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IDENTIFICATION OF ENZYMATIC ACTIVITIES WHICH PROCESS PROTEIN BOUND MONO(ADP-RIBOSE)

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Enzymatic activities have been identified in extracts of cultured mouse cells which catalyze the removal of intact mono(ADP-ribosyl) residues linked to proteins at arginine. Activities that sequentially remove AMP and ribose 5-phosphate have also been identified. These results suggest that mono(ADP-ribosylation) of proteins is a reversible post translational modification.

The presence of mono(ADP-ribosyl)transferase activities has been demonstrated in a number of different animal cell types (1-8). Although the physiological acceptor proteins for these enzymes have not yet been identified, a number of proteins have been found to have altered activities after ADP-ribosylation *in vitro* (7,9-12). To date, the cellular mono(ADP-ribosyl)-transferases that have been well characterized catalyze the transfer of ADP-ribose to arginine residues in acceptor proteins (13). In order for mono(ADP-ribosylation) to have a functional role as a regulatory post-translational protein modification, it must be reversible (14). The only enzyme known at this time that catalyzes the removal of mono(ADP-ribose) from protein is an ADP-ribosyl protein lyase which catalyzes the cleavage of ADP-ribose from glutamic acid residues (15,16). The (ADP-ribose)-glutamate linkage in protein most probably results from the combined action of poly-(ADP-ribose) polymerase and poly(ADP-ribose) glycohydrolase (17) rather than from cellular mono(ADP-ribosyl)transferases. In this paper, we describe

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enzymatic activities in extracts of cultured mouse cells which process ADP-ribose attached to proteins via an arginine linkage.

MATERIALS AND METHODS

Preparation of radiolabelled NAD^+. NAD^+ radiolabelled with [^{14}C] in the nicotinamide proximal ribose was prepared in two steps from [^{14}C] 5-phosphoribosyl-1-pyrophosphate (PRPP) which was provided by Amersham. Step 1 was catalyzed by nicotinamide phosphoribosyltransferase which was partially purified from rat liver after the method of Dietrich (18). The reaction mixture (1 ml) contained 50 mM Tris-HCl, pH 7.4, 65 µM [^{14}C]PRPP (246 Ci/mole), 5 mM nicotinamide, 5 mM $MgCl_2$, 10 mM ATP and 100 µl of partially purified enzyme. Incubation was for 1 hour at 37°C. Step 2 involved the addition of 0.4 mg (1.2 units) of NAD pyrophosphorylase (Boehringer-Mannheim) and an additional 20 µmoles of ATP (final volume 1.4 ml). Incubation was for 1 hour at 37°C. The [^{14}C]NAD formed was purified using a dihydroxyboryl-BioRex 70 affinity resin essentially as described previously (19). The incubation mixture was adjusted to a final volume of 10 ml and a final concentration of 0.25 M ammonium acetate. The pH was adjusted to 9.0 ± 0.2 and the sample was applied to a 0.5 ml boronate column. Following application, the column was washed with 10 ml of 0.25 M ammonium acetate pH 9.0 and eluted with 5 ml of H_2O. The eluate was lyophilized, resuspended in 0.5 ml of H_2O and stored at -15°C. NAD^+ radiolabelled with [^{32}P] in the adenosine proximal phosphate was synthesized from α-[^{32}P]ATP (New England Nuclear). The incubation (50 µl) mixture contained 83 mM Tris-Cl, pH 7.5, 6 µM [^{32}P]ATP (~ 3000 Ci/mmol), 20 mM NMN, 50 mM phosphoenolpyruvate, 1 mM $MgCl_2$, 13 units of pyruvate kinase (Sigma, type II) and 5 µg of NAD pyrophosphorylase. Incubation was for 1 hour at 37°C. The incubation mixture was then subjected to chromatography as described above. The [^{32}P]NAD was lyophilized and redissolved in 2 ml of 10 mM sodium phosphate (pH 6.0) and stored at -15°C.

Preparation of ADP-ribosylated histones. Radiolabelled ADP-ribosyl histones were produced using turkey erythrocyte mono(ADP-ribosyl)transferase (20). The incubations contained 50 mM sodium phosphate buffer, pH 7.0, 0.4 ml/ml of a mixture of histones (Histone preparation II-A, Sigma), 0.68 µg/ml enzyme and either 0.25 µM [^{14}C]NAD or 100 µM [^{32}P]NAD. Incubation was for 1 hour at 30°C. Trichloroacetic acid (TCA) was added to a final concentration of 20%, the precipitate was collected by centrifugation, washed twice with ethyl ether and resuspended in 0.45 ml of 1 mM HCl. Next, 0.05 ml of 1 M sodium phosphate buffer, pH 6.5, was added and the solution was stored at -15°C. Polyacrylamide gel electrophoresis showed co-migration of radioactivity with histone H1 and each of the core histones as determined by autoradiography and Coomassie blue staining.

Preparation of [^{14}C] 5-Phosphoribosyl Histones. This substrate was prepared by incubation of [^{14}C]ADP-ribosylated histones (65 µM in ADP-ribose) with 0.37 units snake venom phosphodiesterase (Sigma) in 50 mM sodium phosphate buffer, pH 8.0, 10 mM $MgCl_2$, in a total volume of 200 µl. Incubation was for 90 min at 37°C. The reaction was monitored by assaying for release of TCA soluble counts in a parallel incubation with [^{32}P]ADP-ribosylated histone. Protein was then precipitated by addition of TCA to 20%. The pellet was washed with ether and resuspended in 1 mM HCl and and stored in phosphate buffer as described above.

Preparation of SVT2 Cell Extracts. Simian virus 40-transformed mouse fibroblasts (SVT2) were cultured as described previously (21). Frozen cell pellets, (approximately 1.5 x 10^7 cells) were lysed by treatment on ice for 2 min with 0.5 ml of 100 mM sodium phosphate buffer, pH 6.5, in an Eppendorf microcentrifuge tube. The lysate was then subjected to centrifugation in a Brinkmann Eppendorf Microcentrifuge for 2 min. The supernatant was removed and used in the assay for ADP-ribosyl-protein processing activity. Protein concentration was measured by the Bradford method (22).

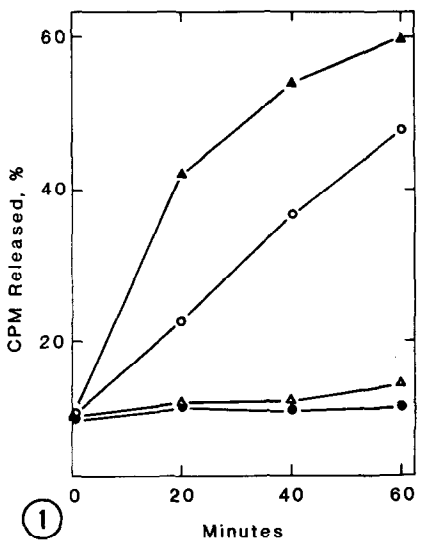
Assay Conditions. Processing activity was assayed by incubating cell extracts (1 mg/ml final protein concentration) with 25 μM (ADP-ribose concentration) [^{32}P or ^{14}C] ADP-ribosylated histone in a typical volume of 75 μl at 37°C in a standard assay. Aliquots of 15 μl were removed and an equal volume of 40% (w/v) TCA was added. Samples were then subjected to centrifugation and 20 μl of the supernatant was counted in 2 ml of Aquasol (NEN) in a liquid scintillation counter to determine total release, or spotted onto paper chromatograms and analyzed.

Chromatographic Analysis. Paper chromatography was performed using 0.12 M Na Citrate, 2.0 M NH_4Cl, pH 4.8, mixed with 95% ethanol (1:3) in a descending system (32). The chromatographic lanes were cut into 1 x 3 cm strips and counted with 2 ml of toluene based scintillation fluid in a liquid scintillation counter. Peaks of radioactivity were compared with unlabelled standards which were visualized under UV light (nucleotides) or by spraying with a solution of glacial acetic acid:aniline:H_2O (1:1:1) (sugars and sugar phosphates).

RESULTS

In order to search for enzymatic activities that act on arginine-(ADP-ribose) residues in protein, mixtures of histones modified with radiolabelled ADP-ribosyl moieties were produced. These preparations were labelled with ^{14}C in the protein proximal ribose or ^{32}P in the adenosine proximal phosphate. Figure 1 shows a typical result obtained when [^{14}C]-ADP-ribosyl-histones were incubated with an SVT2 cell extract and monitored for conversion of radiolabel into an acid-soluble form. A time dependent release of radiolabel was observed which was stimulated by the presence of 5 mM $MgCl_2$. The stimulation could be attributed to Mg^{+2} since the chloride salts of other cations such as Na+, K+ or Ca^{+2} had no appreciable effect on release. The rate of release was proportional to the protein concentration of the cell extract and boiling the extract prior to incubation eliminated release. The presence of 1 mM 5,5'-dithiobis(2-nitrobenzoic acid) inhibited release, but this inhibition was reversed by the subsequent addition of 2 mM dithiothreitol.

The nature of the material released was examined by paper chromatography. Figure 2 shows the results obtained with both [^{14}C] and [^{32}P] labelled substrates in the standard incubation and in the presence of added Mg^{+2}. In the standard incubation, both [^{14}C] and [^{32}P] label were present in two primary peaks, one of which co-migrated with ADP-ribose and a second which migrated more slowly than ADP-ribose. Since this unidentified material contained both radiolabels, it is likely that it is closely related to



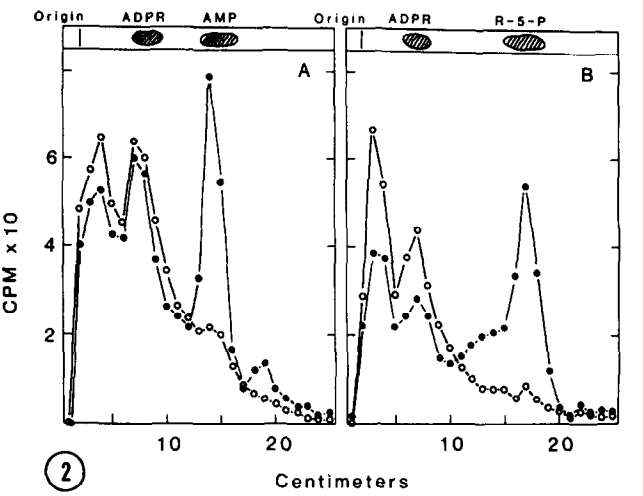


Figure 1. Release of radioactivity from [^{14}C]ADP-ribosylated histones. (○) Standard assay conditions (See Methods); (●) boiled control, extract treated at 100°C for 5 min prior to assay; (▲) standard assay conditions plus 5 mM $MgCl_2$; (△) boiled control plus 5 mM $MgCl_2$.

Figure 2. Paper chromatography of acid soluble products following incubation for 60 min with SVT2 cell lysates. (○) Stardard assay conditions; (●) standard assay conditions plus 5 mM $MgCl_2$. Panel A: [^{32}P]ADP-ribosylated histones; Panel B: [^{14}C]ADP-ribosylated histones. Only regions of chromatograms containing radioactivity are shown.

ADP-ribose and we have termed it ADPR'. In the presence of Mg^{+2}, there was a decrease in the amount of ADP-ribose and ADPR' and a large increase in a peak co-migrating with 5'-AMP for the [^{32}P] labelled substrate (Panel A). For the [^{14}C] labelled substrate (Panel B), a large increase in a peak co-migating with ribose 5-phosphate as well as a material that migrated between ADP-ribose and ribose 5-phosphates was observed.

These data provide evidence for an enzymatic activity that catalyzes the release of intact ADPR from protein and for Mg^{+2} stimulated pyrophosphatase activity that processes ADP-ribose. Since Mg^{+2} stimulated total release, it was likely that the pyrophosphatase activity acted upon both released and protein bound ADPR. Magnesium stimulation of ribose 5-phosphate release also suggested the presence of an activity that catalyzed the release of protein bound ribose 5-phosphate. Figure 3 shows an incubation of [^{14}C] 5-phospho-ribosyl histone under standard conditions and in the presence of added Mg^{+2}.

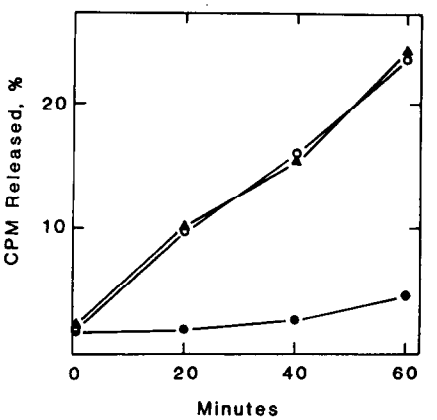


Figure 3. Release of label from [^{14}C] 5-phosphoribosyl histones. (○) Standard assay conditions; (●) boiled control; (▲) standard assay conditions plus 12 mM $MgCl_2$.

Release of radiolabel was shown to be catalyzed by the cell extracts but the presence of added Mg^{+2} had no stimulatory effect. Upon analysis by paper chromatography, the radiolabel co-migrated with ribose 5-phosphate.

DISCUSSION

Since the proteins modified by cellular mono(ADP-ribosyl)transferases in vivo are unknown, we have utilized a mixture of histone proteins modified with labelled ADP-ribose residues in order to increase the probability of detecting processing activity. To our knowledge, this represents the first report of an activity that catalyzes the release of an intact ADP-ribosyl residue bound to arginine residues. In addition to the appearance of ADP-ribose, we have also observed a second product that we have termed ADPR'. This product contains ADP-ribose with an additional moiety associated with the protein proximal ribose (unpublished observations). This product does not appear to result from serine protease activity since addition of 10 mM phenylmethylsulfonyl fluoride to the incubations had no effect on its formation. We are currently characterizing this material further.

This study also provides evidence for a multistep processing pathway for arginine-ADP-ribose. We have observed that the presence of Mg^{+2} stimulated pyrophosphatase activity which acts upon ADP-ribose. The effect of Mg^{+2} was not just on processing of released ADP-ribose and ADPR' since the total

amount of release was increased. Thus, either Mg^{+2} directly stimulated the release and subsequent processing of ADP-ribose and/or ADPR' or it stimulated a pyrophosphatase activity which acted on protein bound ADP-ribose. Our evidence favors the latter possibility. We have observed that the addition of 2 mM free ADP-ribose to the standard incubation did not affect the release of ADP-ribose or ADPR'. However, free ADP-ribose did abolish the effect of added Mg^{+2}. This argues against a direct stimulatory effect of Mg^{+2} on release of ADP-ribose or ADPR' and against an indirect stimulatory effect by release of product inhibition. DeWolf et al. (4) have previously observed the release of AMP from ADP-ribose bound to acid insoluble fractions of bovine thyroid membranes and Soman et al. (5) have reported a Mg^{+2} stimulated activity in rabbit skeletal muscle that acts on ADP-ribose linked to an arginine analog. The presence of activity that catalyzes the removal of ribose 5-phosphate from arginine residues (Fig. 3) provides additional evidence for a multistep processing pathway. We are not aware of any previous reports of an enzymatic activity that catalyzes the removal of ribose 5-phosphate from protein.

Although the physiological relevance of these activities remain to be established, their presence indicates that ADP-ribosylation is a reversible post-translational protein modification. However, in contrast to phosphorylation or acetylation, the possibility of removing the ADP-ribose intact or via a two step (or more) process adds interesting potential regulatory capabilities to mono(ADP-ribosylation).

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