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Prothymosin α interacts with the CREB-binding protein and potentiates transcription

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Prothymosin α (ProTα) is a histone H1-binding protein localized in sites of active transcription in the nucleus. We report here that ProTα physically interacts with the CREB-binding protein (CBP), which is a versatile transcription co-activator. Confocal laser scanning microscopy reveals that ProTα partially colocalizes with CBP in discrete subnuclear domains. Using transient transfections, we show that ProTα synergizes with CBP and stimulates AP1- and NF-κB-dependent transcription. Furthermore, overexpression of ProTα enhances the transactivation potential of CBP. These findings reveal a new function for ProTα in transcription activation, probably through CBP-mediated recruitment to different promoters.

INTRODUCTION

Prothymosin α (ProTα; 109–111 amino acids, pI 3.5) is perhaps the most acidic protein existing in the cell nucleus (Haritos *et al.*, 1984; Clinton *et al.*, 1991; Manrow *et al.*, 1991). The function of ProTα is associated with cell growth (Eschenfeldt and Berger, 1986) and its gene is a target of the c-myc proto-oncoprotein (Eilers *et al.*, 1991).

The unusual chemical features of ProTα provide important clues for its function. The primary structure of ProTα contains 52 acidic residues, 31 of which are clustered in the central part of the molecule (Frangou-Lazaridis *et al.*, 1988). Similar polyacidic tracks are found in several nuclear proteins whose functions are linked to chromatin decondensation, nucleosome assembly/disassembly and establishment of chromatin structures competent for transcription (Earnshaw, 1987). Previous studies have shown that ProTα interacts specifically with linker histone H1, which is a major determinant of the folding of nucleosomal arrays into higher-order structures (Papamarcaki and Tsolas, 1994; Karetsou *et al.*, 1998); under *in vitro* conditions, binding to core histones at high concentrations has also been reported (Diaz-Jullien *et al.*, 1996). In the context of a physiological chromatin reconstitution reaction, we have shown previously that ProTα modulates the interaction of H1 with chromatin (Karetsou *et al.*, 1998). Moreover, other studies have shown that cells overexpressing ProTα have increased levels of histone H1-depleted chromatin (Gomez-Marquez and Rodriguez, 1998). Taken together, these results support the notion that ProTα is involved in chromatin remodeling.

Recently, indirect immunofluorescence labeling and confocal scanning laser microscopy studies have indicated that ProTα is localized close to transcription sites and in nuclear domains that often overlap or engulf promyelocytic leukemia protein-containing bodies (PML bodies) (Vareli *et al.*, 2000). Interestingly, PML bodies contain several transcription factors, CREB-binding protein (CBP)/p300 transcription co-activators and RNA polymerase II (von Mikecz *et al.*, 2000). These morphological observations raise the interesting possibility that ProTα is implicated in transcription. In this study, we report that ProTα interacts with the transcription co-activator CBP both *in vivo* and *in vitro*, and colocalizes with it in subnuclear domains. Using transient transfections, we show that ProTα synergizes with CBP and stimulates AP1- and NF-κB-dependent transcription. In addition, we find that overexpression of ProTα stimulates the transactivation activity of CBP. Based on these results, we propose that ProTα is a new CBP cofactor that potentiates transcription, possibly through reorganization of the chromatin structure.

RESULTS

ProTα interacts with CBP *in vivo* and *in vitro*

Recent studies have shown that ProTα is localized in sites of active transcription (Vareli *et al.*, 2000) and interacts with the

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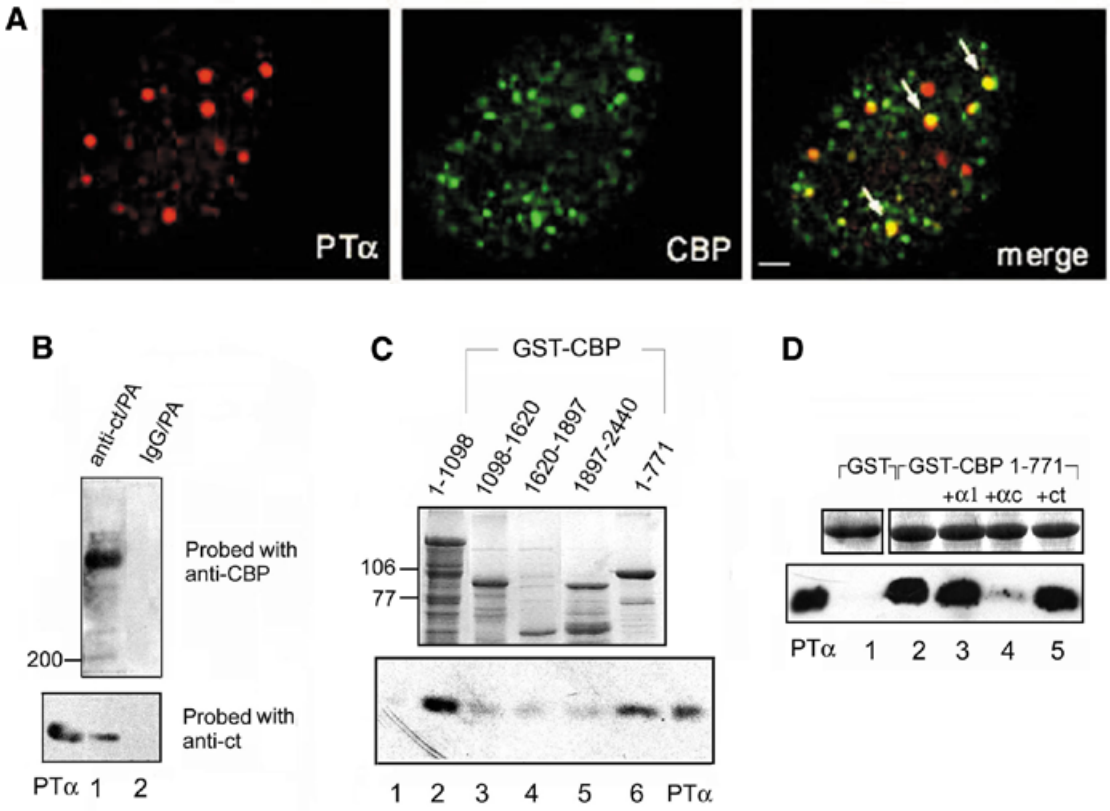

Fig. 1. ProTα binds to CBP *in vivo* and *in vitro*. (**A**) CBP colocalizes with ProTα. HeLa cells were double-labeled with the polyclonal anti-ProTα (anti-ct, red) and the monoclonal anti-CBP antibody C1 (green). The merge represents the overlay of ProTα and CBP staining, and arrows indicate the colocalization of the proteins. Size bar, 2 μm. (**B**) *In vivo* interaction between ProTα and CBP. Whole-cell lysates from HeLa cells were immunoprecipitated using anti-ct antibody–protein A beads (lane 1) or control IgG–protein A beads (lane 2) as indicated in Methods. Immune complexes were analyzed by western blotting with the specific anti-CBP antibody (A-22) diluted 1:200 (upper panel) and the affinity-purified anti-ct antibody diluted 1:500 (lower panel). The blot for ProTα represents 1/5 of the total sample and PTα indicates 0.1 μg of ProTα used as a positive control. Reactions were detected by enhanced chemiluminescence (ECL) (Amersham). (**C**) GST pull-down experiments. Purified GST (lane 1) or GST fusion proteins (2 μg each) encoding different CBP fragments (1–1098, 1098–1620, 1620–1897, 1897–2440, 1–771, lanes 2–6) were immobilized on glutathione–agarose beads and incubated with 2 μg of ProTα as indicated in Methods. The beads were washed and analyzed for ProTα by western blotting. PTα corresponds to 0.1 μg of purified protein. Detection was performed by ECL. The upper panel shows Coomassie Blue staining of the gel to document equal loading. (**D**) Identification of the CBP-binding site within the ProTα molecule. ProTα (2 μg) was incubated with GST (lane 1), or the CBP fragment 1–771 (4 μg each) was immobilized on glutathione–agarose beads in the absence (lane 2) or in the presence of 6 μg of the indicated peptides: thymosin α1, the acidic peptide (ac) or the peptide ct (lanes 3–5, respectively) (for sequences see Methods). The beads were analyzed as indicated in (C) and 0.05 μg of ProTα (PTα) were used as positive control. The upper panel shows Coomassie Blue staining of the relevant part of the gel to show equal loading. The insert shows the loading of GST for comparison.

histone acetyltransferase p300 (Cotter and Robertson, 2000). To gain insight into the potential role of ProTα in transcription, we performed colocalization studies for ProTα and the versatile transcription co-activator CBP, a protein highly homologous to p300, employing specific antibodies in dual labeling experiments followed by confocal microscopy. Using the monoclonal anti-CBP (C1) and the polyclonal affinity-purified anti-ProTα antibodies (anti-ct), we found that CBP staining partially overlapped with that of ProTα (Figure 1A).

To test whether the colocalization of CBP and ProTα correlates with *in vivo* complex formation of these proteins, we performed co-immunoprecipitation assays. Lysates from HeLa cells were immunoprecipitated with the anti-ct antibodies and the presence of CBP was detected by western blotting. This analysis revealed that CBP co-immunoprecipitated with ProTα (Figure 1B, lane 1). Control IgG–protein A beads did not bind CBP and ProTα (Figure 1B, lane 2). Furthermore, no cross-reaction of the anti-ct antibodies with CBP was detected in western blots (data not shown).

Direct evidence for a physical interaction between ProTα and CBP was obtained by glutathione *S*-transferase (GST) pull-down experiments. Purified ProTα was tested for its ability to interact with different fragments of CBP fused to GST. We found that ProTα binds to the N-terminal region of CBP (lane 2) and, more precisely, within the first 771 amino acids (Figure 1C, lane 6). This region includes several functional domains of CBP, such as the binding site for nuclear hormone receptors, the zinc finger domain and the CREB binding domain (Goodman and Smolik, 2000). The binding was specific because ProTα was not retained by GST alone (lane 1), or by other regions of CBP (lanes 3–5).

In addition, the CBP fragments tested ranged in pI from 7.35 (region 1–1098) to 11.35 (region 1897–2440), which argues against non-specific binding of ProTα to basic regions of CBP.

To identify the region of ProTα responsible for the interaction with CBP, we performed additional GST pull-down assays in the presence of three peptides modeled after the published amino acid sequence of bovine thymus ProTα, thymosin α1, the acidic peptide (ac) and the C-terminal peptide ct (residues 1–26, 52–69 and 87–109, respectively). The results shown in Figure 1D indicate that the acidic peptide inhibited strongly the *in vitro* binding of CBP fragment 1–771 with ProTα; a peptide composed exclusively of glutamic residues also inhibited binding, suggesting that this interaction is highly dependent on the presence of polyglutamic stretches in ProTα (see Supplementary data available at *EMBO reports* Online). Similar results were obtained when CBP fragment 1–1098 was substituted for region 1–771 (data not shown). These data allow mapping of the CBP binding site within the acidic region of ProTα, which also binds to linker histone H1.

ProTα stimulates transcription

To examine the role of ProTα in gene expression, we asked whether overexpression of ProTα could affect transcription driven by the AP1 transcription factor, a well-characterized regulator of cell proliferation that is dependent on CBP (Arias *et al.*, 1994; Bannister *et al.*, 1995). HeLa cells were co-transfected with a luciferase reporter gene, 5× coll TRE–tata–luc based on the TPA-responsive element (coll TRE) of human collagenase gene, which binds AP1 (van Dam *et al.*, 1998) and a ProTα-expressing plasmid (pCMVPTα). As shown in Figure 2A, increasing amounts of pCMVPTα stimulated the promoter activity in a dose-dependent fashion. Conversely, the activity of a tata–luc construct, which does not contain the TPA-responsive elements, was not affected by overexpression of ProTα (Figure 2A). Since c-Jun is a major component of AP1, we also checked the effect of ProTα on the collagenase promoter after c-Jun overexpression. As expected, increased c-Jun levels in the cells activated the collagenase promoter. Co-expression of ProTα further enhanced the transcription to an extent similar to that observed in the absence of c-Jun (not shown).

Next, we examined the ability of ProTα and CBP to stimulate 5× coll TRE individually and in combination with each other. As depicted in Figure 2B, ProTα and CBP potentiated the promoter 2.2- and 2.7-fold, respectively. Interestingly, simultaneous addition of ProTα and CBP resulted in a synergistic activation (13-fold). We further extended these studies to two other targets of CBP: the activator NF-κB (Gerritsen *et al.*, 1997) and the MHC class II gene promoter (Kretsovali *et al.*, 1998). ProTα and CBP could individually stimulate p65-mediated activation of an NF-κB–luc reporter. Again, when added simultaneously, they led to a synergistic activation of the NF-κB–luc (Figure 2B). However,

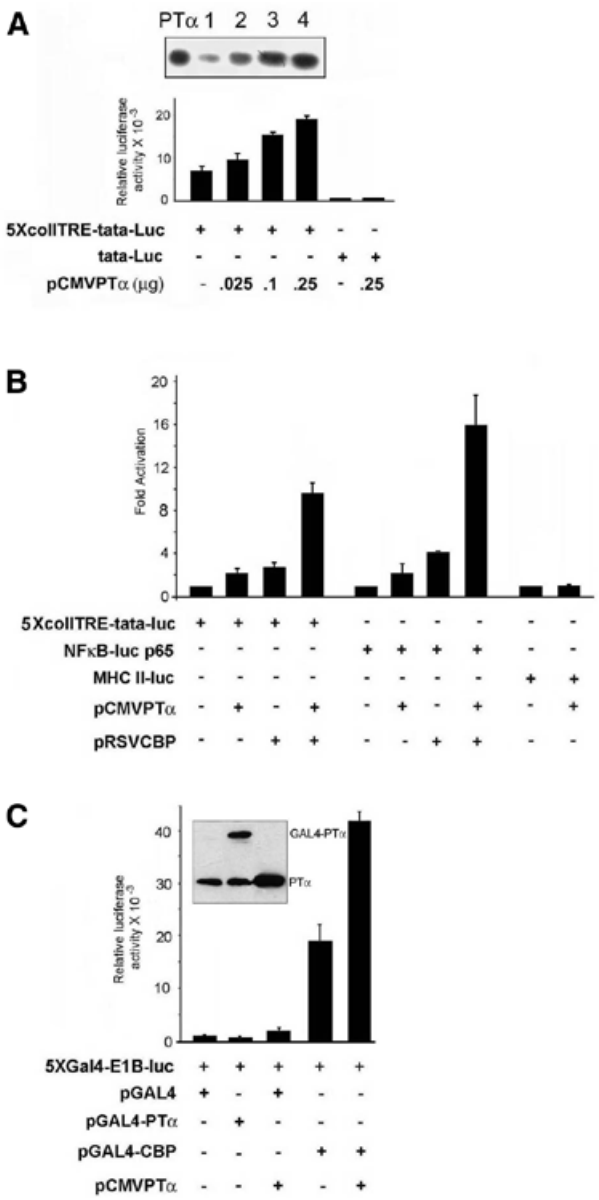

Fig. 2. ProTα potentiates transcription. (**A**) Overexpression of ProTα stimulates transcription from the collagenase promoter. HeLa cells were transiently transfected with the 5× coll TRE–tata–luc or tata–luc constructs (0.1 μg each) in the absence or in the presence of the indicated amounts of pCMVPTα. The upper panel shows western blotting analysis giving the expression levels of ProTα in the transfection experiment described in (A). The experiments were repeated at least three times and the error bars indicate the standard deviations of triplicate values. (**B**) Synergism between ProTα and CBP enhances AP1- and NF-κB-dependent transcription. HeLa cells were transfected using the calcium phosphate method with the reporter plasmids 5× coll TRE–luc (0.5 μg), NF-κB–luc and p65 expression vector (0.5 and 0.1 μg, respectively) or MHC II–luc (0.5 μg) in the presence of pCMVPTα and pRSVCBP (1.0 μg each), as indicated. Error bars represent the standard deviation from three independent experiments. (**C**) Transactivation by CBP is stimulated by ProTα. HeLa cells were transfected with 0.1 μg of 5× GAL4–E1B–luc reporter plasmid and expression vectors encoding either GAL4 alone or GAL4–ProTα or GAL4–CBP (full-length) or GAL4–CBP in the presence of pCMVPTα (0.1 μg each), as indicated. The upper panel shows western blotting analysis giving the expression levels of ProTα and GAL4–ProTα in the transfection experiment. The experiments were repeated at least three times and the error bars indicate the standard deviations of triplicate values. In all cases, 0.1 μg of a plasmid expressing β-galactosidase (pCMV–LacZ) was co-transfected and used as an internal standard, and luciferase values were normalized to each other based on the respective β-galactosidase activity.

the MHC class II promoter was not activated by ProTα (Figure 2B). Therefore, ProTα collaborates with CBP to potentiate AP1- and NF-κB-mediated transcription.

In an attempt to elucidate the mechanism of ProTα action, we fused it to the DNA-binding domain of GAL4. The fusion protein was expressed equally well as the wild-type ProTα (Figure 2C) and its overexpression activated AP1-mediated transcription (not shown). However, GAL4–ProTα could not direct transcription of a GAL4 site-dependent reporter (Figure 2C), in agreement with a previous report (Martini *et al.*, 2000). We next examined whether ProTα could stimulate the activity of CBP when the latter was tethered on DNA through fusion to the GAL4 DNA-binding domain. Indeed, co-transfection of pCMVPTα and GAL4–CBP resulted in a strong enhancement of CBP transactivation potential (Figure 2C). Taken together, these data suggest that ProTα does not act as a 'classical' transcription activator that directly provides promoter contacts with the basic transcription machinery, but rather as a dynamic component, which needs to be recruited through CBP in order to exert its function.

DISCUSSION

We have shown previously that ProTα modulates the interaction of histone H1 with chromatin and proposed a role for this polypeptide in chromatin remodeling (Karetsou *et al.*, 1998). The observations described here provide evidence that ProTα interacts with the versatile co-activator of transcription CBP both *in vivo* and *in vitro* and stimulates gene transcription. The site of interaction was mapped within the N-terminal domain of CBP (residues 1–771) and a region of ProTα composed of two polyglutamic stretches. This binding could be due to the specific sequence characteristics of this acidic domain and/or its local conformation. However, under the experimental conditions employed in the binding studies (pH 7.4 and 150 mM ionic strength), this motif is not expected to adopt any special type of secondary structure, as predicted by the program AGADIR (Munoz and Serrano, 1995). This prediction is in agreement with experimental work showing that native ProTα has a random coil conformation (Gast *et al.*, 1995; Uversky *et al.*, 1999). Therefore, we can conclude that the CBP–ProTα interaction is mediated through the extended acidic clusters of ProTα. This is further supported by the observation that polyglutamic acid (10–20 residues), predicted to be unfolded under the same experimental conditions (Munoz and Serrano, 1995), also inhibited binding.

Recently, the histone acetyltransferase p300, a protein highly homologous to CBP, has been reported to bind to ProTα through its C-terminal region (Cotter and Robertson, 2000). This discrepancy with our results could be due to the GST–ProTα used in those assays, as opposed to the use of purified native ProTα in our system. The recombinant protein might lack potentially important post-translational modifications or could adopt an altered structure due to the presence of the large GST moiety. Alternatively, p300 and CBP might differ in their properties to bind ProTα.

Confocal laser scanning microscopy shows that ProTα partially colocalizes with CBP in discrete subnuclear domains. The pattern and degree of colocalization were similar to the colocalization characteristics of CBP and RNA polymerase II, but also of transcription factors that associate with CBP (von Mikecz *et al.*, 2000), suggesting a dynamic interaction between CBP and ProTα that is spatially and temporally regulated. Furthermore, recent work has shown that ProTα is concentrated in nuclear domains that often overlap or engulf sites of active transcription (Vareli *et al.*, 2000). In this respect, the colocalization of ProTα and CBP and their *in vivo* interaction point to a role of ProTα in gene regulation.

In agreement with this assumption, we provide experimental evidence showing that overexpression of ProTα enhances the transcription activity of CBP-dependent promoters, such as coll TRE and NF-κB, and stimulates the transactivation activity of CBP. On the other hand, another target of CBP, the MHC class II gene promoter, was not affected by ProTα. This result implies that additional factors, such as the chromatin state of the promoters or the composition of the co-activator complex, are implicated in this mechanism. A recent report has shown that ProTα can specifically enhance transcription activation by the estrogen receptor by eliminating the estrogen repressor REA and favoring recruitment of the co-activator SRC-1 (Martini *et al.*, 2000). Since the estrogen receptor is also dependent on CBP (Wang *et al.*, 2001), the possibility that ProTα might provide the receptor with higher amounts of CBP and, consequently, SRC-1 cannot be excluded.

The precise mechanism whereby ProTα increases transcription needs to be studied further. ProTα does not bear an intrinsic transcription activity when tethered to the GAL4 DNA-binding domain (Martini *et al.*, 2000; this study). Thus, the effect of ProTα on transcription might be exerted either on the chromatin state of the promoter or on the enzymatic activity of CBP, which is directly involved in transcription activation (Martinez-Balbas *et al.*, 1998). *In vitro* HAT assays using core histones as substrates revealed that the acetyltransferase activity of CBP was not affected by ProTα (Z. Karetsou, A. Kretsovali and T. Papamarcaki, unpublished data). Therefore, our data are most consistent with the first hypothesis and point to chromatin as the possible target of ProTα's function. We propose that ProTα might be targeted to promoter regions by CBP and subsequently promotes H1 reorganization or displacement leading to unfolding of the compact 30 nm chromatin fiber (Karetsou *et al.*, 1998). ProTα might also prevent histone H1 from rebinding, maintaining the partially H1-depleted and extended chromatin conformation necessary for the passage of RNA polymerase II.

Overall, the interaction of ProTα with the versatile co-activator of transcription CBP, together with its ability to stimulate transcription, suggest an important role for this acidic polypeptide in gene expression.

METHODS

Protein chemical procedures. ProTα was purified from calf thymus as described previously (Karetsou *et al.*, 1998). Thymosin α1 (residues 1–26, Ac-SDAAVDTSSEITTKDLKEKKEVVEEA, pI 4.2), the acidic peptide (residues 52–69, GSGDEEEEEGGEEEEEEEG, pI 3.0) and the ct peptide (residues 86–109, GKRAAEDDEDDD-VDTKKQKTDEDD, pI 3.8) of bovine ProTα were synthesized by the EMBL Protein Sequencing and Peptide Synthesis Facility. Polyglutamic acid (mol. wt 1500–3000, pI 3.1–2.9) was obtained from Sigma.

Plasmids. To generate pCMVPTα, the coding region of the rat ProTα cDNA (Frangou-Lazaridis *et al.*, 1988) was amplified and subcloned into the *Eco*RI–*Xho*I sites of a cytomegalovirus (CMV)

expression cassette p163/7, where the H2 promoter was exchanged with the CMV promoter (Woodroofe *et al.*, 1992). The construct has been verified by sequencing. pGAL4–PTα was made from pCMVPTα that was digested by *Xho*I, filled in with the Klenow fragment of DNA polymerase I, digested with *Eco*RI and cloned into the *Eco*RI–*Sma*I restriction sites of the pBXGI vector containing GAL4–DBD (1–147). The reporter plasmids 5× coll TRE–tata–luc, tata–luc have been described previously (van Dam *et al.*, 1998) and were obtained from A. Pintzas (National Research Institute, Athens, Greece). The mouse class II–353 Ea chloramphenicol acetyltransferase construct has been described previously (Thanos *et al.*, 1988) and was used to generate an equivalent luciferase reporter. GAL4–CBP fusion construct, 5× GAL4–E1B–luc reporter plasmid and p65 expression vector were kindly provided by D. Thanos (Columbia University, New York, NY). NF-κB–luc (κB–TK5–luc) was provided by C. Esslinger (Ludwig Institute for Cancer Research, University of Lausanne, Switzerland).

Cell culture and transient transfections. HeLa cells were grown in Dulbecco's modified Eagle's medium in the presence of 10% fetal calf serum (FCS) and antibiotics. For transient transfections, HeLa cells were grown in 6-well plates to 60–70% confluence and transfected using effectene (Qiagen) or the calcium phosphate method (Thanos *et al.*, 1988), where indicated. Cells were harvested 36–48 h later and luciferase activity was detected in the extracts using a Promega luciferase assay system according to the manufacturer's instructions. A plasmid expressing β-galactosidase (pCMV–LacZ) was co-transfected as an internal standard, and luciferase activity was normalized for transfection efficiency with the respective β-galactosidase activity. Total DNA was adjusted using empty vectors.

Indirect immunofluorescence. HeLa cells grown on coverslips 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. Specimens were blocked in 10% FCS in phosphate-buffered saline for 1 h in a humidified chamber and antibody incubations were carried out for 1 h. Samples were then mounted in Mowiol containing 100 mg/ml DABCO (Sigma). For ProTα and CBP co-staining, primary antibodies from mouse or rabbit were used simultaneously and detected with species-specific secondary antibodies linked to fluorescein (FITC) or rhodamine (TRITC) (Dianova, Hamburg, Germany). ProTα was visualized using the affinity-purified anti-ct antibody followed by FITC-labeled donkey anti-rabbit IgG, and CBP was stained using the monoclonal C1 antibody (Santa Cruz Biotechnology) followed by rhodamine-labeled anti-mouse IgG. Specimens were visualized in a Leica TCS-SP confocal scanning microscope, equipped with an argon/krypton laser and Leica TCS software. Images were exported to Adobe Photoshop.

Immunoprecipitation and western blotting. Whole-cell extracts were prepared from HeLa cells (~24 × 10^6 cells) lysed in 200 µl of buffer containing 20 mM Tris–HCl pH 8.0, 137 mM NaCl, 5 mM EDTA, 5 mM NaF, 25 mM β-glycerolphosphate, 1% NP-40 and 1 µg/ml each of protease inhibitors aprotinin, leupeptin and pepstatin. The extract was centrifuged at 13 000 *g* for 10 min and pre-cleared with 20 µl of IgG–protein A (40 µg of IgG) for 30 min at room temperature. The supernatant was divided into two portions and incubated with (i) 25 µl of anti-ct–protein A beads (20 µg of anti-ct antibody) and (ii) 25 µl of IgG–protein A beads for 12 h at 4°C. After incubation, the beads were washed three times with 1 ml of lysis buffer containing 300 mM NaCl, resuspended in 15 µl of Laemmli buffer and analyzed by western blotting.

ProTα was analyzed by the anti-ct polyclonal antiserum affinity purified over a ct-Affigel (Karetsou *et al.*, 1998). CBP was detected by the rabbit polyclonal A-22 antibody (Santa Cruz Biotechnology).

GST pull-down experiments. Fragments of CBP were subcloned into pGEX vectors (Pharmacia) in frame with GST and were expressed in *Escherichia coli* DH5α (Kretsovali *et al.*, 1998). Fusion proteins (~2 µg each) were immobilized on glutathione beads and incubated with 2 µg of purified ProTα in a buffer containing 20 mM HEPES pH 7.5, 150 mM KCl, 5 mM Mg Cl_2, 0.1% NP-40 and 0.2% bovine serum albumin (BSA), supplemented with protease inhibitors. Reactions were carried out for 5 h at 4°C and the beads were washed three times in the same buffer without BSA. Bound proteins were subjected to 15% SDS–PAGE and ProTα was detected by western blotting.

Supplementary data. Supplementary data are available at *EMBO reports* Online.

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