

Myb induced myeloid protein 1 (Mim-1) is an acetyltransferase

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Abstract We have screened protein extracts from chicken blood cells for acetyltransferases. An in gel acetyltransferase assay revealed that a 32 kDa protein, which is more prevalent in whole blood when compared with erythrocyte cells, possessed an auto-acetylation activity. This protein was purified by a series of chromatographic steps, sequenced by Edman degradation and subsequently identified as Myb induced myeloid protein (Mim-1). Mim-1 has similarities to the conserved acetyltransferase motifs found in the GNAT superfamily of proteins and also contains three minimal GK acetylation motifs. These data identify Mim-1 as an acetyltransferase.

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1. Introduction

Post-translational modifications of proteins, in particular phosphorylation and more recently acetylation and methylation, have been recognised as major mechanisms of regulation. Evidence linking acetylation with active chromatin indicated a key role for such modifications in transcriptional processes ([1], reviewed in [2]). The importance of acetylation and its role in gene regulation was underlined by the identification and cloning of the first histone acetyltransferase *Tetrahymena* p55, a homologue of the yeast transcriptional co-activator Gcn5 [3]. Gcn5 is contained within the Gcn5-related N-acetyltransferases (GNAT) superfamily [4]. This family contains many diverse proteins from different species, and these show a degree of homology over a region of approximately 100 amino acids. Within this region four motifs, A–D, have been identified and linked to acetyl-coenzyme A (acetyl-CoA) binding (A) and enzyme activity (B and D) [4].

Experiments by a number of groups have shown that some of the histone acetyltransferases could acetylate non-histone substrates, examples include P/CAF [5] and p300/CBP [6]. Proteins that are modified include HMG 17 [5], HMG I(Y) [7], p53 [8], TFIIE β and TFIIF [9], GATA1 [10], EKLF [11] and α -importin [12]. That such a diverse set of proteins can be acetylated indicates a broad biological role for acetylation.

Although these proteins can be modified in vitro by histone acetyltransferases, the enzymes responsible for their in vivo acetylation have in many cases not been identified.

In a series of experiments we screened protein extracts from chicken blood cells for histone acetyltransferase activities. This analysis revealed that a protein with a molecular weight of approximately 32 kDa possessed an auto-acetylation activity. This protein was extracted, purified and micro-sequenced. Sequence analysis identified the acetyltransferase as Myb induced myeloid protein 1 (Mim-1), a protein that has previously been described in a number of different cellular locations but not identified as an acetyltransferase or assigned any biological function.

2. Materials and methods

2.1. Cell and nucleus preparation

Fresh chicken blood was collected into phosphate buffered saline (PBS) containing 5 mM Na₂EDTA, 0.1 mM phenylmethylsulphonyl fluoride (PMSF) and 0.1 mM benzamidine. Cells were washed once in PBS followed by two washes in wash buffer (10 mM NaCl, 10 mM Tris–HCl pH 7.5, 6 mM MgCl₂, 250 mM sucrose, 0.1 mM PMSF and 0.1 mM benzamidine). The cells were lysed with 45 volumes (packed cell volume) of cell lysis buffer (10 mM NaCl, 10 mM Tris–HCl pH 7.5, 6 mM MgCl₂, 250 mM sucrose, 0.2% (v/v) Triton X-100, 0.1 mM PMSF and 0.1 mM benzamidine) and stirred for 20 min at 4°C. Nuclei were collected by centrifugation at 1400×g. The nuclei were washed twice in wash buffer and collected by centrifugation at 1400×g. The nuclei were stored in wash buffer containing 40% (v/v) glycerol at –70°C.

2.2. NaCl extraction

Extraction of acetyltransferases from prepared nuclei was achieved using a modification of the method described by Schöler et al. [13]. Nuclei were washed and resuspended at a final concentration of 2 mg/ml in extraction buffer (450 mM NaCl, 20 mM HEPES pH 7.8, 0.5 mM dithiothreitol, 0.2 mM Na₂EDTA, 25% (v/v) glycerol, 0.1 mM PMSF and 0.1 mM benzamidine) and incubated for 30 min at 4°C under constant agitation. Following extraction, nuclear debris was pelleted by centrifugation at 14000×g for 5 min at 4°C. The supernatant was collected and dialysed overnight against 10 mM sodium phosphate buffer pH 8.0, 10% (v/v) glycerol, 5 mM β -mercaptoethanol, 0.1 mM PMSF and 0.1 mM benzamidine. The sample was centrifuged as above and the supernatant was collected.

2.3. In gel acetyltransferase activity assay

In gel acetyltransferase assays were performed essentially as described by Brownell and Allis [14]. Briefly, identical samples were loaded onto duplicate SDS polyacrylamide gels. The acetyltransferase assay gel had calf thymus total histones (Worthington) incorporated into the gel matrix at a concentration of 0.1 mg/ml. All gels were electrophoresed under identical conditions, after which the non-histone gel was stained with Coomassie brilliant blue. The two activity gels, no substrate and histone substrate gels were incubated in buffer A (50 mM Tris–HCl pH 7.9, 1 mM dithiothreitol and 0.1 mM Na₂EDTA pH 8.0) containing 20% (v/v) isopropanol for a total of 1 h

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Abbreviations: acetyl-CoA, acetyl-coenzyme A; GNAT, Gcn5-related N-acetyltransferase; PMSF, phenylmethylsulphonyl fluoride

with three changes of buffer. This was followed by incubation with buffer A for 30 min with three changes of buffer. The gels were denatured by incubation in buffer A containing 8 M urea for a total of 1 h with three changes of buffer. The gels were then incubated overnight in buffer A containing 0.04% (v/v) Tween 40 at 4°C. The gels were subsequently incubated in buffer B (50 mM Tris–HCl pH 7.9, 10 mM Na-butyrate, 10% (v/v) glycerol, 1 mM dithiothreitol and 0.1 mM Na₂EDTA pH 8.0) for 40 min with two buffer changes. The gels were then incubated each with 2 ml buffer B containing 10 μ Ci [³H]acetyl-CoA (2–10 Ci/mmol, Amersham) for 3 h. The gels were washed in 10% (v/v) acetic acid for 2 h with five changes of fresh wash buffer. The activity gels were incubated in Amplify (Amersham) for 30 min and dried down before exposure to film for approximately 3 weeks.

2.4. Column chromatography

A series of different chromatography steps were used to isolate the acetyltransferase. For each of the columns the eluate was continually monitored at 280 nm and the in gel activity assay was used to trace the acetyltransferase.

2.4.1. Hydroxyapatite chromatography. Salt extracted proteins were applied to a 10 ml hydroxyapatite column (Bio-Rad) (pre-equilibrated with 10 mM sodium phosphate buffer pH 8.0, 10% (v/v) glycerol, 5 mM β -mercaptoethanol, 0.1 mM PMSF and 0.1 mM benzamidine) at a flow rate of 1 ml/min. Bound proteins were eluted using a linear gradient (10–300 mM) of sodium phosphate.

2.4.2. Q anion exchange column chromatography. The fractions containing the acetyltransferase from the hydroxyapatite column were pooled and dialysed against 10 mM NaCl, 10 mM Tris–HCl pH 7.5, 5 mM β -mercaptoethanol, 0.1 mM PMSF and 0.1 mM benzamidine before loading onto a pre-equilibrated 1 ml Q column (Bio-Rad) at a rate of 0.5 ml/min. The bound proteins were eluted with a linear NaCl gradient (10–400 mM NaCl).

2.4.3. S cation exchange chromatography. The flowthrough from the Q column was dialysed against 10 mM HEPES pH 7.8, 10 mM NaCl, 10% (v/v) glycerol, 5 mM β -mercaptoethanol, 0.1 mM PMSF and 0.1 mM benzamidine before loading the sample onto a pre-equilibrated 1 ml S column (Bio-Rad) at a rate of 0.5 ml/min. Proteins were eluted across a linear NaCl gradient from 10 mM to 400 mM NaCl at 0.5 ml/min. Fractions of interest were pooled and dialysed against buffer C, 10 mM Tris–HCl pH 7.5, 10% (v/v) glycerol, 5 mM β -mercaptoethanol, 0.1 mM PMSF and 0.1 mM benzamidine.

2.5. Cyanogen bromide cleavage

A sample of the purified 32 kDa acetyltransferase was electrophoresed through a preparative SDS–PAGE gel and the 32 kDa band excised. The protein was then recovered by electro-elution and dialysed overnight to remove salt before lyophilisation. The protein was dissolved in 70% (v/v) formic acid and a single crystal of cyanogen bromide added before incubating for 24 h in the dark for the cleavage reaction. Following cleavage, the sample was diluted to 6 ml with

dH₂O and the sample lyophilised. The sample was resuspended in 1 ml dH₂O. This resuspension and freeze drying was repeated for a total of four times to ensure removal of the formic acid. The final pellet was resuspended in SDS loading buffer, before electrophoresis through a 16% SDS polyacrylamide Tris-tricine gel.

2.6. Protein sequencing

Gel separated proteins and peptides were blotted onto polyvinylidene difluoride (PVDF) membrane (Bio-Rad) and subsequently stained with Coomassie brilliant blue. The membrane was rinsed with dH₂O, dried and the samples sequenced by Edman degradation (Alta Bioscience, University of Birmingham).

3. Results

3.1. Isolation and identification of the acetyltransferase

Proteins were initially extracted from intact chicken blood cells using a modification of a freeze thaw method in the presence of 450 mM NaCl [13]. For this experiment a small volume, 80 μ l, of loosely packed blood cells was extracted in 200 μ l of high salt buffer. Proteins released from the cells into the supernatant were assayed using the in gel acetyltransferase assay as described by Brownell and Allis [14]. Fig. 1 shows the screen for acetyltransferases. The Coomassie stained gels show the total protein content of the extracts. The fluorographs of the activity gels show a number of potential acetyltransferase proteins and their approximate molecular weights. Fig. 1a shows that a number of proteins in the whole cell extract have acetyltransferase activity. We decided to purify the acetyltransferase at approximately 32 kDa, which is significantly smaller than other acetyltransferases previously described.

We next investigated whether this activity could be extracted from a crude nuclear preparation. Nuclei were prepared from 30 ml of packed blood cells, and the proteins extracted. Fig. 1b shows the in gel activity assay of proteins extracted from the nuclei. The 32 kDa acetyltransferase detected in the whole cell extract is present in the crude nuclear extracts and is the major acetyltransferase. In addition, we tested whether the acetyltransferase was present in erythrocytes by removing the buffy coat containing the white blood cells at the time of blood preparation. The result of the in gel activity assay clearly shows that the 32 kDa acetyltransferase is more abundant in whole blood cells than in the fraction depleted of white blood cells. Subsequently, all extracts were

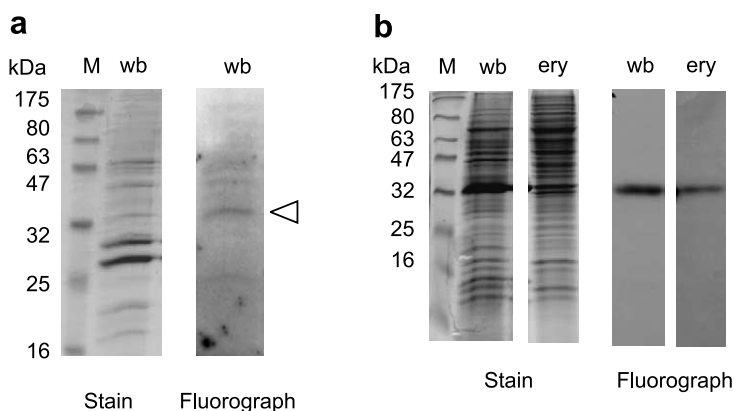


Fig. 1. In gel acetyltransferase activity assays of protein extracts. Proteins in the salt extractions were electrophoresed through two 13% SDS–PAGE gels. One gel was stained with Coomassie brilliant blue, whereas the remaining gel was subjected to an in gel activity assay using histones polymerised into the gel matrix as substrate. The 32 kDa acetyltransferase is marked with an arrowhead. The approximate molecular masses (kDa) are indicated by the marker proteins (M). a: Proteins extracted from intact chicken blood cells (wb). b: Proteins extracted from nuclear preparations from either whole blood (wb) or erythrocyte (ery) cells.

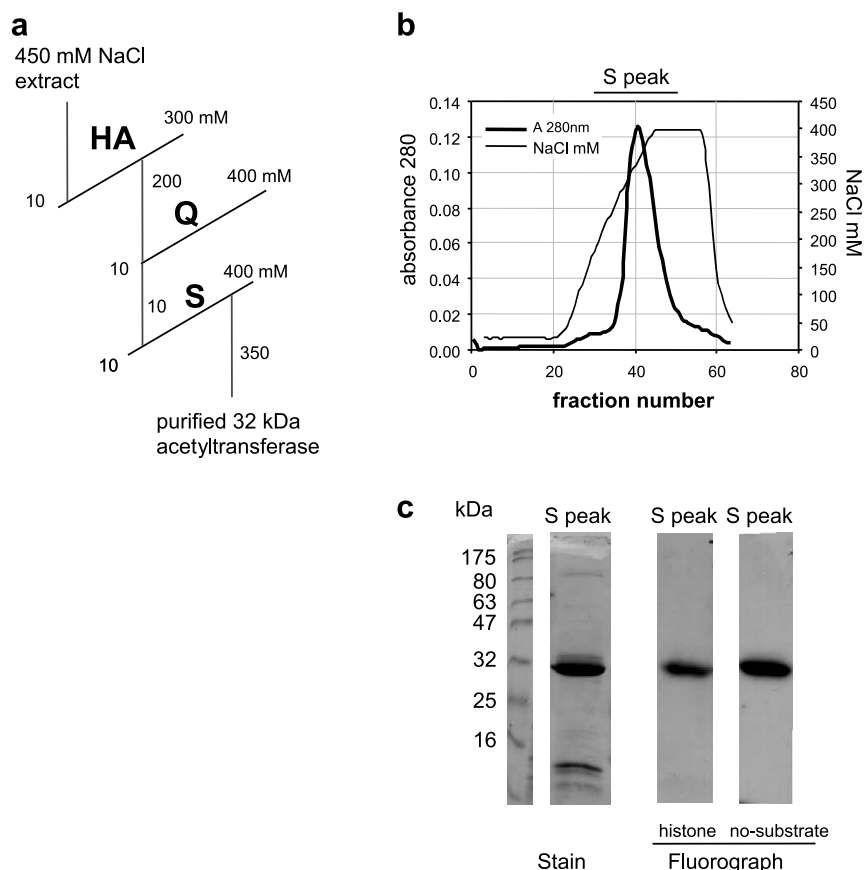


Fig. 2. Purification of the 32 kDa acetyltransferase. a: Flow chart showing the chromatography steps used to purify the acetyltransferase. HA hydroxyapatite, Q anion exchange and S cation exchange chromatography. b: Elution profile from the final S cation exchange column. c: In gel activity assay of proteins pooled from the main peak eluted from the S cation exchange column. One gel was stained with Coomassie brilliant blue. Duplicate samples were assayed for acetyltransferase activity with either histone or no substrate cast into the resolving gel.

made from whole blood preparations to maximise the yield of the acetyltransferase.

The protein was purified from the 450 mM NaCl extract using a series of chromatography steps, these are summarised in Fig. 2a. The acetyltransferase was traced throughout the purification procedure using the in gel activity assay. Fig. 2b,c shows the final stage of the purification. Proteins that were eluted in the main peak from the S cation exchange were pooled and subjected to an in gel activity assay. In this assay we also included an activity gel without substrate to allow us to determine whether the protein was capable of auto-acetylation. The Coomassie stained gel together with the fluorograph of the in activity gels, with and without histone substrate, is given in Fig. 2c. The Coomassie gel shows that the 32 kDa protein has been highly purified, as judged by the lack of other proteins in the sample. The fluorograph shows that the purified protein has acetyltransferase activity in both the histone and no substrate gels.

At a number of different stages in the purification process we were able to blot the partially purified proteins onto PVDF membrane and sequence the 32 kDa acetyltransferase. Internal sequence was also obtained following cyanogen bromide cleavage of a highly purified sample. The sequences obtained are as given in Table 1 and were used to search publicly available databases. The result of BLAST searches showed that all of the sequences obtained match a known protein, Mim-1 [15]. The Mim-1 cDNA was first identified by a differ-

ential hybridisation screening technique to search for genes activated by the *v-myb* oncogene [15]. The cDNA is 1054 bp in length and encodes a protein of 326 amino acids. The protein contains two imperfect direct repeats sharing 71% identity.

3.2. Conserved acetyltransferase domains

Many acetyltransferases are members of the GNAT superfamily of proteins [4]. This family, of which Gcn5 is the archetypal member, contains proteins from a wide variety of species. These proteins share a number of common amino acid sequence motifs A–D [4]. Sub-groups, in particular the MOZ and Myf family, contain some but not all of the sequence motifs. Motif A contains the acetyl-CoA binding domain that is present in all family members.

The data presented in Figs. 1–3 demonstrate that Mim-1 has acetyltransferase activity. We therefore examined the amino acid sequence of Mim-1 for similarities to conserved ace-

Table 1
Protein sequences obtained by Edman degradation from the 32 kDa acetyltransferase at various stages in the purification

Sample	Sequence	Match
HA fraction	HPPQQQGRHWAQIIS	Mim-1
Peak S fraction	HPPQQ	Mim-1
CNBr internal sequence	~KVFPQIV	Mim-1

tyltransferase motifs. Sequence comparison revealed similarities between the conserved motifs present in other acetyltransferases and repeat 2 of Mim-1 more readily than repeat 1.

Global alignment shows that Mim-1 has 20% identity with either Gcn5 or Esa-1, which rises to 34% and 36% respectively allowing for conservative changes (data not shown). The overall level of identity between acetyltransferases is not always high, for example, an alignment of 40 amino acids constituting the catalytic domains of Gcn5 and Esa-1 shows 23% identity. The identity of Esa-1 and Mim-1 is 18% whilst Gcn5 and Mim-1 show 10% identity over the same region.

Further analysis of the Mim-1 sequence reveals similarities to the motifs A, B and D suggesting that Mim-1 is a member of the GNAT superfamily. Fig. 3a gives the alignments of the conserved domains from Gcn5 and Esa-1 with the Mim-1 repeat 2. Neuwald and Landsman [4] used sequence analysis to identify important conserved amino acids within these motifs. These are indicated by an asterisk in Fig. 3a. Kuo et al.

[16] used site directed mutagenesis of yeast Gcn5 to detect those amino acid residues in the active and acetyl-CoA binding sites that are required for HAT activity, these are shown as arrows. A number of these conserved and functional residues are present in the Mim-1.

Close inspection of the alignment between Mim-1 and the Gcn5 acetyl-CoA binding domain (motif A) shows that out of the 10 conserved residues (as proposed by Neuwald and Landsman [4]), Mim-1 shares similarity with six. The same region of Mim-1 aligned with Esa-1 shows that seven out of 10 conserved amino acid residues are similar. Additionally, seven of the eight conserved residues in motif B are present in Mim-1. The identity between Gcn5 and Mim-1 over this region is 95% allowing for conservative changes. The same region of Mim-1 aligned to Esa-1 shows that four out of the eight are similar. In motif D, Mim-1 shares similarity with four of the six conserved amino acids present in Gcn5 (Esa-1 does not have a comparable domain).

The work of Kuo et al. [16] has shown that certain amino acids are required for the HAT function of Gcn5, marked with an arrow in Fig. 3a. Over the acetyl-CoA binding site (motif A) Mim-1 shares similarities with four out of the eight amino acids required for HAT activity.

For motif B, Mim-1 shares similarity with all six amino acids in Gcn5 that are essential for HAT activity [16]. Motif D also has some similarities with Mim-1, sharing three out of the six amino acid residues required for activity. Additionally it was noticed that the catalytic domain of Gcn5 (motif B) lies upstream of the acetyl-CoA binding site (motif A) at the level of the primary structure. This orientation is reversed in both Mim-1 and Esa-1.

Clearly the Mim-1 protein has an acetyl-CoA binding site and shows similarities with the conserved motifs found within the GNAT superfamily of acetyltransferases. It should be noted that very high levels of identity might not be expected since different acetyltransferases have different protein targets, which could be reflected in protein sequence variation. For example, in the bacterial ArgA protein the acetyl-CoA binding domain contains the sequence SRG where many other group members have GYG [4]. Mim-1 contains SKG differing from the ArgA sequence by the conservative change of R to K.

3.3. Auto-acetylation

The in gel activity assay (Fig. 2) shows the presence of a band when no substrate is cast into the gel matrix suggesting that the Mim-1 protein can auto-acetylate. This is not unknown since Herrera et al. [5] have shown that P/CAF can auto-acetylate. A number of studies have suggested a weak consensus sequence GKXXP for acetylation motifs ([8,12,17–19], reviewed in [20]). Mutational analysis indicates that this motif could be as small as the two residues, GK [12]. We therefore examined the Mim-1 sequence for potential GK acetylation sites and found three minimal GK acetylation motifs; these are highlighted in Fig. 3b. We note that the GK sequence is present at the in vivo acetylation sites of histones H2A, H3 and H4. In the gel activity assay, the Mim-1 protein is electrophoresed into a single concentrated band. In this region of the gel the same protein contains both acetyltransferase domains and potential acetylation target sites. It is therefore understandable that we detect auto-acetylation in this assay.

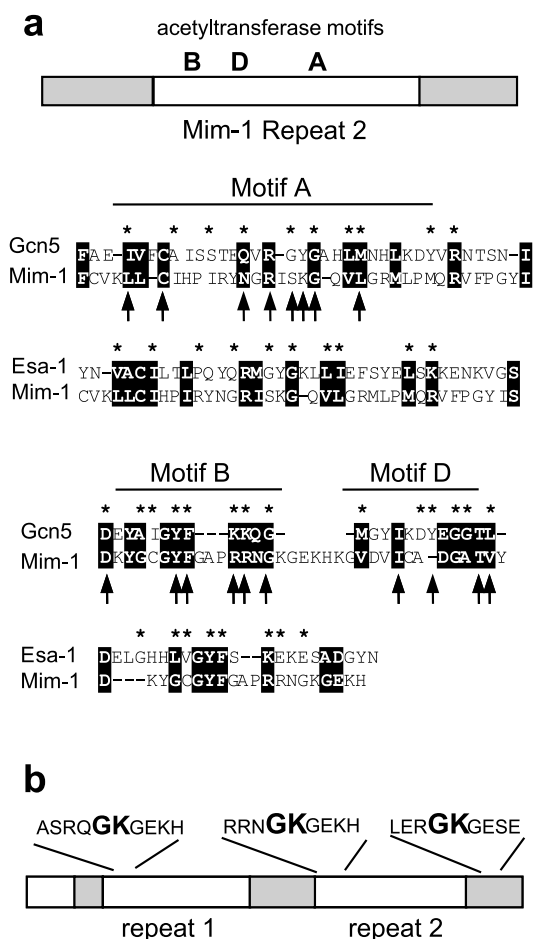


Fig. 3. Sequence motifs in Mim-1. a: The schematic representation of Mim-1 shows the second repeat and the relative positions of the acetyltransferase motifs B, D and A within repeat 2. Sequence alignments of the acetyltransferase motifs of Mim-1 with those of Gcn5 and Esa-1. Similar amino acids are shown in boxes. Asterisks indicate conserved residues in the GNAT family as proposed by Neuwald and Landsman [4]. Residues required for histone acetyltransferase activity of Gcn5 are marked with arrows and are as described by Kuo et al. [16]. b: Schematic representation of the Mim-1 protein showing the location of the three potential GK acetylation sites.

3.4. Is Mim-1 a histone acetyltransferase?

Throughout the purification procedure the Mim-1 acetyltransferase activity was traced by the in gel activity assay. Because Mim-1 shows a strong auto-acetylation in this assay it is not possible to determine whether resulting acetylation is due to histone or auto-acetylation. We therefore tested the Mim-1 sample eluted from the S column for histone acetyltransferase activity using a liquid assay [21]. This highly purified Mim-1 was incapable of acetylating histones in this assay (data not shown). The Mim-1 protein was purified using the S column exploiting the net basic charge of the protein as previously described [22,23]. However, during the purification procedure we noticed that a small proportion (~5%) of Mim-1, as identified by sequencing, was retained by the Q column (see purification scheme in Fig. 2) and eluted at 175 mM NaCl. When this fraction was tested in a liquid HAT assay we found acetylation of histones H4>H3>H2A with free histones, modulated to H4>H2A with nucleosomal substrates (data not shown). This activity is similar to the yeast Nu4A acetyltransferase complex [24]. It is attractive to conclude that the HAT activity was due to Mim-1, in a protein complex retained on the Q column. However, we cannot exclude the possibility that the activity is from another acetyltransferase, which is present in the same fraction but does not function in the in gel assay. Further experiments are needed to test whether Mim-1 can function as a HAT when in an appropriate protein complex.

4. Discussion and conclusions

The data presented here demonstrate that the 32 kDa acetyltransferase detected in protein extracts from chicken blood cells is the protein Mim-1. This protein is encoded by a gene spanning 5.8 kb of DNA and contains seven exons. A putative leader sequence of 23 amino acids has been identified which is never seen on mature proteins suggesting post-translational cleavage. The protein also shows 60% identity (allowing conservative changes) with the 16 kDa human leukocyte chemotactic protein LECT 2 [25], it is therefore possible that LECT 2 could also be an acetyltransferase.

The Mim-1 gene is expressed in myeloid cells of the chicken haematopoietic system, in particular in myeloblasts and promyelocytes [15]. Expression is reduced in terminally differentiated granulocytes but the protein is stored in large amounts as granules. Mim-1 can be secreted from virally transformed cell lines [15] and upon artificially induced exocytosis from chicken polymorphonuclear neutrophils [26]. Another group of workers independently identified the same protein [27]. This protein, p33, originally isolated from hen liver nuclei, was also abundant in chicken heterophils [22] and was found to be identical to Mim-1 with the exception of one amino acid. The Mim-1 protein has also been found in chicken granulocyte preparations and was salt eluted from nuclei together with the chromatin decompaction protein Ment [28]. However, it was thought that Mim-1 was present as a contaminant in nuclear preparations [28]. We have observed that a 32 kDa acetyltransferase is released from 15 day embryonic nuclei by micrococcal nuclease digestion [21]. Subsequent Western blotting has identified it as Mim-1 (data not shown). More recently Mim-1 has been detected in osteoclasts and is either secreted, located in the cytoplasm or found within the peri-nucleus [29]. Further, Falany et al.

[29] have suggested that Mim-1 could be involved in signalling events involved in bone remodelling. Our results demonstrating that Mim-1 is an acetyltransferase could indicate a possible role for acetylation and Mim-1 in such signalling events.

Recently it has been shown that a number of transcription factors can be acetylated. Acetylation of transcription factors by histone acetyltransferases has been implicated as a mechanism of control. For example, P/CAF has been shown to acetylate transcription factors such as p53 [8], TFIIE β and TFIIF [9], whilst p300/CBP has been shown to acetylate GATA-1 [10], EKLF [11] and TFIIE β and TFIIF [9]. Imhof and co-workers [9] have shown that the site of acetylation of TFIIE β is K52, which lies in a sequence that shows homology to the amino tail of histone H3. This site has been determined by site directed mutagenesis of the transcription factor at K52R. However, p300/CBP can still acetylate TFIIE β with this amino acid substitution, suggesting a different site specificity of this HAT [9]. Interestingly, p300/CBP and P/CAF, although similar and associated with each other, appear to have distinct functions. p300/CBP also acetylates GATA-1, which directly regulates the transcriptional activator function of GATA-1 in vivo [10]. GATA-1 has a conserved domain of K/RXKK (where X is any amino acid) in many different species suggesting an important regulatory role. This sequence is also found to be important in p53 [8]. Further work from a different group showed that GATA-1 was acetylated at lysine residues K246 and 252 of one zinc finger and K312 of the second zinc finger [6]. Acetylation of p53 by P/CAF [8] leads to increased DNA binding activity, suggesting that acetylation of p53 leads to an altered conformation of the protein, which in turn leads to the increased DNA binding activity [8]. In summary, it has been demonstrated that the function of a protein may be modulated by acetylation. Our finding that Mim-1 can auto-acetylate in the in gel assay could suggest a role for this modification in its regulation, an important consideration when a protein is relatively abundant and has several potential roles.

The key finding of our work is that Mim-1 is an acetyltransferase. Mim-1 had been identified in numerous studies, in different circumstances and in many different cells and cellular locations. However, a function for the protein has never been described. Whilst we have been able to define Mim-1 as an acetyltransferase, we currently have not yet identified its in vivo targets. Mim-1 (p33) was originally classified as a nuclear protein, but the presence of a leader sequence would indicate that it is secreted and may act as a signalling molecule, especially in the development of chicken blood. A number of groups have found Mim-1 in nuclear preparations; however, a nuclear role for Mim-1 as a histone acetyltransferase is difficult to reconcile with the presence of the leader sequence. However, we find that Mim-1 is released from nuclei by the action of micrococcal nuclease [21]. The presence of a leader sequence does not always exclude a protein from the nucleus since human follistatin-related protein, which also contains a signal sequence, is located within the nuclei of human granulosa cells [30]. The fact that Mim-1 has been reported in numerous cellular locations could indicate that it has multiple roles and multiple targets as is becoming clear for many different proteins (reviewed in [31]). Work can now proceed to determine more precisely the role of Mim-1 in different biological processes.

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