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p33, an endogenous target protein for arginine-specific ADP-ribosyltransferase in chicken polymorphonuclear leukocytes, is highly homologous to *mim-1* protein (*myb*-induced myeloid protein-1)

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We have determined the partial amino acid sequence of p33, an endogenous substrate protein for arginine-specific ADP-ribosyltransferase in chicken polymorphonuclear leukocytes (heterophils), and found that the sequence was completely identical with the regions of amino acid sequences deduced from *mim-1* (named for *myb*-induced myeloid protein-1, which is expressed in chicken promyelocytes) cDNA [(1989) Cell, 59, 1115–1125], except for one amino acid difference (Tyr^{297}→Ile). These results together with data on cellular and subcellular distributions of p33 in heterophils suggest that *mim-1* may encode the precursor protein of p33.

Polymorphonuclear leukocyte (chicken); Myb; Cell differentiation; ADP-ribosylation

1. INTRODUCTION

Arginine-specific ADP-ribosyltransferase (EC 2.4.2.31) catalyzes transfer of the ADP-ribose moiety of NAD to arginine residue(s) of the target protein [1]. Cholera toxin ADP-ribosylates an arginine residue of Gsα, which leads to the activation of adenylate cyclase [2]. Transferases in vertebrates have been isolated, purified and characterized [3], but their physiological role has yet to be determined because much less is known of the endogenous target protein for the transferase. We have reported findings of arginine-specific ADP-ribosyltransferase activity and its possible endogenous substrate protein, which we designated p33, in the granules of chicken peripheral polymorphonuclear pseudoeosinophilic granulocytes (heterophils) [4], cells which correspond to human neutrophils [5]. We purified the ADP-ribosyltransferase and p33 from chicken heterophils and characterized them [4]. The in situ ADP-ribosylation of heterophils suggested that p33 may be a major substrate in these cells [4].

mim-1 (named for *myb*-induced myeloid protein-1) was found to be a cellular gene directly activated by the nuclear oncogene product, v-Myb [6], and *mim-1* protein was detected not only in v-*myb*-transformed cells but also in granules of normal promyelocytes, and in much lesser amounts in those of matured granulocytes in chickens [6].

We report here that the amino acid sequences of the N-terminal region and several internal peptide fragments of p33 are highly homologous to the amino acid sequence deduced from *mim-1* cDNA. Based on the homology of the amino acid sequences between p33 and the predicted *mim-1* protein, we conclude that *mim-1* encodes the precursor protein of p33.

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Abbreviations: EDTA, ethylene diamine tetraacetic acid; TFA, trifluoroacetic acid; PCR, polymerase chain reaction; HPLC, high performance liquid chromatography.

2. MATERIALS AND METHODS

2.1. *Materials*

Chickens (White Leghorn) were obtained from Hara Farms, Shimane, Japan. Lysine-specific *Achromobacter* protease-1 (lysylendopeptidase) was obtained from Wako Chemicals Co., Japan. All other chemicals were obtained from Seiwa Industry Co., Shimane, and were used without further purification. Heterophils were obtained from chicken peripheral blood [4]. Bone marrow cells were collected from chicken leg bones and were used after removal of the erythrocytes by 12.5% Ficoll/metrizoate centrifugation, as described in [4].

2.2. *Purification and amino acid sequencing of p33*

p33 was purified from chicken peripheral heterophils, as described elsewhere [4]. Purified p33 (500 μg) in 3 ml of 7 M guanidine-HCl containing 10 mM EDTA, 0.5 M Tris-HCl buffer (pH 8.5) and 21.6 mM dithiothreitol was incubated for 2 h at room temperature. After adding 25 mg of iodoacetic acid, the solution was kept in the dark for 30 min, dialyzed against H_2O, and lyophilized. The carboxymethylated p33 thus obtained was dissolved in 1 ml of 0.1 M Tris-HCl buffer (pH 9.0) and digested with 5 μg of lysylendopeptidase at 30°C for 6 h. The digest was lyophilized, dissolved in 300 μl of 0.1% TFA and applied on a C4 column (SynChropak RP-4, 4.6 × 250 mm, SynChrom Inc.) and eluted with a linear gradient of acetonitrile (0–60%) in 0.1% TFA at a flow rate of 0.5 ml/min. Some of the peaks obtained were further fractionated with a microbore C4 column (SynChropak RP-4,

2.1 × 250 mm), as described in the legend to Fig. 1. Amino acid sequences of the N-terminal region and lysylendopeptidase-digested internal peptide fragments were determined by automated Edman degradation using a Model 477 gas-phase protein sequencer and a Model 120 PTH amino acid analyzer (Applied Biosystems).

2.3. *Primer synthesis, PCR, and nucleotide sequencing*

Primers for PCR and DNA sequencing were made, based on the nucleotide sequence of *mim-1* cDNA reported by Ness et al. [6], by an Applied Biosystems Model 391 PCR-Mate DNA synthesizer, and desalted with a NAP 5 column (Pharmacia). The nucleotide sequence and positions of primers in *mim-1* cDNA are as follows; primer 1, CGCTCAAAGGCACGAGATGC (11–29); primer 2, CGATCGCAGTTCTCAACATG (999–981); primer 3, CTGTGCTGATG GTGCAAC (695–712). Using the Cetus GeneAmp RNA PCR kit, first-strand cDNA was synthesized from total RNA extracted with acid phenol [7]. The *mim-1* cDNA fragment (989 bp) was amplified from the prepared cDNA with primers 1 and 2, isolated by agarose gel electrophoresis, and purified using a Geneclean kit (BIO 101). With the 989-bp fragment as a template, the sequencing reaction was carried out using a Dye Deoxy Cycle sequencing kit (Applied Biosystems) on a thermal cycler PC-700 (ASTEC, Japan) with primers 2 or 3, and the DNA sequence was determined using an Applied Biosystems Model 373A DNA sequencer.

3. RESULTS AND DISCUSSION

p33 purified from heterophils was reduced, carboxymethylated and digested with lysylendopeptidase, and the digest was applied on reversed phase HPLC with a C4 column. As shown in Fig. 1, several peaks were isolated. The combined fraction of the indistinct peaks, 6 and 7, was further subjected to HPLC with a microbore C4 column, and distinct peaks 6 and 7 appeared (Fig. 1, inset). Amino acid sequences of the N-terminal region and 8 isolated internal peptides corresponding to peaks 1–8 of the p33 were determined (Fig. 2, underlined sequences). An homology search of protein data bases revealed that all of the 9 peptide sequences completely matched the regions of the amino acid sequence deduced from the *mim-1* cDNA [6], except for position 297 (Fig. 2). According to the predicted amino acid sequence of *mim-1* protein, 209 amino acids were determined out of 303 (24–326) amino acids of *mim-1* protein. One amino acid difference (Tyr^{297}→Ile) was detected between the amino acid sequence deduced from *mim-1* cDNA and that derived from p33. As the N-terminal sequence of p33 corresponded to amino acids 24–46 of *mim-1* protein, p33 does not have an N-terminal hydrophobic peptide. These findings support the hypothesis of Ness et al. that the N-terminal region of *mim-1* protein may function as a leader peptide [6].

To examine whether *mim-1* cDNA encodes the Tyr^{297} in the chickens used here, we amplified the *mim-1* cDNA fragment from chicken peripheral heterophil or bone marrow cell RNA by reverse transcription PCR. On analysis of the PCR products with agarose gel electrophoresis, a band of the expected size (989 bp) was detected only with bone marrow cell RNA (data not

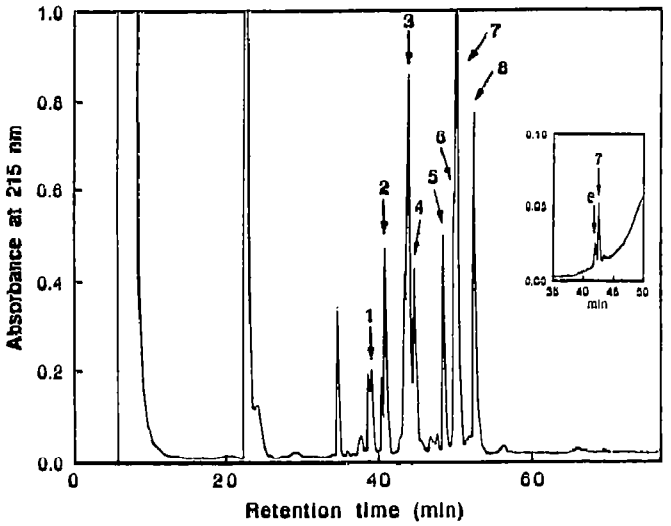

Fig. 1. Lysylendopeptidase-digested peptide map of p33. Carboxymethylated p33 was digested with lysylendopeptidase and analyzed by reversed phase HPLC on a C4 column. Fragments were eluted by linear gradient of acetonitrile in 0.1% TFA at a flow rate of 0.5 ml/min. Absorbance of the eluate was monitored at 215 nm. Further analysis of peaks 6 and 7 was performed using a microbore C4 column. Conditions for elution were the same as for the first HPLC except that the flow rate was 0.2 ml/min. Findings with refractionation of peaks 6 and 7 are shown in the inset.

1 M P A L S L I A L L S L V S T A F A R Q
21 W E V H P P Q Q Q G R H W A Q I C S G N
N-terminal
41 P F N R I R G C D R Y G C G N Y G A S R
61 Q G K G E K H K G V D V I C T D G S I V
Peak 8
81 Y A P F S G Q L S G P I R F F H N G N A
101 I D D G V Q I S G S G Y C V K L V C I H
121 P I R Y H G Q I Q K G Q Q L G R M L P M
Peak 2
141 Q K V F P G I V S H I H V E N C D Q S D
Peak 7
161 P T H L L R P I P D I S P P F P Q Q D A
181 H W A V V C A G N P T N E I R G C D K Y
201 G C G Y F G A P R R N G K G E K H K G V
Peak 1
221 D V I C A D G A T V Y A P F S G E L S G
Peak 5
241 P V K F F H N G N A I D D G V Q I R G S
Peak 4
261 G F C V K L L C I H P I R Y N G R I S K
Peak 3
281 G Q V L G R M L P M Q R V F P G Y I S H
Peak 6
301 I H V E N C D R S D P T S N L E R G K G
321 E S E M E V

Fig. 2. A comparison of the amino acid sequence deduced from *mim-1* cDNA and that derived from p33. The amino acid sequence of *mim-1* protein is that reported by Ness et al. [6]. Amino acid sequences underlined correspond to data obtained from the amino acid sequencing of the N-terminal and of 8 peaks of p33 shown in Fig. 1. Note that the amino acid of p33 corresponding to Tyr^{297} of *mim-1* protein is Ile.

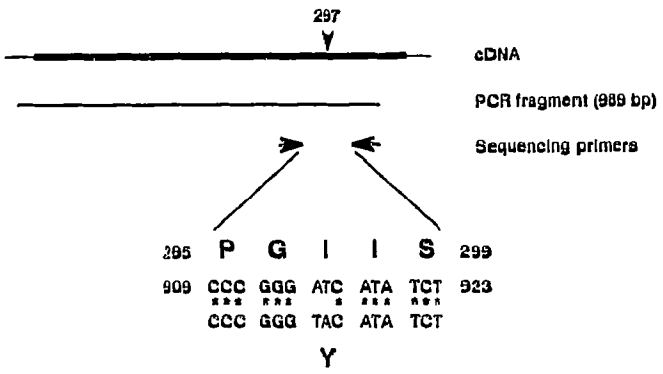

Fig. 3. Nucleotide sequence (909–923) of *mim-1* cDNA. Total RNA from chicken bone marrow cells was reverse-transcribed to cDNA with $(dT)_{16}$. The *mim-1* 989-bp fragment PCR-amplified from cDNA with *mim-1*-specific primers, 1 and 2, was isolated by agarose gel electrophoresis and purified as described in Materials and Methods. With the 989-bp fragment as a template, sequencing reactions were carried out using primers 2 or 3, as indicated by arrows. The arrowhead on the *mim-1* cDNA indicates the position of amino acid 297 in the coding region (bold line). Nucleotide sequences, corresponding to 909–923 of *mim-1* cDNA determined by the method described above (upper) and reported by Ness et al. [6] (lower), were compared. Deduced amino acids are also shown as a single letter code. Identical nucleotides are indicated by asterisks.

shown). This means that the heterophils have no detectable amounts of the *mim-1* transcript, and the notion that the *mim-1* protein level decreases during terminal differentiation into matured granulocytes [6] is given support. The nucleotide sequence encoding the 297th amino acid in the 989-bp amplified fragment was ATC (Ile), consistent with the determined amino acid of p33, while the reported sequence was TAC (Tyr) (Fig. 3). This discrepancy may reflect the differences in chickens used.

Using an antibody against bacterially expressed *mim-1* protein, Ness et al. detected *mim-1* protein in matured leukocyte granules in lesser amounts than that found in promyelocyte granules [6]. We have observed p33 in chicken liver [8]. Immunohistochemical staining of the liver section with a monoclonal antibody against p33 showed that the antibody reacted strongly only with heterophil granules in interlobular connective tissue of the liver, but not with hepatocytes [9]. We also confirmed the granular localization of p33 in subcellular fractionation experiments with Percoll density gradient centrifugation of the peripheral heterophils [4].

These data, taken together, strongly suggest that p33 is the product of the *mim-1* gene. Our findings contribute to the resolution of the physiological role of ADP-ribosylation in animal cells and also to the function of the nuclear oncogene, *myb*, in myeloid cells.

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