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The mammalian G protein *rhoC* is ADP-ribosylated by *Clostridium botulinum* exoenzyme C3 and affects actin microfilaments in Vero cells

P.Chardin, P.Boquet[1], P.Madaule[2], M.R.Popoff[3], E.J.Rubin[4] and D.M.Gill[4]

Institut National de la Santé et de la Recherche Médicale U-248, Faculté de Médecine Lariboisière Saint-Louis, 10, Avenue de Verdun, 75010 Paris, [1]Unité des Antigènes Bactériens, Institut Pasteur, 28 rue du Dr Roux, 75724 Paris, [2]Laboratoire de Physiologie Nerveuse, CNRS, 91198 Gif-sur-Yvette, [3]Unité des Anaérobies, Institut Pasteur, 25 rue de Dr Roux, 75015 Paris, France and [4]Department of Molecular Biology and Microbiology, Tufts University, 136 Harrison Avenue, Boston, MA 02111, USA

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***Clostridium botulinum* C3 is a recently discovered exoenzyme that ADP-ribosylates a eukaryotic GTP-binding protein of the *ras* superfamily. We show now that the bacterially-expressed product of the human *rhoC* gene is ADP-ribosylated by C3 and corresponds in size, charge and behavior to the dominant C3 substrate of eukaryotic cells. C3 treatment of Vero cells results in the disappearance of microfilaments and in actinomorphic shape changes without any apparent direct effect upon actin. Thus the ADP-ribosylation of a rho protein seems to be responsible for microfilament disassembly and we infer that the unmodified form of a rho protein may be involved in cytoskeletal control.**

Key words: actin/ADP-ribosylation/C3/*Clostridium botulinum*/G protein

Introduction

Bacterial enzymes that ADP-ribosylate proteins have proven invaluable for studying the functions of their eukaryotic targets, particularly of G proteins. Exoenzyme C3 is a newly described ADP-ribosyl transferase secreted by C and D strains of *Clostridium botulinum* but distinct from the classical C1 and D neurotoxins. In many eukaryotic cells, C3 has a single protein substrate which is found mostly in the soluble fraction (Aktories *et al.*, 1988; Rubin *et al.*, 1988). The size of this substrate, ~21 000 Mr, and its interaction with GTP led us to suggest that it is a member of the *ras* superfamily of proteins (Rubin *et al.*, 1988). C3-catalysed ADP-ribosylation causes maturational changes in *Xenopus* oocytes and distinct morphological and physiological changes in cultured cells (Rubin *et al.*, 1988) which partially overlap those caused by microinjected activated *ras* protein. The C3 substrate is, however, distinct from $p21^{ras}$ itself. We show now that the bacterially-expressed *rhoC* gene product of human origin is ADP-ribosylated by C3 and coincides with the eukaryotic substrate. The *rho* genes represent a distinct and evolutionarily conserved branch of the *ras* superfamily showing a 30% sequence identity to the classical ras proteins (Madaule and Axel, 1985; Madaule *et al.*, 1987) and a slow GTPase activity (Anderson and Lacal, 1987). At least three *rho* genes have been found in humans, named *rhoA* (formerly clone 1 or 12) (Yeramian *et al.*, 1987), *rhoB* (clone 6) and *rhoC* (clone 9) (Chardin *et al.*, 1988). Hitherto their functions have not been well defined but now some information can be inferred from the effects of ADP-ribosylation. C3 treatment of Vero cells results in the disappearance of actin filaments which suggests that the *rho* product may control microfilament assembly. The change in actin status presumably accounts for some morphological consequences of C3 action.

Results

ADP-ribosylation of rhoC protein

The human *rhoC* cDNA was subcloned in the bacterial expression vector ptac (de Boer *et al.*, 1983) and the *rhoC* protein was expressed in *Escherichia coli* strain JM109 upon the addition of isopropyl-β-D-thiogalactoside (IPTG), as described in Materials and methods. There was too little product to detect directly by gel staining but the presumptive *rhoC* protein was detected by [^{32}P]GTP binding after electrophoretic transfer of proteins to nitrocellulose. The rho protein was readily ADP-ribosylated when a bacterial extract was incubated with [^{32}P]NAD and C3 (Figure 1). At an elevated NAD concentration (100 μM) the amount of ADP-ribosylated *rhoC* protein reached a stable plateau with time of incubation or C3 concentration. The limit was typically ~0.2 pmol/μl (4 ng/μl) of bacterial extract. Two orders of magnitude less *rhoC* protein was available for reaction in uninduced cells. The ADP-ribosylated product had the same electrophoretic mobility as the main C3 product of human cells; the apparent molecular mass of both was ~21 000 (Figure 1). Two-dimensional gel analysis showed that the bacterial and human cell products also had the same charge (pI 5.6). A less intense spot of the same charge (pI 5.6) but of a larger apparent size (Mr 23 000) is present in both cases (Figure 2) and provides additional evidence of identity. It runs closer to the anticipated position of ADP-ribosylated rhoC (Mr ~22 500). Having the same charge the two forms cannot be related by proteolysis and may simply represent conformational isomers. Partial proteolysis by the method of Cleveland *et al.* (1977) generated the same series of radioactive peptides from each of the apparent size variants (not shown).

Homogenized mouse and calf tissues, including thymus, muscle, lung, heart, kidney and spleen, gave patterns similar to that of the HeLa cells in Figure 2.

C3 is highly selective in its choice of protein substrate. No specific C3 substrate was detected in the parental *E.coli* strains JM109 or HB101. Likewise, there was no ADP-ribosylatable substrate in extracts of *E.coli* cells that expressed other members of the *ras*-related family of proteins (*c-H-ras*, *rap1A*, *rap2*, *ral*, *rab2*, *rab4* and *rab5*) (Chardin and Tavitian, 1986; Touchot *et al.*, 1987, expression by P.Chardin, V.Pizon, N.Touchot, and A.Zahraoui, in

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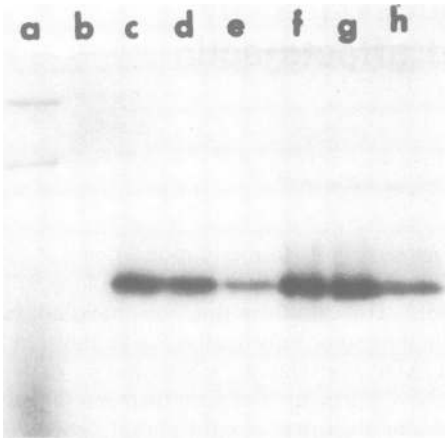

Fig. 1. [^{32}P]ADP-ribosylation of *rhoC* expressed in *E.coli* and in HeLa cells. Incubation conditions are described in Materials and methods. 10 μl of each product, representing 10^8 bacteria or 2×10^5 HeLa cells, was analysed by SDS–polyacrylamide gel electrophoresis and autoradiography (5 h with no screen). **Lane a**, which is a negative control, represents an extract of *E.coli* that expressed *rab4*, a different *ras*-related gene (P.Chardin, V.Pizon, N.Touchot and A.Zahraoui, in preparation). **Lanes b–e** represent extract of JM109 transformed with ptac-*rhoC*. **b**: incubated without C3. **c**: control (with C3), the major product has an apparent Mr of 21 000. **d**: plus 10 mM EGTA. **e**: plus 10 mM EDTA. **f**: HeLa cell extract, control. **g**: HeLa plus 10 mM EGTA. **h**: HeLa plus 10 mM EDTA.

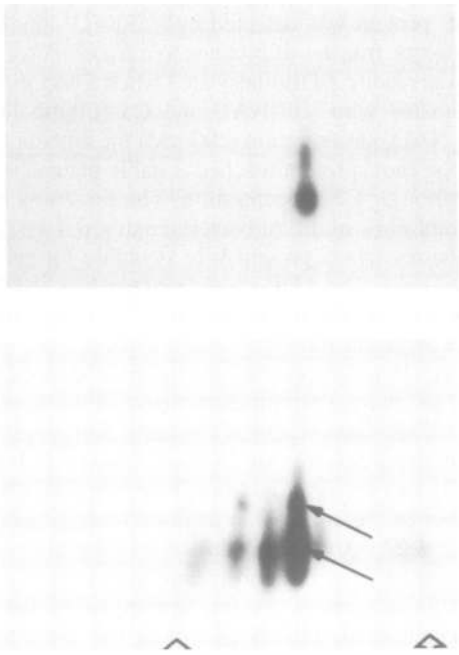

Fig. 2. Two-dimensional polyacrylamide gel analysis of the [^{32}P]-ADP-ribosylated products of HeLa cell extract (above) and *E.coli* JM109 ptac-*rhoC* extract (below) as in Figure 1. The two radioactive HeLa cell spots align precisely with the bacterial spots indicated by slender arrows and have apparent molecular weights of 23 000 and 21 000. The broad arrow heads indicate the horizontal migration of (left) β-lactoglobulin (pI 5.1) and (right) human carbonic anhydrase (pI 6.5).

preparation). We show this for the ptac-rab4 clone in Figure 1, lane a. Gp, a *ras*-related protein purified from placenta (Evans *et al.*, 1986), was not a substrate. As we now expect, the [^{32}P]ADP-ribosylated product of mammalian cells was not precipitated by antisera against various small G proteins, namely Gp (antibody given by John Northup), *SEC4* (Goud *et al.*, 1988; antibody given by Peter Novick), *YPT1* (Segev *et al.*, 1988, antibody given by Nava Segev) or ARF (Kahn *et al.*, 1988, antibody given by Rick Kahn, and also prepared by ourselves).

The *rhoC* protein is susceptible to bacterial proteolysis: several fragments of $>18\,500$, $<21\,000$ Mr are ADP-ribosylated and these differ from the starting material in charge (pI 5.2–5.5) as well as in size (Figure 2). Proteolysis was more severe when the protein was synthesized in bacterial strain HB101 than in JM109. Such fragmentation was not evident in the HeLa cell product, nor was there evidence that the HeLa cells performed any other kind of processing, such as the fatty acylation by which some other proteins of the *ras* family are modified.

Magnesium is required for the ADP-ribosylation

ADP-ribosylation of both the cellular and the cloned forms of *rhoC* protein was inhibited substantially by EDTA but much less by EGTA (Figure 1) suggesting that magnesium is required. One possible interpretation is that the *rhoC* product binds magnesium and that magnesium supports a protein conformation which is suitable for ADP-ribosylation. It is known that magnesium is involved in the binding of guanyl nucleotides to the related protein p21^{ras}. Guanine nucleotides stabilize the rhoC protein against denaturation and prevent loss of ADP-ribosylatable substrate at 45°C for example (Rubin *et al.*, 1988) but we have not encountered any condition in which guanine nucleotides affect the ADP-ribosylation rate *per se*.

Action of C3 upon Vero cells

In cell lines tested previously, C3 altered the shapes of intact cells only if it was inserted into the cells by osmotic lysis of pinosomes or by microinjection (Rubin *et al.*, 1988). We have since found, however, that Vero cells respond directly to C3 added to the culture medium. As was found for NIH3T3 and PC12 cells, the effect on Vero cells is dose-dependent and non-lethal. At the dose used in Figure 3 (5 μg/ml) the Vero cells start changing shape within 30 min of the addition of C3 and gradually contract over the next 12–24 h. The cell bodies condense except for beaded processes which span the area previously covered by the flat cell (Figure 3f). Figure 3, panels b and c, show a field in which the cells are contracted to various degrees (compare to panel a): it is clear that cells in various stages of contraction still contain microtubules (Figure 3b) which are reorganized only as a consequence of the shape change. However, actinomorphic cells can no longer be stained by the fluorescent reagent NBD-phallacidin which is specific for filamentous actin (Figure 3e). The result implies that the cells no longer contain actin microfilaments and that the disassembly of microfilaments accounts for the shape changes. Cells regain their normal morphology and growth rate a few days after the removal of C3 from the bathing medium.

Vero cells treated with C3 were lysed and portions of the lysate were [^{32}P]ADP-ribosylated. The exposure to C3 substantially reduced the amount of rho available for ADP-ribosylation by C3 *in vitro* (Table I). The available actin, in contrast, was changed little–sometimes slightly increased

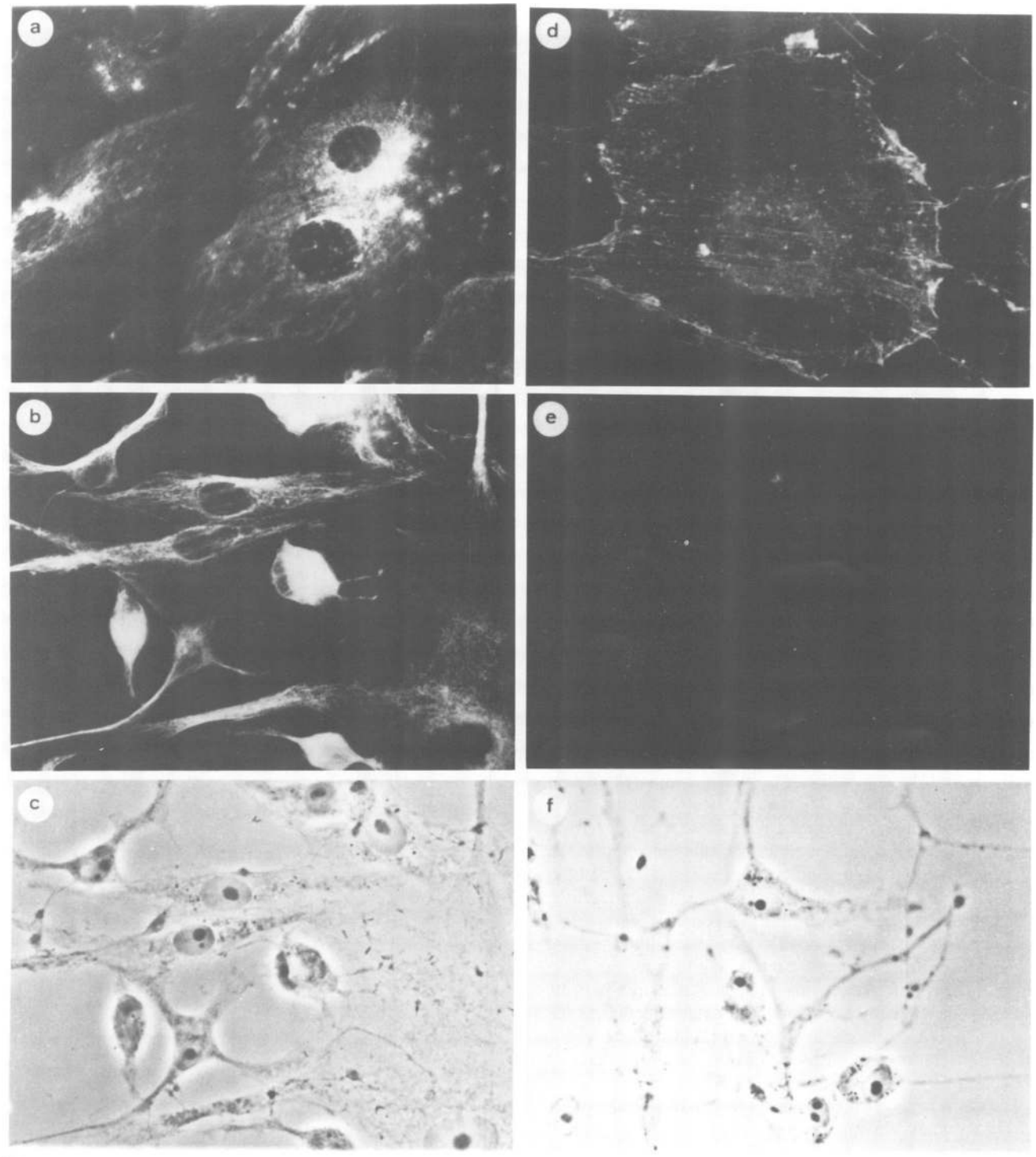

Fig. 3. C3 disrupts actin microfilaments but not microtubules. Vero cells treated with 5 μg/ml purified exoenzyme C3 for 15 h were stained for tubulin and filamentous actin as described in Materials and methods. (**a**) Control cells stained for tubulin: fluorescence microscopy. (**b**) C3-treated cells stained for tubulin: fluorescence. (**c**) Same cells as **b**: phase contrast. (**d**) Control cells stained with NBD phallacidin for actin filaments: fluorescence. (**e**) C3-treated cells stained with NBD phallacidin: fluorescence. (**f**) Same cells as **e**: phase contrast.

or sometimes (as in Table I) slightly decreased. C2-treated cells which change shape have little or no available actin (Ohishi and Tsuyami, 1986).

Discussion

We have shown that a major C3 target protein in many mammalian cells, which we have provisionally named p21.bot, is the product of the *rhoC* gene. Interestingly, *Saccharomyces cerevisiae* extract contains a C3 substrate of 23 500 Mr, the size expected for the *rho1* protein, 209 residues, which has about a 70% sequence similarity to the mammalian *rho* proteins. *Dictyostelium* also has a C3 substrate of ~21 kd. These findings lead to the expectation that the *rho* proteins may be ADP-ribosylated in a highly

Table I. Reduction in the amount of rho available for ADP-ribosylation *in vitro*

	Treatment of intact cells	
	Control	C3
	ADP-ribose incorporated *in vitro*, pmol/mg protein	
Actin	59	54
Rho	1.9	0.6

Vero cell monolayers were exposed to 0 or 5 μg/ml C3 for 24 h, then washed in fresh medium. Cells were scraped from the plates, washed in 130 mM NaCl, 10 mM Hepes, pH 7.4, and collected by centrifugation. Portions of the cells were lysed by detergent and ADP-ribosylated with C3 (for *rho*) or with *C.difficile* transferase for actin at 37°C for 30 min. The *C.difficile* enzyme ADP-ribosylates actin directly with the same specificity as C2 component I (Popoff *et al.*, 1988). Incubation conditions were: 10 μl volume, 7.5×10^4 cells, 10 mM Tris, pH 8.0, 0.07% Triton X-100, either C3 (40 μg/ml) or *C.difficile* transferase (50 μg/ml) and 5 μM [^{32}P]NAD (26 000 c.p.m./pmol). The cells also provided ~7 μM unlabeled NAD. These conditions were expected to be saturating (Rubin *et al.*, 1988) and this was confirmed by finding the same degree of ADP-ribosylation after 10 min as after 30 min of incubation. The ADP-ribosylated proteins were identified by gel electrophoresis and autoradiography and were quantitated by counting appropriate portions of the gels.

conserved region and therefore that the very similar *rhoA* and *rhoB* proteins are likely to be substrates as well as *rhoC*. Indeed two-dimensional gel patterns of the ADP-ribosylation products of certain tissues (brain for example) do contain spots of the size and charge expected for *rhoA* and *rhoB* in addition to *rhoC*. Thus it is possible that C3 modifies *rho* proteins in general and we will now refer to the substrate generically as *rho*.

Hitherto, most information relevant to the function of *rho* concerned the *S.cerevisiae rho1* gene product. Disruption of *rho1* results in loss of viability while a dominant mutation in *rho1* renders cells unable to sporulate (Madaule *et al.*, 1987). Our identification of the C3 substrate provides further insights into the biological role of *rho*. C3 delivered to the cytosol by osmotic lysis of pinosomes caused NIH 3T3 cells to assume an actinomorphic morphology (Rubin *et al.*, 1988). We describe here a similar response of Vero cells, but in this case the cells respond to C3 in the culture medium without any special procedure to allow it to enter the cells. It presumably enters the cytosol unaided at a slow rate. Its effect inside the cells is evident both as a reduction in the amount of *rho* protein available for subsequent [^{32}P]-ADP-ribosylation and as a collapse of the microfilament network.

The C3 was secreted by bacteria which also produce C2, a binary toxin consisting of component II required for cell entry and component I which efficiently ADP-ribosylates actin. Together they cause microfilament breakdown in intact cells. Thus it was important to show that the microfilament disassembly shown in Figure 3 was truly caused by C3 and not by C2. There are three relevant observations. Firstly, the C3 used is electrophoretically pure and we can detect no trace of actin ADP-ribosyl transferase activity. Secondly, whereas C2-II is activated by trypsin treatment, the effect of C3 on Vero cells does not depend on, nor is even increased by, prior trypsinization of the enzyme preparation. Nor is it potentiated by native or trypsin-treated C2-II. Thirdly, affected cells have experienced a reduction in available *rho* protein but have suffered no significant change in available actin. Thus we conclude that C3 is truly responsible for the effects seen. Apparently *C.botulinum* secretes two ADP-ribosyl transferases (exoenzyme C3 and C2 component I) which promote microfilament disassembly in different ways. It should be noted that actin is also ADP-ribosylated by several clostridial enzymes that are distinct from C2 toxin. Such enzymes include the iota toxin of *C.perfringens* type E (Vandekerkhove *et al.*, 1987), *C.spiroforme* toxin (Popoff and Boquet, 1988) and an ADP-ribosyl transferase from a strain of *C.difficile* (Popoff *et al.*, 1988). Furthermore, toxin B of *C.difficile* causes actin filaments to break down by yet another, at present unknown, mechanism (Wedel *et al.*, 1983; Mitchell *et al.*, 1987). Clearly microfilaments are a common target for clostridia.

Since C3 does not appear to affect actin directly, the breakdown of filaments is probably a consequence of ADP-ribosylation of *rho* protein. It is likely, therefore, that *rho* is directly or indirectly involved with microfilament assembly or disassembly. Microfilaments can be disassembled by increasing intracellular calcium or by decreasing intracellular phosphatidylinositol-4,5-bis phosphate (PIP_2) (Janmey and Stossel, 1987). However, PC12 cells had normal calcium levels and experienced a normal increase in intracellular calcium in response to bradykinin even after all of their available *rho* had been ADP-ribosylated by C3 (not shown). This argues against a role for *rho* in the PIP_2-IP3 pathway. Two actin-associated proteins of the approximate size of *rho* were described recently (Shapland *et al.*, 1988) but they appear to have a considerably more basic charge than the C3 substrate. In fact, we have estimated the amount of ADP-ribosylatable rho protein in tissues at 0.01% of the total protein (Rubin *et al.*, 1988) and if this amount truly reflects the total amount of this protein in the cells, rho is much less abundant than other actin-binding proteins and may not be detected easily in actin association. Alternatively, it may act through an intermediate such as a severing protein.

In addition to the actinomorphic changes, C3 has other effects on cells and we may consider whether these depend on the disassembly of microfilaments. Certainly a microfilament defect which blocked cytokinesis could adequately explain the high frequency of binucleate cells seen in cultures treated with C3 (Rubin *et al.*, 1988). Microfilament changes might also be involved in the migration of germinal vesicles which is the principal effect of C3 when injected into *Xenopus* oocytes (Rubin *et al.*, 1988). It is less obvious how microfilament changes might relate to the C3 response of PC12 cells. Shortly after C3 administration, PC12 cells sprout small processes that resemble the initial neurites formed in the presence of nerve growth factor (Rubin *et al.*, 1989). Of course it is possible that the individual rho proteins, and certainly the group of rho proteins, may have several effects of which cytoskeletal control is only one. Thus the phenotypes of yeast underexpressing *rho1* or producing a hyperactive form of *rho1* protein overlap, but only partially, the phenotype observed with actin mutants (Novick and Botstein, 1985). Pleiotropic effects have been described for YPT1 (Schmitt *et al.*, 1986, 1988; Segev and Botstein, 1987) and are well known for $p21^{ras}$.

Some confusion has arisen in the literature because C3 is a minor contaminant of certain preparations of the C1 and D botulinal neurotoxins and mixtures of C3 with a neurotoxin both catalyse ADP-ribosylation and block neurosecretion (Ohashi and Narumiya, 1987; Kurihara and Kubo, 1987; Adam-Vizi and Knight, 1987; Kikuchi *et al.*, 1988). Pure

C1 or D neurotoxins do not, however, catalyse ADP-ribosylation (Rosener *et al.*, 1987; Rubin *et al.*, 1988) and pure C3 does not affect secretion (Rubin *et al.*, 1989; Adam-Vizi *et al.*, 1988).

C3 is the latest addition to the list of bacterial ADP-ribosyl transferases which have GTP binding proteins as substrates. Other examples include diphtheria toxin, cholera toxin and pertussis toxin which have been particularly useful reagents for investigating the biology of elongation factor 2, Gs and Gi, respectively. It may be anticipated likewise that C3 will continue to be of value in the elucidation of the role of rho proteins.

Materials and methods

Construction of ptac-rhoC

Human cDNA encoding *rhoC*, homologous to human *rhoA* (Yeramian *et al.*, 1987) and *Aplysia rho* (Madaule and Axel, 1985) was described by Chardin *et al.* (1988). The cloned cDNA is an *Eco*RI fragment containing ~20 bp of 5'-non-coding region, 579 bp of coding sequence and ~600 bp of 3'-non-coding sequence that includes a *Pst*I site ~100 bp before the poly(A) site. The initiation codon for *rhoC* is part of a *Nco*I site (see Figure 4) which was convenient for subcloning into the bacterial expression vector ptac (de Boer *et al.*, 1983) which has been used successfully to express high levels of $p21^{cHras}$ (Tucker *et al.*, 1986). *RhoC* cDNA was cut with *Nco*I and 5' ends were filled using the Klenow fragment of DNA polymerase I. The 3' end was then trimmed with *Pst*I. The resultant fragment (~1 kb) was subcloned into double-stranded M13 mp11 that had been similarly treated with *Eco*RI, Klenow fragment and *Pst*I. Single-stranded M13 phage DNA was prepared, its sequence was checked, and then hybridized with the oligonucleotide 5'-CAGTGAATTCTATGGCTGCA-3' which was designed to introduce a T residue immediately before the initiating ATG in order to increase expression. Plaques of mutated M13 were screened with kinase-labeled mutagenesis oligonucleotide under stringent conditions (hybridization in 5 × Denhardt's solution, 5 × SSPE, 0.1% SDS at 53°C, final wash in 1 × SSC, 0.1% SDS at 55°C for 15 min). The sequence of probe-positive M13 phages were checked and double-stranded phages were prepared. An *Eco*RI–*Pst*I insert possessing the expected sequence GAATTCTATG was then subcloned into ptac-ral partially digested by *Eco*RI and completely by *Pst*I (the large fragment retaining the tac promoter was recovered by electro-elution). The ptac-ral expression vector includes a 3' *Pst*I site. It was derived from ptac-*cHras* (Tucker *et al.*, 1986) and originally from ptac (de Boer *et al.*, 1983).

Expression of rhoC protein

E.coli strain JM109 (Yanisch-Perron *et al.*, 1985) was transformed with ptac-*rhoC*. An overnight culture was induced with 0.1 mM IPTG. After 10 h of incubation, the cells were washed and lysed with lysozyme exactly as described by Tucker *et al.* (1986). Cell debris was removed by centrifugation.

Exoenzyme C3

The enzyme was purified from the culture supernatant of *C.botulinum* strain 1873-D as described by Rubin *et al.* (1988). The product consists of a single electrophoretic species (Mr 26 000) and exhibits no detectable actin ADP-ribosyltransferase activity.

Preparation of cell and tissue extracts

Pelleted HeLa cells were lysed by freezing and thawing then incubated at 37°C for 5 min to allow some hydrolysis of endogenous NAD. The lysate was extracted with one packed cell volume of 50 mM Hepes buffer, pH 8, and a soluble fraction was prepared by centrifugation. Homogenates of mouse and calf organs were prepared in five vols of 0.32 M sucrose, 0.1 mM phenylmethylsulphonyl fluoride (PMSF), and were centrifuged at 100 000 *g* for 1 h to prepare soluble fractions.

[^{32}P]ADP-ribosylation and gel analysis

Soluble bacterial or mammalian tissue samples, 1 μl, were diluted with 10 μl of 50 mM Hepes buffer pH 8.0, 2.5 μM [^{32}P]NAD (~20 000 d.p.m./pmol) and 3 μg/ml of C3 and incubated for 30 min at 37°C. For extracts of mammalian cells, 10 mM thymidine was also included to suppress poly(ADP-ribose) formation. One-dimensional gel electrophoresis (using 12.5% polyacrylamide) was performed as described by Laemmli (1970).

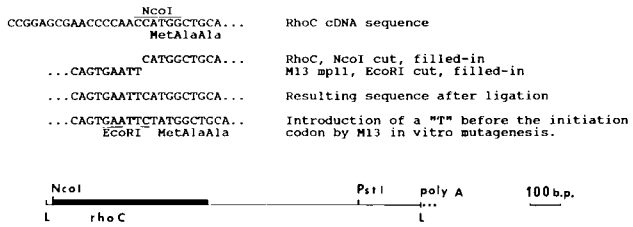

Fig. 4. Principal stages in the construction of ptac-*rhoC*, involving the insertion of a single T residue before the initiating AUG and the creation of an *Eco*RI site. Details are given in Materials and methods. Below is a sketch of the human *rhoC* DNA. L indicates the *Eco*RI linkers at each end of the cDNA. The heavy line indicates coding sequence. A classical AATAAA polyadenylation signal sequence is located 13 bp before the poly(A) tail.

For two-dimensional gel analyses (O'Farrell, 1975, as modified by Ames and Nikaido, 1976) the isoelectric focusing gels contained pH 3.5–10 ampholines (LKB) and the second dimension gels contained 12.5% acrylamide.

Vero cells

Cells were grown as monolayers in Dulbecco's-modified Eagle's medium (DMEM) plus 10% new-born calf serum. Cells to be stained were grown on coverslips. After exposure to C3 they were fixed for 20 min with formalin (3.7%) in phosphate-buffered saline (PBS). They were permeabilized with 0.1% Triton X-100 and covered with 100 μl of either diluted mouse monoclonal anti-tubulin (Amersham) or N-(7-nitrobenz-2-oxa-1,3-diazo-4-yl)phallacidin (NBD phallacidin: Molecular Probes, Eugene, OR) and incubated 20 min at room temperature. After four washes of 5 min each with PBS containing 0.2% gelatin, the cells exposed to anti-tubulin were incubated for 20 min at room temperature with rhodamine-labeled anti-mouse IgG (Sigma). All cells were washed as before, mounted in glycerol, and photographed using a fluorescence microscope.

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