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Prothymosin α modulates the interaction of histone H1 with chromatin

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ABSTRACT

Prothymosin α (ProTα) is an abundant acidic nuclear protein that may be involved in cell proliferation. In our search for its cellular partners, we have recently found that ProTα binds to linker histone H1. We now provide further evidence for the physiological relevance of this interaction by immunoisolation of a histone H1–ProTα complex from NIH 3T3 cell extracts. A detailed analysis of the interaction between the two proteins suggests contacts between the acidic region of ProTα and histone H1. In the context of a physiological chromatin reconstitution reaction, the presence of ProTα does not affect incorporation of an amount of histone H1 sufficient to increase the nucleosome repeat length by 20 bp, but prevents association of all further H1. Consistent with this finding, a fraction of histone H1 is released when H1-containing chromatin is challenged with ProTα. These results imply at least two different interaction modes of H1 with chromatin, which can be distinguished by their sensitivity to ProTα. The properties of ProTα suggest a role in fine tuning the stoichiometry and/or mode of interaction of H1 with chromatin.

INTRODUCTION

Prothymosin α (ProTα) is a highly conserved acidic polypeptide (1–3) localized in the cell nucleus (4–6). The physiological role of this protein remains unclear, despite its wide distribution and abundance (3,7,8). Several pieces of evidence suggest a link between ProTα and cell proliferation. ProTα expression is elevated in proliferating cells (9–11) and in response to increased levels of c-myc (12). A correlation between ProTα and c-myc mRNA expression has been found in human colon cancers (13) and in other *in vivo* systems (14), while high protein levels have been reported in intestine and breast malignant tissues (15). ProTα mRNA is present throughout the cell cycle, increases at S/G2 phase and can be induced by transcription factor E2F (16). Moreover, antisense oligonucleotides directed against ProTα mRNA inhibit cell division in myeloma cells (17), suggesting an essential role in cell proliferation.

The unusual structure of ProTα might be indicative of its function. Calf thymus ProTα (12.5 kDa) contains an acidic region in the centre of the molecule (18). Histidine, aromatic and sulphur amino acids are entirely absent, whereas there are only seven widely dispersed hydrophobic residues. Based on these sequence features, an extended non-folded conformation is expected that might be favourable for interaction of the protein with other cellular components (4,19). Nucleoplasmin and high mobility group protein 1 (HMG1) also contain extended acidic domains and have well-documented functions related to the organization of chromatin (20).

ProTα binds selectively to the linker histone H1 through its acidic domain (21). Histone H1 is responsible for the organization and stabilization of nucleosomal arrays into higher order structures and there is substantial evidence that the differential association of H1 affects gene expression (for a review see 22). A number of biochemical studies have shown that active chromatin in nuclei is partially depleted of linker histone H1 (23,24), suggesting that H1 may exhibit reduced affinity for these regions. However, H1 may also be found associated with active genes, but an altered mode of interaction has been proposed (24–26). Currently, evidence is accumulating that H1, rather than being a global repressor of transcription, affects the expression of selected genes (27–30). The factors that modulate the stoichiometry of H1 association or its mode of interaction with chromatin at a given genomic site are unknown. Conceivably, H1 interactions may be affected by binding of non-chromosomal proteins (20,31). In this vein, the study of proteins such as ProTα that interact with histone H1 may help to elucidate the regulatory mechanisms of H1 distribution.

In this study we present a detailed analysis of the interaction between ProTα and histone H1 and its effect on the association of H1 with chromatin and DNA. On the basis of our results, we propose a role for ProTα in chromatin, where it may modulate the extent and/or mode of interaction of H1 with nucleosomal fibres.

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MATERIALS AND METHODS

Protein and peptide work

ProTα was purified by a new method, based on published procedures (1,4), as follows. Freshly excised calf thymus was immediately frozen in liquid nitrogen and stored at –80°C. Frozen tissue (30 g) was pulverised in liquid nitrogen and immediately added to 400 ml boiling water. The suspension was homogenized with a Polytron homogenizer and acidified with 1 vol buffer A (1 M HCOOH, 0.2 M pyridine, pH 2.8). The acidic extract was centrifuged at 10 000 *g* for 25 min at 4°C and the supernatant was kept. The pellet was resuspended in an equal volume of buffer A and centrifuged as before. The two supernatants were combined, concentrated to a jelly-like material and dissolved in 5 ml 7.5 mM sodium borate, 1 mM DTT, pH 8.8. The sample was applied to a CM-Sephadex G-25 column (2.2 × 39 cm) pre-equilibrated with the same buffer. The column was run at 18 ml/h and ProTα was recovered in the first 80 ml of the flow-through volume. The pooled fractions were concentrated to dryness in a Speed-Vac, dissolved in 3 ml 5 mM HCl, 0.02% NaN_3 and dialysed over the same solution for 3 h. Subsequently, the sample (6 ml) was concentrated, dissolved in 1 ml 10 mM sodium acetate, 8 M urea, 0.02% NaN_3, pH 4.5 and applied to a SP-Sephadex G-25 column (1.0 × 43 cm), previously equilibrated with 10 mM sodium acetate, 0.02% NaN_3, pH 4.5. ProTα was eluted in the first fractions (9–13, 1.2 ml each) of the flow-through volume. All steps were carried out at room temperature, unless indicated.

The preparation was analysed by 15% SDS–PAGE and stained as a single protein band with silver. High performance liquid chromatography (HPLC) gave a single peak and the amino acid analysis was in agreement with the published values (3,18). This method yields ~1.5 mg ProTα/30 g tissue.

ProTα concentration was determined by amino acid analysis (3).

Drosophila histone H1 was purified as described (32). Histone H1 and core histones from calf thymus were obtained from Boehringer Mannheim. The globular region of histone H1 was prepared by partial trypsinolysis of 630 µg H1 in 630 µl 20 mM Tris–HCl, 1 M NaCl, pH 7.4, for 20 min at 20°C (33).

Histone H1 (2 µg) was phosphorylated with 0.024 U protein kinase C (Boehringer Mannheim) in the presence of 20 µCi [γ-^{32}P]ATP, 50 µM ATP, 0.2% BSA, 1% phospholipids for 30 min at 37°C.

Thymosin α1, acidic peptide and ct peptide corresponding to residues 1–28, 52–69, 86–109 respectively of bovine thymus ProTα (18) were synthesized by the EMBL Protein Sequencing and Peptide Synthesis Facility. Peptide biotinylation has been described elsewhere (34).

Crosslinking experiments using dimethylpimelimidate (DMP; Pierce) were performed as follows. Aliquots of 1 µg histone H1 were incubated with different amounts of ProTα, thymosin α1, the acidic peptide or the ct peptide in 10 µl 0.1 M sodium phosphate buffer, pH 6.7, for 10 min at room temperature. Then, 10 µl 0.2 M sodium borate buffer, pH 9.0, and 2.2 µl 0.2 M DMP were added and the samples were further incubated for 30 min. The reaction was stopped by addition of 2 µl 1 M Tris–HCl, pH 7.4, 7 µl Laemmli buffer and boiling.

Chromatin reconstitution

Chromatin assembly extract was prepared from *Drosophila* embryos as described (35). Plasmid immobilization (pdHSP70 XX3.2) on paramagnetic beads, chromatin assembly reactions and micrococcal nuclease analysis were performed as described (29). One unit of H1 is the amount needed to increase the repeat length of chromatin from 180 to 200 bp (29). Incorporation of H1 into chromatin was as follows. H1 (2 U) was mixed with assembly extract prior to addition of DNA beads. Chromatin was assembled for 6 h at 25°C and beads were concentrated in a magnetic field. Immobilized chromatin was washed with 650 mM NaCl to strip bound H1, then three times with 100 mM NaCl, and finally equilibrated in binding buffer (20 mM Tris–HCl, 100 mM NaCl, 1 mM $MgCl_2$, 0.02% NP-40, pH 7.4). Then, histone H1 and traces of ^{32}P-labeled H1 were added to the binding buffer (15 µl total volume) and incubated with the chromatin beads for 30 min at 25°C. Histone H1–DNA complexes were formed in a binding buffer consisting of 20 mM Tris–HCl, 150 mM NaCl, 5 mM $MgCl_2$, 0.02% NP-40, pH 7.4, in 15 µl total volume.

To assay the interaction of ProTα with H1-containing chromatin, chromatin was reconstituted in the presence of 2 U histone H1 on 100 ng DNA which had been immobilized on paramagnetic beads. Magnetically purified chromatin was incubated with 0.4 µg ProTα in 100 µl 20 mM Tris–HCl, 150 mM NaCl, pH 7.4. After incubation, the supernatant was removed and the chromatin beads were subjected to salt extractions with 50, 400 and 650 mM NaCl. The proteins were precipitated with 6 vol acetone overnight at –20°C and analysed by western blotting using an anti-ct antibody.

Immunochemical techniques

Anti-H1 antibodies were raised in rabbits according to Srebreva and Zlatanova (36) and the serum was affinity-purified over immobilized H1.

Antibodies against peptide ct were raised in rabbits and were affinity-purified as described (21). For construction of the immunoaffinity and the non-specific IgG columns, 1 mg affinity-purified anti-ct or non-immune IgGs were bound to 0.7 ml protein A–Sepharose. The antibodies were covalently coupled to the beads using DMP as a crosslinker (37). ProTα–agarose was prepared by coupling 220 µg of the protein to 1.0 ml Affigel 15 (BioRad).

Immunoisolation of the ProTα–H1 complex. NIH 3T3 cells were grown as monolayers in DMEM supplemented with 10% fetal calf serum. Samples of 50 × 10^6 cells were lysed with 3 ml 20 mM Tris–HCl, pH 7.4, 800 mM NaCl, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM dithiothreitol, protease inhibitors (pepstatin, leupeptin, aprotinin, 1 µg/ml each) and incubated on ice for 15 min. After centrifugation at 10 000 *g* for 10 min at 4°C, the supernatant was adjusted to 150 mM NaCl, divided into four portions (corresponding to 12.5 × 10^6 cells) and incubated with (i) anti-ct protein A–agarose, (ii) IgG protein A–agarose or (iii) ProTα–agarose beads for 3 h at 4°C and further for 1 h at 25°C, with gentle rocking. Then, the beads were poured into columns, washed with 10 ml 20 mM Tris–HCl, pH 7.4, containing 150 mM NaCl, 1 mM EDTA, 1 mM PMSF and protease inhibitors and then with 0.3, 0.6 and 1.0 M NaCl (1.5 ml each). The fractions were dialysed over 1 mM phosphate buffer, pH 7.4, 1 mM EDTA, 1 mM PMSF, concentrated, divided into two and analysed by western blotting for histone H1 and ProTα.

Western Blotting for ProTα. Samples were resolved by 15% SDS–PAGE (0.75 mm thickness) and, after electrophoresis, the gels were equilibrated in 20 mM sodium acetate, pH 4.5 (300 ml with three changes, 15–20 min). Electrotransfer was performed

onto activated nitrocellulose treated with glutaraldehyde (21,38) at 7 V, 45 mA, for 7 h in the above buffer. The membranes were washed with TBST (20 mM Tris, 0.9% NaCl, 0.1% Tween 20, pH 7.4) and incubated in blocking buffer (TBST with 0.1% gelatin) for 2–12 h. Finally, the filters were incubated with anti-ct antibody (1:1000), then with anti-rabbit IgG–peroxidase (1:5000) and detected by enhanced chemiluminescence (ECL; Amersham Life Science).

Protein bandshifting on agarose gels

ProTα was incubated with histone H1 or with the GH1 peptide in 20 mM NaH_2PO_4/Na_2HPO_4, pH 6.7, for 30 min at 25°C in 23 µl total volume. After addition of the sample buffer (2 µl glycerol, 4 µl bromophenol blue), the samples were loaded on an agarose gel (0.75% in 20 mM NaH_2PO_4/Na_2HPO_4, pH 6.7) and electrophoresed at 34 V, 15 mA, for 3 h at room temperature, using 0.1 M NaH_2PO_4/Na_2HPO_4, pH 6.7, as the running buffer. The gels were stained with Coomassie blue for 20 min (1 vol Coomassie blue diluted in 3 vol destaining solution), destained in 25% methanol, 7% acetic acid for 3 h and finally equilibrated in distilled H_2O for 3–12 h. Ligand blotting assays were performed as described (21).

SDS–polyacrylamide gels (39) were stained with silver as described (40).

RESULTS

Isolation of the ProTα–H1 complex from NIH 3T3 cell extracts

To obtain further evidence for the interaction between ProTα and histone H1, we have attempted to co-isolate ProTα and histone H1 from crude cell extracts by immunoaffinity chromatography. Affinity-purified anti-ProTα antibodies (anti-ct) were covalently linked to protein A–agarose and the column matrix was incubated with extracts of NIH 3T3 cells (for details see Materials and Methods). The bound proteins were eluted by applying to the column 0.3, 0.6 and 1.0 M NaCl and analysed by western blotting using the affinity-purified anti-ct and anti-H1 antibodies. ProTα was detected in the 0.6 M fraction of the immunoaffinity column (Fig. 1A, lane 2, upper panel), while histone H1 was detected in the 0.3 and 0.6 M fractions (Fig. 1A, lanes 1 and 2). Arguing for the specificity of the ProTα–H1 interaction is the fact that neither ProTα nor H1 was detected when anti-ct antibodies were substituted by non-immune rabbit IgG (Fig. 1A, lanes 4 and 5). In an alternative approach, purified ProTα was covalently linked to agarose beads and incubated with the cell extract under identical conditions. Consistently, histone H1 was eluted from the ProTα–agarose column (Fig. 1B, lane 2). The specificity of the affinity-purified anti-H1 and anti-ct antibodies is demonstrated in Figure 1C. These data suggest the presence of the ProTα–H1 complex under physiological conditions.

Identification of interacting sites between ProTα and histone H1

To further characterize the interaction of ProTα with histone H1, we employed a bandshifting assay. Due to their charge characteristics, histone H1 and ProTα migrate to opposite electrodes when electrophoresed on an agarose gel at neutral pH (Fig. 2B, lanes 1 and 8). Mixing of these proteins resulted in the formation of a

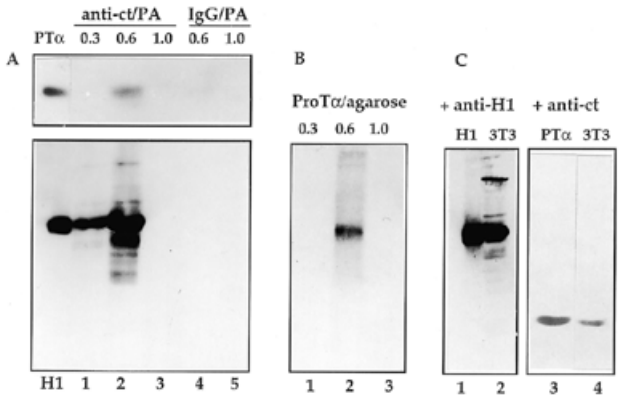

Figure 1. Immunoisolation of the ProTα–histone H1 complex from NIH 3T3 cell extracts. (**A**) Western blotting analysis of the material eluted from the anti-ct protein A–agarose column. The fractions eluted from the anti-ct protein A–agarose column with 0.3, 0.6 and 1.0 M NaCl (lanes 1–3) or the non-specific IgG protein A–agarose column with 0.6 and 1.0 M NaCl (lanes 4, 5) were divided into two and analysed as follows. (Upper) The samples were fractionated by 15% SDS–PAGE and transferred onto glutaraldehyde-activated nitrocellulose membranes as described in Materials and Methods. The blot was probed with affinity-purified anti-ct antibody (1:1000) and then with anti-rabbit IgG–peroxidase (1:5000). Lane PTα indicates 0.1 µg purified ProTα as a control. (Lower) The second part of the samples was analysed by western blotting using affinity-purified anti-H1 antibody (1:20) and then with anti-rabbit IgG–peroxidase as described in Materials and Methods. Lane H1 contains 0.2 µg histone H1 as a control. (**B**) Western blotting analysis of the material eluted from the ProTα–agarose column with 0.3, 0.6 and 1.0 M NaCl (lanes 1–3). The blot was probed with affinity-purified anti-H1 antibody as described above. (**C**) Specificity of the affinity-purified anti-H1 and anti-ct antibodies. An aliquot of 0.2 µg H1 (lane 1) and cell extract from 8×10^5 NIH 3T3 cells (lane 2) were fractionated by 15% SDS–PAGE, electrotransferred onto nitrocellulose membranes and probed with affinity-purified anti-H1 antibody (1:20). For detection of ProTα, 0.2 µg ProTα and NIH 3T3 cell extract (8×10^5 cells) were electrotransferred onto glutaraldehyde-activated nitrocellulose membranes and probed with affinity-purified anti-ct antibody (lanes 3 and 4), as described in Materials and Methods. Reactions were revealed by ECL.

complex which migrated with an intermediate velocity (Fig. 2B, lanes 2 and 3). The specificity of this interaction was highlighted by the fact that cytochrome c, a small equally basic protein (lane 7), does not retard ProTα under identical conditions (lanes 4–6). The migration of cytochrome c was affected at ProTα:cytochrome c ratios of 2 or 4, possibly due to a non-specific effect of the excess of negative charges (lanes 5 and 6).

In order to gain an insight into the binding site of ProTα within the H1 molecule, we treated H1 with trypsin. Limited trypsinization of H1 results in degradation of the non-folded protein tails, while the central globular domain (GH1) resists degradation (33; Fig. 2A). Like intact H1, the GH1 peptide retards ProTα, revealing complex formation (Fig. 2B, lane 9). These data suggest that the globular domain of histone H1 could be a target for ProTα. Whether additional contacts between the extensively charged H1 tails and ProTα further stabilize this interaction remains to be tested.

In a previous work we suggested that the acidic domain of ProTα was involved in the interaction with H1, based on the inhibitory effect of polyglutamic acid on ProTα–H1 binding (21). In order to test this prediction directly, we used as diagnostic tools three peptides modeled after the published amino acid sequence of bovine thymus ProTα (18): the acidic peptide, comprising the first 18 amino acid residues of the acidic region (residues 52–69), and the peptides thymosin α1 and ct (residues 1–28 and 87–109

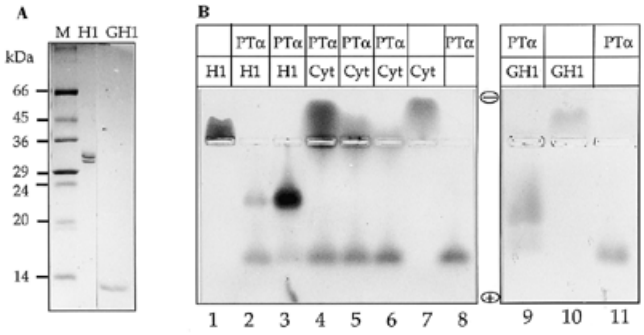

Figure 2. ProTα interacts with the globular domain of histone H1. (**A**) Purity of histone H1 and GH1 derived from H1 by trypsin digestion. Aliquots were resolved by 15% SDS–PAGE and stained with Coomassie blue. M, molecular weight markers. (**B**) An electrophoretic mobility shift assay reveals contacts between ProTα and GH1. The following amounts of proteins were incubated as detailed in Materials and Methods and electrophoresed on a 0.75% agarose gel. Lane 1, 3 µg H1; lane 2, 1.5 µg H1 + 6 µg ProTα; lane 3, 3 µg H1 + 6 µg ProTα; lanes 4–6, 6 µg ProTα + 6, 3 or 1.5 µg cytochrome c (Cyt) respectively; lane 7, 3 µg cytochrome c; lanes 8 and 11, 6 µg ProTα; lane 9, GH1 peptide equimolar to 8 µg H1 + 6 µg ProTα; lane 10, GH1 as in lane 9.

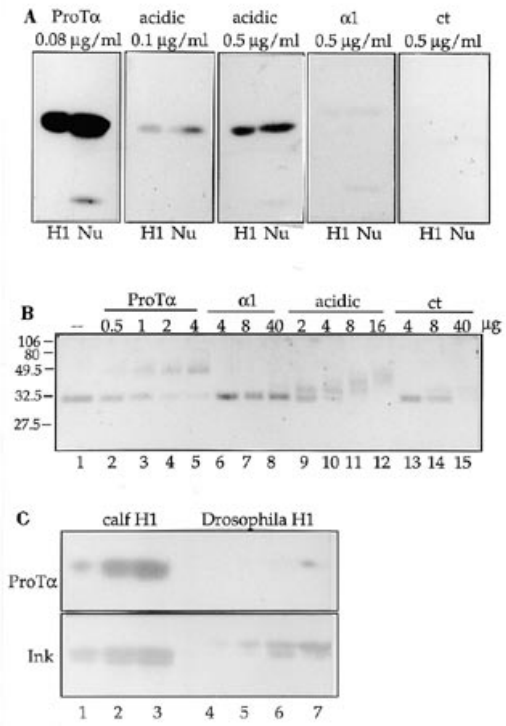

Figure 3. Identification of interacting sites within ProTα. (**A**) Far western analysis. Aliquots of 1 µg histone H1 (H1) and 40 µg total nuclear protein (Nu) were resolved by 15% SDS–PAGE and electrotransferred onto nitrocellulose membranes. The filters were incubated with the indicated concentrations of biotinylated ProTα or the biotinylated peptides acidic peptide, thymosin α1 and ct. Bound peptides were visualized with a streptavidin–peroxidase conjugate and the ECL technique. (**B**) Crosslinking analysis. An aliquot of 1 µg histone H1 (lane 1) was incubated with the indicated amounts of ProTα (lanes 2–5), thymosin α1 (lanes 6–8), acidic peptide (lanes 9–12) or ct peptide (lanes 13–15). Interacting partners were crosslinked with DMP and analysed by SDS–PAGE and Coomassie blue staining. Numbers to the left indicate the molecular weight markers in kDa. (**C**) Species specificity of the interaction. Aliquots of 0.1, 0.2 and 0.4 µg calf H1 (lanes 1–3) and 0.05, 0.1, 0.2 and 0.3 µg *Drosophila* histone H1 (lanes 4–7) were separated by 15% SDS–PAGE and transferred onto nitrocellulose filters. The membranes were incubated in blocking buffer (TBST with 0.2% gelatin) for 12 h and then with 0.2 µg/ml ProTα for 1.5 h at room temperature. The filters were probed with affinity-purified anti-ProTα antibody (anti-ct) and anti-rabbit IgG–peroxidase conjugate (panel ProTα). Reactions were revealed by ECL. Subsequently the membrane was stained with india ink to check for protein loading (panel ink).

respectively), which served as controls. Binding of these peptides to histone H1 was analysed by two independent methods: (i) ligand blotting assays, employing biotinylated peptides as probes; (ii) chemical crosslinking using DMP as a crosslinker. Ligand blotting assays showed that only the acidic peptide bound to histone H1 in a dose-dependent manner (Fig. 3A, acidic). Identical results were obtained from the crosslinking experiments (Fig. 3B). DMP addition to a mixture of histone H1 and increasing amounts of ProTα or the acidic peptide resulted in crosslinked species of higher molecular weight (Fig. 3B, lanes 2–5 and 9–10 respectively). The additional bands which appear when the acidic peptide is added in 40- or 80-fold molar excess are likely to be due to non-specific binding (Fig. 3B, lanes 11 and 12). In contrast, equivalent amounts of thymosin α1 and ct peptide did not crosslink to histone H1 (Fig. 3B, lanes 6 and 7, 13 and 14 respectively). When a vast excess of these peptides (40 µg) was incubated with H1, faint crosslinked species were observed (Fig. 3B, lanes 8 and 15). These results establish binding between the acidic region of ProTα and histone H1. Judging from the concentrations of ProTα and the peptides used, it is apparent that binding of the acidic peptide to H1 is significantly weaker compared with that of full-length protein. Therefore, it seems that intact ProTα contains additional binding determinants or folding of the acidic domain could be perturbed in such a way that some H1 binding sites are masked.

The interaction between histone H1 and ProTα displayed a remarkable species specificity: ProTα exhibited considerably reduced affinity for *Drosophila* H1 compared with calf H1 (Fig. 3C, compare lanes 1–3 and 4–7, ProTα) when equivalent amounts of linker histones were used (ink staining).

ProTα does not interact with H1 in chromatin

Our analysis of the ProTα–histone H1 interaction in chromatin was based on a chromatin reconstitution system using extracts derived from *Drosophila* embryos (35). Because of the low affinity binding of ProTα to *Drosophila* histone H1, we used calf histone H1 for all chromatin reconstitution experiments. Faithful incorporation of calf H1 into chromatin was verified by visualizing the characteristic increase in nucleosome repeat length (NRL) (29,35,41). Analysis of reconstituted chromatin by partial digestion with micrococcal nuclease (MNase) demonstrated a gradual increase in NRL from 185 to 205 and 215 bp when increasing amounts of calf histone H1 were titrated (Fig. 4A). The amount of H1 required to increase the NRL from 180 to 200 or 215 bp was defined as 1 or 2 U respectively. One unit of H1 corresponds to ~0.1 µg calf histone H1 in this experimental system.

In order to investigate whether ProTα was able to associate directly with H1-containing chromatin, we reconstituted chromatin on immobilized DNA (29), purified it magnetically and incubated it with ProTα. The chromatin beads were re-isolated from the reaction and the associated non-histone proteins were eluted with buffer containing 50, 400 or 650 mM NaCl. Immunoblotting analysis detected ProTα exclusively in the supernatant of the

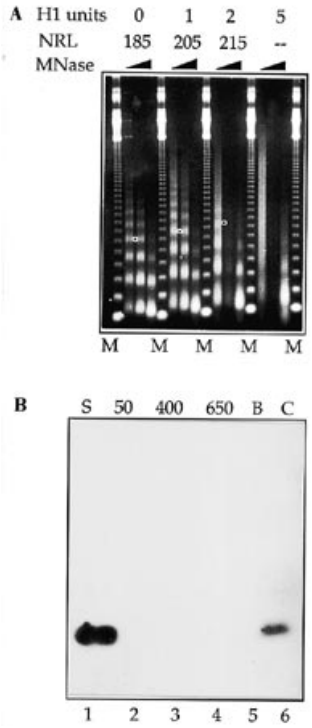

Figure 4. ProTα does not interact stably with H1-containing chromatin. (**A**) Reconstitution of chromatin with varying stoichiometries of H1. Plasmid DNA was assembled into chromatin with *Drosophila* extracts without H1 (0 units) or in the presence of the indicated units of calf histone H1. Incorporation of H1 was determined from the accompanying change in nucleosome repeat length (185, 205 and 215 bp). Reconstituted chromatin was digested with MNase for 0.5, 1 or 5 min. Purified DNA was analysed on a 1.3% agarose gel and stained with ethidium bromide. White circles mark the tetranucleosome-derived DNA, visualizing the increase in NRL. M, 123 bp ladder (BRL). (**B**) ProTα does not interact stably with H1-containing chromatin. Chromatin was reconstituted in the presence of 2 U calf histone H1 on 100 ng linearized plasmid DNA, immobilized onto paramagnetic beads. Magnetically purified chromatin was incubated with ProTα and purified again. Proteins in the supernatant were precipitated with acetone and the chromatin beads were subjected to salt extraction with 50, 400 and 650 mM NaCl. Western blot analysis with anti-ct antibody detects ProTα exclusively in the supernatant (lane 1) and not in the salt-extracted proteins or the remaining chromatin beads (lane 5). Lane 6 contains 0.2 µg ProTα as a positive control (C).

binding reaction (Fig. 4B), suggesting that the protein was unable to associate tightly with H1-containing chromatin.

ProTα modulates the interaction of H1 with chromatin

The fact that the GH1 domain of H1 is a potential binding site for ProTα raises the possibility that ProTα may actually modulate the interaction of H1 with chromatin. To visualize this competition, *Drosophila* chromatin was first assembled on immobilized DNA in the presence of histone H1, which was subsequently washed off the beads again with 650 mM NaCl, in order to create a nucleosomal array with available binding sites for H1. After equilibration at a lower salt concentration, the chromatin beads were incubated with 5 U H1 (or 2 U, data not shown) in the absence or presence of increasing concentrations of ProTα (see Materials and Methods). When ProTα was titrated into the reaction it efficiently prevented interaction of bulk H1 with chromatin, which was recovered in the supernatant (Fig. 5).

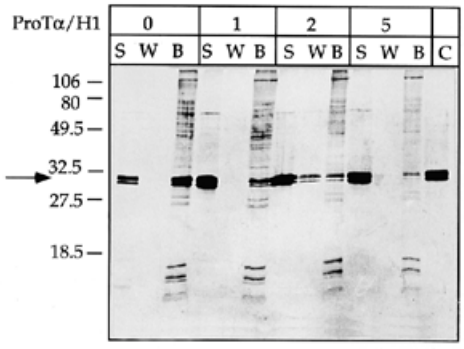

Figure 5. ProTα affects the binding of histone H1 to chromatin. Chromatin with free H1 binding sites was reconstituted on immobilized DNA and then incubated with 5 U calf histone H1, in the absence of ProTα (panel 0) or in the presence of ProTα at ProTα:H1 ratios of 1, 2 and 5 (w/w) (panels 1, 2 and 5). Supernatants were removed and the beads were washed three times with 20 mM Tris–HCl, 100 mM NaCl, 0.2% NP-40. Proteins in the supernatant (S), the last wash (W) and remaining on the beads (B) were fractionated by 15% SDS–PAGE and stained with silver. Numbers to the left indicate the molecular masses in kDa. The arrow points to histone H1.

Remarkably, when *Drosophila* H1 was used in a similar experiment, ProTα did not affect its binding to chromatin (not shown).

In a second approach, ProTα was directly added to a chromatin assembly mixture with or without histone H1. This time incorporation of H1 was followed by the characteristic change in NRL (Fig. 6). ProTα itself did not affect the NRL of chromatin (panels 1 and 2). Incorporation of 2 U histone H1 in the absence of ProTα increased the NRL from 180 to 215 bp, as expected (Fig. 6, panels 1 and 4). When, however, ProTα was included in the reaction with H1, the NRL increased only to 200 bp, rather than to 215 bp (panel 3). It must be noted that even a large excess of ProTα did not prevent interaction of an amount of H1 able to increase the NRL to 200 bp. These results indicate that ProTα selectively affects the interaction of H1 with chromatin.

ProTα extracts a fraction of histone H1 from chromatin but not from free DNA

Finally, we asked whether ProTα could detach histone H1 from chromatin or free DNA. Chromatin was assembled with 5 U histone H1, purified magnetically and excess histone H1 was washed off the beads. Incubation of these beads with increasing concentrations of ProTα released significant amounts of H1 from chromatin (Fig. 7A). The sensitivity of detection was increased by including traces of ^{32}P-phosphorylated H1 (upper panel). Identical results were obtained when 4 U H1 were used (data not shown). However, lower amounts of histone H1 (1 and 2 U) resisted extraction by ProTα (Fig. 7B), even at ProTα:H1 ratios of 10 or 20 (not shown). Remarkably, the interaction of *Drosophila* H1 with chromatin, even at high H1 inputs, was not affected by ProTα (Fig. 7C).

Importantly, the H1 molecules that were stripped from chromatin by ProTα did not represent linker histones bound to non-nucleosomal DNA, because ProTα was unable to dissociate histone H1 from naked DNA. H1–DNA complexes were formed after incubation of 1.0 µg immobilized DNA with 1.3 µg histone H1 and challenged with increasing concentrations of ProTα (Fig. 8). Even with a 5-fold excess of ProTα no release of H1 was

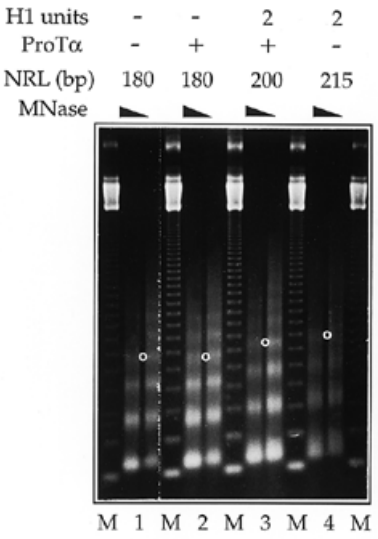

Figure 6. ProTα modulates the interaction of H1 with chromatin. DNA was assembled into chromatin in the *Drosophila* extracts without histone H1 (panels 1 and 2) or with 2 U histone H1 (panels 3 and 4) in the absence (panels 1 and 4) or presence of 6 µg ProTα (panels 2 and 3). The NRL was determined as in Figure 4**A**.

observed. Lower amounts of DNA (0.2 or 0.5 µg) gave identical results (not shown).

These results point to a particular mode of interaction of H1 with chromatin that is sensitive to extraction by ProTα.

DISCUSSION

ProTα is amongst the most abundant proteins in the mammalian nucleus (4–6), at amounts matching those of core histones (17). Yet, the function of this conserved, small and acidic polypeptide remains elusive. Using a variety of assays we have demonstrated an interaction between ProTα and linker histone H1, which is dependent on concentration (0.05–0.2 µg/ml) and temperature (21). In this concentration range ProTα does not bind to other highly charged proteins, such as core histones (21) or cytochrome c (this study), but in a higher concentration range binding of ProTα to core histones has been reported (42). It is noteworthy that biotinylated ProTα also retains its ability to interact with histone H1. In this study, we have isolated ProTα by immuno-affinity chromatography from crude cell extracts. By analysing the immunopurified material, we have shown that histone H1 has been retained by the anti-ProTα column. The ability of ProTα to interact with histone H1 may therefore provide insight into its physiological function.

In this work we analysed the effects of ProTα on the interaction of histone H1 with chromatin using a cell-free system for chromatin reconstitution under physiological conditions (35) on bead-immobilized DNA (29,43). ProTα failed to interact stably with chromatin in the presence or absence of H1. In addition, it did not bind DNA or H1-coated DNA directly (not shown). Furthermore, ProTα and purified calf thymus polynucleosomes did not co-fractionate during sedimentation in a sucrose gradient (data not shown).

Incorporation of increasing amounts of histone H1 into chromatin results in a characteristic increase in the nucleosome

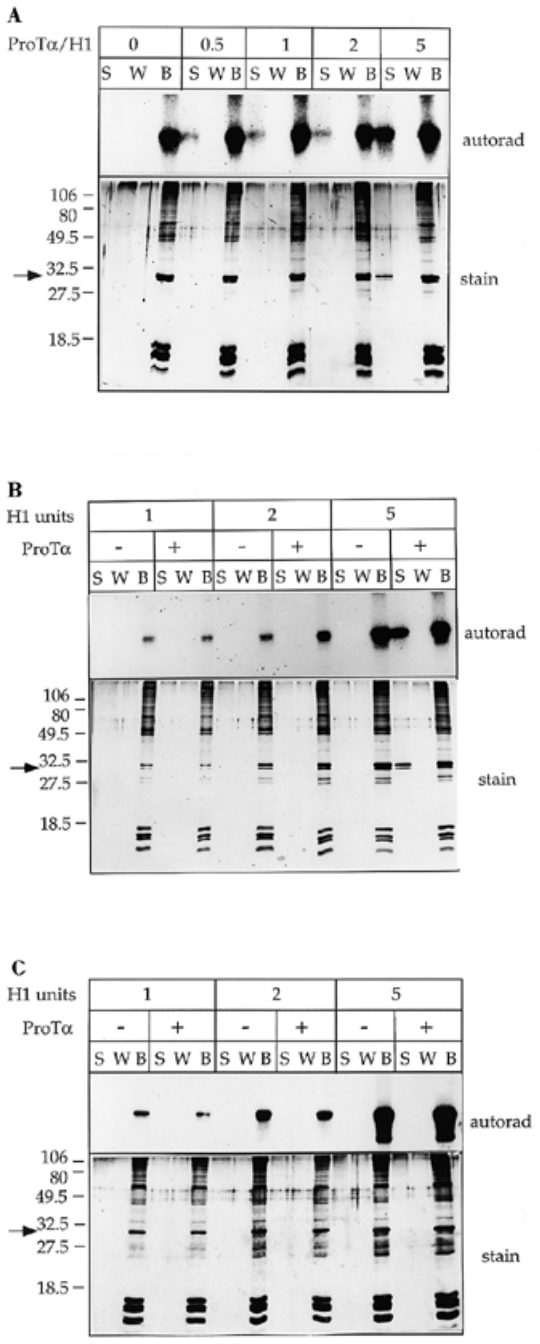

Figure 7. ProTα strips histone H1 from chromatin. (**A**) Chromatin was assembled on immobilized DNA with 5 U histone H1 and traces of ^{32}P-phosphorylated H1, purified magnetically, washed with 100 mM NaCl and incubated with buffer alone (panel 0) or increasing amounts of ProTα [indicated ratios of ProTα:H1 (w/w)]. Chromatin was isolated again and the proteins in the supernatant (S), those removed by washing with 100 mM salt (W) and those remaining stably bound on the beads (B) were separated by 15% SDS–PAGE. The gel was autoradiographed (upper) and stained with silver (lower). Numbers to the left indicate positions of molecular weight markers in kDa. The arrow marks the position of H1. (**B**) As (A) with the following modifications. Chromatin was reconstituted with 1, 2 and 5 U histone H1 and challenged with ProTα at a ProTα:H1 ratio of 5 (w/w). (**C**) As (B) except that *Drosophila* H1 was substituted for calf H1. In contrast to calf H1, *Drosophila* H1 is not released from chromatin in the presence of ProTα (see B).

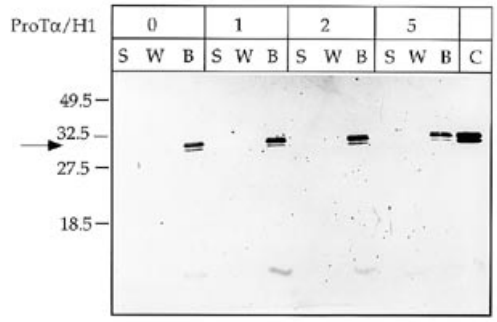

Figure 8. ProTα does not extract histone H1 from naked DNA. Histone H1 (1.3 µg) was incubated with 1.0 µg DNA to form H1–DNA complexes (see Materials and Methods). After incubation, the beads were washed with 150 mM NaCl and incubated with buffer alone (panel 0) or with ProTα, at ProTα:H1 ratios of 1, 2 and 5 (w/w). Proteins in the supernatant (S), those released during the last wash step (W) and those remaining on the beads were analysed by 15% SDS–PAGE and stained with silver. (**C**) Input of histone H1 (1.3 µg).

repeat length (NRL), a measure of the linker length that separates adjacent nucleosomes (35,41). If H1 is titrated into a chromatin assembly reaction, the NRL is increased to the physiological value of ~200 bp, but further addition of H1 results in even wider nucleosome spacing, with NRLs reaching 220 bp (35). Remarkably, the presence of ProTα during chromatin assembly did not affect incorporation of sufficient H1 to increase the NRL to the physiological value of ~200 bp, but prevented association of excess H1. In a complementary experiment, ProTα was able to detach a fraction of H1 from chromatin if excess H1 (over the amount required to establish the physiological spacing, i.e. 4–5 U) had been incorporated. In contrast, the amount of H1 required to increase the NRL to 200 bp resisted extraction by ProTα. These results suggest the presence of at least two distinct interaction modes of H1 with chromatin that can be distinguished by their sensitivity to ProTα extraction.

Since substoichiometric amounts of H1 gradually increase the nucleosomal spacing (44), it is difficult to derive information about the H1/nucleosome stoichiometry from NRL measurements. However, there is evidence for varying H1 stoichiometry *in vivo* with values exceeding one per nucleosome (26) and several studies have suggested that NRLs beyond 200 bp may reflect the binding of a second H1 molecule to a nucleosome core (45,46). Recently, Nightingale *et al.* (47) showed that more than one molecule of histone H1 may associate with a nucleosome, with secondary sites having a lower affinity, comparable with that of the association of histone H1 with naked DNA. Importantly, despite the lower affinity of H1 for naked DNA compared with chromatin (48), ProTα was not able to release H1 from naked DNA. This suggests that *in vitro* association of excess H1 with chromatin is not simply due to binding to naked DNA, but is rather due to a different type of interaction. ProTα may, therefore, regulate the extent and mode of association of H1 with chromatin.

The structural and functional significance of varying histone H1 stoichiometry in chromatin is presently unclear. However, given the fact that H1 is a major determinant of chromatin fibre folding (25,49–51) and that H1–nucleosome interactions are profoundly altered in active chromatin (24), varying H1 stoichiometry may indicate distinct higher order structures and/or functional states. Evidence is accumulating for a key role of histone H1 in transcriptional repression of a selected group of genes (22,27,28,30,52). Gene activation during development or in rapid response to inducers may require the selective dissociation of H1 from specific target sites. While our data do not support a role of ProTα in active release of all histone H1 from chromatin, they are compatible with a role as an acceptor for H1 molecules that are stripped from chromatin by other activities. Recently, Dimitrov and Wolffe (53) showed that nucleoplasmin, an abundant protein in early developmental stages of *Xenopus*, was able to release histone H1 from chromatin. Interestingly, ProTα resembles nucleoplasmin with respect to the highly acidic region that associates with histone H1.

Another potential function for ProTα is suggested from the observation that its concentration is particularly high in proliferating cells (3,9–11,17). The assembly of newly replicated DNA into chromatin in dividing cells requires the faithful incorporation of histone H1. By analogy to the core histones, which are kept soluble in complexes with acidic carriers that ensure their faithful assembly into nucleosomes (54–57), it is possible that freshly synthesized linker histones associate with carrier molecules that ensure the proper association of H1 with nucleosomal fibres. The highly charged linker histone, if not safeguarded by a protein, is expected to associate with DNA and other proteins in a manner that could be harmful to the cell. We have shown that ProTα will allow association of sufficient H1 to increase the NRL to the physiological value of 200 bp, but will prevent further association of H1. It will also detach H1 from low affinity binding sites on chromatin that differ from naked DNA. These results are compatible with a role of ProTα as an H1 carrier molecule. This hypothesis is supported by recent studies which showed that ProTα is present in the cell nucleus throughout the cell cycle, with highest levels at S phase, and its gene promoter can be strongly induced by transcription factor E2F (16). E2F is an important component of the mechanism which controls progression of cells into S phase, while the targets for its action include genes encoding proteins directly involved in DNA replication and cell cycle regulation (58).

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