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Prothymosin α associates with the oncoprotein SET and is involved in chromatin decondensation

Zoe Karetsou[a], Goran Martic[a], Sotiria Tavoulari[a], Savvas Christoforidis[a], Matthias Wilm[b], Claudia Gruss[c], Thomais Papamarcaki[a,*]

[a]*Laboratory of Biological Chemistry, Medical School, University of Ioannina, 451 10 Ioannina, Greece*
[b]*EMBL Heidelberg, Gene Expression Programme, Meyerhofstrasse 1, 69117 Heidelberg, Germany*
[c]*Department of Biology, University of Konstanz, Konstanz 78457, Germany*

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Abstract Prothymosin α (ProTα) is a histone H1-binding protein that interacts with the transcription coactivator CREB-binding protein and potentiates transcription. Based on coimmunoprecipitation and mammalian two-hybrid assays, we show here that ProTα forms a complex with the oncoprotein SET. ProTα efficiently decondenses human sperm chromatin, while overexpression of GFP–ProTα in mammalian cells results in global chromatin decondensation. These results indicate that decondensation of compacted chromatin fibers is an important step in the mechanism of ProTα function.

Keywords: Prothymosin α; CREB-binding protein; Oncoprotein SET; Chromatin decondensation

1. Introduction

Mammalian genomic DNA is organized into chromatin that presents a barrier to DNA-dependent processes such as transcription, replication and DNA repair [1]. It is now established that DNA-binding factors regulate recruitment and further assembly of multicomponent coactivator complexes on the promoter regions to establish a local chromatin structure that is permissive for subsequent events [2].

Intense research in the past decade has advanced our understanding on the basic subunit of chromatin, the nucleosome; however, much less is known about the chromatin structure beyond the ''30-nm'' fiber. Linker histone H1 is the major factor that stabilizes both intramolecular folding and fiber–fiber interactions [3]. Perturbation of the interaction of H1 with chromatin has been shown to increase the accessibility of nucleosomes [4] and facilitate the action of remodeling complexes [5]. In this context, proteins that destabilize H1 interaction with chromatin might function as chromatin 'opening' factors.

A recent example is prothymosin α (ProTα), an acidic protein involved in cell proliferation [6–8] and apoptosis [9–11]. Biochemical studies have shown that H1 co-immunoprecipitates with ProTα from crude cell extracts, while a fraction of H1 is released when chromatin is challenged with ProTα [12]. More recent work has shown that ProTα potentiates transcription through interaction with the transcriptional coactivator CREB-binding protein (CBP) [13] and selectively enhances estrogen receptor transcriptional activity [14]. Furthermore, ProTα has been found to modulate histone acetyltransferase activity through interaction with Epstein Barr nuclear antigen 3C [15].

To understand better the function of ProTα, we attempted to identify proteins that associate with this acidic polypeptide using immunoprecipitation assays. In this work, we identified the oncoprotein SET (also known as TAF-Iβ, PHAPII, I_2^{PP2A}) as a ProTα-interacting protein. Our data suggest that the interaction of ProTα with SET is promoted and/or stabilized by the transcriptional coactivator CBP in vivo. Furthermore, we show that ProTα and SET induce global chromatin decondensation.

2. Materials and methods

2.1. Plasmids

pFlag–SET was a gift from Dr. D.C. Tkachuk (VA Puget Sound Health Care System, Seattle, Washington). To generate pGAL4–SET, the SET gene was amplified and cloned into the *Eco*RI–*Xba*I restriction sites of the pBXGI vector. For pGFP–SET and pGFP–NSET, the SET and NSET (amino acid residues 1–210) genes were cloned into the *Eco*RI–*Sal*I sites of pEFGP-C3 vector (Clontech). pGFP–ProTα was made by inserting ProTα gene into *Eco*RI–*Sma*I cleavage sites of pEGFP–C1 expression vector. For pVP16-ProTα, ProTα gene was cloned into *Eco*RI-*Sal*I restriction sites of pVP16 vector (Clontech). All constructs have been verified by sequencing.

2.2. Antibodies and recombinant proteins

A polyclonal antibody recognizing the NH_2-terminus of SET (residues 1–23) was raised in rabbits (Davids Biotechnology, Germany) and the serum was purified over a peptide-agarose affinity column. Anti-CBP rabbit polyclonal antibodies (A-22) were purchased from Santa Cruz Biotechnology. ProTα was detected by the affinity-purified anti-ct antibody [12]. Baculovirus expressed glutathione-*S*-transferase-tagged SET was prepared according to Bac-To-Bac baculovirus expression system procedure (Invitrogen).

2.3. Cell culture, transient transfection and luciferase assays

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) in the presence of 10% fetal calf serum and transfected using the calcium phosphate method [13]. Luciferase activity was detected in the cell extracts using a Promega luciferase assay system, according to the manufacturer's instructions.

* Corresponding author. Fax: +30 26510 97868.
E-mail address: thpapama@cc.uoi.gr (T. Papamarcaki).

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2.4. Immunoprecipitation

Whole cell extracts or S100 extracts were prepared from HeLa cells [16]. The extracts were pre-cleared with 20 μl of IgG–protein A (40 μg of IgG) for 30 min at room temperature and then incubated with 20 μl of anti ct–protein A beads (30 μg of anti-ct antibody) or with 20 μl IgG–protein A beads for 4 h at 4 °C. The antibodies were covalently coupled to the beads using DMP as a crosslinker [13]. After incubation, the beads were washed, resuspended in 15 μl Laemmli buffer and analyzed as indicated.

2.5. Mass spectroscopy and peptide sequencing

Proteins bound on the anti-ct/Protein A beads were separated by SDS/PAGE and visualized with silver staining. Protein bands were excised from the silver-stained gel, reduced, alkylated and digested overnight with trypsin. Peptide sequences were obtained by nano-electrospray tandem mass spectrometry [17].

2.6. Chromatin decondensation assays

Human semen from a healthy fertile donor and kept frozen until use. Demembraned sperm chromatin was incubated with 6 μg of each protein and after the incubation, 1-μl aliquot of the reaction mixture was added to 1 μl of PBS containing 50% glycerol, 7.4% formaldehyde and 5 μg/ml Hoechst 33258 [18]. The DNA was visualized under a fluorescent microscope.

HeLa cells were transiently transfected with pGFP-expression plasmids. 36h post-transfection cells were fixed with methanol at −20 °C for 5 min, followed by incubation in 3.8% paraformaldehyde for 20 min at room temperature and quenched in 50 mM ammonium chloride for 15 min. Cell nuclei were stained with 2.5 ng/ml Hoechst and observed under a fluorescence microscope.

Immunofluorescence images of sperm or Hela cell nuclei were imported as TIFF files into the AutoCAD program. The perimeter of the nuclei and their area were measured using the *spline* command of the program.

3. Results and discussion

3.1. Identification of a complex containing ProTα and the oncoprotein SET

To search for ProTα-interacting proteins, we performed immunoprecipitation experiments using HeLa cell extracts. Specific antibodies against ProTα (anti-ct) or non-specific IgG used as a negative control were cross-linked on Protein-A beads and subsequently incubated with the cell extracts. After incubation, the beads were washed with 0.6 M NaCl and the bound proteins were eluted with 1 M NaCl. SDS–PAGE and silver staining of the immunoprecipitated material revealed two major bands of approximately 39 and 42 kDa (p39, p42) that were not present in the control beads (Fig. 1A). The immunoprecipitation of these proteins could be inhibited by pre-incubation of the antibody beads with the antigenic peptide ct (not shown). The protein bands were subjected to mass spectrometric analysis and we managed to obtain sequence data from p39, which was identified as the myeloid leukemia associated oncoprotein SET, also known as template activating factor-1β (TAF-1β) or inhibitor of protein phosphatase 2A (I_2^{PP2A}) [19,20]. The presence of SET was confirmed by Western blotting analysis of the immunoprecipitated material using specific polyclonal antibodies (Fig. 1A). Furthermore, the anti-ct antibody did not cross-react with SET, suggesting that the oncoprotein is pulled down through specific interactions with the components of the complex (not shown).

The oncoprotein SET belongs to the evolutionarily conserved family of histone chaperones (nucleosome assembly factors, NAPs) that mediate histone storage, nucleosome assembly and sperm chromatin decondensation and it contains a long polyacidic track located at its carboxyterminal region [21] (Fig. 1B). SET was first identified as a gene that was fused to the *CAN* gene in acute undifferentiated leukemia as a result of a translocation [22]. Subsequent work has shown that SET binds to histones, remodels chromatin structure [23,24] and inhibits histone acetylation as a component of the INHAT complex [25]. Recently, SET has been found to inhibit the granzyme A activated-DNase activity of the tumor metastasis suppressor NM23-H1, which induces caspase-independent DNA damage characterized by single-stranded DNA nicks and other features of apoptosis [26]. Therefore, two independent studies point to the key roles of ProTα and SET in the regulation of apoptosis providing new evidence for the mechanisms that regulate cell life and death [11,26]. Our results suggest a physical interaction between ProTα and SET, which might be important for the physiological role of these acidic proteins. However, we were unable to show direct binding between ProTα and SET using GST pull-down or in vitro binding assays (not shown). Therefore, we suggest the presence of at least one bridging factor between SET and ProTα. This

Fig. 1. ProTα co-immunoprecipitates with the oncoprotein SET. (A) Immunoprecipitation experiments in Hela cell lysates (S100) using control IgG/protein A beads (lane 1) or anti-ct antibody/protein A beads (lane 2). The beads were washed with 0.6 M NaCl and bound proteins were eluted with 1M NaCl. The sample was divided and analyzed by 15% SDS–PAGE and silver staining or Western Blotting with the anti-PTα and anti-SET antibodies. Reactions were revealed by enhanced chemiluminesence (ECL). (B) Mass spectrum analysis of 39 kDa protein band. The sequenced peptides are underlined within the SET amino acid sequence. The long carboxyterminal polyacidic track of SET is shown in bold and italics.

molecule could be the co-immunoprecipitated protein (p42) or alternatively other factors which are not detectable by silver staining.

3.2. ProTα-SET interaction is promoted and/or stabilized by CBP

To demonstrate further the in vivo interaction between ProTα and SET, we employed mammalian two-hybrid cell based assays using VP16 activation domain-tagged ProTα and GAL4-SET. When GAL4-SET and VP16-ProTα were co-transfected in HeLa cells, 1.25-fold stimulation of the activity of the reporter 5× GAL4-E1B-luc was observed, suggesting the formation of a transient and dynamic complex on the promoter (Fig. 2A). Identical results were obtained when GAL4-ProTα was co-transfected with VP16-SET (not shown). Our previous work has shown direct interaction between ProTα and the transcription coactivator CBP [13], which is a multifunction protein that interacts with a variety of transcription factors, signaling molecules and nuclear hormone receptors [27]. Furthermore, direct binding of SET with CBP has been detected (Karetsou et al., unpublished data). On the basis of these findings, we suspected that CBP might exist in the ProTα/SET complex possibly acting as a bridging molecule between these acidic proteins. However, the coactivator was not detected by silver staining in our gels, which could be due to the fact that CBP is a high molecular weight protein (2441 aa) extremely susceptible to proteolysis in cell extracts [28]. Alternatively, CBP might be a minor or transient component of this complex not detectable by protein staining.

When CBP was cotransfected with GAL4-SET and VP16-ProTα, the activity of the reporter was significantly enhanced (Fig. 2A, left panel). Furthermore, CBP did not activate the promoter either alone or in the presence of Vp16-ProTα (Fig. 2A, right panel). These results propose that the interaction of SET with ProTα might be promoted and/or stabilized by CBP in vivo. Supporting evidence to this suggestion was provided by immunoprecipitation experiments in HeLa cell extracts, which identified CBP in ProTα/SET immunoprecipitated material (Fig. 2B).

Collectively, our data indicate the existence of a novel complex containing ProTα, the histone chaperone SET and the histone acetylase CBP that may be involved in the regulation of chromatin structure.

3.3. ProTα and SET induce global chromatin decondensation

The function of decondensation factors in promoting chromatin remodeling accompanied by nuclear swelling has been known for many years [29,30]. SET exhibits chromatin decondensation activity, while a mutant form of SET that lacks the carboxyterminal acidic region is not functional [18]. The long polyacidic region mainly composed of glutamic acid stretches is a common structural feature between SET and ProTα. This raises the interesting possibility that the two acidic proteins have similar and/or complementary functions in modulating chromatin structure. To support this idea, we used sperm chromatin as a well-established model system that represents a physiologically compacted chromatin template [18,30]. Demembraned human sperm nuclei were incubated with purified ProTα, baculovirus expressed SET, or BSA as control. After incubation, chromosomal DNA was stained with Hoechst 33258 and visualized under a fluorescence microscope. As shown in Fig. 3A, ProTα was able to decondense sperm chromatin in vitro, with efficiency comparable to that of SET. Using the AutoCAD program, we estimated that 50% of total sperm nuclei displayed approximately a 1.5–2.0-fold increase in their area upon challenging with ProTα (Fig. 3B). Taking into account the in vivo interaction between ProTα and SET, these results support the notion that these acidic proteins might be recruited to gene promoters in order to decondense the compacted chromatin fibers in mammalian cells.

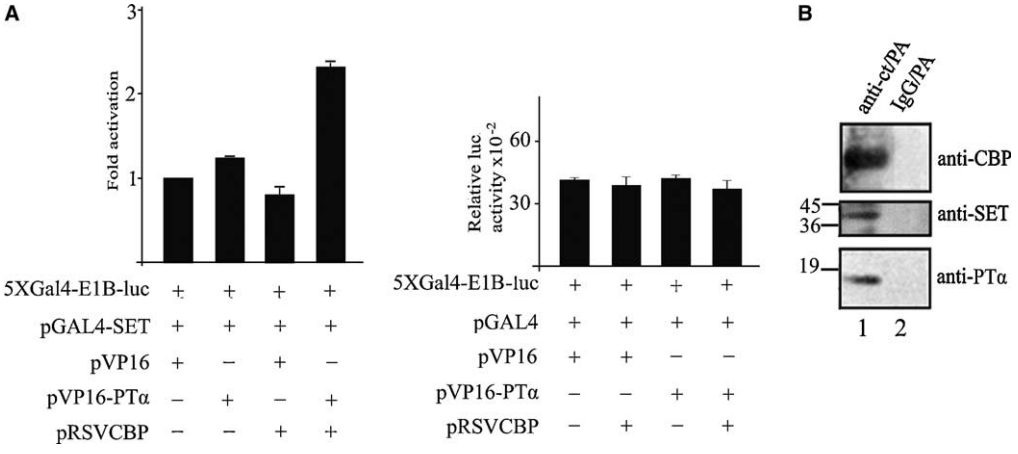

Fig. 2. ProTα binds to SET in the presence of CBP. (A) (left panel) A mammalian two-hybrid assay was performed with HeLa cells transfected with 0.5 μg of 5× GAL4-E1B-luc reporter plasmid, 2 μg of pVP16-PTα expression vector, and 2 μg of pGAL4-SET in the presence or absence of 3 μg of pRSVCBP. The experiments were repeated at least three times and the error bars indicate the standard deviations of triplicate values. (right panel) HeLa cells were co-transfected with 0.5 μg of 5× GAL4-E1B-luc, 2 μg of pGAL4 and 2 μg of pVP16-PTα in the presence or absence of 3 μg of pRSVCBP, as indicated. In all assays, 0.1 μg of a plasmid expressing β-galactosidase (pCMV-LacZ) was cotransfected and used for internal standard. Total DNA was adjusted using empty vectors. Luciferase values were normalized to each other based on the respective β-galactosidase activity. The assays were repeated at least three times and the values shown are derived from triplicate readings. (B) Whole cell lysates from HeLa cells were immunoprecipitated with anti-ProTα antibody/Protein A beads. Immune complexes were analyzed by Western blotting with anti-CBP (A-22), anti-SET and anti-ProTα antibodies. Reactions were visualized by ECL.

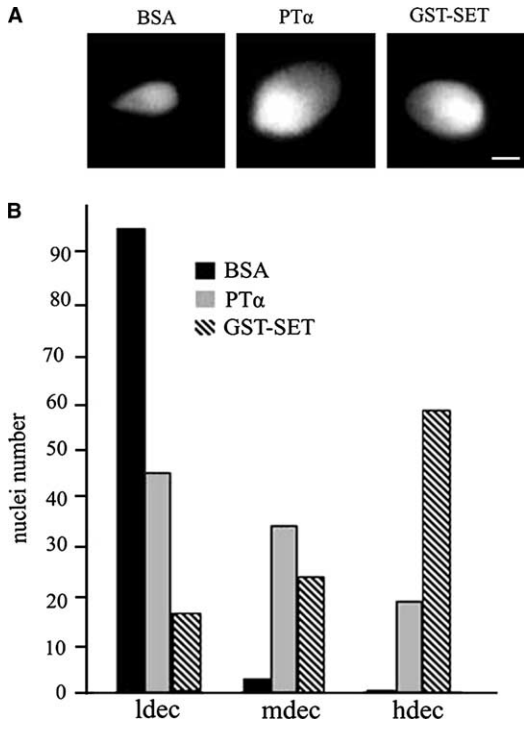

Fig. 3. Decondensation of human sperm chromatin by SET and ProTα. (A) Sperm chromatin was incubated with 6 μg of ProTα, SET and BSA, as control. After incubation, aliquots were mixed with the fixation buffer containing the Hoechst dye and chromosomal DNA was visualized under a fluorescence microscope. Bar size 5 μm. (B) Quantitation of the nuclei area of the experiment described in (A) with the AutoCAD 2000 program. The measurements were classified into three groups (1–1.5, 1.5–2 and >2), indicating low, medium and highly decondensed nuclei (*ldec*, *mdec*, and *hdec*), respectively.

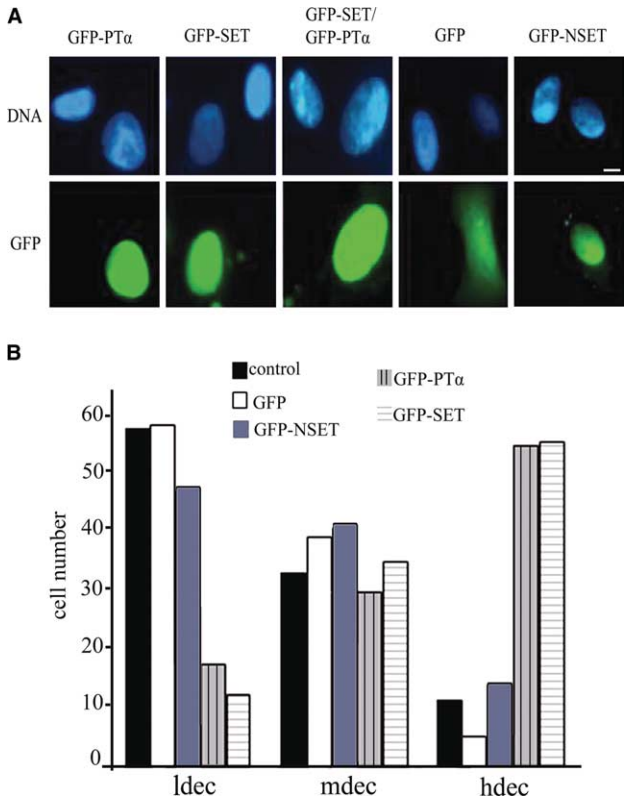

Fig. 4. SET and ProTα induce global chromatin decondensation. (A) HeLa cells were transiently transfected with 3 μg of pGFP, pGFP–PTα, pGFP–SET, and pGFP–NSET expression plasmids, as indicated. 36h post-transfection, cells were fixed, stained with Hoechst 33258 and observed under a fluorescence microscope. Bar size 10 μm. (B) Quantitation of the area of 100 transfected nuclei with AutoCAD 2000. The measurements were classified into three groups (<6, 6–8 and >8), indicating low, medium and highly decondensed nuclei (*ldec*, *mdec*, and *hdec*), respectively.

To further address this hypothesis, GFP–ProTα and GFP–SET expression plasmids were transiently transfected into HeLa cells and 36 h post-transfection, cells were fixed and stained with Hoechst 33258. We observed that overexpression of the fusion proteins resulted in enlargement of the nuclei compared to non-transfected cells, cells transfected with either the GFP vector alone, or with GFP–NSET that lacks the polyacidic stretch of SET. Co-expression of both chaperones resulted in an additive decondensation effect (Fig. 4A). Measurements of the nuclei area revealed that the average area of 100 nuclei transfected with pGFP–ProTα or pGFP–SET or with both proteins was increased by 1.5-, 1.3- and 2.1-fold, respectively, compared with the controls (Fig. 4B). The transfected cells exhibited normal cell cycles, which rules out the possibility that enlargement of nuclei could be due to cell arrest in late S phase. Similar results were obtained when non-tagged or Flag-tagged ProTα and SET were overexpressed in HeLa cells (not shown). The mechanisms that regulate the condensation/decondensation state of chromatin fibers are not known and at present, few experimental approaches allow dissection of the decondensation activities of nuclear factors. Overexpression is a first attempt to study the decondensation activity of ProTα and SET in vivo. Recent studies have visualized focal decondensation of chromatin after tethering the glucocorticoid receptor (GR) [31] or the VP16 acidic activation domain [32] within condensed chromatin fibers, in mammalian cells.

It is widely accepted that the assembly of multicomponent coactivator complexes that alter the compaction state of chromatin structure is an obligatory step for several DNA-dependent processes [33]. The presence of ProTα and SET in gene promoters might contribute to the disruption of higher-order chromatin folding through interactions with the key elements that stabilize the compact chromatin fibers, namely linker histone H1 and core histones. The recruitment and the formation of such a remodeling complex is possibly regulated by the histone acetyltransferase and transcription coactivator CBP. Furthermore, since both SET and ProTα enhance the transactivation potential of the coactivator (unpublished work), it is tempting to speculate that these acidic proteins might also modulate the activity of CBP, which has been reported to be necessary for decondensation events in other systems [33]. Future work should map the genomic sites of ProTα and SET and investigate whether these proteins, besides their global effect on chromatin folding, influence the activity of specific genes.

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