Vol. 144, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1987 Pages 536-542

MULTIPLE ISOFORMS OF ADP-RIBOSYLATED G-LIKE PROTEINS FROM MAMMALIAN THYROID MEMBRANES

William J. Deery, Fernando Rebeiro-Neto, and James B. Field

Baylor College of Medicine, Diabetes Research Laboratory,
St. Luke's Episcopal Hospital,
P. O. Box 20269, Houston, TX 77225-0269

Received March 4, 1987

SUMMARY: Bovine, canine, and porcine thyroid membrane proteins which were [^{32}P] ADP-ribosylated by cholera and pertussis toxin in vitro were analyzed by one and two-dimensional polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. These three mammalian species have similar cholera toxin substrates (M_r 42,000 and 48,000) and pertussis toxin substrates (M_r 40,000). Resolution by two dimensional gel electrophoresis of these ribosylated proteins revealed that they each consist of at least 6 distinct polypeptides with similar isoelectric points ranging from approximately 5.5 - 7.0.

The activity of the catalytic subunit of adenylate cyclase is regulated both positively and negatively by a complex which consists of guanine nucleotide binding proteins termed N_S or G_S and N_i or G_i, respectively (1-7). These proteins have a heterotrimeric composition consisting of α (M_r 39,000 - 55,000), β (M_r 35,000), and γ (M_r 8,000) subunits (8). The α subunits, which bind guanine nucleotides and possess GTPase activity, are also sites for toxin-dependent ADP-ribosylation (9-12). The G proteins are intimately involved in transducing receptor signals to the catalytic subunit of adenylate cyclase (1,2,4). Using [^{32}P] NAD^+ and specific in vitro buffer conditions, it is possible to preferentially [^{32}P] ADP-ribosylate α_s and α_i with cholera toxin (CT) and pertussis toxin (PT), respectively (13). Stoichiometry of such labeling has been reported to be 1 mol ADP-ribose/mol α protein (2,14-17). We have exploited this phenomenon to detect these quantitatively minor proteins in thyroid membrane preparations and to determine whether they exist as multiple polypeptide species. While essentially all previous studies examined [^{32}P] ADP-ribosylated membrane proteins from various tissues by one dimensional SDS-PAGE,

Abbreviations: CT, cholera toxin; PT, pertussis toxin; SDS - PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; CHES, cyclohexylamino-ethane sulfonic acid; NAD^+, nicotinamide adenine dinucleotide; TRIS, TRIS (hydroxymethyl) amino methane; EDTA, ethylenediamine-tetraacetic acid.

0006-291X/87 $1.50

isoelectric points of multiple CT substrates from wild type and UNC variant S49 lymphoma cell membranes have been reported (18). Recently, isoelectric points of some ADP-ribosylated α subunits (19), and evidence for 35 kilodalton β subunit isoforms have also been reported (20). Our results indicate that several species of $G\alpha_s$ and $G\alpha_i$-like proteins exist in each of the three different mammalian thyroid membranes examined, and that the molecular weights, pI values, and relative number of species appear to be relatively conserved.

MATERIALS AND METHODS

[^{32}P] NAD^+ was synthesized, purified, and provided by the Molecular Endocrinology Core Laboratory of the Diabetes and Endocrinology Research Center of Baylor College of Medicine. GTP was purchased from Boehringer-Mannheim Biochemicals and ATP from Sigma Chemical Company. Cholera and pertussis toxins were obtained from List Laboratories. The reagents used for SDS-PAGE were from Bio-Rad and ampholine pH 3.5-10 from LKB Bromma.

Partially purified thyroid membranes were prepared essentially as described previously (13,21). Briefly, fat and connective tissue were removed from the thyroid in ice cold Krebs-Ringer bicarbonate buffer containing 1 mg/ml bovine serum albumin and glucose, following which the gland was sliced and then well minced with scissors. After washing with the above buffer, the mince was suspended in 10 volumes of 20 mM Tris HCl, 1 mM EDTA (pH 7.5), and homogenized with 13-15 strokes in a loosely fitting Dounce homogenizer. After centrifugation at 2°C at 800 x g for 10 min, the resulting supernatant was centrifuged at 2°C for 30 min at 20,000 x g. The pellet containing the membranes was resuspended in the Tris-EDTA buffer, passed through a 21 then 27 gauge needle twice, and recentrifuged at 20,000 x g for 30 min. The pellet was resuspended to 3-6 mg/ml in Tris-EDTA buffer containing 10% sucrose and stored at -70°C.

Labeling of the CT and PT substrates with [^{32}P]NAD was done according to the procedure described previously (13). Briefly, incubations were for 30 min at 33°C in a final reaction mixture volume of 60 ul containing 25 ug of thyroid membranes, 10 uM [^{32}P] NAD^+ (10-20 x 10^6 cpm), 10 mM thymidine, 1 mM GTP and 1.2 ug PT or 12 ug CT. When ribosylation was catalyzed by CT, 300 mM potassium phosphate and 10 mM $MgCl_2$ were present. They were replaced by 1 mM ATP for PT catalyzed ribosylation. Other components are described in detail elsewhere (13). The reaction was terminated by precipitation with 20% ice cold TCA. After washing the precipitate with ethyl ether, it was immediately solubilized at room temperature for one and two dimensional electrophoretic analysis.

One dimensional SDS-PAGE was performed according to the method of Laemmli (22.) Each sample was solubilized in 50 ul of 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.002% bromophenol blue and 62 mM Tris-HCl (pH 6.8), and electrophoresis was performed using a 5% stacking gel and 10% resolving gel. Two dimensional PAGE was performed according to the methods of Anderson et al (23) and Dunbar (24). Precipitated samples were solubilized in 50 ul of either 9 M urea, 4% Nonidet P-40, 2% 2-mercaptoethanol, 2% 3.5-10 ampholine, or 0.05 M CHES, 2% SDS, 10% glycerol, 2% 2-mercaptoethanol (25), and after isoelectric focusing, resolved according to molecular weight on 10% polyacrylamide SDS gels. In certain cases PT ribosylation reactions were terminated and protein directly solubilized by addition of 4-fold concentrated CHES-SDS sample buffer to the reaction mixture. All gels were stained with Coomassie blue, destained, dried and subjected to autoradiography at -70°C using XAR-5 or XS-5 film (Kodak) and two Dupont lightning plus BE intensifying screens.

RESULTS

One-dimensional SDS-PAGE analysis: Figure 1 shows a 1-D SDS-PAGE analysis of proteins from bovine (Fig. 1 A,D), canine (Fig. 1 B,E), and porcine (Fig. 1 C,F) thyroid membranes incubated with [^{32}P]NAD^{+} and either CT (Fig. 1 A,B,C) or PT (Fig. 1 D,E,F). A similar pattern of major membrane proteins is observed in these three mammalian species. Autoradiography reveals that polypeptides from bovine (Fig. 1 G), canine (Fig. 1 H), and porcine (Fig. 1 I) membranes with approximate molecular weights of 42,000 and 48,000 are ADP-ribosylated by CT. Likewise, all three animal species exhibit a common PT-catalyzed ribosylated protein(s) migrating in a molecular weight region of approximately 40,000 (Fig. 1 J,K,L). The labeled material at the bottom of lanes G, H and I is autoribosylated CT.

Two dimensional PAGE analysis: In order to determine whether the [^{32}P]ADP-ribosylated protein bands observed by 1-D PAGE consist of polypeptides with multiple isoelectric points, labeled membrane samples were subjected to 2-D PAGE. The stained protein patterns in Figure 2 A (bovine),D (canine) and G (porcine) demonstrate that, like in 1-D PAGE, the three animal species have similar major membrane proteins. These proteins also have similar pI values. Autoradiography of the CT substrates (Figure 2 B,E,H) reveals that the 42K and 48K species each consists of at least six isoforms with similar pI values. The number of species and their

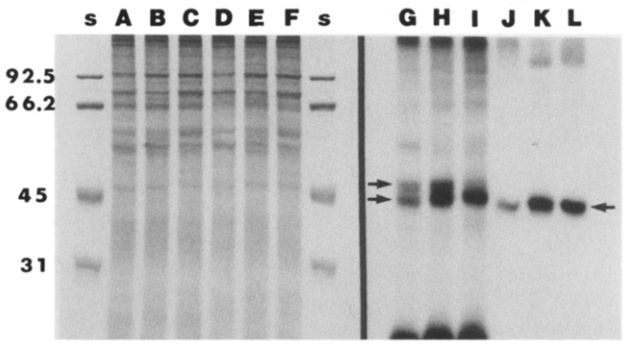

Figure 1. One dimensional (1-D) SDS-PAGE of thyroid membrane proteins ADP-ribosylated by CT and PT. Membrane preparations were ribosylated as described in Materials and Methods. Lanes A and D (bovine), B and E (canine), and C and F (porcine) were samples incubated with CT and PT, respectively, electrophoresed and Coomassie stained. Lanes G and J (bovine), H and K (canine), and I and L (porcine) are autoradiographs of above samples incubated with CT and PT, respectively. Double arrows point to M_r 42,000 and 48,000 CT substrates; single arrow points to M_r 40,000 PT substrates. Lanes S are molecular weight standards expressed in kilodaltons.

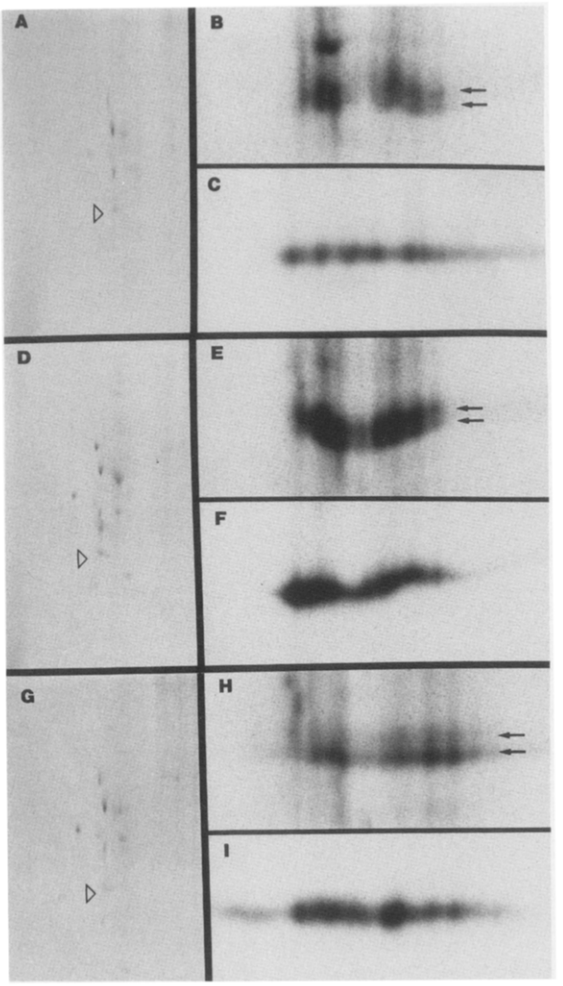

Figure 2. Two dimensional (2-D) PAGE of thyroid membrane proteins ADP-ribosylated by CT and PT. Membrane preparations were ribosylated as described in Material and Methods. Samples were precipitated and solubilized in CHES-SDS sample buffer except for panel C which was directly solubilized by 4-fold concentrated CHES-SDS buffer as described in Materials and Methods. Panels A (bovine), D (canine), and G (porcine) were samples focused (pH 3.5-10) and electrophoresed on 10% SDS-PAGE and Coomassie stained. Acidic region is left, basic region is right for all panels. Arrowheads point to membrane actin (M_r 46,000). Panels B (bovine), E (canine), H (porcine) and C (bovine), F (canine), I (porcine) are autoradiographs of above samples incubated with CT and PT, respectively. Double arrows point to M_r 42,000 and 48,000 CT substrates.

isoelectric points are also similar between the three animals. With respect to the membrane-associated actin (pI 5.6), the most acidic species appear to be 5.5; the most basic are approximately 7.0. Figure 2 (C,F and I) show that the substrates from the three animals labeled with PT also consist of at least 6 similar isoforms, and interestingly, with pI values similar to the CT substrates.

DISCUSSION

The present results from 2-D PAGE indicate that several species of CT and PT substrates exist in each of the mammalian thyroid membranes examined. Based upon the criteria of toxin-catalyzed ADP-ribosylation, molecular weight and reconstitution of cyc$^-$ mutants using membrane extracts (18,26,27), these CT and PT substrates could be regulatory $G\alpha_s$ and $G\alpha_i$ subunits of adenylate cyclase, respectively. The possibility that the multiple species observed is an artifact of protein solubilization is reduced since the same pattern is observed either using urea or CHES-SDS sample buffers, or by directly solubilizing an unprecipitated reaction mixture with the addition of 4-fold concentrated CHES-SDS sample buffer. It is also unlikely that the variety of labeled species is due to multiple ribosylations of one or a few proteins resulting in more than 1 mol of NAD^+/mol of α polypeptide, since the labeling observed is quite uniform, and does not increase in intensity for the more acidic pI species. Furthermore, multiple sites for CT or PT-catalyzed ADP-ribosylation have not been reported (2,14-17). It is possible, however, that variation in the number of protein species could be observed with differences in membrane preparation or ribosylation conditions, for example.

Recent data from studies using guanine nucleotides and toxins, for example, have indicated that, in addition to adenylate cyclase, G α subunits have multiple regulatory functions in processes such as K^+ (28,29) and Ca^{++} (30,31) channels, arachidonic acid release (32), and phospholipase C action (33). The possibility that the various systems utilize different isoforms of G α subunits remains to be explored. Since significant occurrence of intrinsic ADP-ribosylation and a possible role of ribosylation in the function of G proteins in vivo have not been clearly established, a more precise determination of pI values for ribosylated species has somewhat limited importance and was not attempted in the present report. The results do indicate, however, that the number of different CT and PT-substrate isoforms and their pI values are quite conserved in the mammalian thyroid membranes examined. It clearly remains to be determined whether these species are produced from posttranslational modifications, or whether they differ at the genetic level. Regardless of the nature of their origin, it is tempting to propose that distinct multiple functions might

exist for these species, particularly in light of the programmable messengers theory of hormone action recently put forth by Rodbell (34).

ACKNOWLEDGEMENTS: This work was supported by United States Public Health Grants DK 26088 and DK 27685. The authors are grateful to Dr. Bonnie S. Dunbar and Ms. Donnie S. Bundman for the helpful discussions concerning 2-D PAGE. We wish to thank Ms. Mary Ann Farabee for the excellent secretarial assistance.

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