Evidence of Endogenous Mono-ADP-Ribosylation of Cardiac Proteins *Via* Anti-ADP-Ribosylarginine Immunoreactivity (44506)

CHRISTOPHER J. SCHWAB,[2] MARTHA J. COLVILLE,[3] AARON T. FULLERTON,[4] AND KATHRYN K. MCMAHON[1]

Department of Pharmacology, Texas Tech University Health Sciences Center, Lubbock, Texas 79430

Abstract. **Arginine-specific mono-ADP-ribosylation of proteins and arginine-specific mono-ADP-ribosyltransferase occur in heart. We developed a polyclonal antiserum, R-28, against ADP-ribosylpolyarginine that recognized mono-ADP-ribosylated proteins and identified the major mono-ADP-ribosylation products of quail heart. Treatment of immobilon-bound ADP-ribosylated G_s protein with hydroxylamine under conditions that remove ADP-ribose from its arginines eliminated R-28 immunoreactivity to G_s. Also, R-28 immunoreactivity to quail heart proteins was removed by NaOH and phosphodiesterase I treatments. Similar treatment with mercuric chloride did not remove the immunoreactivity but did remove exogenously (*via in vitro* pertussis toxin treatment) added ADP-ribose from cysteine of cardiac G_i/G_o proteins. The antiserum did not appear to react with ADP-ribosylasparagine of Rho (formed by C_3 toxin), ADP-ribosyldiphthamide of elongation factor 2 (formed by diphtheria toxin) in quail heart preparations, or polyADP-ribosylated proteins of a neonate rat cardiac nuclear preparation. Thus, the R-28 antiserum appears to contain predominantly antibodies directed against ADP-ribosylarginine. To test the usefulness of R-28, immunoblotting of subcellular fractions of quail heart was performed. R-28 showed the greatest immunoreactivity in the sarcolemma with significant immunoreactivity in denser membrane fractions. The cytosol also contained an immunoreactive band distinct from those found in the membranes. Hydroxylamine treatment eliminated immunoreactivity in the sarcolemma and denser membrane fractions but not the cytosol, suggesting the membranous immunoreactive bands contain ADP-ribosylarginine. In conclusion, a polyclonal antiserum that recognizes ADP-ribosylarginine proteins has been raised. The usefulness of the antiserum is demonstrated by the characterization of endogenous arginine mono-ADP-ribosylation products in quail heart. The quail heart has several sarcolemmal and denser membrane fraction proteins that appear to be mono-ADP-ribosylated on arginines.** [P.S.E.B.M. 2000, Vol 223:389–396]

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[1]To whom requests for reprints should be addressed at Department of Pharmacology, Texas Tech University Health Sciences Center, Lubbock, TX 79430. E-mail: phrkkm@ttuhsc.edu

Current addresses: [2]Department of Microbiology and Immunology, Texas Tech University Health Sciences Center, Lubbock, TX 79430; [3]Family Medicine Clinic, Cox Medical Center, Springfield, MO 65802; and [4]Crime Laboratory, Texas Department of Public Safety, Lubbock, TX 79408.

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Protein mono-ADP-ribosylation uses NAD^+ as a substrate and covalently attaches ADP-ribose to particular amino acids of proteins. This reaction was recognized first to occur potentially endogenously in eukaryotes when Moss and Vaughan characterized a mono-ADP-ribosyltransferase from turkey red blood cells (1). Since then, significant work has uncovered several forms of this enzyme (2), but little is known about substrate proteins for this class of reactions. This laboratory has demonstrated endogenous mono-ADP-ribosylation in cardiac tissue including evidence that an arginine-specific mono-ADP-ribosyltransferase is present in the sarcolemma of cardiac tissue from several mammalian and an avian species (3, 4). The purpose of the present work is to produce an antiserum

that can be used to study proteins that contain arginines modified by mono-ADP-ribose.

There have been three major approaches used to detect proteins that act endogenously as substrates for mono-ADP-ribosylation. One approach has been to radiolabel the protein product by incubating cells or tissue with radiolabeled orthophosphate (5), NAD (6), or adenine (7). This approach seems to be most successful when using cultured cells rather than tissues or whole animals simply because of the specific activity of the NAD that can be achieved inside the cells. A second approach involves chemical release of the ADP-ribose followed by fluorometric detection of the released and later derivatized ADP-ribose (8). This method suffers from the need for large amounts of the protein source for detection. The third approach has been to detect mono-ADP-ribosylated proteins by immunoreactivity to antibodies specific to the ADP-ribose moiety. Two laboratories have previously produced such antibodies (9, 10). Both antibodies have had limited success in attempts to be used to identify proteins that act as substrates for this reaction. The structure of the antigens used for these antibodies may give a clue to the reason for the lack of usefulness of these antibodies. In both antigens, ADP-ribose is attached to a protein through bonds that do not resemble the native form of ADP-ribosylated proteins. That is, in naturally occuring ADP-ribosylated proteins, the ADP-ribose is attached to the particular amino acid residue via the C_1 of the terminal ribose in ADP-ribose. In the antigen used by Meyer and Hilz (9), the ADP-ribose moiety was attached to serum albumin *via* a (carboxyethyl)thiomethyl residue attached to the N^6 of the adenine ring. In the antigen produced by Eide *et al.* (10), the ADP-ribose was randomly conjugated to BSA *via* the hydroxyl groups of the ribose rings using periodate oxidation. In both cases, the ADP-ribose is in an unnatural steric configuration relative to the protein, and there is random attachment to the amino acids that act as the linkage sites. Both of these characteristics of the antigens predict low specificity of the resulting antibodies.

In this paper, we describe the production of an antiserum that shows specificity to proteins ADP-ribosylated on arginines. The antigen used to produce this antiserum was ADP-ribosylated polyarginine formed by the cardiac ADP-ribosyltransferase. The use of the ADP-ribosyltransferase ensured the formation of an ADP-ribosylarginine linkage that was identical to that found in the endogenous situation.

Materials and Methods

Experimental Animals. Japanese quail were obtained from an internal source. New Zealand white rabbits were purchased from Myrtle Rabbitry (Thompson Station, TN). Rat tissues were prepared previously (3). All animals were housed in the Texas Tech University Health Sciences Center Lab Animal Research Center and cared for as stated in the policies and regulations of the Institutional Animal Care and Use Committee.

Cardiac Subcellular Fractionation. Subcellular fractions were generated from rat and quail heart following the procedures of Hosey and Fields (11) as described in Piron and McMahon (3). In brief, homogenates were subfractionated by differential and sucrose gradient centrifugation steps. The fractions used in these experiments included the following: nuclear (pellet of a 10-min centrifugation at 600*g* of the homogenate), non-nuclear (supernatant of a 10-min centrifugation at 600g of the homogenate), cytosolic (supernatant of a 1-hr centrifugation of non-nuclear fraction at 100,000*g*), microsomal (pellet of a 1-hr centrifugation of non-nuclear fraction at 100,000*g*), $F_1 - F_7$ (protein in 8.5%, 15%, 28%, 32%, 36%, 40% or pellet of a sucrose discontinuous gradient following centrifugation of the microsomal fraction at 100,000*g* for 2 hr on a swinging bucket rotor, respectively). The F_2, F_3, and F_4 fractions were enriched with G proteins (3) and muscarinic receptors (11) and therefore were considered enriched in sarcolemmal membranes. The F_5 and F_6 fractions were those collected from the denser (36% and 40%) sucrose and had lower enrichment of plasma membrane proteins (3, 11). These fractions were considered enriched in sarcoplasmic reticulum membranes, and the F_7 fraction was considered enriched in mitochondria.

Antibody Formation. The antigen, ADP-ribosyl-polyarginine, was formed in 400 µl by incubating 0.25 m*M* polyarginine (5–15 kDa, estimated to be polypeptides of 29–86 arginine residues) in the presence of 5.5 µg quail heart membranes (F_2 or F_3), 10 m*M* NAD, and 20 m*M* Tris-HCl, pH 8.0 at 30°C for 1 hr. The incubation was terminated by the addition of 200 µl of SDS sample buffer (0.2 *M* Tris-HCl, pH 6.8, 6% SDS, 30% glycerol, 0.006% bromophenol blue, 10 m*M* EDTA, pH 7.4, and 10% β-mercaptoethanol). The mixture was applied to a discontinuous SDS-PAGE (3% stacking gel, 11.5% resolving gel) and electrophoresed until the bromophenol blue exited the resolving gel. The polyarginine (both ADP-ribosylated and unmodified) was retained in and tightly bound to the stacking gel whereas the proteins and free NAD migrated into the resolving gel. The stacking gel was fragmented (13) and injected into New Zealand white rabbits. Ten days after antigen injections, the rabbits were bled. Serum was collected from the rabbit blood (13) and stored at – 20°C, and this process was repeated every 6 weeks. Three rabbits were injected (R-27, R-28, and R-29), and all gave positive sera as defined below after the fourth injection.

Chemical- and Biochemical-Sensitivity Blot Assay. Quail and rat heart sarcolemma (plasma membrane) (F_4) fractions were ADP-ribosylated in the presence of [^{32}P]NAD as described in Piron and McMahon (3). Following the ADP-ribosylation reaction, the samples were separated by 8% or 10% SDS-PAGE electrophoresis and then transferred to Immobilon membranes. The Immobilon membranes were then subjected to chemical or biochemical sensitivity assays. To test for hydroxylamine sensitivity and therefore an ADP-ribosylarginine linkage (14), Immobilon membranes were incubated with either 1 *M* NH_2OH or

NaCl in 50 m*M* HEPES, pH 7.4 and 0.5% SDS (Buffer A) for 16–20 hr at 37°C. To test for mercuric chloride sensitivity and therefore ADP-ribosylcysteine linkages (14), Immobilon membranes were incubated with either 1 m*M* $HgCl_2$ or NaCl in Buffer A for 1 hr at 37°C. To test for phosphodiesterase I sensitivity and therefore to confirm the presence of a diphosphate bond in the immunoreactive bands, Immobilon membranes were incubated with 50 m*M* HEPES, pH 9.0, and 10 m*M* $MgCl_2$ in the presence or absence of 5 U/ml of phosphodiesterase I at 37°C for 4 hr. Following any one of the various chemical or biochemical sensitivity incubations, the Immobilon membrane was rinsed with water to terminate the reaction. To determine the efficiency of the biochemical or chemical sensitivity incubation, the control and treated Immobilon membranes were then simultaneously exposed to autoradiography film at −80°C.

Immunoblotting Procedure. Immobilon membranes were blocked with 3% milk (from dry milk) in 500 m*M* NaCl in 20 m*M* Tris, pH 7.5 (TBS) for 1 hr at room temperature. Following a 20-min wash with TBS, the membranes were incubated with primary antibody diluted 1:5000 in 1% milk overnight at room temperature. The membranes were again washed two times with TBS for at least 20 min and then exposed to secondary antibody (donkey anti-rabbit IgG conjugated to horseradish peroxidase, Amersham) diluted 1:5000 in 1% milk in TBS for 1 hr at room temperature. The immunoreactivity was detected by chemiluminescence (ECL, Amersham) onto autoradiography film.

Toxin Treatments. Cholera toxin treatment of cardiac microsomes was described previously (15). Pertussis toxin treatment of cardiac membrane preparations was also described (3). Diphtheria toxin treatment was done following the method of Carroll and Collier (16). C_3 toxin treatment was performed according to the method of Fritz and Aktories (17).

Materials. Cholera, pertussis, diphtheria, and clostridium C_3 toxins were from Sigma Chemical Co. (St. Louis, MO), List Biochemicals (Campbell, CA), Calbiochem Novabiochemicals (La Jolla, CA), and Upstate Biotechnology Incorp. (Waltham, MA), respectively. [^{32}P]NAD was from New England Nuclear (Boston, MA). Immobilon PVDF was purchased from Millipore (Marlborough, MA). Kaleidoscope prestained molecular weight markers were from Bio-Rad (Hercules, CA). Phosphodiesterase I from snake venom was purchased from Worthington Enzymes (Freehold, NJ; enzyme # 3.1.4.1). All other reagents were reagent grade.

Results

Antigen Formation. The proposed antigen for the production of ADP-ribosylarginine antibodies was ADP-ribosylpolyarginine. The antigenicity to the ADP-ribose region of the molecule was presumed to be dependent on the stoichiometry of ADP-ribose to polyarginine molecules. To estimate the stoichiometry of ADP-ribose incorporation into polyarginine, [^{32}P]NAD(1–3 μCi/reaction tube) was included in the antigen formation reaction (see Materials and Methods). Following the incubation and electrophoresis of the ADP-ribosylpolyarginine, the stacking gel, which contained the polyarginine, was collected and fragmented, and an aliquot was analyzed by scintillation counting for radioactivity. It was estimated that 1.9 ± 0.7 moles of ADP-ribose were incorporated per mole of the polyarginine molecule (n = 6). The commercial polyarginine used had a molecular size range of 5,000–15,000 Daltons. Assuming an average molecular size of 10,000 Daltons for the polyarginine and a molecular weight of 174 g/mole for arginine suggests 1 ADP-ribose for every 44 arginines. Extending the time of reaction from 1 to 3 hr or changing the pH of the reaction to 9.0 did not improve the incorporation of ADP-ribose. Using a 139,000-Dalton form of polyarginine (799 arginines), the incorporation of ADP-ribose was 3.1 mole/mole of polyarginine or 1 ADP-ribose for every 258 arginines. Because the stoichiometry of ADP-ribose to arginine moieties was greater with the smaller polyarginine, the 5,000–15,000-Dalton polyarginine was used for antigen production for all injections.

Screening for Antibody Production. The pre- and postimmune sera from the three rabbits (R-27, R-28, and R-29) were screened for antibodies by Western blotting to rat and quail cardiac F_4 sarcolemmal fractions (Fig. 1). The three preimmune sera did not show significant immunoreactivity to any quail membrane proteins. R-27 preimmune serum reacted slightly with a band of rat proteins with a molecular weight of ≈ 42 kDa. It was noted that all three preimmune sera (as well as postimmune sera) cross-reacted with the ≈ 42 kDa molecular-weight standard. There were immunoreactive bands to all three postimmune sera in both quail and rat cardiac membranes. In quail, an immunoreactive band common to all three sera was seen at 110 kDa. Serum R-28 showed another major band at 173 kDa and a minor band at 78 kDa. Serum R-29 showed the 78-kDa band as a major immunoreactant in the quail membranes. In rat cardiac membranes, three immunoreactive bands were detected. R-27 and R-29 sera showed a band at 207 kDa, and R-28 and R-29 showed a band at 165 kDa. The R-29 serum also showed a band at 78 kDa. The R-28 serum gave the most intense immunoreactivity in quail whereas R-29 gave the most intense immunoreactivity in rat. The R-28 antisera were used for further characterization.

Chemical and Biochemical Sensitivity-Blot Assays. To test if the R-28 antiserum was cross-reacting with ADP-ribosylated proteins, a series of chemical and biochemical sensitivity-blot tests were performed. The chemical sensitivity-blot tests were designed from known selective ADP-ribosylamino acid sensitivity (14). The biochemical sensitivity assay was designed from the established (14) removal of AMP from ADP-ribosylated proteins by phosphodiesterase I. Previously, these chemical and biochemical sensitivity assays for ADP-ribose/amino acid linkages were done in solution. Therefore, it was necessary first

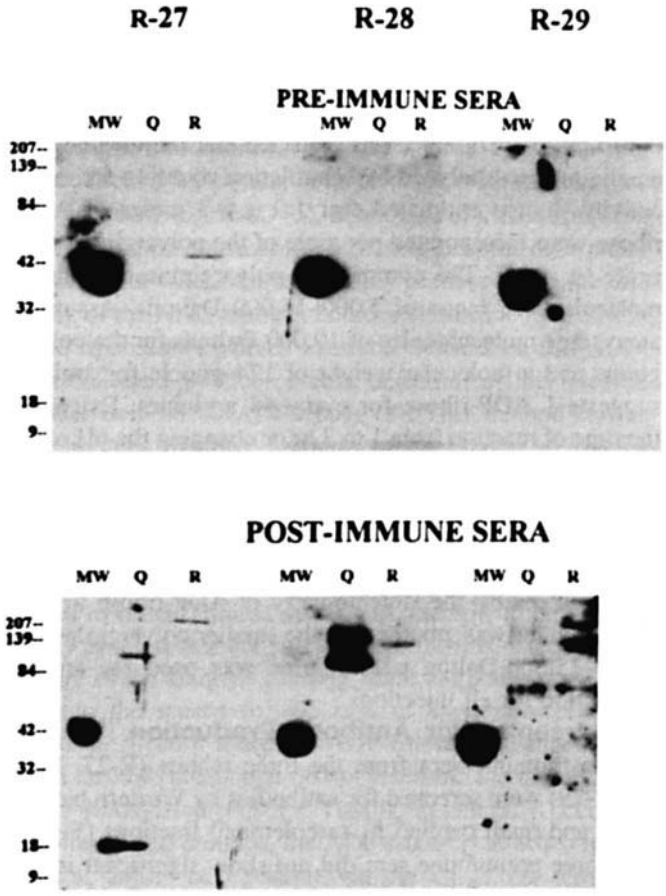

Figure 1. Immunoreactivity of (upper) preimmune and (lower) postimmune sera from rabbits R-27, R-28, R-29. Quail (Q), rat (R) cardiac sarcolemma fractions (F_4) and prestained molecular-weight marker proteins (MW) were separated on 11.5% SDS-PAGE, transferred to Immobilon, and immunoblotted with sera (diluted 1:5000). The quail and rat samples each contained 25 μg of protein.

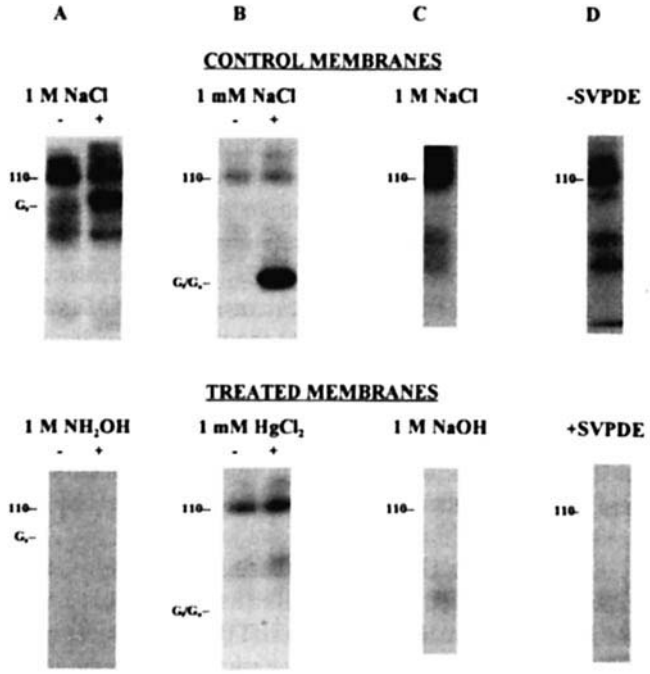

Figure 2. Chemical and biochemical sensitivity-blot assay. Duplicate samples of (A) quail microsomes or (B–D) sarcolemma (25 μg of protein) were incubated with [^{32}P]NAD to ADP-ribosylate proteins, separated on 10% SDS-PAGE, and transferred to Immobilon. One of the duplicates was then treated with NaCl at the concentrations shown (Control Membranes), and one was treated with NH_2OH, $HgCl_2$, NaOH, or snake venom phosphodiesterase (SVPDE) as described in Experimental Procedures. In (A) and (B), the quail membranes were preincubated without (–) or with (+) either cholera toxin or pertussis toxin, respectively, under conditions that allow ADP-ribosylation of the arginine of G_s (12) or the cysteines of the G_i/G_o (3) proteins. The Immobilon strips were then exposed to autoradiography film.

to establish that these same chemical and biochemical sensitivity assays could be done as sensitivity-blot assays, that is, after the ADP-ribosylated proteins had been electrophoresed and transferred onto Immobilon membranes. Figure 2A shows the autoradiogram of control and hydroxylamine-treated Immobilon membranes that contained quail heart microsome proteins that had been treated with [^{32}P]NAD in the presence or absence of cholera toxin. The control treatment (1 *M* NaCl) shows that ^{32}P-labelled proteins can be detected in the absence (–) and presence (+) of cholera toxin in the incubation. The predominant protein labeled in the absence of cholera toxin was the 110-kDa protein previously identified to be enriched in the sarcolemma (4, 12). In the presence of cholera toxin, the specific labeling of a 52-kDa band (presumed to be $G_{s\alpha}$) was seen as expected. In the sensitivity-blot assay, neutral hydroxylamine removed [^{32}P]ADP-ribose from arginine linkages of the cholera toxin ADP-ribosylated cardiac $G_{s\alpha}$ as well as [^{32}P]-labeled proteins from endogenous reactions (i.e., present in the absence of cholera toxin) (Fig. 2A) as it has been shown to do in solution in rat heart preparations (3). Mercuric chloride removed the [^{32}P]ADP-ribose from the cysteine linkage of the $G\alpha_{i/o}$ of pertussis toxin-pretreated quail cardiac sarcolemma (Fig. 2B) but did not remove the [^{32}P]-labeling from the 110-kDa band (or other proteins) formed by the endogenous arginine-specific mono-ADP-ribosyltransferase. Similar to neutral hydroxylamine, sodium hydroxide reduced the amount of [^{32}P]ADP-ribose from the [^{32}P]ADP-ribosylated proteins of quail heart sarcolemma (Fig. 2C) in the sensitivity-blot assay. It should be noted that sodium hydroxide treatment of the Immobilon potentially could remove protein from the Immobilon and thus give a false or misleading result. This was tested by monitoring the loss of color of the prestained protein molecular weight markers used in the experiment. Because of little loss of color from those protein markers by the treatment with sodium hydroxide, it is believed that protein loss was minimal. Phosphodiesterase I removes AMP from the ADP-ribose from all known amino acid linkages (14) when done in solution. In the sensitivity-blot assay, the phosphodiesterase I significantly removed the radioactivity in ADP-ribosylated quail cardiac proteins (Fig. 2D). Thus, the sensitivity-blot assay appeared to be usable for the screening of immunoreactive bands for ADP-ribosyl/amino acid linkages.

Screening of Antiserum for Amino Acid Selectivity of the Immunoreactivity. Using quail heart preparations, the presence of immunoreactivity following

the various chemical and biochemical sensitivity blot assays was determined. The hydroxylamine sensitivity-blot assay was used for initial screening of the R-28 antiserum for antibodies that recognize the ADP-ribosylarginine moiety in proteins from quail cardiac membranes pretreated with cholera toxin. Figure 3A shows that R-28 antiserum reacted with several bands both in the presence and absence of cholera toxin. In the absence of the toxin, the major immunoreactivity was with a protein of 110 kDa. Note that this major immunoreactivity in the quail membranes with the antiserum appears to be the major radio-labeled protein also (Fig. 2A). In the presence of cholera toxin, the immunoreactivity included a band at the exact mobility as the toxin-specific [^{32}P]-incorporation (Fig. 2A). Hydroxylamine treatment greatly diminished detectable immunoreactivity, including the cholera toxin–specific response, from the quail heart membranes. A time course of the hydroxylamine sensitivity of immunoreactivity was also done (unpublished data). Quail heart sarcolemmal preparations demonstrated immunoreactivity similar to that seen in Figure 1 (R-28 postserum blot). At 9 hr of hydroxylamine treatment, about 50% of the 110-kDa immunoreactivity remained. At 18 hr, less than 10% of the 110-kDa immunoreactivity remained, and at 24 hr all of that activity was eliminated. Quail cardiac membranes treated with pertussis toxin and [^{32}P]NAD were used to form ADP-ribosylcysteine on the $G\alpha_{i/o}$ pool of the heart. After this pretreatment of the cardiac samples, the incorporation of [^{32}P]-ADP-ribose into $G\alpha_{i/o}$(39–41 kDa) was monitored by autoradiography of the electrophoresed and transferred proteins (Fig. 2B). A radioactive band at 40 kDa was present that was dependent on the presence of pertussis toxin. The mercuric chloride sensitivity blot assay was then used to determine if this ADP-ribosylcysteine band cross-reacted with the R-28 antiserum. As shown in Fig. 3B, there was a lack of immunoreactivity with the antiserum at the size of ≈ 40 kDa. This suggested that the R-28 antiserum did not recognize or cross-react with ADP-ribosylcysteine. It was also noted that the major immunoreactive bands (110 kDa and other larger bands) were not removed by mercuric chloride treatment suggesting that those bands are not due to ADP-ribosylcysteine bonds in proteins.

Sodium hydroxide would be expected to remove ADP-ribose from arginines, cysteines, and lysines but not diphthamide (a histidine derivative), asparagine, serine, or threonine (14). As seen in Figure 3C, treatment with NaOH removed the immunoreactivity of all major bands. This confirmed the NH_2OH sensitivity-blot assay results and suggested that the antiserum recognized ADP-ribosylarginines. Because all immunoreactivity was removed by treatment with NaOH, this also suggested that none of the immunoreactive bands were ADP-ribosyl-diphthamides, -asparagines, -serines or -threonines.

Since phosphodiesterase I removes AMP from all known ADP-ribosylamino acid linkages (14), it would be expected to eliminate immunoreactivity if an antibody had in its epitope the AMP region of the ADP-ribose moiety. As seen in Figure 3D, treatment with phosphodiesterase I under conditions that completely removed [^{32}P]AMP from ADP-ribosylated proteins (Fig. 2D) decreased R-28 immunoreactivity but did not eliminate it. An interpretation is that this polyclonal antiserum has a high proportion of antibodies that react with the AMP moiety and a small proportion of antibodies that do not react with that portion of the ADP-ribosylarginine moiety.

Diphtheria toxin and clostridium C_3 toxin were used to ADP-ribosylate the diphthamide of elongation factor-2 (EF-2) and the asparagine of the Rho protein in quail heart microsomes. Figure 4 shows the [^{32}P]ADP-ribosylation pattern of quail cardiac microsomes in the presence or absence of diphtheria toxin. A toxin-specific ^{32}P band was identified at 120 kDa. As also seen in Figure 4, this ^{32}P-labeling was close to but not identical to immunoreactivity at 110 kDa. Also it is noted that the immunoreactivity was not changed by the diphtheria toxin treatment. This suggests that while the diphtheria toxin substrate/product has similar but not identical mobility, it does not appear to be the same protein

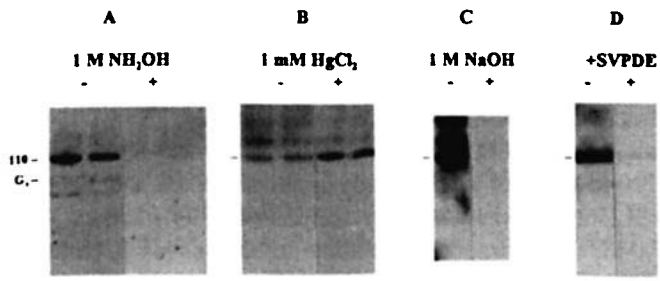

Figure 3. Chemical and biochemical sensitivity of quail sarcolemma immunoreactivity to R-28. Quail microsomes or sarcolemma (25 µg of protein) were electrophoresed and transferred to Immobilon. The Immobilon strips were then exposed to (A) NH_2OH, (B) $HgCl_2$, (C) NaOH, and (D) snake venom phosphodiesterase (SVPDE), as described in Experimental Procedures and then immunoblotted with R-28. In (A), the quail microsomes were preincubated without (–) or with (+) cholera toxin under conditions that allow ADP-ribosylation of G_s (12). In (B), the quail membranes were preincubated without (–) or with (+) pertussis toxin under conditions that allow ADP-ribosylation of cysteines of the G_i/G_o proteins (3).

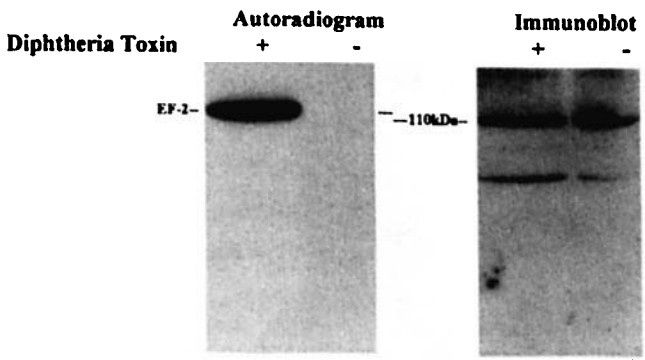

Figure 4. (Left) Diphtheria toxin–dependent [^{32}P]ADP-ribosylation and (right) R-28 immunoblot of quail cardiac microsomal EF-2. 25 ng of quail heart microsomes were incubated in the absence (–) or presence (+) of 1 µg of activated diphtheria toxin as described elsewhere (16). The samples were then electrophoresed, transferred, immunoblotted with R-28, and then exposed to autoradiography film.

as the immunoreactive protein. It should be noted that there is a possibility that the stoichiometry of ADP-ribosylation by the diphtheria toxin might be small. Potentially immunoreactivity of the toxin-labeled EF-2 might be present but not detectable above the background immunoreactivity at that molecular-weight range. Figure 5 shows autoradiography and immunoreactivity data for C_3 toxin ADP-ribosylated Rho protein in quail heart microsomes. [^{32}P]-ADP-ribose was incorporated in a C_3 toxin–specific manner into a protein at the expected size (17) of ≈ 29 kDa (Fig. 5, left). Immunoreactivity was not detected at this size irrespective of the toxin (Fig. 5, right). Three notable immunoreactive bands (> 110, 110, and 78 kDa) were detected with or without C_3 toxin treatment in the microsomal pellet of the heart as previously shown in sarcolemma (Fig. 1). As with the diptheria toxin labeling studies, the stoichiometry of the C_3 ADP-ribosylation reaction was not determined. It is possible that the antibody recognized the C_3 ADP-ribosylated Rho protein but that immunoreactivity was undetectable on the blot due to low stoichiometry of the reaction. These results with diphtheria and C_3 toxins suggest that the R-28 antiserum does not appear to contain antibodies that recognize either ADP-ribosyldiphthamide or ADP-ribosylasparagine. These results are consistent with the results of the immunoreactivity following the NaOH sensitivity-blot assay.

The nuclear fraction (P600) of neonate rat heart has been shown by this laboratory to contain polyADP-ribosylated proteins (4). This subcellular fraction of rat neonate heart was used to test R-28 antiserum for cross-reactivity to polyADP-ribose where the amino acid linkage is presumed to be through the C-terminal glutamate. In the nuclear fraction, the major [^{32}P]ADP-ribose was incorporated into proteins of 116 kDa and higher (Fig. 6) as seen previously (4). Presumably, the 116 kDa protein is the poly-

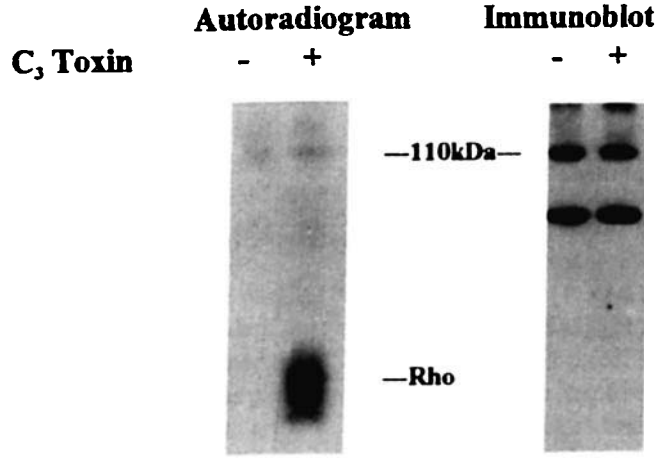

Figure 5. C_3 toxin–dependent [^{32}P]ADP-ribosylation (left) and R-28 immunoblot (right) of quail heart microsomes containing Rho protein. Quail cardiac postnuclear pellets (20 µg of protein) were incubated in the absence (–) or presence (+) of 0.1 µg C_3 toxin as described elsewhere (17). The samples were then electrophoresed, transferred, and immunoblotted with R-28 antiserum, and then exposed to autoradiography film.

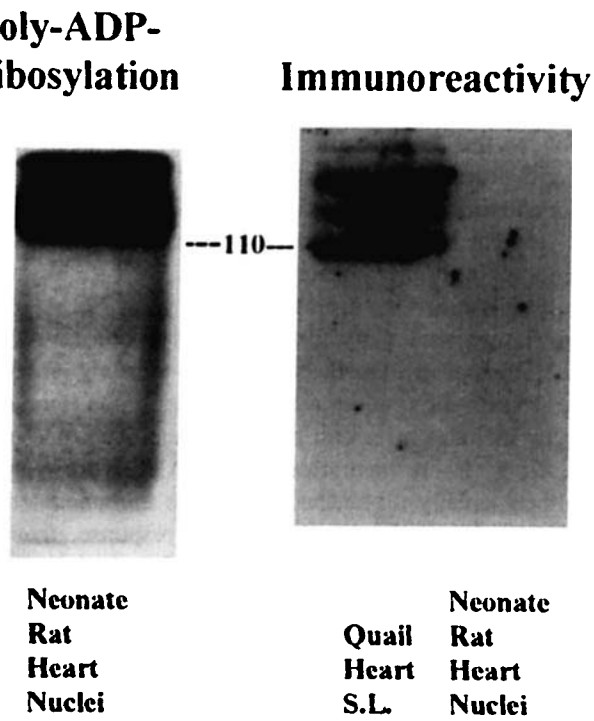

Figure 6. Auto-polyADP-ribosylation (left) and immunoblot (right) of neonate rat cardiac nuclei. Neonate (1-day-old) rat heart nuclei (50 µg) were incubated with [^{32}P]NAD under conditions that allow auto-polyADP-ribosylation of the polyADP-ribose polymerase as described elsewhere (3). The sample was then electrophoresed on 10% SDS-PAGE with an accompanying lane containing 25 µg of quail cardiac sarcolemma. Following electrophoresis, the proteins were transferred, immunoblotted with R-28, and exposed to autoradiography film.

ADP-ribose polymerase (18). There was no R-28 antiserum immunoreactivity with the nuclear proteins, suggesting that the antibodies do not recognize the ADP-ribosyl-ADP-ribose of the ADP-ribose polymer or the ADP-ribosyl-glutamate. As there was no immunoreactivity in the neonate rat cardiac nuclear fraction, these results also suggest that the neonate rat cardiac nuclear fraction does not contain any ADP-ribosylarginine–containing proteins.

Immunoreactivity in Cardiac Subcellular Fractions. Subcellular fractions of quail heart were tested for immunoreactivity to the R-28 antiserum. As seen in Figure 7 (left) all fractions except the nuclear and mitochondrial fractions contained some immunoreactivity. The whole homogenate (H) showed bands to be of 110 and 60 kDa. This 110-kDa major product was localized to the sarcolemmal (SL_1 and SL_2) and denser membrane (SR) fractions. The 60-kDa protein seen in the homogenate was localized to the cytosolic (C) fraction. Besides these two major immunoreactive bands, the denser membrane fractions and sarcolemmal-enriched fractions also contained two other bands (about 137 and 173 kDa). All of the immunoreactive bands of the sarcolemmal and denser membrane fractions were sensitive to hydroxylamine (Fig. 7, right), suggesting that they contain ADP-ribosylarginines. The 60-kDa immunoreactive band of the cytoplasmic fraction was not sensitive to hydroxylamine. This would suggest that the protein does

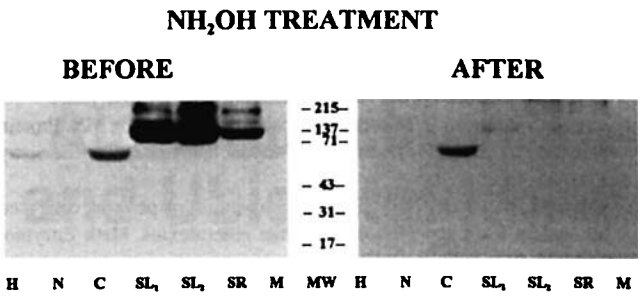

Figure 7. Immunoreactivity of quail heart subcellular fractions before and after NH_2OH treatment. Quail homogenate (H), nuclei (N), cytosol (C), sarcolemma (SL_1 and SL_2), denser membrane fractions (SR), and mitochondria (M) (25 μg of each) were electrophoresed, transferred, and immunoblotted with R-28 antiserum. The Immobilon was then stripped of antibodies following the suggested protocol of the ECL kit (Amersham), treated with 1 *M* NH_2OH for 18 hr, and immunoblotted a second time with R-28 antiserum.

not contain an ADP-ribosylarginine moiety or that it is somehow protected from the hydroxylamine treatment in this particular protein. The presence of this hydroxylamine-insensitive band enforces the need for appropriate control experiments with this antiserum.

Discussion

An antiserum, R-28, has been developed that is specific to ADP-ribosylated proteins where this moiety has been attached to an arginine residue. This antiserum was raised against ADP-ribosylated polyarginine. The reactivity of the R-28 to macromolecules (presumably proteins) was sensitive to treatment with phosphodiesterase I, which is consistent with immunoreactive molecules containing a phosphodiester bond as is present in ADP-ribose. This suggests that indeed the antiserum recognizes ADP-ribosylated proteins. The antiserum appears to be more sensitive to ADP-ribosylarginine than the other known ADP-ribosylated amino acids (cysteine, asparagine, and glutamate) as well as the intrapolymer ADP-ribosyl-ADP-riboses of polyADP-ribose. Several criteria have been used to establish this specificity including attempting to remove immunoresponsiveness by chemical treatments known to disrupt specific amino acid linkages. The R-28 antiserum recognized the cholera toxin ADP-ribosylation product, ADP-ribosyl-$G_{s\alpha}$. Hydroxylamine and sodium hydroxide treatments removed immunoreactivity whereas mercuric chloride did not. This is consistent with the linkage of the amino acid to an arginine or glutamine but not to a cysteine, diphthamide, asparagine, threonine, or serine. Proteins containing ADP-ribosylcysteine, -diphthamide, -asparagine, and -glutamate were not detected by the antiserum whereas proteins containing ADP-ribosylarginine were. There is a possibility that the diphthamide and asparagines ADP-ribosylation by diptheria and C_3 toxins had very low stoichiometry. It is possible that immunoreactivity to these two products might have occurred but were below the level of detection of the blot sensitivity assay even though [^{32}P]ADP-ribose labeling was detected. Thus this antiserum appears to be capable of differentiating proteins containing ADP-ribosylarginine from proteins containing most other possible ADP-ribosyl amino acid linkages.

Although the R-28 antiserum appears to differentiate clearly the type of ADP-ribosyl amino acid linkage contained in proteins, there is some immunoreactivity with molecules that do not appear or are not known to contain ADP-ribose. Two examples are seen in Figures 1 and 7. The 42-kDa molecular-weight marker of the prestained standards, which is a modified carbonic anhydrase, is immunoreactive (Fig. 1). This immunoreactivity and the purple color are sensitive to hydroxylamine treatment (unpublished data) suggesting that the modification done by the company to make it visually perceivable may contain a hydroxylamine-sensitive linkage. This immunoreactivity was detected in the preimmune serum of the R-28 rabbit (Fig. 1). This suggested that the antigen may not be ADP-ribosylarginine. Unmodified carbonic anhydrase is not immunoreactive (unpublished data). As seen in Figure 7, the quail heart contains a 60-kDa cytosolic immunoreactive band that was insensitive to hydroxylamine treatment. Currently, it is unknown what causes the immunoreactivity of the protein(s), but by the criteria of hydroxylamine insensitivity, it would appear not to be ADP-ribosylarginine. Use of hydroxylamine sensitivity assays as controls will be necessary with this antiserum.

In conclusion, this work produced an antiserum that potentially will be a tool for further purification and characterization of mono-ADP-ribosylated proteins. In addition, Graves *et al.* (19) used this antiserum for the detection of mono-ADP-ribosylated desmin. The antiserum detected both ADP-ribosylated desmin in chick skeletal muscle cells and purified avian desmin, and this reactivity was shown to be arginine-specific. Thus, the antibody described in this study is useful for characterization of arginine-specific mono-ADP-ribosylation of proteins and further studies into the cellular function of endogenous mono-ADP-ribosylation.

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