

## A 22 kDa *ras*-related G-protein is the substrate for an ADP-ribosyltransferase from *Clostridium botulinum*

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Received 1 August 1988

A ribosyltransferase from *C. botulinum* type D ADP-ribosylated a protein of 22 kDa (p22) in human astrocytoma (1321N1) cells. ADP-ribosylation of membrane-bound p22 was potentiated by 2 mM MgCl<sub>2</sub> or guanine nucleotides but was much reduced in the presence of 10 mM Mg<sup>2+</sup> plus GTP $\gamma$ S. p22 was immunoprecipitated by a monoclonal antibody (142-24E05) raised against a peptide sequence common to the *ras* gene family but not by other *ras* or G-protein antibodies. p22 was also ADP-ribosylated in *Drosophila* but was not detected in *Dictyostelium*. These data suggest that the 22 kDa botulinum toxin substrate is a GTP-binding protein and a member of the *ras* protein family.

ADP-ribosylation; Botulinum toxin; GTP-binding protein; *ras* gene

### 1. INTRODUCTION

The abilities of cholera and pertussis toxins to ADP-ribosylate the GTP-binding regulatory proteins, G<sub>s</sub> and G<sub>i</sub>, that couple stimulatory and inhibitory receptors, respectively, to adenylate cyclase have been instrumental in identifying these proteins as well as in determining their function [1]. The *ras* oncogene products are a family of 21 kDa, membrane-associated, GTP-binding proteins, the biological function of which is uncertain [2]. The identification of a toxin that could ADP-ribosylate and acutely modulate the function of p21<sup>ras</sup> would be of great experimental value. Botulinum toxin types C and D were recently shown to ADP-ribosylate a protein of approx.

21 kDa in several tissues [3,4] and this activity has been shown to be due to the presence of a 26 kDa ADP-ribosyltransferase (exoenzyme C3) in the toxin preparations [5,6]. We show here that the 22 kDa protein (p22) ADP-ribosylated by a preparation of botulinum toxin type D is a G-protein immunologically related to but distinct from Ha-, Ki- and N-*ras*.

### 2. MATERIALS AND METHODS

Botulinum toxin type D was purchased from Wako. Pertussis toxin was from List. GTP $\gamma$ S and GDP $\beta$ S were from Boehringer Mannheim. [<sup>32</sup>P]NAD (800 Ci/mmol) was from DuPont-New England Nuclear and <sup>35</sup>S TRANSLabel (1200 Ci/mmol) was from ICN. Rabbit polyclonal antipeptide sera to G $\alpha$  and G $\beta$  [7] were a gift from S. Mumby (University of Texas, Dallas). Monoclonal antibodies to peptides representing p21<sup>ras</sup> amino acids 29–44 (6B7 [8], F. McCormick, Cetus Corp.) and 96–118 (142-24E05 [9], NCI Repository, Microbiologicals, Bethesda, MD) were used with goat anti-mouse secondary antibody and collected on fixed *S. aureus* (Pansorbin, Calbiochem-Behring). Monoclonal antibody Y13-259, which reacts with all known p21<sup>ras</sup> proteins through an epitope encompassing amino acids 63–73 [10], was used as described [11].

1321N1 astrocytoma cells were cultured as in [12]. To prepare membranes, cells were lysed in hypotonic buffer [12] and centrifuged (800  $\times$  g, 5 min). The supernatant was further cen-

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*Abbreviations:* BTx, botulinum toxin type D preparation; G-protein, GTP-binding protein; G<sub>s</sub>, G<sub>i</sub>, stimulatory and inhibitory G-proteins of adenylate cyclase; GTP $\gamma$ S, guanosine 5'-O-(3-thiotriphosphate); GDP $\beta$ S, guanosine 5'-O-(2-thiodiphosphate); p22, 22 kDa BTx substrate

trifuged ( $40000 \times g$ , 20 min) and the pellet obtained was resuspended in buffer containing EDTA (1 mM), dithiothreitol (10 mM), sucrose (10%, w/v) and Hepes (20 mM), pH 7.6. Lysates from 1321N1 cells and *Drosophila* were prepared by freeze-thawing cells twice in liquid nitrogen or by 10 strokes of a Dounce homogenizer, respectively. Nuclei and unbroken cells were removed by centrifugation ( $800 \times g$ , 5 min).

The standard ADP-ribosylation reaction mixture contained thymidine (10 mM), nicotinamide (50 mM), dithiothreitol (10 mM), EDTA (0.25 mM),  $MgCl_2$  (2 mM), GTP (0.5 mM), Hepes (50 mM) pH 7.6, and [ $^{32}P$ ]NAD (5  $\mu$ Ci, 30  $\mu$ M). Modification of the reaction mixture is described in fig.1. For ADP-ribosylation by pertussis toxin, nicotinamide and  $MgCl_2$  were omitted and ATP (1 mM) was added. Membranes (100  $\mu$ g membrane protein in a final assay volume of 100  $\mu$ l) were incubated with BTx (250  $\mu$ g/ml) or vehicle (45 min,  $37^\circ C$ ) and reactions were terminated by the addition of ice-cold Tris (10 mM)/EDTA (1 mM), pH 7.5, and centrifugation ( $10000 \times g$ , 2 min). Incubations containing lysates were terminated by addition of 1 ml trichloroacetic acid (20%, w/v) centrifuged and the precipitate was washed with 1 ml diethyl ether. To examine  $^{32}P$ -labeled toxin substrates, pellets were resuspended in sample buffer, boiled and approx. 40  $\mu$ g protein separated on 15% SDS-polyacrylamide gels as described [11]. Gels were dried and exposed to Kodak X-Omat AR film. Metabolic labeling and immunoprecipitation of G proteins were carried out as in [7]. Protein was determined by the method of Bradford [13] using bovine serum albumin as standard.

### 3. RESULTS AND DISCUSSION

The botulinum toxin type D preparation ADP-ribosylated a protein of approx. 22 kDa in 1321N1 astrocytoma cell membranes. The extent of ribosylation was concentration dependent, increasing between 10 and 250  $\mu$ g BTx protein/ml (not shown). The ADP-ribosylation of p22 in 1321N1 cell membranes was stimulated by guanine nucleotides (fig.1, lanes 2–5). The inclusion of 2 mM  $Mg^{2+}$  in the reaction mixture resulted in a 5–10-fold increase in labeling (lane 6) and the response to 2 mM  $Mg^{2+}$  was potentiated by GDP $\beta$ S, GDP, GTP or ATP (lanes 7–10). In contrast to the other nucleotides tested, GTP $\gamma$ S attenuated the ADP-ribosylation induced by 2 mM  $Mg^{2+}$  by approx. 40% (lane 11). The addition of 10 mM  $Mg^{2+}$  also greatly reduced the level of ribosylation compared to that obtained with 2 mM  $Mg^{2+}$ . The inclusion of GTP $\gamma$ S with 10 mM  $Mg^{2+}$  resulted in a further decrease in ribosylation of p22 (lanes 12,13). The regulation of ribosylation by  $Mg^{2+}$  and guanine nucleotides, shown here and in other recent studies [3–5], resembles that seen with pertussis and cholera toxin [14,15]. These observations suggested that p22 was a G-protein.

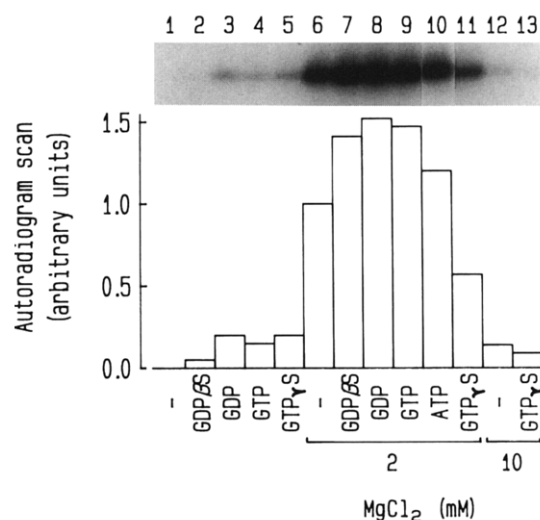


Fig.1. Effects of nucleotides and  $Mg^{2+}$  on the ADP-ribosylation of a 22 kDa protein in 1321N1 cell membranes. Membranes were incubated with BTx (100  $\mu$ g/ml) in the absence or presence of  $Mg^{2+}$  and various nucleotides as indicated. Nucleotide concentrations were 500  $\mu$ M except for GTP $\gamma$ S (100  $\mu$ M). Data shown are representative of at least 3 experiments.

The size and apparent GTP-binding properties of p22 suggested that the toxin substrate might be p21<sup>c-ras</sup> or a related protein. A panel of antibodies was used to immunoprecipitate G proteins from 1321N1 cells labeled metabolically with  $^{35}S$  TRANSlabel or from cell lysates incubated in vitro with BTx and [ $^{32}P$ ]NAD. ADP-ribosylated p22 was immunoprecipitated by antibody 142-24E05 (fig.2, lane 3). This provided additional evidence that p22 was a G-protein because the 142 antibody was generated against a peptide encompassing part of the GTP-binding domain of p21<sup>c-ras</sup> [9,16]. However, the ADP-ribosylated p22 was not immunoprecipitated by two other p21<sup>ras</sup> antibodies, Y13-259 and 6B7 (lanes 4,5). Similarly, the BTx substrate has been reported not to be immunoprecipitated by two polyclonal antibodies raised against p21<sup>c-ras</sup> [6,17]. Both the 259 and 6B7 antibodies recognized p21<sup>c-ras</sup> in  $^{35}S$ -labeled 1321N1 cells (lanes 7–9 and not shown), and control experiments demonstrated that ADP-ribosylation by BTx did not interfere with the immunoprecipitation of p21<sup>c-ras</sup> by Y13-259 (lanes 7,8). On SDS-polyacrylamide gels the mobility of p21<sup>c-ras</sup> from 1321N1 cells was clearly different

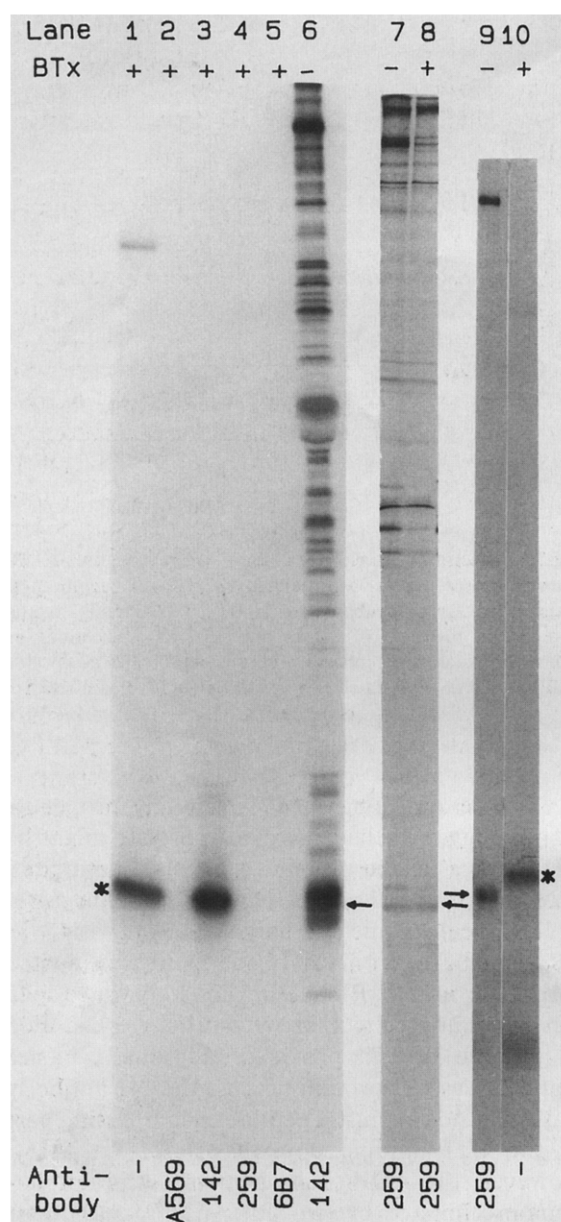


Fig.2. Immunoprecipitation of p21<sup>ras</sup> and ADP-ribosylated p22 by G-protein antibodies. Intact 1321N1 cells were labeled with <sup>35</sup>S TRANSLabel (lanes 6–9) or cell membranes incubated with [<sup>32</sup>P]NAD (lanes 1–5,10) or unlabeled NAD (lanes 7,8) in the presence or absence of BTx as indicated. p21<sup>c-ras</sup> (→) and p22 (\*) or membranes (lanes 2–5,7,8) with the indicated antibodies. Samples were displayed on SDS-polyacrylamide gels. Lanes 1,10 show total membrane proteins labeled by BTx without immunoprecipitation.

from that of the ribosylated p22 (lanes 9,10). Therefore, p22 is not p21<sup>c-ras</sup> itself but is probably a related member of the *ras* gene family. The antibodies A569 and A572 immunoprecipitate G<sub>i</sub>α and G<sub>s</sub>α in 1321N1 cells [7] but did not recognize p21 or p22 (fig.2, lane 2, and not shown), suggesting that p22 is not closely related to G<sub>s</sub> or G<sub>i</sub>.

Phosphoinositide hydrolysis is sensitive to inhibition by pertussis toxin in some cell types, however in many others, including 1321N1 cells, the toxin has no effect [18]. It is therefore presumed that a different G-protein (G<sub>p</sub>) unrelated to G<sub>i</sub> couples receptors to phospholipase C in these cells. Studies on *ras*-transfected cell lines have suggested that p21<sup>ras</sup> may be the G-protein that couples hormone and growth factor receptors to phosphoinositide hydrolysis [19,20]. Pretreatment of 1321N1 cell membranes with BTx failed to affect guanine nucleotide-stimulated inositol phosphate formation, the only observed changes [21] being attributable to the toxin buffer. These data suggest that p22 is not involved in the regulation of phospholipase C.

Preliminary experiments were performed to determine the intracellular location and abundance of p22. Following ADP-ribosylation of a 1321N1 cell lysate, p22 was found to be approx. 65% cytosolic (fig.3A). This property also differentiates p22 from the other 1321N1 cells' G-proteins, p21<sup>c-ras</sup> and G<sub>i</sub>α (no G<sub>o</sub>α is present in 1321N1 cells [7]), which are largely membrane-bound (Buss, J.E., unpublished). The abundance of p22 was estimated by comparing the amount of p22 that was ribosylated by BTx to the amount of G<sub>i</sub>α that could be ribosylated by pertussis toxin in a 1321N1 cell lysate. The ribosylation conditions were optimized for each toxin to allow maximal incorporation of [<sup>32</sup>P]NAD into each protein. The intensity of the 22 kDa band labeled by BTx was similar to that of the 41 kDa band(s) labeled by pertussis toxin (fig.3B). This suggests that p22 in the 1321N1 cells is likely to be present in similar abundance to the G<sub>i</sub>α-proteins.

The BTx substrate has been detected in all vertebrate cells tested thus far [6,22]. We found that the toxin substrate was also present in a lysate prepared from *D. melanogaster* (fig.3C) and could be immunoprecipitated by the 142 antibody (not shown). However, BTx did not ribosylate a 22 kDa protein in growing or differentiated cells (5–20 h,

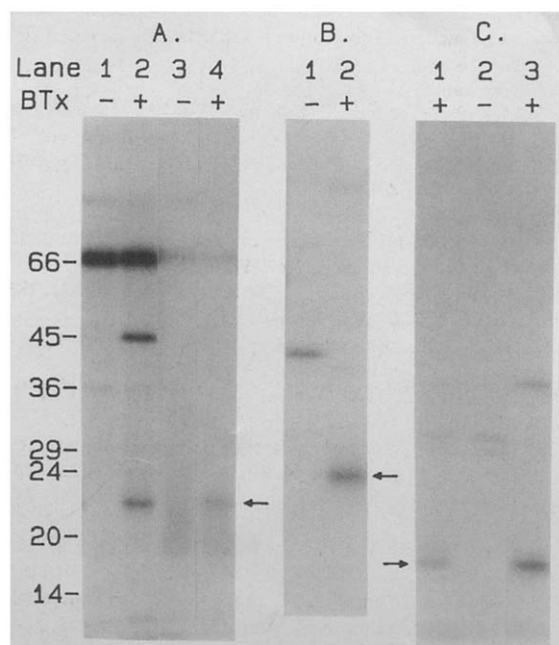


Fig.3. ADP-ribosylation of p22 from 1321N1 cells and *D. melanogaster*. (A) Cellular distribution of p22: a 1321N1 cell lysate was incubated with [ $^{32}$ P]NAD then separated into soluble (lanes 1,2) and particulate fractions (lanes 3,4) by centrifugation ( $10000 \times g$ , 2 min) prior to electrophoresis. Contamination of the BTx with C2 binary toxin, that ADP-ribosylates actin [23], is probably responsible for the labeled band at 45 kDa in this figure. The 66 kDa band is bovine serum albumin. Molecular mass markers refer to panel A only. (B) Comparison of ADP-ribosylation of  $G_{i\alpha}$  by pertussis toxin (lane 1) and p22 by BTx (lane 2) in a 1321N1 cell lysate. (C) A lysate prepared from adult *Drosophila* was ADP-ribosylated by BTx. Lane 3 shows p22 from a 1321N1 cell lysate run on the same gel.

following starvation) of the lower eukaryote, *Dictyostelium discoideum* (not shown).

The effects of  $Mg^{2+}$  and guanine nucleotides on ADP-ribosylation and the immunological data reported here are consistent with p22 being a G-protein related to  $p21^{ras}$ .  $p21^{ras}$  has been shown to induce differentiation of PC12 cells [24] and Rubin et al. [6] recently reported that introduction of purified botulinum exoenzyme C3 into these cells also induced changes in cell morphology. We observed a small increase in ADP-ribosylated p22 in PC12 cells differentiated by exposure to nerve growth factor (not shown) suggesting that, like  $p21^{ras}$ , p22 could play a role in this process. However, it is possible that these two members of

the *ras* gene family affect cell function via different pathways, since we were unable to immunoprecipitate ADP-ribosylated p22 with an antibody (6B7) to the putative  $p21^{c-ras}$  effector region [8,10].

$p21^{ras}$  has been found in all eukaryotic cells, including yeast and *Dictyostelium* [2]. Our inability to detect ADP-ribosylated p22 in *Dc. discoideum* suggests that its function may be more specialized than  $p21^{c-ras}$  and perhaps restricted to higher eukaryotes. Since p22 was detected in *D. melanogaster* it is possible that mammalian cells possess a homologue of one of the *Drosophila ras* proteins [2]. BTx has recently been reported to ADP-ribosylate a 22 kDa protein in rabbit neutrophils; the function of this protein remains uncertain, since toxin treatment did not affect several functions of these cells including superoxide generation and release of  $\beta$ -glucosamidase [25]. Several 20–30 kDa GTP-binding proteins have recently been described, some of which may be involved in membrane trafficking and secretion [26–30]. We have no evidence for the involvement of p22 in these processes, however, since p22 was not found exclusively at the plasma membrane, it may regulate processes other than signal transduction.

This is the first report describing the ADP-ribosylation of a product of the *ras* gene family. The possibility that BTx-induced ADP-ribosylation may acutely perturb p22 function might help elucidate the role(s) of this *ras* protein in mammalian cells.

**Acknowledgements:** We thank Patricia Solski for excellent technical assistance, Dr C. Der for PC12 cells, Dr R. Firtel for *Dc. discoideum*, Dr F. McCormick for antibody 6B7 and Dr M. McKeown for *D. melanogaster*. Supported by University of California Cancer Research Coordinating Committee funds and NIH grants GM 36927 to J.H.B. and CA42348 to J.E.B.

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