Acetylation of importin-α nuclear import factors by CBP/p300

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Histone acetylases were originally identified because of their ability to acetylate histone substrates [1–3]. Acetylases can also target other proteins such as transcription factors [4–7]. We asked whether the acetylase CREB-binding protein (CBP) could acetylate proteins not directly involved in transcription. A large panel of proteins, involved in a variety of cellular processes, were tested as substrates for recombinant CBP. This screen identified two proteins involved in nuclear import, Rch1 (human importin-α) and importin-α7, as targets for CBP. The acetylation site within Rch1 was mapped to a single residue, Lys22. By comparing the context of Lys22 with the sequences of other known substrates of CBP and the closely related acetylase p300, we identified G/SK (in the single-letter amino acid code) as a consensus acetylation motif. Mutagenesis of the glycine, as well as the lysine, severely impaired Rch1 acetylation, supporting the view that GK is part of a recognition motif for acetylation by CBP/p300. Using an antibody raised against an acetylated Rch1 peptide, we show that Rch1 was acetylated at Lys22 *in vivo* and that CBP or p300 could mediate this reaction. Lys22 lies within the binding site for a second nuclear import factor, importin-β. Acetylation of Lys22 promoted interaction with importin-β *in vitro*. Collectively, these results demonstrate that acetylation is not unique to proteins involved in transcription. Acetylation may regulate a variety of biological processes, including nuclear import.

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Results and discussion

Table 1 shows that two nuclear import factors, Rch1 and importin-α7, were acetylated by recombinant CBP, as was the transcription factor HMG1. The fact that many proteins were not acetylated by CBP in this assay testifies to the specificity of the observed acetylation of import factors. The acetylation of HMG1 serves as a positive control, as it is already known to be acetylated *in vivo* at Lys2 and Lys11 [8]. A negative result in this screen does not, however, rule out the possibility that these substrates are acetylated by CBP or another acetylase *in vivo*.

Rch1 is an importin-α isoform that recognises classical nuclear localisation signals (NLSs) and functions as an import adapter, allowing access to the importin-β-dependent import pathway. There are at least six importin-α isoforms in human [9,10]. They all comprise two functional domains, an amino-terminal importin-β binding (IBB) domain that confers binding to and import by importin-β [11,12] and an arm-repeat domain that harbours the NLS-binding site (Figure 1a). The acetylase domain of CBP acetylated Rch1 and importin-α7 at levels comparable to the acetylation of histones (Figure 1b). Another family member, importin-α3 was, however, not acetylated by CBP, suggesting that acetylation may be relevant to the function of certain importin isoforms. Deletion analysis of Rch1 indicated that a domain composed of the amino-terminal 95 residues (1–95) was sufficient for acetylation by CBP, whereas a construct lacking this region (56–529) was not a substrate (Figure 1c).

The domain of Rch1 acetylated by CBP overlaps with the IBB domain [9]. The NLS-bound Rch1 has to interact with the nuclear pore-associated importin-β in order for nuclear import to take place [13,14]. We therefore asked if the Rch1–importin-β association was affected by acetylation. We prepared in parallel two preparations of bacterially expressed Rch1: one acetylated by CBP using ^{14}C-acetyl-CoA and the other metabolically labelled with ^{35}S-methionine to serve as the control for binding.

Table 1

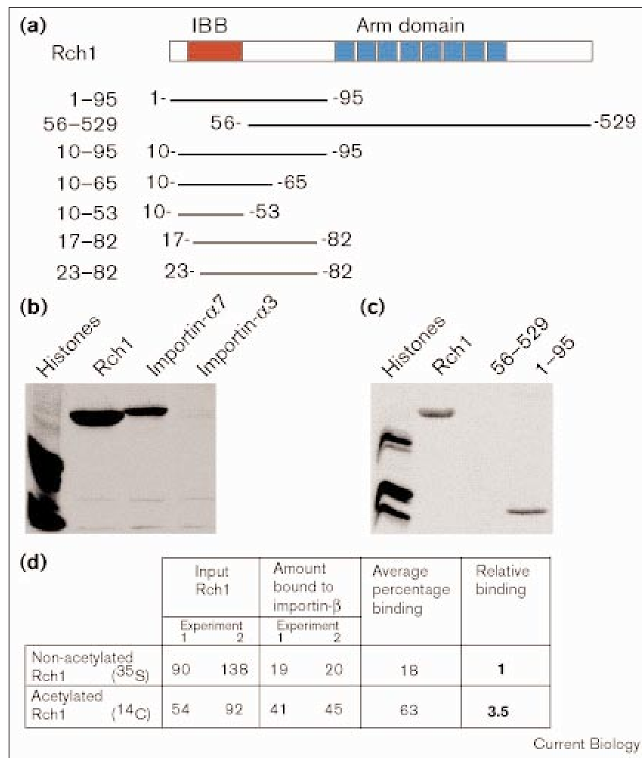
Panel of proteins tested as substrates for CBP.

Substrate	Acetylated by CBP	Function
HMG1	Yes	Transcription factor
Rch1	Yes	Nuclear import
Importin-α7	Yes	Nuclear import
Importin-α3	No	Nuclear import
TRF1	No	Chromosome structure
TRF2	No	Chromosome structure
RPA	No	Nuclear excision repair
HHR23B	No	Nuclear excision repair
hENDO1	No	DS break repair
hAPendo1	No	DS break repair
Ku70/80	No	DS break repair
PRP2	No	RNA splicing

Approximately 0.5–1 μg of each recombinant protein was incubated with glutathione-S-transferase (GST)–CBP (residues 1098–1758) and ^{14}C-acetyl-CoA in a standard histone acetyltransferase reaction [17]. DS, double-stranded.

Equimolar amounts of each Rch1 preparation were then used in importin-β binding assays and the relative binding to importin-β was determined for each preparation (Figure 1d). We found that the CBP-dependent acetylation of Rch1 increased its ability to bind importin-β by approximately 3–4-fold, suggesting that the acetylation of Rch1 seen here *in vitro* could regulate nuclear import.

Figure 1



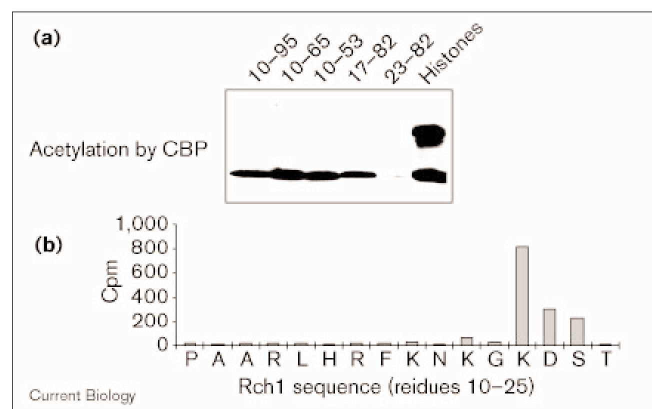
	Input Rch1		Amount bound to importin-β		Average percentage binding	Relative binding
	Experiment 1	2	Experiment 1	2		
Non-acetylated Rch1 (^{35}S)	90	138	19	20	18	**1**
Acetylated Rch1 (^{14}C)	54	92	41	45	63	**3.5**

Identification of substrates for the CBP acetylase. **(a)** Schematic representation of Rch1 and deletion constructs. **(b)** Rch1 and importin-α7 but not importin-α3 are acetylated by CBP. Approximately 1 μg of each protein was incubated with GST–CBP (residues 1098–1758) and ^{14}C-acetyl-CoA in a standard HAT reaction [18]. Reaction products were resolved by SDS–PAGE and viewed following autoradiography. Histones serve as a positive control. There was no detectable radiolabelling of substrate when CBP was omitted (data not shown). **(c)** CBP acetylates Rch1 within its amino terminus. The indicated Rch1 regions were tested as substrates of CBP. Reactions were performed as in (b). **(d)** Acetylation of Rch1 increases its binding to importin-β. Two preparations of bacterially expressed His-tagged Rch1 were prepared in parallel. One was metabolically labelled in *Escherichia coli* using ^{35}S-methionine, purified using Ni^{2+}–agarose, eluted from the beads and then mock-acetylated, thereby serving as the control for binding. The other was prepared identically except that it was not labelled with ^{35}S-methionine but was radioactively acetylated by CBP using ^{14}C-acetyl-CoA. Z-tagged importin-β was also expressed in, and purified from, bacteria and was left attached to immunoglobulin G–agarose beads. A binding reaction (binding buffer: 50 mM Tris.Cl pH 7.5, 500 mM NaCl, 5 mM magnesium acetate, 10 mM β-mercaptoethanol) was then set up containing excess agarose-bound importin-β and an equimolar amount of either ^{35}S-labelled Rch1 or ^{14}C-acetylated Rch1. Reactants were slowly rotated at room temperature for 2 h. The reactions were then pelleted, washed extensively with binding buffer, resolved by SDS–PAGE and blotted. The relative binding to importin-β was determined for each preparation of Rch1 using a PhosphorImager. The results of two independent experiments are presented.

To establish whether the acetylated lysine residue falls within the importin-β binding interface, we carried out a further deletion analysis. Figure 2a shows that this is the case. Crucially, residues 23–82 were not acetylated by CBP but when an additional six residues were included amino-terminally (17–82), acetylation was observed. This indicates that the motif FKNKGK can confer acetylation to Rch1. Protein sequence analysis of CBP-acetylated Rch1 confirmed that the acetylation site maps to this motif and involves only Lys22 (Figure 2b). This mapping places the acetylated lysine at the interface between Rch1 and importin-β. Recently, the crystal structure of an Rch1 domain bound to importin-β has been reported [15]. The structure indicates that Lys22 is an unusual basic residue within the IBB domain because it does not form a stabilising salt bridge with any acidic residues from importin-β unlike the nearby residues Lys18 and Lys20. Thus, neutralisation of the basic charge of Lys22 by acetylation would not be predicted to be detrimental to the interaction. Perhaps the observed stabilisation of the interaction (Figure 1d) comes about because acetylation of Lys22 repositions its neighbours into a more favourable binding conformation.

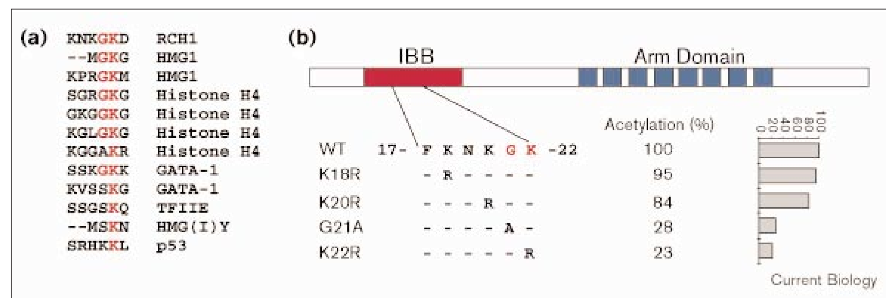
Having established Lys22 as the site for acetylation in Rch1, we asked whether the context of this lysine is conserved

Figure 2



Lys22 of Rch1 is acetylated by CBP. **(a)** Rch1 residues 17–22 are crucial for acetylation by CBP. The indicated Rch1 deletions (see Figure 1a) were tested as substrates of CBP. Reactions were performed as described in Figure 1b and the resulting autoradiogram is shown. **(b)** CBP acetylates Rch1 residue Lys22. A peptide fragment of Rch1 corresponding to amino acids 10–82 was ^{14}C-acetylated by CBP using standard conditions [18]. The reaction products were resolved by SDS–PAGE and blotted to PVDF membrane. Automated Edman degradation was performed on the blotted material and each cycle fraction was collected and counted using a liquid scintillation counter.

Figure 3

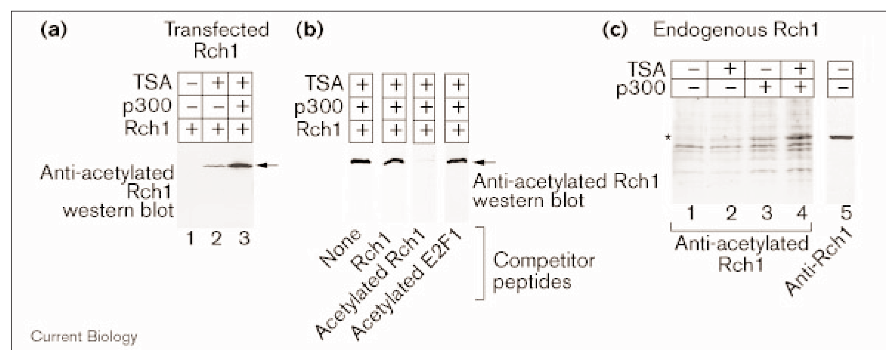


The GK motif is required for efficient acetylation of Rch1 by CBP. **(a)** Alignment of known CBP acetylation sites within different substrates. The GK motif (see text) is shown in red. The lysine residues shown in red are known to be acetylated *in vivo* (HMG1 [8], histone H4 [19], GATA-1 [5], HMG(I)Y [7] and p53 [4]) and/or by CBP *in vitro* (Rch1 (this study), histone H4 [20], GATA-1 [5], TFIIE [21], HMG(I)Y [7] and p53 [4]). **(b)** Schematic representation of Rch1 indicating the wild-type (WT) protein sequence (amino acids 17–22). The wild-type (WT) protein sequence (amino acids 17–22) and the positions and sequence of the mutations introduced into Rch1 are shown below. A dash indicates no change. Rch1 17–82 (or the indicated mutant) was used as substrate for CBP essentially as described for Figure 1, except that following SDS–PAGE the Rch1 band was excised and the amount of acetylation determined by liquid scintillation counting. The amount of acetylation is given relative to that of wild-type Rch1. These data are expressed graphically on the right.

relative to CBP-acetylated lysines in other proteins. An alignment of other known CBP targets (Figure 3a) indicates that the only detectable preference is a glycine or serine residue directly preceding the acetylated lysine. The exception is p53, which has a lysine at this position and one of the sites in histone H4 that has an alanine.

To assess the importance of the conserved GK sequence in Rch1, we carried out a mutagenic analysis. Mutating Gly21 severely disrupted acetylation by CBP; the severity of this mutation was comparable to mutating the acetylated lysine, Lys22 (Figure 3b). In contrast, mutating Lys18 and Lys20 had little or no effect. Essentially identical results were obtained using p300 (data not shown). The importance of the GK sequence for CBP/p300 acetylation is consistent with the finding that importin-α3, which does not have a GK sequence, was not acetylated by CBP (Figure 1b). Moreover, these results are consistent with the recently reported crystal structure of the *Tetrahymena* GCN5 acetylase bound to a histone H3 peptide, which indicates that a glycine residue immediately preceding the acetylated lysine within H3 is important for recognition by the acetylase [16]. These results indicate that residues other than lysines are necessary as a recognition site for a mammalian acetylase. We suspect that other determinants, less obvious than the glycine or serine residue, may also be necessary to determine the specificity, because some of the proteins that are not

Figure 4



Rch1 is acetylated by p300 at Lys22 *in vivo*. **(a)** Western blot showing Rch1 acetylation *in vivo*. U2OS cells were transiently transfected with an expression vector driving the expression of full-length Rch1 with carboxy-terminal Myc and His_6 tags. An expression vector encoding p300 was co-transfected as indicated. After washing off the DNA precipitate, the cells were allowed to recover for 12 h before TSA was added as indicated. Partial purification of the Rch1 protein was then performed using Ni^{2+}–agarose. The Rch1 was resolved by SDS–PAGE, blotted and probed with the AcK22 antibody. The blot was then stripped and re-probed with an anti-Myc antibody, which confirmed equal amounts of Rch1 in each lane (data not shown). No signal was detected when non-transfected cells treated with TSA were used (data not shown). **(b)** Peptide competition confirms that Rch1 is acetylated at Lys22 *in vivo*. Rch1 and p300 were transfected into U2OS cells. The cells were then exposed to TSA, the Rch1 protein purified as in (a) and then western blotted. Equal amounts of the same extract were loaded in each lane. The blot was probed with antibody AcK22 in the presence or absence of 1 μg/ml competitor peptide as indicated. The arrow in (a,b) indicates the position of Rch1–His_6. The antibody was raised against Rch1 16-RFKNKGK*DSTEM-27, where K* indicates the acetylated lysine. The unacetylated peptide was identical except there was no acetyl group at Lys22. **(c)** Endogenous Rch1 is acetylated *in vivo*. 293T cells were either mock-transfected (–) or transfected with an expression vector encoding p300 (+). Cells were either treated or not treated with TSA as in (a). Total cellular extract was prepared and resolved by SDS–PAGE, blotted and probed with AcK22 (lanes 1–4) or a control anti-Rch1 antibody (lane 5). The asterisk indicates the position of Rch1.

substrates for CBP in Table 1 do contain G/SK residues. Nevertheless, the G/SK sequence can serve as a guide for potential CBP/p300 acetylation sites once a protein is demonstrated to be a CBP/p300 target.

We next asked whether Rch1 was acetylated at the GK motif *in vivo*. In order to do this, we raised a rabbit polyclonal antibody (AcK22) that specifically recognises acetylated Rch1 (Figure 4). To test the specificity of the AcK22 antibody we first transfected a carboxy-terminally His-tagged version of Rch1 into cells and, following purification, we western blotted with AcK22. The antibody recognized Rch1 efficiently when a deacetylase inhibitor, TSA, was included (Figure 4a, compare lanes 1 and 2). Introduction of exogenous p300 into cells increased dramatically the recognition of acetylated Rch1 by AcK22 (lane 3). These results confirm that AcK22 recognises acetylated Rch1 *in vivo*. To confirm that the acetylation on Rch1 occurred at the GK motif, we used a peptide competition assay. Figure 4b shows that recognition of acetylated Rch1 by the AcK22 antibody was not inhibited by a peptide containing an unacetylated GK motif (Figure 4b, compare lanes 1 and 2). When the peptide was acetylated specifically at Lys22, however, it could effectively compete for the recognition of Rch1 by the AcK22 antibody. In contrast, an acetylated control peptide derived from the transcription factor E2F1 (which is known to be acetylated *in vivo*, [17]) did not compete for the recognition of acetylated Rch1. These results therefore show that the AcK22 antibody recognises specifically the acetylated version of Rch1 and that this recognition is context-dependent, that is, it recognises acetylated Lys22 only in the context of Rch1. We can therefore conclude that Rch1 can be acetylated *in vivo* by p300 and that this acetylation takes place at Lys22.

Finally, we asked whether the AcK22 antibody could detect endogenous acetylated Rch1 in a total cellular extract in which Rch1 had not been overexpressed (Figure 4c). AcK22 weakly recognized a band (lane 1, asterisk) that comigrated with Rch1 (lane 5). The other proteins recognized by the AcK22 antibody in lane 1 may correspond to importin-α isoforms. Significantly, the asterisked band in lane 1 has the characteristics of acetylated Rch1, as determined for transfected Rch1 in Figure 4a. Thus, detection of endogenously acetylated Rch1 was augmented slightly by the addition of TSA or by p300 transfection (lanes 2 and 3) and synergistically when both TSA and p300 were present (lane 4). These observations, plus the characterised specificity of AcK22 (Figure 4b), confirm that endogenous Rch1 is acetylated. However, the acetylated form could be detected only when the balance was biased towards the acetylated state. This is presumably because Rch1 acetylation is tightly controlled *in vivo*.

Our results point to the possibility that nuclear import may be subject to regulation by acetylation. Acetylation of Rch1 by CBP/p300 is likely to take place in the nucleus where CBP/p300 are localised, but may also affect the function of Rch1 when it shuttles to the cytoplasm. How acetylation regulates nuclear import functions remains to be established, but the results presented here suggest that acetylation may not necessarily be confined to the regulation of transcription, but may well affect other cellular processes, such as nuclear import.

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