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Identification of *rho* as a substrate for botulinum toxin C_3-catalyzed ADP-ribosylation

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Recombinant *Aplysia rho* and a GTP-binding protein purified from human neutrophil membranes (G_{22K}) were ADP-ribosylated by botulinum toxin C_3 with stoichiometries of 0.8 and 0.6, respectively. *Rho* and G_{22K} appeared to be different proteins since (i) *rho* migrated faster on polyacrylamide gels, (ii) unlike G_{22K}, *rho* did not require the presence of cytosol to be ADP-ribosylated, (iii) G_{22K} was not recognized by an anti-*rho* antiserum, and (iv) antibody 142-24E05 recognized G_{22K} effectively but only poorly cross reacted with *rho*. ADP-ribosylation had no effect on the ability of *rho* to bind or hydrolyse GTP. Therefore, it appears that there are multiple botulinum toxin C_3 substrates and that the toxin exerts its effects on cell function by a mechanism other than modulating the GTPase activity of *rho*.

GTP-binding protein; Gene, *ras*; Protein, Rho; Botulinum toxin; ATP-ribosylation

1. INTRODUCTION

A family of GTP-binding (G) proteins, with an $\alpha\beta\gamma$ structure, serve as transducers for hormone receptors at the plasma membrane. This family includes G_s and G_i, which regulate adenylate cyclase, G_o and G_i proteins that appear to regulate phospholipases and ion channels, and the transducins which couple light-activated rhodopsin to retinal cGMP phosphodiesterase [1,2]. A characteristic of these proteins is that their α-subunits are targets for bacterial toxin-catalysed ADP-ribosylation. $G_s\alpha$ is ADP-ribosylated by cholera toxin, $G_o\alpha$ and $G_i\alpha$ are ADP-ribosylated by pertussis toxin, and transducin α-subunits are targets for both toxins [1,2].

Another family of G-proteins exists that are monomers of approx. 21 kDa. These include the H-, K- and N-*ras* and the *ras*-related *rho, ral, rab* and *rap* gene products [3–7]. It has recently been reported that the botulinum toxin C_3 (BTx C_3), produced by *Clostridium botulinum* types C and D, ADP-ribosylates proteins of 20–26 kDa [8,9]. We have previously demonstrated that a 22 kDa botulinum toxin substrate (which we have termed G_{22K}), present in the tissues of species from several phyla, is a *ras*-related GTP-binding protein that is distinct from H-, K- and N-ras [10–12].

Injection of the active subunit of BTx C_3 into various cell types has been shown to induce changes in morphology and differentiation characteristic of p21-*ras* activation [13]. This suggests that the toxin activates process(es) regulated by *ras*-gene products. Further, treatment of neutrophils and platelets with a preparation of botulinum toxin D, contaminated with BTx C_3, resulted in potentiation of agonist-stimulated secretion [14]. Thus, *ras*-related BTx C_3 substrates may be involved in membrane trafficking and/or secretion in addition to their effects on proliferation and differentiation. It is anticipated that acute activation of *ras*-related GTP-binding protein(s) by BTx C_3-catalyzed ADP-ribosylation may provide a better understanding of the role of these proteins in cell function, as well as of p21-*ras* in tumorigenesis.

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We report here that bacterially expressed *Aplysia rho* is stoichiometrically ADP-ribosylated by BTx C_3. This ADP-ribosylation produces no effect on the guanine nucleotide binding capability or GTPase activity of the purified protein. Evidence is presented that *rho* is distinct from the neutrophil BTx C_3 substrate, G_{22K}. It seems likely that, analogous to pertussis toxin, there are multiple BTx C_3 substrates in eukaryotic cells.

2. MATERIALS AND METHODS

2.1. *ADP-ribosylation by botulinum C_3 toxin*

Samples (15 μl volume of protein ± cytosol) were incubated in a total volume of 50 μl with final concentrations of 100 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10 mM dithiothreitol, 1 mM ATP, 0.5 mM L-α-dimyristoylphosphatidylcholine, 10 mM thymidine, 2.5 mM $MgCl_2$, 2 or 4 mM NAD (5000–20000 cpm/pmol) and 0.5 μg/ml botulinum C_3 toxin. Incubations were for 120 min at 30°C, and were terminated by addition of Laemmli sample buffer [15]. For estimation of stoichiometry, incubations were terminated by addition of 1 ml of 1.0% SDS, 100 μg/ml bovine serum albumin, and 15% trichloroacetic acid. Precipitated proteins were vacuum filtered over BA85 nitrocellulose filters and washed 5 times with 2 ml of 6% trichloroacetic acid, then counted by liquid scintillography. Neutrophil cytosol was passed over a Sephadex G25 column to separate proteins from endogenous NAD.

2.2. *Immunoblotting*

Western blots were performed essentially as described by Towbin [16]. Anti-*ras* monoclonal antibodies Y13-259 [3] and 142-24E05 [17] were obtained from Oncogene Science, Inc., and the National Cancer Institute Repository, Microbiological Associates, respectively. Anti-*rho* antibody was prepared against the intact bacterially expressed *rho*, as described in [18].

2.3. *Proteins*

Bacterially expressed H-*ras* was a generous gift from Channing Der, La Jolla Cancer Research Foundation, La Jolla, CA. *Rho* was expressed and purified as in [18]. G_{22K} was purified as described in [12]. Botulinum C_3 toxin and anti-C_3 toxin antibody were prepared as previously described [19,20].

2.4. *GTPγS binding/GTPase assay*

Binding of [^{35}S]GTPγS was essentially as described in [21]. For estimation of GTP hydrolytic activity, *rho* was incubated for 60 min in the presence or absence of BTx C_3 under standard ADP-ribosylation conditions. 20 μl of the ribosylation reaction (10 pmol GTP-binding protein) was then incubated at 30°C for the indicated times in a reaction mix (50 μl final volume) containing 25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 150 mM NaCl, 3 mM L-α-dimyristoylphosphatidylcholine, 5 mM $MgCl_2$, and 1 μM GTP (1.5×10^5 cpm/tube). Reactions were terminated by the addition of 750 μl of ice-cold charcoal (5%, w/v) in 20 mM phosphoric acid. Tubes were kept on ice for 10 min prior to centrifugation (1 min, Beckman Microfuge E) at room temperature. The amount of $^{32}P_i$ released was then determined by counting a 0.5 ml aliquot of the clear supernatant.

2.5. *Miscellaneous*

Gels were run according to Laemmli [15] and were 15% polyacrylamide. Silver staining was as described [22]. Protein values were obtained by amido black assay [23]. All chemicals were of the highest grade available. The GTPase data have been presented in terms of pmol GTPγS binding, which is a measure of active G-protein. The stoichiometry of ADP-ribosylation of G_i by pertussis toxin was shown to correlate with the extent of guanine nucleotide binding by the protein [29] and denatured *rho* (100°C, 3 min or repeated freeze thawing) did not serve as a substrate for BTx C_3. These findings suggest that normalization to GTPγS binding is also valid for ADP-ribosylation. There were 0.2 and 0.5 pmol GTPγS bound/pmol *rho* and G_{22K}, respectively.

3. RESULTS

The *Aplysia rho* protein, purified from a bacterial expression system [18], is shown in fig.1, lane 2. The expressed protein has an apparent molecular mass of 20500 Da, similar to that reported for *rho* purified from bovine brain [24] and consistent with the molecular mass predicted by the cloned *Aplysia* gene [4]. Bacterially expressed H-*ras* (lane 1) and two separate preparations of human neutrophil G_{22K} protein (lanes 3,4) are shown for comparison. Both of these proteins migrate slower than *rho* on SDS-polyacrylamide gels, with apparent molecular masses of 22–23 kDa.

3.1. *ADP-ribosylation of* rho *by botulinum C_3 toxin*

We examined the ability of each of these *ras*-related proteins to serve as substrates for ADP-ribosylation by BTx C_3 toxin. G_{22K}, as previously reported [12], was able to serve as a BTx C_3 substrate in the presence but not in the absence of neutrophil cytosol (fig.2, lanes 6,7). Under these same conditions, bacterially expressed *Aplysia rho* protein (lane 4) was also ADP-ribosylated in the presence of toxin. Labeling of *rho* by BTx C_3 toxin was not dependent upon cytosolic factor, but the extent of ADP-ribosylation was somewhat enhanced in the presence of cytosol (lane 5). As presented in table 1, the enhancement of labeling by cytosol was typically much less than that shown in fig.2. In contrast to G_{22K} and *rho*, bacterially expressed H-*ras* did not serve as a toxin substrate, either in the presence or absence of cytosol (lanes

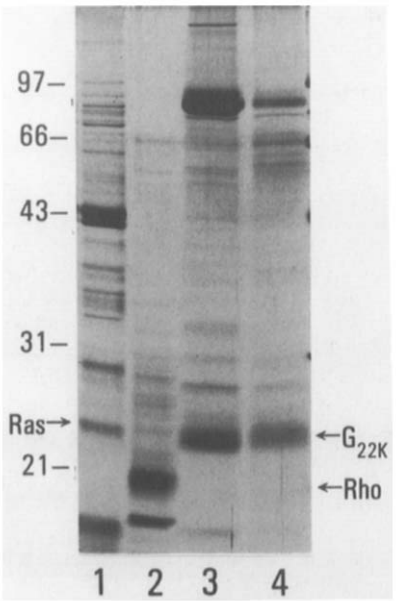

Fig.1. Silver stain visualization of low molecular mass GTP-binding proteins. Lanes: 1, 100 ng H-*ras*; 2, 100 ng *rho*; 3, 200 ng G_{22K} (Prep 1); 4, 100 ng G_{22K} (Prep 2).

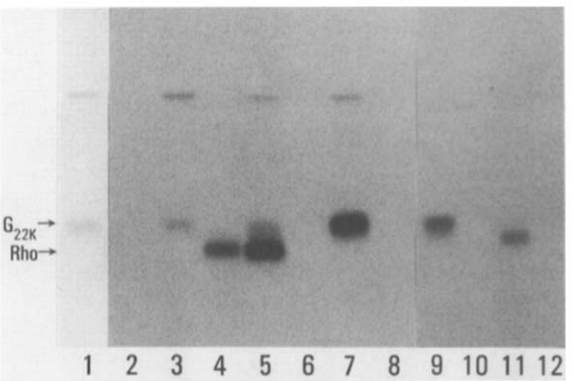

Fig.2. ADP-ribosylation of low molecular mass GTP-binding proteins by botulinum C_3 toxin. Samples were ADP-ribosylated in the presence of [^{32}P]NAD as described in section 2. Lanes: 1, cytosol alone; 2, 60 ng H-*ras*; 3, 60 ng H-*ras* + cytosol; 4, 30 ng *rho*; 5, 30 ng *rho* + cytosol; 6, 60 ng G_{22K}; 7, 60 ng G_{22K} + cytosol; 8, protein blank (toxin alone); 9, 60 ng G_{22K} + cytosol; 10, 60 ng G_{22K} + cytosol + 1:200 dilution of anti-C_3 antibody; 11, 30 ng *rho*; 12, 30 ng *rho* + 1:200 dilution of anti-C_3 antibody. (Note: lanes 9–12 are from a separate experiment than lanes 1–8).

2,3). We have also determined that other low molecular mass GTP-binding proteins, including bovine ARF [25] and G_{24K} or G_{26K} from human neutrophils [11,12], do not serve as BTx C_3 toxin substrates (not shown). The labeling of both *rho* and G_{22K} by BTx C_3 is specific for that component, as evidenced by the ability of an anti-C_3 toxin antibody to inhibit ADP-ribosylation of both proteins (fig.2, lanes 9–12).

The stoichiometry of the ADP-ribosylation of *rho* and G_{22K} by BTx C_3 was assessed, these data being shown in table 1. We were able to ADP-ribosylate *rho* to an extent of approx. 0.8 mol/mol of GTPγS binding both in the absence and presence of neutrophil cytosol. There thus appears to be a single site for ADP-ribosylation on *rho*. Ribosylation of G_{22K} to a level of 0.63 mol/mol of GTPγS binding was only achieved in the presence of neutrophil cytosol. In contrast, H-*ras* was not significantly ADP-ribosylated: values of less than 0.06 mol/mol of GTPγS binding were obtained.

3.2. *Immunological distinction between G_{22K} and* rho

In order to assess possible relationships between the neutrophil botulinum toxin substrate, G_{22K}, and *rho*, we examined the ability of several antibodies to cross-react with these proteins. A polyclonal antibody prepared against the bacterially expressed *Aplysia rho* protein clearly recognized *rho*, but not H-*ras* or G_{22K} (fig.3, lanes 1–3). In contrast, monoclonal antibody 142-24E05 recognized both G_{22K} and H-*ras* effectively, but only poorly cross-reacted with an equivalent amount of *rho* protein (fig.3, lanes 4–6). Monoclonal antibody Y13-259 only detected H-*ras* (fig.3, lanes

Table 1

Stoichiometry of ADP-ribosylation by botulinum toxin C_3

Protein	Cytosol	ADP-ribose incorporated/ GTPγS-binding site (mol/mol)[a]
G_{22K}	–	0.03 (0.03, 0.04)
	+	0.60 (0.66, 0.53)
Rho	–	0.73 (0.60, 0.86)
	+	0.82 (0.76, 0.87)
H-ras	–	0.02 (0.02, 0.02)
	+	0.06 (0.08, 0.04)

[a] Values are averages of two separate experiments, with samples assayed in duplicate. Values determined from each experiment are shown in parentheses. Buffer and cytosol blanks were subtracted in order to estimate incorporation into the proteins of interest

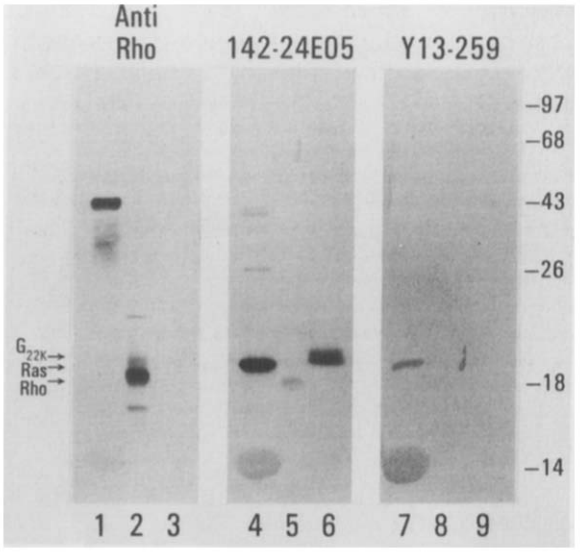

Fig.3. Recognition of G_{22K}, *rho*, and H-*ras* by various antibodies. (Lanes 1,4,7) 100 ng H-*ras*; (lanes 2,5,8) 100 ng *rho*; (lanes 3,6,9) 100 ng G_{22K}. Anti-*rho* antibody was used at 1:200 dilution, 142-24E05 at 1:500 dilution, and Y13-259 at 1:250 dilution. Blotting was performed as described in section 2. Molecular mass markers are indicated at the right of the figure.

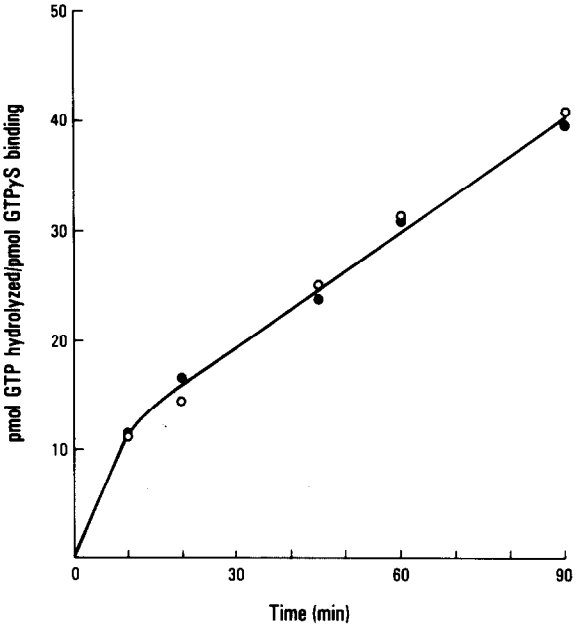

Fig.4. Time course of GTP hydrolysis by control and ADP-ribosylated *rho*. The GTPase activity of 10 pmol *rho* protein was determined following incubation in the presence (●) and absence (○) of BTx C_3 as indicated in section 2. The results shown are the mean of triplicate samples and are representative of three independent experiments. Standard deviations were less than 5%.

7–9). These proteins therefore appear to be immunologically distinct from each other. This is consistent with partial amino acid sequence information we have previously obtained from G_{22K} [12].

3.3. *Effect of ADP-ribosylation of* rho *on guanine nucleotide binding and hydrolysis*

We examined the effect of ADP-ribosylation of *rho* by BTx C_3 on the ability of *rho* to bind [^{35}S]GTPγS. The extent of binding was not significantly modified by ribosylation. An equilibrium value of 580 pmol/ml was obtained for non-ribosylated *rho* as compared to 520 pmol/ml (average of duplicate determinations, n = 2 experiments) for protein ribosylated to an extent of 0.86 mol/mol. The rate of [^{35}S]GTPγS binding to the ADP-ribosylated *rho* was not significantly different from that of the non-ribosylated protein (not shown). The rate of hydrolysis of GTP by *rho* was approx. 0.5 pmol/pmol GTPγS binding per min. This activity was not altered by prior ADP-ribosylation of *rho* (fig.4).

4. DISCUSSION

We have used a bacterially expressed *Aplysia rho* protein to investigate the possibility that *rho* is a substrate for BTx C_3 and to compare the properties of this GTP-binding protein with those of G_{22K}, purified from human neutrophils. *Aplysia rho* was indeed ADP-ribosylated by BTx C_3 with a stoichiometry close to unity, suggesting that there is a single site for ribosylation on the protein. The difference in mobility of *rho* and G_{22K} on polyacrylamide gels and the lack of an absolute requirement for cytosol to ADP-ribosylate *rho* suggested that G_{22K} and *rho* were distinct proteins. An additional indication that the neutrophil G_{22K} botulinum toxin substrate and *rho* are not the same protein comes from the observation that *rho* was not as immunoreactive as G_{22K} or H-*ras* with the 142-24E05 antibody and G_{22K} was not recognized by the anti-*rho* polyclonal antiserum. This lack of recognition by the anti-*rho* antibody seems unlikely to result from species differences in *rho*, as the

antibody was prepared against the whole *Aplysia rho* protein, which has an overall homology of ~85% with the human forms of *rho* [4], although we cannot rule out this possibility.

Injection of BTx C_3 into several cell types has been reported to cause effects similar to the introduction of activated (oncogenic) forms of *ras*. Activated forms of *ras* exist in the GTP bound state and frequently possess a decreased ability to hydrolyze GTP [3]. ADP-ribosylation of $G_s\alpha$ by cholera toxin results in inhibition of its intrinsic GTPase activity, maintaining the protein in a state able to cause persistent activation of adenylate cyclase [1]. However, near stoichiometric ADP-ribosylation of *rho* did not affect its GTP-binding or GTPase activity. Other mechanisms therefore need to be considered for botulinum C_3 toxin-mediated effects on cell function. It is possible that the interaction of *rho* with some effector molecule(s) might be modified by ADP-ribosylation, without inhibition of its intrinsic GTPase activity. Some transforming mutants of p21 *ras* have similar GTPase activity to normal *ras* in vitro but still cause cellular transformation [26]. However, it has recently been found that the GTPase activities of these mutants is much lower in vivo than normal p21 because they are resistant to stimulation by the GTPase-activating protein, GAP, the putative effector of *ras* [27]. ADP-ribosylation of *rho* might similarly affect its interaction with a GAP-like regulator of its GTPase activity in vivo.

During the preparation of this manuscript, it was reported by Kikuchi et al. [28] that *rho* protein, purified from bovine brain, also served as a substrate for a botulinum toxin. Our data have confirmed their finding that ADP-ribosylation does not attenuate the GTPase activity of bovine *rho*. Kikuchi et al. used a preparation of botulinum toxin C_1, presumably contaminated with the C_3 component that is expressed by the C and D strains of *Clostridium botulinum*. We demonstrate here that the C_3 toxin is responsible for ADP-ribosylation of both *rho* and G_{22K} by using a purified toxin preparation and a C_3-specific antiserum.

Thus, *rho* protein is a substrate for BTx C_3 but there are additional substrates present in human neutrophils (G_{22K}), and possibly in other tissues. There are at least three human *rho* genes [4], the products of which could each be BTx C_3 substrates. In addition, an amino acid sequence homologous to *rap1* [7] has been obtained from a preparation of G_{22K} [12], suggesting that it may also be ADP-ribosylated by botulinum toxin. This situation is analogous to pertussis toxin, which ADP-ribosylates the three G_i proteins as well as G_o and transducin. The total number and identity of BTx C_3 substrates remains to be determined.

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