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THE rho GENE PRODUCT EXPRESSED IN E.COLI IS A SUBSTRATE OF BOTULINUM ADP-RIBOSYLTRANSFERASE C3

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SUMMARY: The ras-related rho A protein expressed in E. coli, was ADP-ribosylated by botulinum ADP-ribosyltransferase C3. C3 also modified the valine-14 mutant rho protein but not the products of H-ras, R-ras, ral, ypt, and rap 1 genes. A ras-rho chimaera consisting of 60 amino acids from the amino terminus of ras fused to 133 amino acids from the carboxy terminus of rho was not modified by C3. Antibodies raised against the porcine brain cytosolic substrate of C3 cross reacted with the rho, valine-14 rho and ras-rho proteins, but not with the gene products of H-ras, R-ras, ral or rap 1. Polyclonal anti-H-ras antibodies cross reacted with H-ras but not with ral, rho, or the C3 substrate purified from porcine brain.

Botulinum ADP-ribosyltransferase C3 is an exoenzyme produced by Cl. botulinum Type C and D (1, 2, 3). This enzyme is distinct from the actin-ADP-ribosylating botulinum C2 toxin (1) and is also structurally and functionally unrelated to botulinum neurotoxins C1 and D (4, 5). The ADP-ribosyltransferase activity of botulinum neurotoxins C1 and D is most probably due to contamination with C3. Botulinum ADP-ribosyltransferase C3 has been shown to modify 21-24 kDa proteins in all eukaryotic cells studied so far (1-6). ADP-ribosylation by C3 is regulated by divalent cations and by guanine nucleotides suggesting that the C3 substrate is a GTP-binding protein (2, 4, 6). Recently, it has been shown that C3 modifies a GTP-binding protein purified from porcine brain (7) homologous to or identical with the rho gene

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product. Here we report that C3 ADP-ribosylates the rho A gene product expressed in E. coli.

MATERIALS AND METHODS

Materials. Botulinum ADP-ribosyltransferase C3 was purified as described (2). [^{32}P]NAD was obtained from NEN (Dreieich, FRG). Platelet membranes were prepared as described (8). The substrate of C3 was purified from porcine brain cytosol by acetone precipitation, CM-Sephadex, Octyl-Sepharose and TSK Phenyl-5 PW HPLC chromatography to apparent homogeneity. The purification will be described in detail elsewhere (7).

Preparation of rho A, valine-14 rho, ras-rho- and R-ras proteins were as described (9).

The H-ras and ral proteins were kindly donated by Dr. A. Wittinghofer (Heidelberg, FRG). ypt Protein was a gift of Dr. D. Gallwitz (Göttingen, FRG) and rap 1 protein was donated by Dr. P. Chardin (Paris).

ADP-ribosylation assay. ADP-ribosylation was performed as described (1, 2, 7) in a medium containing 50 mM triethanolamine-HCl (pH 7.5), 1 mM DTT, 0.5 mM ATP, 1 mM GDP, 1 mM $MgCl_2$, 0.5 µM [^{32}P]NAD (about 0.6 µCi/tube), 0.3 µg C3 and 5 to 10 µg of the various tested GTP-binding proteins or about 200 µg of platelet membrane preparations. Incubation was in a total volume of 100 µl for 30 min at 37° C. Labelled proteins were analysed by SDS-polyacrylamide (15%) gel electrophoresis according to Laemmli (10).

Immunoblotting was performed according to Towbin et al. (11) with rabbit anti-C3 serum (1:250) or rabbit anti-H-ras serum (1:500), peroxidase-coupled swine IgG to rabbit IgG (Dakopatts) as second antibody and 4-chloro-1-naphtol and H_2O_2 as peroxidase substrate.

RESULTS AND DISCUSSION

Botulinum ADP-ribosyltransferase C3 modifies a cytosolic protein from porcine brain, which by partial amino acid analysis showed high homolgy with the rho gene product (7). Similarly, in rat adrenal gland an ADP-ribosyltransferase found in botulinum neurotoxin D preparations, which is most probably identical with C3 (4), ADP-ribosylates a GTP-binding protein related to or identical with rho gene products (12, 13). Therefore, we studied whether the rho protein expressed in E. coli was ADP-ribosylated by C3. Fig. 1 shows an autoradiogram of a SDS polyacrylamide gel electrophoresis of proteins incubated in the presence of [^{32}P]NAD and botulinum ADP-ribosyltransferase C3. Lane 1 shows the labelling of human platelet membrane proteins (about 22 kDa) by botulinum ADP-ribosyltransferase C3 . The

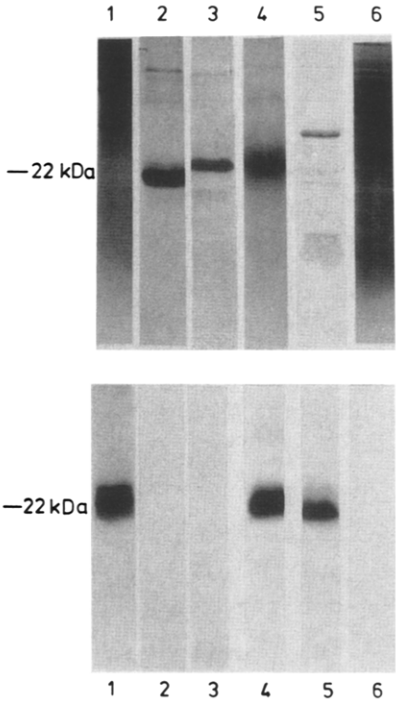

Fig. 1: ADP-ribosylation of 22 kDa proteins by botulinum ADP-ribosyltransferase C3. Platelet membranes (200 µg, lane 1); H-ras protein (5 µg, lane 2); ral protein (3 µg, lane 3); purified C3-substrate from brain cytosol (2 µg, lane 4); partially purified rho protein (10 µg, lane 5), the major band on the SDS gel is a contaminant, the rho A protein expressed in E. coli is clipped at the C terminus and runs as a smear at around 21 kDa; and sonificated E. coli extract (200 µg, lane 6) were ADP-ribosylated by 0.3 µg C3 in the presence of 0.5 µM [^{32}PNAD]. Samples were subjected to SDS PAGE and ^{32}P-ADP-ribosylated proteins were analyzed by autoradiography (shown).

^{32}P-ADP-ribosylated C3-substrate purified from brain cytosol is shown in lane 4. As depicted in lane 5, C3 ^{32}P-ADP-ribosylated the partially purified rho protein expressed in E.coli. A control extract from non-transfected E. coli was not modified by C3 (lane 6). In the amino acid sequence of the rho protein, glycine-14 corresponds to glycine-12 of the ras proteins and mutation in this position have been related to the transforming activities of ras oncogenes (14). We find that the mutated valine-14 rho protein is also a substrate of C3 (not shown). In contrast, neither the Ha-ras (lane 2), nor ral (lane 3) proteins were labelled. Also the R-ras-, ypt and rap 1 proteins did not serve as substrates

for ADP-ribosyltransferase C3. Furthermore, we studied the C3-induced ADP-ribosylation of a chimaeric protein, ras-rho, consisting of 60 amino acids of the ras protein sequence at the N-terminus and 133 amino acids from rho at the C-terminal. This protein was biologically active when microinjected into cells and could still bind guanine nucleotides but was not ADP-ribosylated by C3. We tentatively conclude therefore that ribosylation probably occurs within the amino terminal 60 amino acids of rho A.

The apparent molecular weight of the labelled rho protein was somewhat lower than the molecular weight of the labelled protein found in human platelet membranes or of the C3-substrate purified from brain cytosol. The reason for this difference is that E.coli expressed rho protein is clipped at the C-terminal end and also lacks posttranslational modifications. In order to examine further the relationship between the rho protein and the C3-substrate found in brain cytosol, we studied whether antibodies raised against the C3 substrate purified from porcine brain recognized the rho proteins expressed in E. coli. Figure 2 shows that these antibodies cross reacted with the partially purified C3 substrate (lane 3), rho (not shown), valine-14 rho (lane 4) and ras-rho chimaeric protein (not shown), but not with H-ras and ral proteins. In contrast, rabbit anti-H-ras antibodies cross reacted only with H-ras protein. Thus,

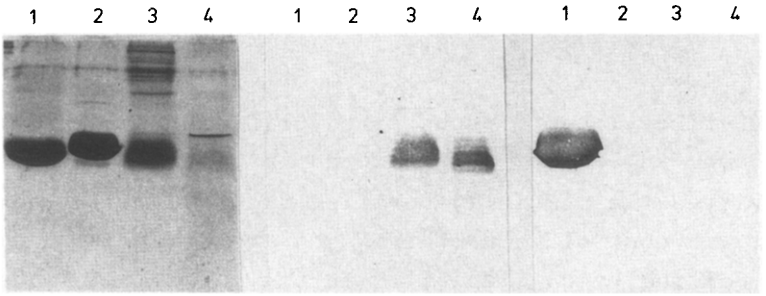

Fig. 2: SDS gel and immunoblot of GTP-binding proteins. H-ras-protein (lane 1, 20 µg), ral-protein (lane 2, 20 µg), C3 substrate purified from porcine brain cytosol (lane 3, 10 µg), valine-14 rho-protein (lane 4, 2 µg) were analyzed by SDS PAGE and thereafter, immunobloting was performed with anti-C3 substrate antibodies (ANTI-C3S AB) or with anti-H-ras antibodies (ANTI-ras AB) as described.

these data strongly support the view that the rho protein is very closely related or perhaps identical with the C3 substrate in brain cytosol.

At least 3 different rho genes have been described in human tissue (15, 16). The functions of these GTP-binding proteins are not known at present. ADP-ribosyltransferases such as cholera- and pertussis toxins have been very useful in studying the functions of their respective G-protein substrates (17). It is likely that the finding of an ADP-ribosylation of the rho protein expressed in E. coli by botulinum ADP-ribosyltransferase C3 will facilitate the analysis of the functions of this family of ras-related gene products.

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