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Immunochemical Detection of Guanine Nucleotide Binding Proteins Mono-ADP-ribosylated by Bacterial Toxins

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ABSTRACT: Rabbits immunized with ADP-ribose chemically conjugated to carrier proteins developed antibodies reactive against guanine nucleotide binding proteins (G proteins) that had been mono-ADP-ribosylated by bacterial toxins. Antibody reactivity on immunoblots was strictly dependent on incubation of substrate proteins with both toxin and NAD and was quantitatively related to the extent of ADP-ribosylation. G_i, G_o, and transducin (ADP-ribosylated by pertussis toxin) and elongation factor II (EF-II) (ADP-ribosylated by pseudomonas exotoxin) all reacted with ADP-ribose antibodies. ADP-ribose antibodies detected the ADP-ribosylation of an approximately 40-kilodalton (kDa) membrane protein related to G_i in intact human neutrophils incubated with pertussis toxin and the ADP-ribosylation of an approximately 90-kDa cytosolic protein, presumably EF-II, in intact HUT-102 cells incubated with pseudomonas exotoxin. ADP-ribose antibodies represent a novel tool for the identification and study of G proteins and other substrates for bacterial toxin ADP-ribosylation.

Mono-ADP-ribosylation is a posttranslational modification in which the ADP-ribose moiety of NAD is covalently linked to one of a defined group of protein substrates (Ueda & Hayaishi, 1985). Mono-ADP-ribosylation of protein substrates from eukaryotic cells may be catalyzed either in whole cells or in broken cell preparations by a variety of microbial toxins (Honjo et al., 1968; Iglewski & Kabat, 1975; Cassel & Pfeuffer, 1978; Gill & Meren, 1978; Moss & Richardson, 1978; Katada & Ui, 1982) or endogenous enzymes (Moss & Vaughan, 1978; Moss & Stanley, 1981; DeWolf et al., 1981; Richter et al., 1983; Tanigawa et al., 1984; Lee & Iglewski, 1984). These reactions have been monitored in broken cell preparations by using radioactively labeled NAD. In toxin-treated intact cells, only indirect assays which measure either functional effects or the extinction of toxin substrate availability have been used. We sought an alternative method which would permit direct detection of mono-ADP-ribosylation of substrates, even in whole cells treated with bacterial toxins. We therefore immunized rabbits with ADP-ribose chemically conjugated to bovine serum albumin (BSA)[1] in an attempt to obtain antibodies capable of recognizing ADP-ribosylated proteins.

Materials and Methods

Materials. EF-II, pseudomonas exotoxin, rabbit reticulocyte lysate, and HUT-102 cells were generously provided by Dr. D. Fitzgerald (NCI). Pertussis toxin was contributed by Dr. R. Sekura (NICHD). Glutamine synthetase was the gift of Dr. S. G. Rhee (NHLBI). Sources of other materials used in protein determination, SDS gel electrophoresis, toxin-cat-

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[1] Abbreviations: ADPR, ADP-ribose; KLH, keyhole limpet hemocyanin; BSA, bovine serum albumin; G proteins, guanine nucleotide binding regulatory proteins; G_i, inhibitory G protein associated with adenylate cyclase; G_o, guanine nucleotide binding protein isolated from bovine brain; EF-II, elongation factor II; kDa, kilodalton(s); ELISA, enzyme-linked immunosorbant assay; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid.

alyzed ADP-ribosylation of protein substrates, immunoblotting, autoradiography, and ELISA were as described previously (Gierschik et al., 1984, 1985; Pines et al., 1985).

Antisera. Antibodies against ADPR were elicited in six New Zealand white rabbits (PG 1-6) by intradermal injection of ADPR conjugated to BSA by the periodate method (Erlanger & Beiser, 1964). Each rabbit was immunized with 200 μg of the ADPR–BSA conjugate in complete Freund's adjuvant and boosted 4 weeks later with 100 μg of antigen in incomplete Freund's adjuvant. The animals were bled before initial immunization and at 1-week intervals beginning 2 weeks after boosting, and heat-inactivated sera were collected. Affinity-purified antibodies were prepared by incubating the whole sera with NAD–agarose beads (periodate ribose linkage) (Sigma) for 18 h at 4 °C and eluting by incubation with 1 mM ADP in a 20 mM Tris-HCl, pH 7.5, and 500 mM NaCl buffer. The ADP was subsequently removed by dialysis.

Protein Purification. Transducin was prepared from bovine rod outer segments as previously described (Gierschik et al., 1985). A purified mixture of G_i/G_o was prepared from bovine forebrain as described previously (Milligan & Klee, 1985).

Membrane and Cell Preparation. Neutrophils were isolated from the blood of healthy human donors by Ficoll-paque sedimentation, dextran precipitation, and hypotonic lysis (Boyum, 1968; Borregaard et al., 1983). Neutrophil membranes were prepared by nitrogen cavitation and Percoll sedimentation by the method of Borregaard et al. (1983).

Toxin Treatment of Purified Proteins, Lysates, and Membranes. Pertussis toxin labeling of purified protein substrates was performed by activating toxin in a 100 mM Tris-HCl, pH 8.0, and 50 mM DTT buffer (70 μg of toxin/mL of buffer) for 1 h at room temperature. The activated toxin (35 μg/mL), or an equivalent buffer for "no toxin" control, was then added to a reaction mixture containing 100 mM Tris-HCl, pH 8.0, 4 mM ATP, 2 mM NAD with or without 70 μCi/mL [^{32}P]NAD, and a 100 μg/mL sample of the appropriate purified protein. Pseudomonas exotoxin labeling of rabbit reticulocyte lysates was performed by activating toxin in 300 mM DTT (30 μg of toxin/mL of buffer) for 30 min at room temperature. The activated toxin (15 μg/mL), or an equivalent buffer for "no toxin" control, was then added to a reaction mixture containing 1500 μg/mL rabbit reticulocyte lysate, 36 mM Tris-HCl, pH 8.1, 0.7 mM EDTA, and 10 mM NAD with or without 35 μCi/mL [^{32}P]NAD.

Toxin Treatment of Intact Cells. Freshly isolated neutrophils from human donors (Boyum, 1968) were incubated with 4 μg of pertussis toxin/mL of culture medium for 4 h at 37 °C. HUT-102 cells (HTLV-I-infected human leukemic cell line) were incubated with 200 ng of pseudomonas exotoxin per milliter of culture medium for 18 h at 37 °C.

Other Methods. ELISA, protein determination, SDS gel electrophoresis, immunoblotting, and autoradiography were performed as described previously (Gierschik et al., 1984, 1985; Pines et al., 1985).

Results

We immunized six rabbits (PG 1–6) with an ADP-ribose BSA conjugate (see Materials and Methods). The specificity of one antiserum (PG1) for ADP-ribose was tested by ELISA (Figure 1). ELISA plates were coated with BSA and keyhole limpet hemocyanin (KLH) and with the same proteins conjugated to ADP-ribose. The data show that the antiserum had approximately equal reactivity against ADP-ribose-conjugated and uncojugated BSA and with ADP-ribose-conjugated KLH (dilution of antiserum giving half-maximal reactivity, approximately 1:30 000) while its reactivity against uncojugated

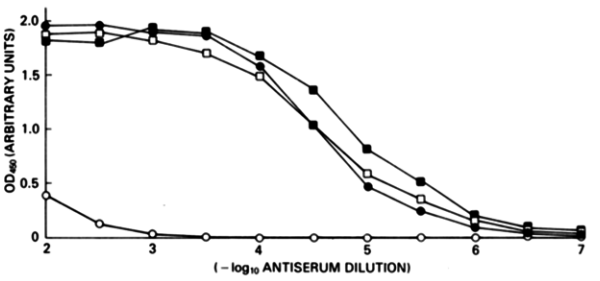

FIGURE 1: Antiserum reactivity against synthetic ADP-ribose–protein conjugates. ADP-ribose-conjugated BSA (■) and KLH (●) and unconjugated BSA (□) and KLH (○) were coated on ELISA plates and incubated with the indicated dilutions of PG1 antiserum for 4 h at room temperature. Reactivity was assayed by using *o*-phenylenediamine as substrate and peroxidase-conjugated goat anti-rabbit IgG as described under Materials and Methods. The optical density at 450-nm incident light is expressed on the ordinate in arbitrary units.

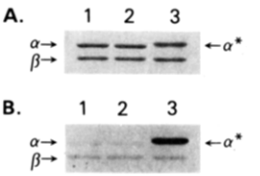

FIGURE 2: Antibody detection of mono-ADP-ribosylated transducin. Transducin was incubated in the presence of 1 mM NAD (lane 1), pertussis toxin (35 μg/mL) (lane 2), and pertussis toxin and NAD (lane 3). The samples (5 μg of transducin/lane) were separated on SDS–polyacrylamide gels and either (A) stained with Coomassie blue or (B) transferred to nitrocellulose and immunoblotted using a 1:100 dilution of PG1 antiserum as the first antibody.

KLH is much lower. This demonstrates the ability of the antiserum not only to recognize the protein–nucleotide conjugate and carrier protein used in immunization but also to recognize ADP-ribose conjugated by the periodate method to a protein not used in immunization.

We next tested the antisera for reactivity on an immunoblot against transducin mono-ADP-ribosylated in a reaction catalyzed by pertussis toxin. Figure 2 shows that recognition of transducin α on immunoblots by PG1 antiserum is absolutely dependent on treatment of transducin with both pertussis toxin and NAD. Notice that on the Coomassie blue stained gel (Figure 2A) the mono-ADP-ribosylated transducin α band is seen to migrate more slowly to the anode, reflecting the increase in molecular weight of the ADP-ribosylated entity. All six antisera showed varying levels of reactivity against ADP-ribosylated, but not unribosylated, transducin α.

To establish more clearly the relationship between the extent of PG1 antiserum reactivity on the immunoblot and the extent of ADP-ribosylation of transducin, we performed two additional experiments. In the first experiment, we performed immunoblots on equal amounts of transducin which had been ADP-ribosylated with increasing concentrations of pertussis toxin. The results, displayed in Figure 3, demonstrate that the extent of antibody staining on the immunoblot increases in direct proportion with the amount of toxin in the reaction mixture. Notice again that the extent of antibody staining on the immunoblot corresponds with a change in mobility of trasducin α on the Coomassie blue stained gel, and with an increase in radiolabel incorporation as seen on autoradiography.

The second experiment demonstrates the ability of the antiserum to detect differences in the extent of ADP-ribosylation of transducin caused by variations in the duration of the toxin labeling reaction. pertussis toxin was incubated with transducin for times ranging from 0 to 180 s. As can be seen in

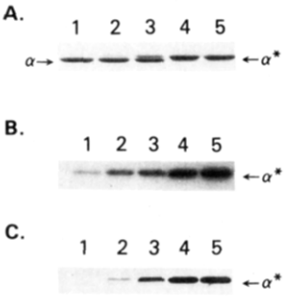

FIGURE 3: Antibody detection of increasing ADP-ribosylation of transducin as a function of increasing pertussis toxin concentration. Transducin was incubated for 60 min at 37 °C with various concentrations of pertussis toxin plus 1 mM NAD (A and C) or 1 mM NAD plus 35 μCi/mL [^{32}P]NAD (B). The samples were separated on SDS–polyacrylamide gels (5 μg of transducin/lane) and assayed by (A) staining with Coomassie blue, (B) autoradiography, or (C) immunoblotting with a 1:100 dilution of PG1 antiserum as the first antibody. The lanes correspond to reaction mixtures containing pertussis toxin concentrations of (1) 0, (2) 1.5, (3) 5, (4) 15, and (5) 50 μg/mL.

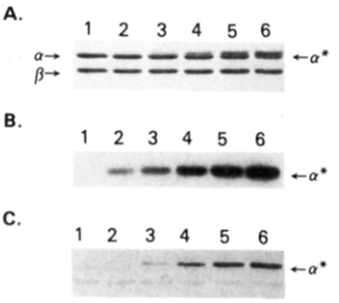

FIGURE 4: Antibody detection of increasing ADP-ribosylation of transducin as a function of increasing time of ADP-ribosylation. Transducin was incubated with pertussis toxin (35 μg/mL) and 1 mM NAD (A and C) or 1 mM NAD plus 35 μCi/mL [^{32}P]NAD (B). Samples were run on SDS–polyacrylamide gels (5 μg of transducin/lane) and assayed by (A) staining with Coomassie blue, (B) autoradiography, or (C) immunoblotting with a 1:100 dilution of PG1 antiserum as the first antibody. The lanes correspond to reaction mixtures incubated for (1) 0, (2) 30, (3) 60, (4) 90, (5) 120, and (6) 180 s.

Figure 4, the increase in transducin ADP-ribosylation, as assayed by the shift in mobility on the Coomassie blue stained gel and by autoradiography, correlates well with the increase in immunoreactivity on the nitrocellulose blot, going from no incorporation at $t = 0$ to a maximum incorporation by $t = 3$ min.

We can use the data displayed in Figure 3 to make a rough estimate of the sensitivity of the antiserum. the ADP-ribosylated transducin α band first becomes detectable on immunoblot C in lane 2. Inspection of the corresponding lane on the Coomassie blue stained gel reveals that this ADP-ribosylated band constitutes no more than 5% of the total transducin α in this lane. A rough calculation shows that the amount of ADP-ribosylated transducin α (TDα) in lane 2 would be (0.05)(40-kDa TDα)/(85-kDa TDαβγ)(5 μg of total protein) = approximately 120 ng of protein. If we assume 1 mol of ADP-ribose/mol of transducin α, the antiserum can detect as little as 3 pmol of ADP-ribose under the conditions used to immunoblot.

Immunoblots with crude PG antisera frequently showed reactivity against proteins in the 67-kDa range, presumed to represent albumin, and reflecting the use of albumin as the carrier protein for ADPR in immunization. To test this, we affinity purified PG antisera on an NAD–agarose column (see Materials and Methods). Affinity-purified antibodies maintained a high level of reactivity against ADP-ribosylated

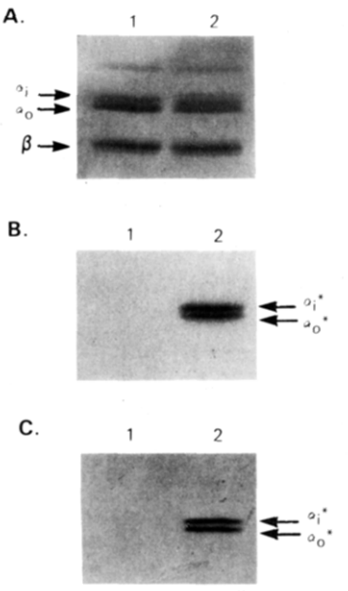

FIGURE 5: Antibody detection of ADP-ribose moiety on G_i and G_o. A mixture of purified G_i and G_o was incubated in the absence (lane 1) or presence (lane 2) of pertussis toxin (35 μg/mL). The reaction mixtures contained either 1 mM NAD (A and C) or 1 mM NAD plus 35 μCi/mL [^{32}P]NAD (B). The samples (5 μg of G_i/G_o per lane) were separated on SDS–polyacrylamide gels and assayed by (A) staining with Coomassie blue, (B) autoradiography, or (C) immunoblotting with a 1:25 dilution of affinity-purified PG5 antibodies as the first antibody.

transducin while retaining only minimal reactivity against BSA. Attempts to affinity purify the antisera using ADP–agarose (ribose linkage) were unsuccessful (not shown).

Having demonstrated specific antibody reactivity with ADP-ribosylated transducin, we assessed the reactivity of the antisera with other proteins ADP-ribosylated by pertussis toxin. A mixture of G_i and G_o purified from bovine brain was ADP-ribosylated by using pertussis toxin and NAD and then tested on immunoblots with affinity-purified antibodies from PG5 antiserum. The results, as seen in Figure 5, show that significant ADP-ribosylation of both G_i and G_o has occurred as assayed by changes in mobility on the Coomassie blue stained gel (A) and by ^{32}P labeling (B) and that this protein modification is detected on an immunoblot with affinity-purified antibodies.

We next sought to determine if antibody reactivity extended to substrates for mono-ADP-ribosylation catalyzed by a different bacterial toxin. We chose to examine the ADP-ribosylation of rabbit reticulocyte elongation factor II (EF-II) by pseudomonas exotoxin and NAD. Figure 6 demonstrates the ability of affinity-purified PG5 antibodies to detect the toxin- and NAD-dependent ADP-ribosylation of the approximately 93-kDa EF-II on an immunoblot.

Finally, we tested whether ADP-ribose antibodies could detect proteins ADP-ribosylated by toxins in intact cells. In the first of these experiments, we incubated freshly isolated human neutrophils for 4 h in culture with or without 4 μg/mL pertussis toxin and then isolated membranes from these cells. These membranes either were then processed by immunoblotting or were ADP-ribosylated using pertussis toxin and [^{32}P]NAD. The results of this experiment are shown in Figure 7. PG1 antiserum visualizes a neutrophil substrate ($M_r \sim$ 40K) in the membranes from pertussis toxin treated (lane 2) but not untreated (lane 3) cells. That significant ADP-ribosylation of this protein entity has been achieved in culture is confirmed by the near total extinction of approximately 40-kDa pertussis toxin substrate availability for [^{32}P]NAD

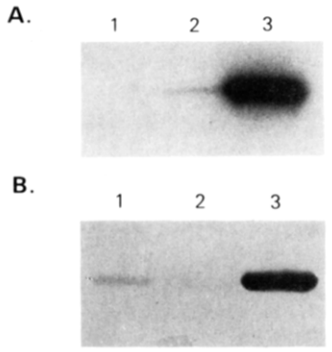

FIGURE 6: Antibody detection of mono-ADP-ribosylated EF-II. Rabbit reticulocyte lysate was incubated (lane 1) with NAD [35 μCi/mL [^{32}P]NAD (A) or 1 mM NAD (B)], (lane 2) with pseudomonas exotoxin (15 μg/mL), and (lane 3) with both toxin and NAD. The samples (150 μg of lysate protein per lane) were separated on SDS–polyacrylamide gels and assayed either by (A) autoradiography or by (B) immunoblotting using a 1:25 dilution of affinity-purified PG5 antibodies as the first antibody.

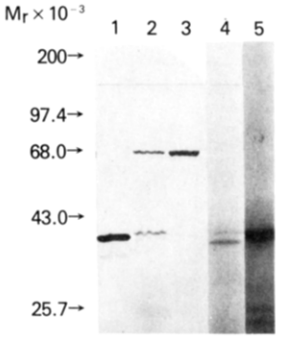

FIGURE 7: Antibody detection of pertussis toxin substrates in intact human neutrophils. Freshly isolated human neutrophils were incubated for 4 h in culture in the presence or absence of 4 μg/mL pertussis toxin. Membranes were prepared from these cells (see Materials and Methods) and either were processed by immunoblotting (using a 1:100 dilution of PG1 antiserum as the first antibody) or were radioactively labeled by incubation with pertussis toxin (35 μg/mL) and [^{32}P]NAD (35 μCi/mL), separated on SDS–polyacrylamide gels, and assayed by autoradiography. Lane 1, immunoblot of mono-ADP-ribosylated purified transducin (5 μg), which serves as an approximately 39-kDa molecular weight marker. Lane 2, immunoblot of membranes from pertussis toxin treated cells (150 μg of protein). Lane 3, immunoblot of membranes from untreated cells (150 μg of protein). Lane 4, autoradiograph of radiolabeled membranes from toxin-treated cells (150 μg of protein). Lane 5, autoradiograph of radiolabeled membranes from untreated cells (150 μg of protein). The stained bands at about 67 kDa (lanes 2 and 3) presumably represent albumin present in the neutrophil membrane preparation and detected by PG1 antiserum.

labeling in membranes from toxin-treated as compared with untreated cells.

In our second intact cell experiment, we incubated HUT-102 cells for 18 h in culture with or without pseudomonas exotoxin (200 ng/mL). A cytoplasmic fraction was obtained from these cells, and samples either were processed by immunoblotting or were ADP-ribosylated by using pseudomonas exotoxin and [^{32}P]NAD. The results, shown in Figure 8, demonstrate the ability of affinity-purified PG5 antibodies to recognize an approximately 93-kDa entity in treated (lane 1) but not untreated (lane 2) cells. Extinction of the radioactive labeling of an approximately 93-kDa entity using [^{32}P]NAD and pseudomonas exotoxin is seen in the cytoplasmic fraction from toxin-treated as compared with untreated cells.

Discussion

Members of the signal-transducing G-protein family (Gilman, 1984; Spiegel et al., 1985) and related guanine nu-

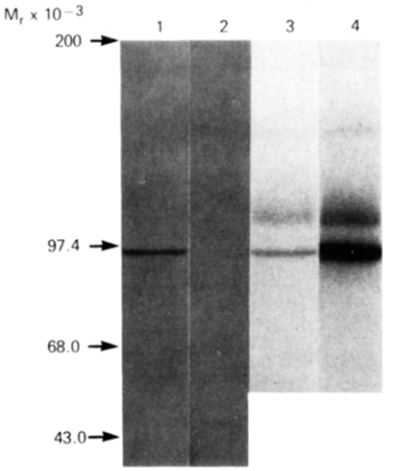

FIGURE 8: Antibody detection of pseudomonas exotoxin substrates in intact cells. HUT-102 cells were incubated for 18 h in culture in the presence or absence of 200 ng/mL pseudomonas exotoxin. Cytoplasmic fractions were obtained from these cells and either were processed by immunoblotting (using a 1:25 dilution of affinity-purified PG5 antibodies as the first antibody) or were radioactively labeled by incubation with pseudomonas exotoxin (15 μg/mL) and [^{32}P]NAD (35 μCi/mL), separated on SDS–polyacrylamide gels, and assayed by autoradiography. Lane 1, immunoblot of cytoplasmic fraction from toxin-treated cells. Lane 2, immunoblot of cytoplasmic fraction from untreated cells. Lane 3, autoradiograph of radiolabeled cytoplasmic fraction from toxin-treated cells. Lane 4, autoradiograph of radiolabeled cytoplasmic fraction from untreated cells.

cleotide binding proteins such as EF-II (Honjo et al., 1968) serve as substrates for mono-ADP-ribosylation by various bacterial toxins (Honjo et al., 1968; Iglewski & Kabat, 1975; Cassel & Pfeuffer, 1978; Gill & Meren, 1978; Moss & Richardson, 1978; Katada & Ui, 1982). Although toxin-catalyzed ADP-ribosylation of G proteins is readily demonstrable (using radioactively labeled NAD) in broken cell preparations, identification of toxin substrates in intact cells is more difficult and has relied on indirect methods (e.g., loss of substrate availability for labeling by toxin and radioactive NAD in broken cell preparations from cells treated with toxin).

We sought a method for direct identification of substrates for toxin-catalyzed ADP-ribosylation and therefore attempted to elicit antibodies capable of recognizing ADP-ribose. Since immunization with enzymatically ADP-ribosylated protein was impractical, we used a chemical method (Erlanger & Beiser, 1964) to conjugate ADP-ribose to a carrier protein. Hapten-specific reactivity was evident on ELISA; more importantly, immunoblots showed specific reactivity with the ADP-ribosylated form of transducin; this indicates that the mode of linkage of ADP-ribose to amino acid was not a critical determinant of antibody reactivity. This is further emphasized by the ability of ADP-ribose antibodies to recognize substrates for mono-ADP-ribosylation that differ in the specific amino acid [cysteine for transducin (West et al., 1985) and diphthamide for EF-II (VanNess et al., 1980)] to which the ADP-ribose is bound.

Although ADP-ribose antibodies clearly react specifically with ADP-ribosylated rather than unmodified G proteins, the precise specificity of these polyclonal antibodies remains to be defined and is likely heterogeneous. While they would appear to be relatively specific for adenine rather than guanine nucleotides,[2] it is unlikely that they are absolutely specific for mono-ADP-ribose. Thus, positive reactions were seen on immunoblots with adenylated glutamine synthetase[2] (Chung & Rhee, 1984). The ability of ADP to elute antibodies bound

[2] B. Eide, unpublished observations.

to NAD–agarose, moreover, indicated that such antibodies are not exclusively specific for ADP-ribose. For this reason, proteins recognized by the present antisera cannot be assumed to represent exclusively substrates for mono-ADP-ribosylation. Although immunological detection methods could prove valuable in identification of substrates for endogenous ADP-ribosyltransferases (Moss & Vaughan, 1978; Moss & Stanley, 1981; DeWolf et al., 1981; Richter et al., 1983; Tanigawa et al., 1984; Lee & Iglewski, 1984), antibodies with a more limited and defined specificity than the present ones will be required.

After this work was completed, a report appeared concerning ADP-ribose antibodies capable of recognizing mono-ADP-ribosylated proteins (Meyer & Hilz, 1986). These authors immunized rabbits with an ADP-ribose derivative linked to albumin that differed in two respects from our own. First, a hydrolysis-resistant methylene diphosphonate linked form of ADP was used, and second, the linkage to protein was through the N^6-position of the adenine ring. The resultant polyclonal antibodies were relatively, but not absolutely, specific for ADP-ribose and detected mono-ADP-ribosylated histones on immunoblots with a sensitivity in the picomole range, comparable to ours.

The antisera we describe here appear quite useful in studying G protein substrates of bacterial toxins. These proteins are not ADP-ribosylated without toxin action and are not recognized by the present antisera unless treated with both toxin and NAD. ADP-ribose antibodies proved capable of recognizing the three known G protein substrates of pertussis toxin, G_i, G_o, and transducin, as well as EF-II ADP-ribosylated by pseudomonas exotoxin. Although cholera toxin substrates were not specifically tested, it is reasonable to assume that antibody reactivity would extend to these as well.

Most significantly, ADP-ribose antibodies proved capable of detecting substrates that had been ADP-ribosylated in intact cells treated with bacterial toxins. With both human neutrophils treated with pertussis toxin and HUT-102 cells treated with pseudomonas toxin, immunoblotting identified a single toxin-dependent protein substrate. These results were confirmed by the indirect method involving extinction of substrate availability.

Recent evidence suggests that pertussis toxin substrates in some cells such as C6 glioma (Milligan et al., 1986) and human neutrophils (Gierschik et al., 1986) may be G proteins related to, but distinct from, G_i, G_o, and transducin. The approximately 40-kDa protein detected by ADP-ribose antibodies in pertussis toxin treated human neutrophils may represent such a novel G protein which reacts poorly, or not at all, with antibodies against G_i, G_o, and transducin, the known pertussis toxin substrates (Gierschik et al., 1986). The availability of ADP-ribose antibodies should prove useful in the delineation of substrates for toxin-catalyzed ADP-ribosylation in intact cells and in identification of novel G-protein toxin substrates.

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