is chromatographed on an S300 gel filtration matrix (not shown), and ii) the H1-lacZ' fusion protein produced in E. coli displays the phosphate transfer capability. Acid-purified H1 was catalytically inactive, but it should be noted that PCA extraction and trichloroacetic acid precipitation should lead to irreversible denaturation of the protein.

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