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DNA-REGULATED ARGININE-SPECIFIC MONO(ADP-RIBOSYL)ATION AND DE-ADP-RIBOSYLATION OF ENDOGENOUS ACCEPTOR PROTEINS IN HUMAN NEUTROPHILS[1]

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SUMMARY Arginine-specific mono(ADP-ribosyl)ation and de-ADP-ribosylation reactions of endogenous acceptor proteins were examined using human neutrophils. The cells contained arginine-specific ADP-ribosyltransferase, acceptor proteins and hydrolase catalyzing the release of ADP-ribose from the ADP-ribose/acceptor conjugate. One major acceptor protein with an apparent molecular mass of 27 kDa was detected in the neutrophils. The ADP-ribosylation of this protein was greatly enhanced when double-stranded DNA was added. The release of ADP-ribose from the ADP-ribosyl core-histones was suppressed. These findings provide clues as to the physiological function of neutrophil ADP-ribosyltransferase.

Arginine-specific mono(ADP-ribosyl)transferase catalyzes transfer of the ADP-ribose moiety from NAD to acceptors such as arginine, other guanidino compound, and arginine residue(s) in specific acceptor proteins. In vertebrates, this enzyme activity is present in different tissues and cells including hen and rat livers (1,2), turkey and human erythrocytes (3) and rat skeletal and cardiac muscles (4). Most of these experiments were done using exogenous acceptors and much less is known of the endogenous acceptors for ADP-ribosylation.

Most recently, we observed a non-histone acceptor protein for ADP-ribosyltransferase in hen liver nuclei. We purified this protein, found that it had a molecular mass of 33 kDa and we called it p33 (5). During studies on ADP-ribosylation and de-ADP-ribosylation reactions, we observed that human neutrophils contained arginine-specific ADP-ribosyltransferase, acceptor proteins and hydrolase catalyzing release of ADP-ribose from the ADP-ribose/acceptor conjugate.

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Abbreviation: SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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We report here evidence for the DNA regulated arginine-specific ADP-ribosylation and de-ADP-ribosylation reactions of endogenous acceptors in human neutrophils.

MATERIALS AND METHODS

[adenylate-^{32}P]NAD (29.6 TBq/mmol) was obtained from New England Nuclear, poly(L-arginine) (Mr 24,000) was purchased from Sigma and NAD was a product from Boeringer Mannheim. All other reagents were purchased from Miyata Chemical Co. Ltd, Shimane.

Preparation of neutrophils --- Leucocyte-enriched populations were obtained from 1 L of heparinized peripheral blood of normal adult volunteers, by dextran sedimentation. After the contaminating erythrocytes were punctured by hypotonic lysis, the cell pellets were collected by centrifugation at 150 x g, and the neutrophils separated by Ficoll-Metrizoate sedimentation. Neutrophils were washed twice with Hanks' balanced salt solution before use.

Fractionation of neutrophils --- Neutrophils suspended in Hanks' balanced salt solution were disrupted by sonication on ice for 2 min with a Branson sonifier at 60-W output, followed by centrifugation at 35,000 x g. The pellets were collected, suspended with 0.35 M NaCl, the preparation stirred at 4°C for 30 min, followed by centrifugation at 40,000 x g for 20 min and the supernatant obtained. Four M NaCl was added to each of the 35,000 x g and 40,000 x g supernatants at a concentration of 1.0 M, and the preparations were applied to a phenyl-Sepharose column (3 x 2 cm) previously equilibrated with 200 ml of 20 mM potassium phosphate buffer (pH 6.8) containing 1 M NaCl and 1 mM 2-mercaptoethanol, respectively, followed by washing with 100 ml of pre-equilibrating buffer containing 2mM 2-mercaptoethanol and 35% propylene glycol. The ADP-ribosyltransferase was eluted with 100 ml of the same solution, except that 65% glycerol was present. The phenyl-Sepharose fractions of 35,000 x g supernatant and salt extract from the precipitate were termed PS1 and PS2, respectively. The eluate of 35,000 x g supernatant from phenyl-Sepharose with buffer containing 35% propylene glycol contained ADP-ribose-acceptor hydolase activity. This fraction was termed PS3. In some experiments, 0.35 M NaCl extracts (50 mg protein) of human neutrophils were applied to phenyl-Sepharose and the ADP-ribosyltransferase was eluted as described above. The fraction (5 mg protein) containing ADP-ribosyltransferase was then applied to a Cm-cellulose column (2 x 3 cm) pre-equilibrated with 50 mM potassium phosphate buffer (pH 6.8) containing 2 mM 2-mercaptoehanol and 20% propylene glycol. After washing the column with 100 ml of same equilibrating buffer, the preparation was eluted with a linear gradient of NaCl from 0 to 0.5 M, containing the same medium in a total volume of 100 ml, at a flow rate of 50 ml/h. The enzyme was eluted at approximately between 0.15 and 0.25 mM NaCl, as a single peak (Fig. 2).

Preparation of labeled ADP-ribosyl core-histones --- Core histones were prepared from hen liver nuclei, as described (6). The mixture containing 3.5 mg of core histones, 0.1 μg of ADP-ribosyltransferase purified from hen liver nuclei and 0.1 mM [^{32}P]NAD (185 kBq) was incubated at 25°C for 60 min and the acid-insoluble fraction washed with 10% trichloroacetic acid was used for the hydrolase substrate.

Assay of ADP-ribosyltransferase --- ADP-ribosyltransferase activity was measured by the incorporation of radioactivity from [^{32}P]NAD to the poly(L-arginine) in the following standard incubation system : 50 mM Tris-HCl, pH 8.5, 5 mM dithiothreitol, 0.1 mM [^{32}P] NAD (18.5 kBq), 40 μg poly(L-arginine) and appropriate amounts of the enzyme preparation in a total volume of 0.2 ml at 25°C for 60 min. The radioactivity of the acid-insoluble material was counted using a filter assay with a liquid scintillation spectrometer.

ADP-ribosylation of endogenous acceptor proteins --- Appropriate amounts of the ADP-ribosyltransferase preparation from phenyl-Sepharose or Cm-cellulose fraction were incubated for 60 min at 25°C with 50 mM Tris-HCl buffer (pH 8.5), 10 μM [^{32}P] NAD (185 kBq), 5 mM dithiothreitol and indicated amounts of DNA or potassium phosphate in a total volume of 0.3 ml. The acid-insoluble material was subjected to 17.5% SDS-PAGE. After electrophoresis, the gel was stained, dried and autoradiographed.

Assay of the hydrolase --- The hydrolase activity was measured by the radioactive ADP-ribose released from [adenylate-^{32}P]ADP-ribosyl core-histone conjugates in the following standard incubation system : [^{32}P]ADP-ribosyl core-histones (100 μg, 100,000 cpm), 10 mM $MgCl_2$, 10 mM dithiothreitol, 50 mM Tris-HCl buffer (pH 7.5), 12 μg of PS3 and various amounts of DNA in a total volume of 0.2 ml at 37°C for 60 min. The reaction was terminated by adding 0.1 ml of 30% trichloroacetic acid and the radioactivity of the acid-soluble fraction was counted with a liquid scintillation spectrometer.

RESULTS AND DISCUSSION

ADP-ribosylation of poly(L-arginine) by PS1 and PS2 --- Approximately equal ADP-ribosyltransferase activity was detected in the phenyl-Sepharose fraction (PS1) of human neutrophil 35,000 x g supernatant and in that fraction (PS2) of 0.35 M NaCl extract of the 35,000 x g precipitate, when poly(L-arginine) was the exogenous acceptor. The specific activities of PS1 and PS2 were calculated to be 1.2 x 10^{-5} μmol/min/mg and 7.4 x 10^{-5} μmol/min/mg, respectively.

The acid-insoluble fractions of PS1 and PS2 incubated with the ADP-ribosylation system containing [^{32}P]NAD and poly(L-arginine) were treated with 50 mM glycine-NaOH buffer, pH 10.0 at 37°C for 16 hours and the supernatants were analyzed by reverse-phase HPLC, respectively, as described (7). In both cases, the major radioactive peak appeared at the time when the authentic ADP-ribose was retained (data not shown).

ADP-ribosylation of endogenous acceptor proteins --- To detect endogenous acceptor proteins for the ADP-ribosyltransferase and to determine whether nucleic acids enhance this ADP-ribosylation, as was seen with hen liver p33 (5), PS1 and PS2 were incubated with [^{32}P]NAD in the presence of different concentrations of double-stranded DNA and acid-insoluble fractions were subjected to SDS-PAGE, respectively, followed by staining and autoradiography (Fig. 1). A autoradiogram of PS2 showed that the major labeled band corresponded to protein of 27 kDa and other labeled bands to proteins of 76 and 30 kDa. The labeling of these proteins was greatly enhanced with increases in DNA (lanes a'-e'). In case of PS1, the major labeled band corresponded to protein of 27 kDa. Other minor labeled bands were evident, but the labeling of these proteins did not vary with the DNA (lanes f'-j'). Further experiments were carried out with another enzyme preparation from a salt extract of neutrophils by subsequent chromatography, successively on phenyl-Sepharose and Cm-cellulose as described under "Methods". The enzyme elution profile from the

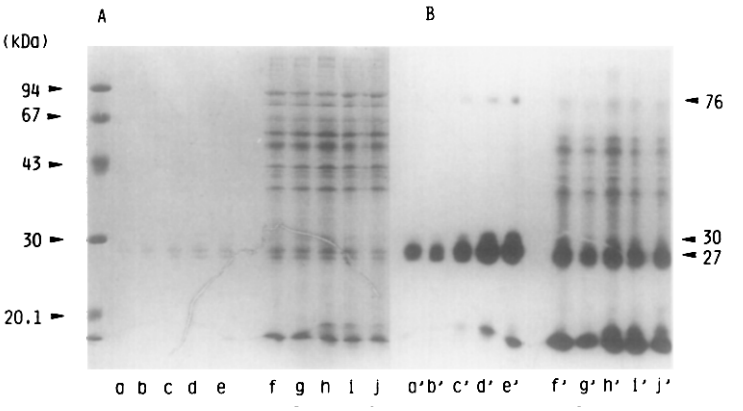
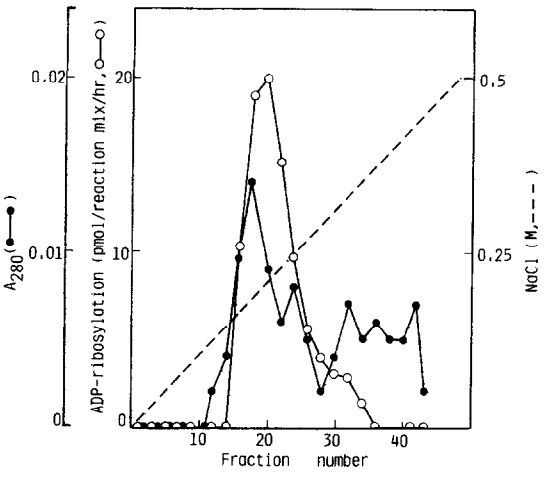


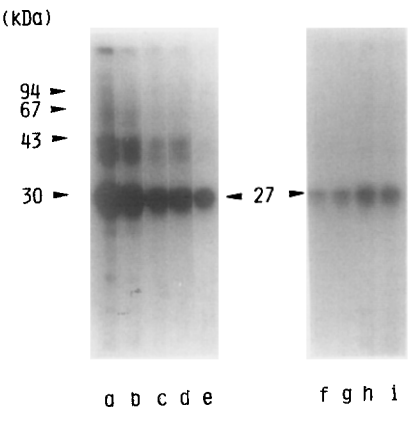
Fig. 1. Autoradiograms of the [^{32}P]NAD-treated phenyl-Sepharose fraction with or without DNA. 30 μg PS2 (lanes a-e and a'-e') and 180 μg PS1 (lanes f-j and f'-j') were incubated at 25°C for 60 min with 10 μM [^{32}P]NAD (185 kBq) and 0, 2, 5, 10 or 20 μg DNA in a total volume of 0.3 ml. Radio labeling of the acid-insoluble fraction from each sample was analyzed by SDS-PAGE, as described in "Methods". The Coomassie brilliant blue-staining pattern (A; lanes a-j) and autoradiograms of the sample gel (B; lanes a'-j') are shown. Molecular weight markers: phosphorylase b (94 k), bovine serum albumin (67 k), ovalbumin (43 k), carbonic anhydrase (30 k) and soybean trypsin inhibitor (20.1 k).

Cm-cellulose is shown in Fig. 2. The enzyme eluted in the range 0.15 - 0.25 M NaCl. Samples of the enzyme fraction were incubated with labeled NAD in the presence of different concentrations of DNA and the labeled proteins were analyzed by SDS-PAGE, followed by autoradiography. The results showed the major labeled band corresponding to proteins of 27 kDa and labeling of these proteins increased with increasing concentrations of DNA (Fig. 3, lanes a-e). Vedia et al. reported the phosphate-enhanced ADP-ribosylation of endogenous proteins in human platelets (8). We tested this point with the Cm-cellulose fraction and observed rather suppression of the modification (Fig. 3, lanes f-i).

Hydrolysis of ADP-ribosyl core-histones ---- To determine whether or not human neutrophils contain the ADP-ribose-acceptor hydrolase, [^{32}P]ADP-ribosyl core-histones were prepared as described under "Methods" and incubated with PS1, PS2 and PS3, respectively. Among the three preparations, PS3 contained predominant ADP-ribosylhistone hydrolase activity. Thus, soluble fraction of the human neutrophils contains the hydrolase activity, as was seen with turkey erythrocytes (9) and rabbit skeletal muscle (10). In contrast to the DNA-enhanced ADP-ribosylation, nucleic acid suppressed the hydrolysis of ADP-ribosyl core-histones (Fig. 4). We confirmed with reverse-phase HPLC that the labeled product formed from radioactive ADP-ribosyl core-histones by the hydrolase is ADP-ribose and that PS3 does not contain pyrophosphatase activity catalyzing 5'-AMP formation from the substrate (data not shown).



(2)



(3)

Fig. 2. Chromatography on a Cm-cellulose column. Phenyl-sepharose fraction (5 mg protein) of 0.35 M NaCl extract from human neutrophils were applied to a Cm-cellulose column. The elution conditions are described in "Methods". Enzyme activity (o—o), 280 nm absorbance (●—●) and NaCl concentration (— — —).

Fig. 3. Effect of DNA and potassium phosphate on ^{32}P incorporation into the Cm-cellulose fraction. The Cm-cellulose fraction (10 μg protein) were incubated with 10 μM [^{32}P]NAD (185 kBq) in the presence of 0, 5, 10, 20 or 40 μg DNA (lanes a-e) or 100, 50 , 10 or 0 mM potassium phosphate (pH 7.5) (lanes f-i). Radioactivity of the acid insoluble fraction from each sample was analyzed by SDS-PAGE, followed by autoradiography, as described in "Methods". Molecular weight markers are as shown in Fig. 1.

We obtained evidence that ADP-ribosyltransferase, endogenous acceptor proteins and hydrolase are present in human neutrophils and that double-stranded DNA regulates the ADP-ribosylation and de-ADP-ribosylation

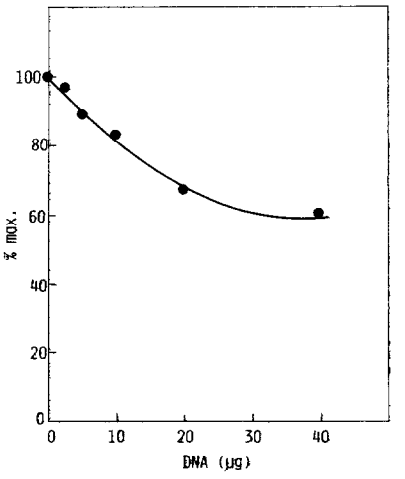


Fig. 4. Effect of DNA on the release of ADP-ribose from ADP-ribosyl core-histones. [^{32}P]ADP-ribosyl core-histones (100 μg, 100,000 cpm)) were incubated with 12 μg of PS3 in the presence of indicated amounts of DNA. Other conditions were as described under "Methods". The reaction was halted by adding trichloroacetic acid and the radioactivity in the acid-soluble fraction was determined. The radioactivity in the acid soluble fraction without DNA was 12,000 cpm. This value was set at 100%.

of the endogenous acceptor protein, in vitro. If it is true in vivo, the DNA-suppressed hydrolysis of the ADP-ribosyl acceptor conjugate will be amenable to the DNA-enhanced ADP-ribosylation. We propose that ADP-ribosylation and de-ADP-ribosylation are coordinated, within the cell.

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