Identification of a novel p300-specific-associating protein, PRS1 (phosphoribosylpyrophosphate synthetase subunit 1)

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CBP [CREB (cAMP-response-element-binding protein)-binding protein] and p300 play critical roles in transcriptional co-activation, cell differentiation, proliferation and apoptosis. Multiple transcription factors associate with CBP/p300. With the exception of the SYT oncoprotein, no proteins have been identified that specifically associate with p300, but not CBP. In the present study, we isolated a novel p300-associated protein for which no interaction with CBP was observed by GST (glutathione S-transferase) pull-down assay using Jurkat cell lysates metabolically labelled with [³⁵S]methionine. This protein bound the KIX (kinase-inducible) domain of p300. Following resolution by two-dimensional acrylamide gel electrophoresis, we identified

the KIX-domain-bound protein by MS analysis as PRS1 (phosphoribosylpyrophosphate synthetase subunit 1), a protein essential for nucleoside biosynthesis. This is the first report to demonstrate the existence of a p300 KIX-domain-specific-interacting protein that does not interact with CBP. Thus p300 may play a role in the regulation of DNA synthesis through interactions with PRS1.

Key words: CBP [CREB (cAMP-response-element-binding protein)-binding protein], KIX (kinase-inducible) domain, p300, PRS1 (phosphoribosylpyrophosphate synthetase subunit 1).

INTRODUCTION

p300, a 2414-amino-acid-residue protein, was initially identified as an adenoviral E1A-associating protein. The highly related protein CBP [CREB (cAMP-response-element-binding protein)-binding protein], containing 2441 amino acids, was isolated as a CREB-binding protein [1–3]. CBP and p300 exhibit similar functions, such as transcriptional co-activation and intrinsic HAT (histone acetyltransferase) activity [4–6]. The complex of CBP and p300 contains several common functional domains, including C/H (cysteine/histidine-rich) domain, a bromodomain, a KIX (kinase-inducible) domain, a glutamine-rich domain and a HAT domain. Multiple transcription factors and components of the transcriptional machinery, such as TBP (TATA-box-binding protein) [7], TFIIB (transcription factor IIB) [8] and RNA polymerase II [9,10], interact with CBP/p300, resulting in enhanced transcription. A number of nuclear hormone receptors and viral oncoproteins also associate with CBP/p300 [11,12].

In addition to functioning as transcriptional co-activators, CBP/p300 participate in cell differentiation, proliferation and apoptosis. These proteins also act as signal integrators. Gene silencing analyses of *p300* and *cbp* have demonstrated functional differences between the roles *in vivo*. Mice with homologous allelic deletions of *p300* or *cbp* were embryonically lethal, exhibiting defects in haematopoiesis [13,14]. Monoallelic inactivation of *cbp* in mice, however, leads to abnormalities in haematopoietic differentiation and an increased incidence of malignancy with aging. In addition, abnormal skeletal patterning is detected in embryos carrying only a single copy of the *cbp* allele, whereas mice lacking one allele of *p300* display no such phenotype [15,16]. The self-renewal of HSC (haematopoietic stem cells) depends on wild-type levels of CBP, whereas p300 is required for proper

haematopoietic differentiation [17]. Thus p300 and CBP play essential, but distinct, roles in haematopoiesis.

Following stimulation with RA (retinoic acid), p300 and CBP differentially affect F9 cell differentiation. Stable F9 transformants expressing a p300-specific ribozyme are resistant to stimulation by RA. In contrast, transduction with a CBP-specific ribozyme remains sensitive to RA-dependent differentiation [18]. This suggests that p300, but not CBP, participates in RA-stimulated cell differentiation. Yao et al. [13] also demonstrated that p300^{−/−} MEF (mouse embryo fibroblasts) are resistant to RA-stimulated cell differentiation by analysing BrdUrd (bromodeoxyuridine) incorporation.

Cellular responses to ionizing radiation are also differentially affected by p300 and CBP expression [19]. The effect of p300 and CBP on the induction of apoptosis was examined in MCF7 cells expressing p300- or CBP-specific ribozyme following the induction of DNA damage by ionizing radiation. Although cell death was inhibited in p300-depleted cells, knockdown of CBP did not affect the cellular sensitivity to radiation damage [19]. These results indicate that p300 has a role in the regulation of apoptosis induced by DNA damage.

Although p300 and CBP possess many distinct functions, the molecular mechanisms controlling these differences remain to be clarified. A variety of cellular proteins interact with p300 or CBP through common domains. The C/H1–KIX region of CBP/p300 is one such domain recognized by transcription factors [20] and viral proteins, such as HTLV-1 (human T-cell leukaemia virus type 1) Tax [21], HPV (human papillomavirus) E2 [22] and HIV Tat protein [23]. Due to the differences in amino acid sequences between these regions, however, we hypothesized that a subset of cellular proteins may possess different affinities for the p300 and CBP C/H1–KIX regions; these differences may contribute to the

Abbreviations used: C/H, cysteine/histidine-rich; CBP, CREB (cAMP-response-element-binding protein)-binding protein; D188E etc., Asp¹⁸⁸ → Glu etc.; DAPI, 4,6-diamidino-2-phenylindole; DBD, DNA binding domain; DTT, dithiothreitol; FBS, fetal bovine serum; GST, glutathione S-transferase; HAT, histone acetyltransferase; KIX, kinase-inducible; MALDI, matrix-assisted laser desorption/ionization; MEK1, MAPK (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated kinase) kinase kinase 1; PRPP, phosphoribosylpyrophosphate; PRS1, PRPP synthetase subunit 1; RA, retinoic acid; 2D, two-dimensional.

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distinct functions of p300 and CBP. To examine this possibility, we searched for novel cellular factors that discriminate between the C/H1–KIX regions of p300 and CBP.

EXPERIMENTAL

Cell culture

Jurkat and MCF7 cells were maintained in RPMI 1640 medium (Nissui) supplemented with 10 % (v/v) heat-inactivated FBS (fetal bovine serum) at 37 °C in a humidified 5 % CO₂ atmosphere. HEK-293T cells were cultured in DMEM (Dulbecco's modified Eagle medium; Nissui) supplemented with 10 % (v/v) FBS.

Plasmid constructions

To construct pcDNA3-PRS1 [where PRS1 is PRPP (phosphoribosylpyrophosphate) synthetase subunit 1], mRNA was obtained from Jurkat cell lysates with an mRNA isolation kit (Roche). Reverse transcription was performed using Superscript II RNase H⁻ Reverse Transcriptase (Invitrogen) with the primer 5'-TGTGGGATGTAGAAAGCTAC-3'. Subsequent PCR steps were performed using *pfu* DNA polymerase (Promega) with the following primers, 5'-CGGGATCCAGGATGCCGAATATC-AAAATC-3' (forward) and 5'-CCGCTCGAGTTATAAAGGGA-CATGGCTGAATAG-3' (reverse). The PCR-amplified fragment for PRS1 mRNA was inserted into the BamHI–XhoI site of the pcDNA3 vector (Invitrogen). pcDNA3-Myc-PRS1 was constructed by inserting annealed synthetic oligonucleotides encoding the sequence for Myc into the HindIII–BamHI site of pcDNA3 and then inserting the sequence for the PRS1 gene into the BamHI–XhoI site of pcDNA3-Myc. GST (glutathione S-transferase) fusion proteins containing CBP(C/H1–KIX)_{aa362–682} (where aa is amino acid) or CBP(KIX)_{aa451–682} were subcloned by inserting the appropriate PCR-amplified fragments into the BamHI–XhoI site of pGEX-6P-1 (Amersham Biosciences). GST fusions of p300(C/H1–KIX)_{aa338–661} and p300(KIX)_{aa567–661}, _{aa567–652}, _{aa576–661}, _{aa576–652} and _{aa604–661} were constructed by insertion of the appropriate PCR-amplified fragments into the SmaI–XhoI site of pGEX-6p-2 (Amersham Biosciences). GST fusions of CBP(KIX) sequences containing point mutations were constructed by PCR using synthetic oligonucleotide primers containing the desired mutations. The PCR-amplified fragments were inserted into pGEX-6P-2. Fusions of Gal4(DBD) (DNA binding domain) or VP16 with PRS1, CBP_{aa1–683}, CBP_{aa668–1667}, p300_{aa1–1000} and p300_{aa668–1667} were constructed by inserting the corresponding PCR-amplified fragments into pM-MCS or pVp16-MCS vectors. pM-MCS and pVp16-MCS were established by inserting the DNA fragment obtained by annealing two complementary synthetic oligonucleotides (5'-AATTCG-GATCCGATATCGCGCCGCTCGAGG-3' and 5'-TCGACCTCGAGGCGGCCGCGATATCGGATCCG-3') into the EcoRI–SalI sites of the pM (Clontech) and pVp16 (Clontech) vectors respectively. The coding regions for p300 and CBP were amplified from pCMVp300 and pCMVCBP respectively. The appropriate DNA fragments were inserted into either the NotI/HindIII or the BamHI site of the pVp16-MCS vector.

Expression and purification of recombinant proteins

Expression plasmids encoding GST fusion proteins were transformed into *Escherichia coli* (BL21 codon plus RIL; Stratagene). The production of GST or GST fusion proteins was induced by adding 0.5 mM IPTG (isopropyl β-D-thiogalactoside) to the culture medium, followed by an additional incubation at 20 °C for 14 h. Bacteria were pelleted by centrifugation and

resuspended in sonication buffer [50 mM Tris/HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA, 1 mM DTT (dithiothreitol) and 1 mM PMSF], and then homogenized using a FRENCH® Press. Following centrifugation, supernatants were adsorbed on glutathione–Sephadex 4B resin (Amersham Biosciences). The resin was washed four times with 600 µl of PBST (PBS containing 0.5 % Triton X-100), then twice with binding buffer [10 mM Tris/HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.5 % Nonidet P40, 1 mM DTT and 1 mM PMSF].

GST pull-down assay

Jurkat cells were metabolically labelled with Trans ³⁵S-label (3.7 MBq/ml; ICN) at 37 °C for 6 h. Cells were lysed in binding buffer, and the soluble protein fraction was incubated for 1 h with GST fusion proteins bound to glutathione–Sephadex 4B resin at 4 °C. After washing four times with 600 µl of binding buffer, the resin was boiled for 5 min in 1 × Laemmli sample buffer [50 mM Tris/HCl (pH 6.8), 2 % SDS, 0.1 % Bromophenol Blue, 10 % glycerol and 5 % 2-mercaptoethanol]. The released proteins were fractionated by SDS/PAGE [10 % (w/v) acrylamide] and detected by autoradiography. The *in vitro*-synthesized PRS1 protein was incubated for 1 h with either GST or GST fusion proteins bound to resin at 4 °C, then washed five times in 600 µl of binding buffer. After elution from the resin by boiling in 1 × Laemmli sample buffer, the bound proteins were separated by SDS/PAGE and detected by autoradiography.

Transfection and luciferase assay

Appropriate plasmids were transfected into MCF7 cells using FuGENE6 transfection reagent (Roche). Luciferase assays were performed at 72 h post-transfection. All transfections were equalized for total DNA by adding an empty plasmid. The relative luciferase activity represents the average of four independent experiments.

Immunoprecipitation

HEK-293T cells (1.5 × 10⁶ cells) were scattered on to 100-mm-diameter dishes on the day before transfection. pcDNA3-Myc-PRS1 (5 µg) was transfected into HEK-293T cells using FuGENE6. At 48 h post-transfection, cells were harvested and solubilized in lysis buffer A [10 mM Tris/HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.1 % Nonidet P40, 1 mM DTT and 1 mM PMSF]. Insoluble material was removed by centrifugation. The supernatants were incubated overnight at 4 °C with 2 µg of anti-Myc (9E10; Santa Cruz Biotechnology), anti-CBP (A22; Santa Cruz Biotechnology) and anti-p300 (N15; Santa Cruz Biotechnology) antibodies, and a mixture of anti-mouse and anti-rabbit IgG (Zymed Laboratories). Following the addition of 30 µl of Protein G–Sephadex (Amersham Biosciences), mixtures were incubated at 4 °C for 1 h. Immune complexes were washed three times with lysis buffer A; the resin was then boiled for 5 min in 1 × Laemmli sample buffer. Immunoprecipitates were analysed by SDS/PAGE [10 % (w/v) acrylamide], followed by immunoblotting with an anti-Myc antibody.

Immunoblot analysis

HEK-293T cells (1.5 × 10⁶ cells) were plated on to 100-mm-diameter dishes on the day before transfection. pcDNA3-Myc-PRS1 or pcDNA3 (5 µg) was transfected into HEK-293T cells and, 48 h post-transfection, cells were harvested and lysed in lysis buffer B [50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 4 mM EDTA, 1 % Nonidet P40, 0.1 % SDS, 1 mM DTT and 1 mM PMSF]. After boiling in 1 × Laemmli sample buffer, proteins were separated by SDS/PAGE on a 5–20 % (w/v) acrylamide gradient

Figure 1 Proteins associated with GST-CBP(C/H1-KIX) or GST-p300(C/H1-KIX)

(A) Schematic illustration of the GST fusion proteins of CBP/p300 C/H1-KIX domains. Br, bromodomain. (B) A 34 kDa protein specifically associates with p300(C/H1-KIX). Left-hand panel, lysates from 1×10^7 Jurkat cells metabolically labelled with [35 S]methionine were affinity-precipitated with GST-CBP(C/H1-KIX) or GST-p300(C/H1-KIX). Bound proteins were separated on an SDS/10% (w/v) polyacrylamide gel and analysed by autoradiography. The arrow shows the 34 kDa protein that associated with p300, but not with CBP. Right-hand panel, Coomassie Blue staining to show equal amounts of GST fusion proteins used for the precipitation.

gel, followed by immunoblotting with anti-Myc, anti-CBP, anti-p300 and anti-actin (Sigma) antibodies.

Indirect immunofluorescence analysis

COS-7 cells (5×10^3 cells) were plated in each well of a four-well Lab-Tek chamber slide system (Nalge Nunc International) on the day before transfection. pcDNA3-Myc-PRS1 (0.2 μ g) was transfected with FuGENE6 transfection reagent. At 48 h post-transfection, cells were fixed with 3.5% (v/v) formaldehyde, permeabilized with 0.1% Nonidet P40 in PBS and treated with anti-Myc or anti-p300 antibodies. The secondary antibodies conjugated with Alexa 488 and 594 series (Molecular Probes) were used to visualize the primary antibody stained p300 and Myc-PRS1 respectively, and were analysed with confocal laser microscopy. Nuclei were stained with DAPI (4,6-diamidino-2-phenylindole).

2D (two-dimensional) gel electrophoresis

GST pull-down complexes from 35 S-labelled Jurkat cell lysates were lysed in buffer containing 8M urea, 2% Nonidet P40, 10% 2-mercaptoethanol and 3.6% Pharmalyte pH 3–10 (Amersham Biosciences) at 37°C for 30 min. After centrifugation, supernatants were loaded on to Immobiline DryStrips (pH 3–10; 13 cm; Amersham Biosciences) rehydrated in a buffer containing 8 M urea, 0.5% Triton X-100, 10 mM DTT, 0.5% Pharmalyte pH 3–10 (Amersham Biosciences) and 1% Orange G. Isoelectric focusing was performed on a Multiphor II electrophoresis chamber (Amersham Biosciences). After isoelectric focusing, DryStrips were equilibrated three times with 15 ml of equilibration buffer [62.5 mM Tris/HCl (pH 6.8), 2% SDS, 0.7 mM 2-

Figure 2 Purification of p300-associated proteins by 2D gel electrophoresis

Proteins associating with the C/H1-KIX domains of either CBP or p300 were precipitated from [35 S]methionine-labelled Jurkat cell lysates with GST-CBP(C/H1-KIX) and GST-p300(C/H1-KIX). Bound proteins were separated by 2D gel electrophoresis using an isoelectric focusing gel strip covering pH 3–10. Autoradiography was used to visualize the bound protein profile for GST-CBP(C/H1-KIX) (A) and GST-p300(C/H1-KIX) (B). The 34 kDa protein was identified as a single spot (indicated by a star) at an isoelectric point of 7.5.

mercaptoethanol and 0.002% Bromophenol Blue] at 37°C for 15 min. The equilibrated DryStrips were applied on to an SDS/10% (w/v) polyacrylamide gel to separate the proteins by molecular mass. Bound proteins were detected by autoradiography.

In vitro translation

To prepare PRS1 protein *in vitro*, 0.4 μ g of pcDNA3-PRS1 was added to 30 μ l of rabbit reticulocyte lysate (T_N T Quick Coupled Transcription/Translation system; Promega) and incubated in the presence of L-[35 S]methionine (Amersham Biosciences) for 90 min at 30°C.

MS

Proteins resolved by 2D gel electrophoresis were digested with lysylendopeptidase (Wako Pure Chemical Industries). The

Figure 3 Mapping of associating regions of p300 and PRS1

(A) *In vitro*-synthesized ³⁵S-labelled PRS1 was incubated with *E. coli*-derived GST (lanes 2 and 6), GST-CBP(C/H1-KIX) (lane 3), GST-p300(C/H1-KIX) (lane 4), GST-p300(KIX) (lane 7), GST-CBP(KIX) (lane 8) and GST-p300_{aa567-661} (lane 9), GST-p300_{aa567-652} (lane 10), GST-p300_{aa576-661} (lane 11), GST-p300_{aa576-652} (lane 12), and GST-p300_{aa604-661} (lane 13). A portion (10%) of the input PRS1 volume was loaded for reference (lanes 1 and 5). Bound proteins were separated by SDS/PAGE [10% and 12.5% (w/v) acrylamide] and analysed by autoradiography. (B) Coomassie Blue staining of the gel demonstrated the use of equal amounts of GST fusion proteins for each precipitation. (C) Summary of p300(KIX)-PRS1 interactions *in vitro*. ○, interaction; ×, no interaction. (D) Schematic illustration of chimaeric proteins combining PRS1 with PRS2. The open and shaded bars represent regions of PRS1 and PRS2 respectively. Four chimaeric proteins were constructed: C1 (PRS2_{aa1-170} and PRS1_{aa171-318}), C2 (PRS2_{aa1-190} and PRS1_{aa191-318}), C3 (PRS2_{aa1-245} and PRS1_{aa246-318}) and C4 (PRS2_{aa1-265} and PRS1_{aa266-318}). (E) *In vitro* synthesized [³⁵S]methionine-labelled PRS1 and chimaeric proteins (C1, C2, C3 and C4) were incubated with GST (lanes 6–10) or GST-p300(KIX) (lanes 11–15). A portion (10%) of the input chimaeric protein was loaded as a reference (lanes 1–5). Associated proteins were resolved by SDS/PAGE [10% (w/v) acrylamide] and detected by autoradiography.

resultant peptides were measured using a MALDI (matrix-assisted laser desorption/ionization) time-of-flight mass spectrometer (Voyager Elite XL; PerSeptive Biosystems) or a nano-flow ESI (electrospray ionization) tandem mass spectrometer (MS/MS) (Q-TOF II; Micromass). The ESI-MS/MS spectra were interpreted by *SeqMS*, a software aid for *de novo* sequencing by MS/MS [24]; the peptides observed by MALDI MS were assigned to sequences by *MS-Match*, a software aid for protein identification (<http://www.protein.osaka-u.ac.jp/rcsfp/profiling>).

RESULTS

Presence of p300(C/H1-KIX)-associating proteins that do not bind CBP(C/H1-KIX) in Jurkat cell lysates

We sought to identify novel p300- or CBP-binding proteins using a GST pull-down assay. We constructed GST-CBP(C/H1-KIX) and GST-p300(C/H1-KIX) in *E. coli* (Figure 1A). GST, GST-CBP(C/H1-KIX) and GST-p300(C/H1-KIX) were added to lysates from metabolically labelled Jurkat cells. Bound proteins

Figure 4 Replacement of CBP(KIX) with p300(KIX) amino acid residues confers PRS1 binding

Mutational analysis of the CBP(KIX) domain revealed that GST-CBP(G590Q/H594D/S601N) and CBP(G590Q/H594D/D647A) acquired the ability to associate with PRS1 *in vitro*. (A) Sequence alignments of p300_{aa567-652} and CBP_{aa587-672}. p300_{aa567-652}, which corresponds to amino acids 587–672 in CBP, is the minimal region necessary to interact with PRS1. Amino acid differences within this region are indicated (*). Underlined residues indicate the sites in CBP substituted with amino acids from p300. (B) *In vitro* synthesized ³⁵S-labelled PRS1 was incubated with GST (lane 2), GST-p300(KIX) (lane 3), GST-CBP(KIX) (lane 4), GST-p300_{aa567-652} (lane 5), GST-CBP_{aa587-672} (WT; lane 6), GST-CBP_{aa587-672}(G590Q) (lane 7), GST-CBP_{aa587-672}(H594D) (lane 8) and GST-CBP_{aa587-672}(G590Q/H594D) (lane 9). A portion (10%) of the input PRS1 volume was loaded as a reference (lane 1). Bound proteins were separated on an SDS/10% (w/v) polyacrylamide gel and detected by autoradiography. (C) ³⁵S-labelled PRS1 was incubated with GST (lane 2), GST-p300(KIX) (lane 3), GST-CBP(KIX) (lane 4), GST-CBP_{aa587-672}(G590Q/H594D) (WT; lane 5), GST-CBP_{aa587-672}(G590Q/H594D/S601N) (lane 6), GST-CBP_{aa587-672}(G590Q/H594D/K633R) (lane 7), GST-CBP_{aa587-672}(G590Q/H594D/S645N) (lane 8), and GST-CBP_{aa587-672}(G590Q/H594D/D647A) (lane 9). A portion (10%) of the input PRS1 volume was loaded as a reference (lane 1). Bound proteins were fractionated on an SDS/10% (w/v) polyacrylamide gel and detected by autoradiography.

were isolated using a glutathione-Sepharose 4B resin, removed from the beads by heating, and resolved by SDS/PAGE. Following staining with Coomassie Brilliant Blue (Figure 1B, right-hand panel), electrophoresed proteins were assessed by autoradiography (Figure 1B, left-hand panel). Equivalent amounts of GST and GST fusion proteins were used for each pull-down (Figure 1B, right-hand panel). Although multiple bands were observed in all three lanes, a number of proteins were only observed in association with GST-CBP or GST-p300 (Figure 1B, left-hand panel). Proteins migrating with apparent molecular masses of 34 and 48 kDa were detected specifically in the GST-p300 lane. In contrast, three bands of approx. 50, 62 and 175 kDa were detected in the GST-CBP lane.

Thus a subset of proteins interact specifically with either CBP or p300. In particular, a protein of 34 kDa appears to have a higher affinity for the C/H1-KIX domain of p300 than for CBP.

Identification of 34 kDa protein as PRS1 by MS

To characterize the 34 kDa protein further, proteins that bound to CBP(C/H1-KIX) and p300(C/H1-KIX) were isolated from Jurkat cells metabolically labelled with [³⁵S]methionine and subjected to 2D gel electrophoresis, followed by autoradiography (Figure 2). The 34 kDa protein (indicated by a star in Figure 2B) was clearly detectable complexed with GST-p300(C/H1-KIX), but not GST-CBP(C/H1-KIX). This protein had an isoelectric point of 7.5 (Figure 2B). The gel spot corresponding to the 34 kDa protein was excised, subjected to *in situ* digestion with lysylendopeptidase and analysed by both MALDI MS and nano-flow ESI-MS/MS. The ESI-MS/MS revealed the partial sequences of two peptides as SGSSHQDLSQK and VGDVK, which could be readily assigned to PRS1 [25]. In addition, peptides corresponding to approx. 38 % of the sequence (318 amino acids) were detected by MALDI MS (results not shown). PRS1

catalyses the formation of PRPP from ribose 5-phosphate and ATP. PRPP is an important substrate in the synthesis of purine and pyrimidine nucleosides [26]. Human PRS exists as a complexes containing PRS1, PRS2 [27], PAP39 (39 kDa PRPP synthetase-associated protein) [28] and PAP41 (41 kDa PRPP synthetase-associated protein) [29].

PRS1 interacts with the KIX domain of p300 *in vitro*, but not with that of CBP

To confirm the physical association of p300(C/H1-KIX) with PRS1 *in vitro*, we isolated cDNA for PRS1 from Jurkat cells. Following further cloning, PRS1 was translated *in vitro* for use in GST pull-down assays. The *in vitro* synthesized PRS1 specifically interacted with GST-p300(C/H1-KIX), but not with GST-CBP(C/H1-KIX) (Figure 3A). These results confirm that the 34 kDa protein identified by 2D gel electrophoresis was PRS1. Subsequently, we analysed the binding of the p300 C/H1-KIX domain to PRS1; a KIX domain of 226 amino acid residues was sufficient to interact with PRS1 (results not shown). To identify the minimal region of p300(KIX) capable of interacting with PRS1, we constructed progressive deletion mutants of GST-p300(KIX). Pull-down assays (Figure 3A) revealed that amino acids 567–652 of p300 were sufficient for the association (Figure 3B). The results of the *in vitro* interaction of p300(KIX) with PRS1 are summarized in Figure 3(C).

Mapping the region of association of PRS1 with p300(KIX)

Next we attempted to identify the minimal region of PRS1 that associates with p300(KIX). As we experienced technical difficulties in producing PRS1-deletion constructs (e.g. instability and low solubility), we elected to construct PRS1/PRS2 chimaeras (Figure 3D). PRS2, a PRS1-related protein containing the same number of amino acid residues as PRS1, has 96 % homology with

Figure 5 PRS1 associates with p300 *in vivo*, but not with CBP

(A) PRS1 interacts with p300 *in vivo*. HEK-293T cells were transfected with 5 μ g of pcDNA3-Myc-PRS1. At 48 h post-transfection, cells were lysed, and supernatants were immunoprecipitated (IP) with anti-Myc (lane 1), a mixture of anti-mouse and anti-rabbit IgG (lane 2), anti-CBP (lane 3) or anti-p300 (lane 4) antibodies. Proteins contained within immune complexes were resolved on an SDS/10% (w/v) polyacrylamide gel. A portion (10%) of the anti-Myc immunoprecipitate was loaded as a reference. We then examined the isolated proteins by immunoblot (IB) analysis with an anti-Myc antibody. (B) Protein expression profiles of endogenous p300, CBP and ectopic Myc-PRS1. pcDNA3-Myc-PRS1 or pcDNA3 (5 μ g) was transfected (Tf) into HEK-293T cells and, at 48 h post-transfection, cells were harvested. Cell lysates were subjected to SDS/PAGE on a 5–20% (w/v) acrylamide gradient gel, followed by immunoblotting with anti-Myc, anti-p300, anti-CBP and anti-actin antibodies. (C) PRS1 and p300 co-localize in the nucleus as well as in cytoplasm. pcDNA3-Myc-PRS1 (0.2 μ g) was transfected into COS-7 cells. At 48 h post-transfection, indirect immunofluorescence analysis was performed with primary antibodies, anti-Myc antibody for PRS1 (α Myc; red) and anti-p300 antibody (α p300; green), and analysed with confocal laser microscopy. The merged image of green and red signals is shown. DAPI was used to visualize nuclear staining. (D) PRS1 associates with the KIX domain of p300 *in vivo*. MCF7 cells were co-transfected with 0.1 μ g of pFR-luc reporter plasmid (Stratagene), 0.1 μ g of the expression plasmid encoding Gal4(DBD) or Gal4(DBD)-PRS1, and 0.1 μ g of VP16-CBP, -p300, -PRS1, or p300 or CBP mutants.

PRS1 at the amino acid level. It did not, however, interact with p300(KIX) *in vitro* (results not shown). An *in vitro*-synthesized [³⁵S]methionine-labelled chimaeric protein, C1, associated with p300(KIX) (Figure 3E, lane 12). Proteins C2, C3 and C4 failed to interact with p300(KIX) (Figure 3E, lanes 13–15), suggesting that the region spanning amino acids 171–190 of PRS1 is important for p300 binding. Within this region, there is only one amino acid that differs between PRS1 and PRS2 [Asp¹⁸⁸ → Glu (D188E)]. We constructed a vector encoding PRS1 containing this mutation, pcDNA3-PRS1(D188E), and prepared mutant protein by *in vitro* translation. A GST pull-down assay of PRS1(D188E) with GST–p300(KIX) revealed that PRS1(D188E) failed to bind to p300(KIX) (results not shown). Thus Asp¹⁸⁸ is essential for the interaction of PRS1 with p300.

Identification of PRS1-binding residues in the p300 KIX domain

PRS1 association is determined by residues 567–652 in the KIX domain of p300; this region corresponds to amino acids 587–672 in CBP. There are eight amino acid residue differences between the two proteins in this region (indicated by * in Figure 4A). To clarify the amino acid residue(s) critical for PRS1 association, we progressively replaced these amino acids in CBP with the corresponding p300 residues. The constructs GST–CBP(KIX)_{aa587–672}(G590Q) and GST–CBP(KIX)_{aa587–672}(H594D) associated weakly with PRS1, whereas CBP(KIX)_{aa587–672}(G590Q/H594D) displayed a stronger interaction (Figure 4B). Additional mutation of CBP(KIX)_{aa587–672}(G590Q/H594D) at S601N or D647A bound with approximately the same affinity as the wild-type p300(KIX). These results indicate that the combination of Gln⁵⁷⁰ and Asp⁵⁷⁴ is critical in the PRS1–p300 association, whereas the presence of either Asn⁵⁸¹ or Ala⁶²⁷ is important for the full-strength association of p300 with PRS1 (Figure 4C).

PRS1 interacts with p300 *in vivo*, but not with CBP

To demonstrate the interaction between PRS1 and p300 *in vivo*, we immunoprecipitated PRS1 from cells overexpressing Myc-tagged PRS1 in the presence of endogenous CBP and p300. PRS1 formed a complex with p300 *in vivo* (Figure 5A, lane 4), but did not associate with CBP (Figure 5A, lane 3). Ectopically expressed Myc–PRS1 was not expected to interfere with the co-immunoprecipitation assay and did not alter the expression of endogenous p300 and CBP (Figure 5B). Co-expression of MEKK1 [MAPK (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated kinase) kinase kinase 1] enhanced the association of PRS1 with p300 (results not shown), suggesting that either modification of PRS1 or p300, or a physiological association of MEKK1 with those molecules, might contribute to the binding between PRS1 and p300. We then analysed subcellular localization of ectopic Myc–PRS1 and endogenous p300 (Figure 5C). Overproduced Myc–PRS1 localized mainly in the cytoplasm, although a very small amount was detected in the nucleus. On the other hand, the subcellular localization of p300 was predominantly in the nucleus, with a small amount in the cytoplasm. These results suggest that PRS1 and p300 could co-localize weakly in the nucleus and cytoplasm (Figure 5C). Moreover, we analysed the interaction of p300 with PRS1 by

measuring the transcriptional activity of a reporter gene. We constructed fusions of Gal4(DBD) or VP16 with CBP_{aa1–2441}, CBP_{aa1–683}, CBP_{aa668–1667}, p300_{aa1–2414}, p300_{aa1–1000}, p300_{aa668–1667}, p300_{Δaa466–682}, p300_{Δaa567–652} and PRS1. Luciferase activity was measured after co-transfection of these constructs and pGAL4-Luc into MCF7 cells. Cells producing Gal4(DBD)–PRS1 did not exhibit any luciferase activity, indicating that PRS1 itself had no transactivation function. PRS1 forms complexes with additional PRS1-related proteins, including PRS1, PRS2, PAP39 and PAP41; thus the fusion of Gal4(DBD) with PRS1 would enhance transcription of the GAL4-Luc gene upon co-expression of the VP16–PRS1 fusion. As expected, we observed a strong transcriptional activation in cells expressing these proteins. Cells producing Gal4(DBD)–PRS1 and VP16–PRS1 were used as positive controls. High luciferase activity was detected in cells producing Gal4(DBD)–PRS1 in conjunction with VP16–p300_{aa1–2414}, VP16–p300_{aa1–1000} or VP16–PRS1, suggesting a physical interaction between these proteins. The deletion mutant encoding the minimal associating region of p300, VP16–p300_{Δaa567–652}, failed to enhance transactivation. The constructs derived from CBP also failed to enhance transactivation. These results suggest that PRS1 associates with p300 through interactions with the KIX domain *in vivo*, but not with CBP (Figure 5D).

DISCUSSION

In the present study, we identified a novel p300-associated protein, PRS1, which specifically recognizes the p300, but not the CBP, KIX domain. Previously, only a few cellular proteins had been identified that specifically interacted with only one of these related transcriptional co-activators. SYT, a proto-oncoprotein, interacts with p300 through its C/H1 and C/H3 domains, but does not interact with CBP [30]. JMY, a novel p300-interacting protein identified by yeast two-hybrid analysis, has not been analysed for potential interactions with CBP [31]. PRS1 is conserved among many species from *E. coli* to mammals. As both p300 and CBP are also conserved from *Xenopus* to mammals, it is likely that the distinct physiological roles of p300 and CBP are regulated by differential associations of cellular proteins. In this regard, PRS1 may be one such protein.

Recent studies have suggested several novel roles for p300, primarily in DNA synthesis and repair. In p300-deficient mouse embryos, a dramatic reduction in DNA synthesis was observed [13]. p300 also associates with either PCNA (proliferative cell nuclear antigen) [32] or DNA polymerase β [33], suggesting that p300 might contribute to DNA repair. CBP/p300 also interacts with and acetylates thymine DNA glycosylase to activate base-excision repair activity [34]. Our present results suggest that the association between PRS1 and p300 might also contribute to the p300-regulated metabolism of DNA synthesis or repair. Upon ectopic expression in cells, Myc-tagged PRS1 localized primarily to the cytoplasm and a small amount was observed in the nucleus, whereas p300 was detected primarily in the nucleus and a small amount in cytoplasm. These results suggest that PRS1 and p300 co-localize in the nucleus as well as in the cytoplasm. Overexpression of MEKK1 alters the localization of

At 72 h after transfection, cell lysates were examined by luciferase assay. Gal4(DBD)–PRS1 alone did not demonstrate transcriptional activation activity (lane 5). Co-transfection of Gal4(DBD)–PRS1 and VP16–p300_{wt}, VP16–p300_{aa1–1000} (containing the KIX domain) and VP16–PRS1 exhibited high luciferase activity (lanes 9, 10 and 14). In contrast, no luciferase activity was detected when VP16–p300_{Δaa567–652} or VP16–p300_{Δaa466–682} was co-transfected (lanes 12 and 13). VP16 fusions of CBP or CBP mutants did not display any interaction with Gal4(DBD)–PRS1 (lanes 6–8). The fold activation represents the relative luciferase activity in comparison with Gal4(DBD) and VP16 alone. These results represent the means ± S.E.M. of four independent experiments. wt, full length.

a subset of p300 to the cytoplasm [35]. This suggests that MEKK1 modulates PRS1 binding ability by altering subcellular localization of p300. Since interaction of PRS1 and p300 was enhanced in cells upon ectopic expression of MEKK1 (results not shown), the association of these proteins may also regulate cellular proliferation.

The three-dimensional structure of the KIX domain of CBP has been shown to contain three α -helices [36]. Progressive amino acid substitutions replacing amino acids in CBP with those from p300 conferred PRS1 binding to mutant CBP (Figure 4). The S601N and D647A mutations dramatically enhanced CBP association with PRS1. In wild-type CBP, these residues are localized to the $\alpha 1$ and $\alpha 3$ helices respectively, suggesting that the secondary structure of the KIX domain of CBP/p300 contributes to the creation of a PRS1 binding site. We also determined that mutation of PRS1 residue 188 (D188E) abrogated the association with p300. Further studies will be necessary to clarify the physiological significance of the interaction of p300 with PRS1, and such an examination will hopefully probe the subtle, yet important, differences between p300 and CBP. Taken together, the association between PRS1 and p300 might contribute to the p300-regulated metabolism of DNA synthesis or repair by sequestering the roles of p300 or by yet unidentified mechanism. Alternatively, a function of PRS1 may be modified by the interaction, which may result in affecting a pool of nucleotides in cells, and altering the pool, in turn, may affect repair or DNA synthesis.

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