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The 65-kDa protein from pig heart

A new substrate for *Clostridium botulinum* ADP-ribosyltransferase (exoenzyme C_3)

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In the pig heart sarcolemma, a 65 kDa protein is found to be ADP-ribosylated by *Clostridium botulinum* ADP-ribosyltransferase (exoenzyme C_3). ADP-ribosylation of this protein is regulated by guanyl nucleotides and cytosol factor in a fashion similar to that for other C_3 substrates. The new exoenzyme C_3 substrate was partially purified. This protein is supposed to be a GTP-binding one.

ADP-ribosyltransferase; GTP-binding protein; Pig heart sarcolemma; *Clostridium botulinum*

1. INTRODUCTION

Several bacterial toxins are known to be competent in the ADP-ribosylation of eukaryote proteins. Some strains of *Clostridium botulinum* of type C and D produce ADP-ribosyltransferase which is called exoenzyme C_3 [1,2]. Ras-related GTP-binding proteins of the rho and rac families can be substrates of this enzyme [3–7]. It is also found that C_3 can ADP-ribosylate proteins of molecular weight 21–24 kDa in all tissues and cells assayed [2–10]. In some tissues electrophoresis data yielded two or more proteins affected by the exoenzyme C_3 [11,12]. The exoenzyme C_3 substrates are found in both membrane and cytosol fractions [11]. ADP-ribosylation of these proteins is regulated by bivalent cations and guanyl nucleotides [5,8,11,13,14]. In the present study, we identify a new substrate of the exoenzyme C_3 in sarcolemma from pig heart ventricles. This protein is similar to GTP-binding proteins by some properties, but it differs from known substrates by its large molecular weight.

2. MATERIALS AND METHODS

Methods of purification of GTP-binding proteins from heart sarcolemma and their ADP-ribosylation were reported earlier [12]. In short, sarcolemma from pig heart ventricles preliminarily treated with sodium perchlorate was solubilized by 1% sodium cholate. Subsequent chromatography was performed in 0.9% cholate. Protein elution from ion-exchange columns was performed with NaCl gradient between 0 and 300 mM using low pressure chromatography and up to 400 mM during FPLC. Gel-filtration was carried out in 150 mM NaCl. Protein elution during hydrophobic chromatography was performed with NaCl gradient ranging from 250 down to 50 mM and parallel cholate gradient between 0.3% and 2%. [α-^{32}P]GTP binding or ADP-ribosylation of the protein by exoenzyme C_3 revealed GTP-binding proteins in fractions. ADP-ribosylation was carried out in the medium containing 50 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 1 mM EDTA, 10 μM [^{32}P]NAD^+, 1 mM DTT, 1 mM ATP and 20 mM thymidine. Concentration of guanyl nucleotides in the medium was 0.1 mM in those experiments where these compounds were used. Assay medium contained 4.5 μg of partially purified protein and 3.8 μg of cytosolic protein. Aliquots were subjected to SDS-PAGE; the gels were fixed, and autoradiography was performed.

Proteolysis was performed within 1 h at the trypsin concentration equal to 10 μg/ml. The electrophoresis was performed in 12% SDS-polyacrylamide gel. Proteins were visualized by Coomassie blue G250. Exoenzyme C_3 was purified from cultural filtrate of *Clostridium botulinum* (type C, strain C20). The purification included ammonium sulfate precipitation, DEAE-Sephadex A-50 chromatography, chromatography on a column with antibodies to *Clostridium botulinum* hemagglutinin and gel-filtration through Sephadex G-100. Details of the procedure will be published elsewhere.

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3. RESULTS

A protein from pig heart ventricles sarcolemma was purified up to homogeneous state by chromatography on DEAE-Trisacryl, Sephacryl S-300, octyl-Sepharose, DEAE-Sepharose and Mono-Q columns using the ability of the protein to bind GTP. Fig. 1 shows electrophoresis of protein preparations of various purification degrees. This figure clearly demonstrates that alongside with GTP-binding activity the protein of molecular weight 65 kDa is also purified. Trypsin proteolysis of homogeneous protein revealed two major peptides of molecular weight 36 and 23 kDa (Fig. 2). In contrast to the known GTP-binding proteins [12], added guanyl

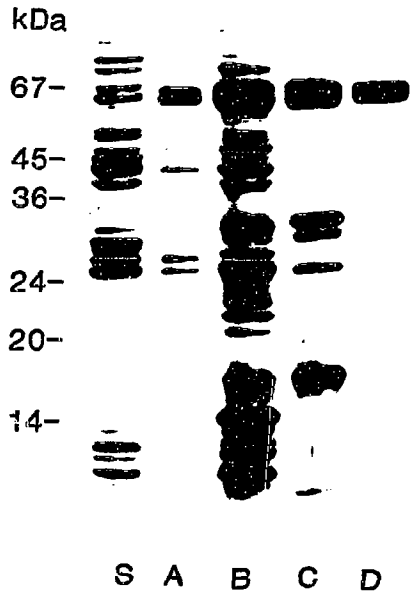

Fig. 1. Purification of the 65 kDa protein according to its GTP-binding activity. SDS-polyacrylamide gel electrophoresis of proteins obtained at different purification steps. (Lane S) Cholate solubilisate of sarcolemma. (Lanes A,B,C,D) Proteins from corresponding steps of purification: (A) DEAE-Trisacryl; (B) Sephacryl S-300; (C) octyl-Sepharose; (D) DEAE-Sepharose.

nucleotides were without effect on the proteolysis. This might be due to the presence of tightly bound nucleotides and/or the necessity of some guanyl exchange factors [15]. In the next series of experiments, it was found that the sarcolemmal 65 kDa protein can be ADP-ribosylated by exoenzyme C_3 from *Clostridium botulinum*. It was also shown that there are 25 kDa proteins which were also affected by the exoenzyme C_3

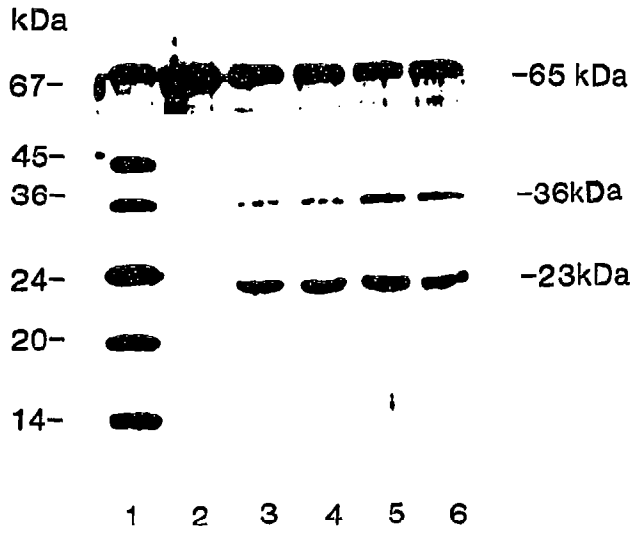

Fig. 2. Proteolysis of purified protein. See section 2 for details of proteolysis and electrophoresis. Lane 1) Protein standards. (Lane 2) The purified 65 kDa protein without trypsin treatment. (Lanes 3,4,5,6) Products of proteolysis of the 65 kDa protein in the absence of any additives (3), or in the presence of 10 mM $MgCl_2$ (4), $MgCl_2$ plus GTP (5), and $MgCl_2$ plus Gpp(NH)p (6).

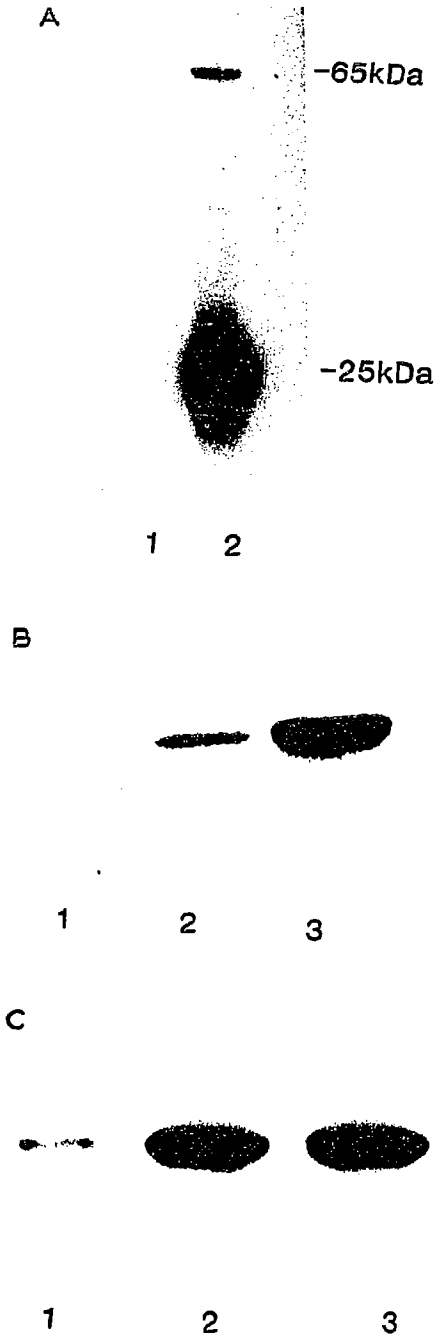

Fig. 3. Exoenzyme C_3-dependent ADP-ribosylation of pig heart 65 kDa protein. (A) ADP-ribosylation of proteins in cholate solubilisate of pig heart sarcolemma in the absence (lane 1) or in the presence of C_3 (lane 2). (B) Influence of cytosol fraction on ADP-ribosylation of 65 kDa protein by C_3: (lane 1) cytosol fraction from NG108-15 cells; (lane 2) partially purified 65 kDa protein from pig heart sarcolemma; (lane 3) mixture of cytosol fraction and 65 kDa protein. ADP-ribosylation medium contained 0.1 mM GTP. (C) Influence of guanyl nucleotides on ADP-ribosylation of 65 kDa protein by C_3: (lane 1) in the absence of guanyl nucleotides; (lane 2) in the presence of GTP; (lane 3) in the presence of GTPS. ADP-ribosylation medium contained cytosol fraction from NG108-15 cells.

(Fig. 3A). During purification the 65 kDa protein preserved its ability for ADP-ribosylation by C_3 up to octyl-Sepharose step (data not shown). ADP-ribosylation of the partially purified 65 kDa protein was stimulated by addition of a cytosol fraction from NG108-15 cells (Fig. 3B). In cytosol 25 kDa protein was shown to be ADP-ribosylated by the exoenzyme C_3. ADP-ribosylation of the 65 kDa protein was stimulated by guanyl nucleotides (Fig. 3C).

4. DISCUSSION

The ability of bacterial ADP-ribosyltransferases to covalently modify certain GTP-binding proteins is well known. However, substrate specificity of ADP-ribosyltransferases is, as a rule, rather broad. For instance, pertussis toxin is capable of ADP-ribosylating α-subunits of transducin, G_i and G_o proteins [16]. Cholera toxin shows a wide spectrum of substrates, including subunits of G_s, G_i and transducin, as well as arrestin (S-antigen) from the retina, A1 subunit of the toxin itself and some other proteins [16–19]. Exoenzyme S from *Pseudomonas aeruginosa* ADP-ribosylates $p21^{c\text{-}H\text{-}ras}$ protein, vimentin and a number of unidentified proteins [20].

ADP-ribosylation of GTP-binding proteins depends on some additional factors. Mechanism of ADP-ribosylation with cholera toxin is the well studied one. The process is activated in the presence of a small molecular weight protein, which is called ADP-ribosylating factor (ARF). The protein is a GTP-binding one and is mainly present in the cytosol [19,21,22]. ADP-ribosylation of small molecular weight substrates by exoenzyme C_3 is also stimulated by cytosol activator of protein origin [23].

The above data showed that in pig heart sarcolemma there is also a 65 kDa substrate besides the well-characterized low molecular weight protein substrates of C_3 [12,24]. Co-purification of the 65 kDa protein with the GTP-binding activity, dependence of activation of the protein ADP-ribosylation on guanyl nucleotides and on cytosol factor, similar to those which occur in the case of some other GTP-binding proteins, seem to suggest that the 65 kDa protein from heart sarcolemma is a GTP-binding protein.

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