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GTP-dependent ADP-ribosylation of a 22 kDa protein in the endoplasmic reticulum membrane

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Treatment of salt-stripped rough microsomal membranes from pancreas or liver with NAD and cholera toxin in the presence of GTP yields an ADP-ribosylated non-ribosomal 22 kDa protein. Membranes containing the modified protein are less active in the co-translational processing of secretory preproteins translated from isolated mRNA in a reticulocyte translation system, but signal peptidase activity is unchanged, suggesting that the 22 kDa protein is involved in the targetting or translocation of secretory proteins at the membrane of the endoplasmic reticulum.

Signal sequence; ADP-ribosylation; Endoplasmic reticulum; GTP-binding protein; Protein translocation

1. INTRODUCTION

Nucleotide triphosphates have been implicated in signal sequence-mediated translocation of newly synthesised proteins across the membrane of the endoplasmic reticulum (ER) [1–4]. Perara et al. [1] showed that post-translational translocation of a ribosome-bound hybrid protein consisting of a signal sequence from β-lactamase ligated to α-globin was dependent on energy substrates including ATP and GTP. Mueckler and Lodish [2] concluded that phosphoanhydride bond cleavage in ATP or GTP was required to translocate a truncated form of the glucose transporter into microsomal vesicles in vitro. GTP has been shown to be required for the step in which the ribosome carrying the secretory polypeptide chain becomes attached to the ER membrane [4], suggesting that a novel GTP-binding protein is involved. Here we describe the identification of a potential GTP-binding protein in the ER membrane by its ADP-ribosylation catalysed by cholera toxin in the presence of GTP. In addition, we show that ADP-ribosylation of microsomal membranes interferes with translocation of secretory proteins in vitro.

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2. EXPERIMENTAL

2.1. *Isolation of intracellular membranes*

Canine and guinea-pig pancreatic rough and smooth microsomes were purified and stripped with salt plus EDTA followed by micrococcal nuclease as described in [5,6]. Mitochondria, Golgi membranes, rough and smooth microsomes were obtained from homogenates of rat liver [7,8]. Detergent extracts were prepared by addition of 1 vol. of 10% (w/v) sodium deoxycholate to 19 vols membranes, vortex-mixing for 1 min, incubation for 15 min at 0°C, and degranulation at 400000 × g for 20 min at 4°C.

2.2. *ADP-ribosylation*

Membranes or deoxycholate extracts (100 μg protein) were incubated with [^{32}P]NAD (NEN) and preactivated cholera toxin (Sigma) or pertussis tox-

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in (kindly provided by Dr A. Dolphin, Dept of Pharmacology, St. George's Hospital Medical School) for 3.5 h as in [9]. Labelled proteins were precipitated in 20% (w/v) trichloroacetic acid, and analysed by SDS-PAGE [5].

Larger amounts of stripped rough microsomes (1 mg protein) were incubated for 50 min with 1 mM unlabelled NAD^+, 1 mM GTP with cholera toxin in its activation buffer [9], and a control batch of membranes was prepared by incubation with the same volume of activation buffer but without toxin. These batches of membranes were washed by centrifugation through a 0.5 M sucrose cushion in 50 mM Hepes-KOH (pH 7.4) at $220000 \times g_{av}$ for 30 min, then the pellets were resuspended and re-pelleted in 20 mM Hepes-KOH (pH 7.4) at $12000 \times g_{av}$ for 20 min at 4°C, and again resuspended in 0.24 M sucrose to $50A_{280}$/ml. Co- and post-translational processing of ^{35}S-preprolactin were assessed after gel electrophoresis and fluorography by scanning on a Joyce-Loebl chromoscan 3 densitometer as described [5,10].

3. RESULTS

3.1. *Proteins in canine pancreatic stripped microsomes [^{32}P]ADP-ribosylated by cholera toxin*

Proteins in canine pancreatic stripped microsomes [^{32}P]ADP-ribosylated by cholera toxin are shown in an autoradiogram (fig.1a). When GTP was present during ADP-ribosylation, one protein of 22 kDa was labelled strongly (track 7), whilst with the non-hydrolysable analogue Gpp(NH)p instead of GTP, labelling of the 22 kDa protein still occurred but was much less intense (track 8). With ATP and no GTP (track 6) or without added toxin (track 5) the 22 kDa labelled band was not produced. The 22 kDa protein was ADP-ribosylated almost as strongly in deoxycholate extracts of canine pancreatic microsomes degranulated by centrifugation, as in intact membranes, showing that it is unlikely to be a ribosomal protein (fig.1a, track 9). In the presence of GTP, labelling of the 22 kDa protein was slightly enhanced by increasing substrate concentration of NAD^+ up to 10 μM (tracks 10–12), whereas at higher concentrations of NAD^+ the label was diluted out (tracks 13,14). The Coomassie blue-stained profiles corresponding to tracks 4 and 5 are shown in tracks 2 and 3. The additional bands in track 2 are the 23 and 10.6 kDa subunits of cholera toxin.

Because of the proximity in molecular mass of the A_1 cholera toxin subunit and the ADP-ribosylated 22 kDa protein, it was important to distinguish between them. Fig.1b (track 3) shows the extent of self-ADP-ribosylation by cholera toxin in a longer exposed autoradiogram; the weakly labelled 23 kDa subunit is shown to be resolved from the strongly labelled 22 kDa protein from stripped rough microsomes (track 1). Pertussis toxin labelled only the ovalbumin added to the incubate in excess to reduce non-specific labelling, and did not ADP-ribosylate the 22 kDa protein (track 2). A time course of incorporation of label showed that in the presence of GTP, the extent of ADP-ribosylation of the 22 kDa protein increased rapidly up to 90 min, and slowly thereafter up to 3.5 h (not shown).

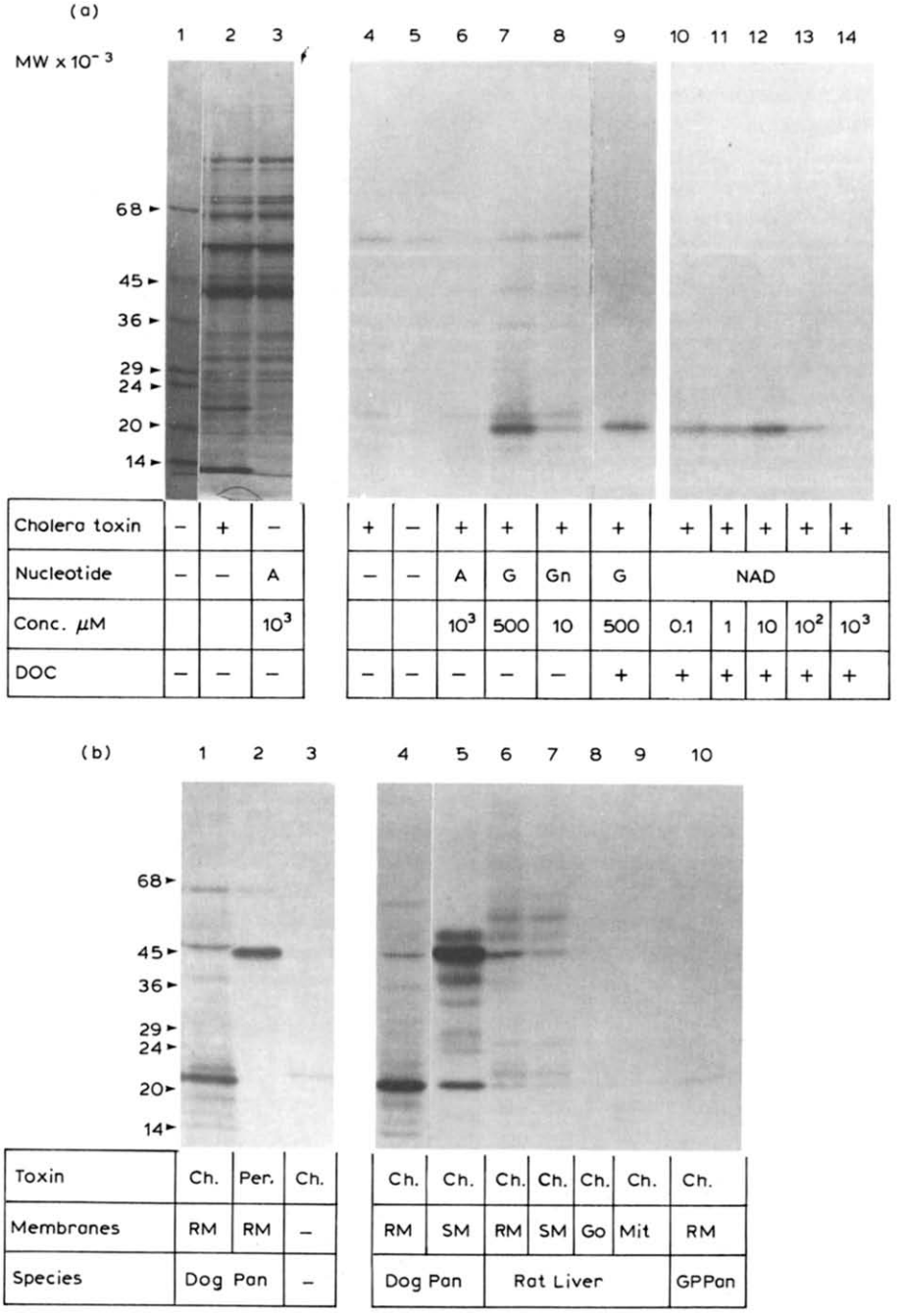
In smooth microsomal membranes isolated from the 0.25–1.65 M sucrose interface after centrifugation of the post-mitochondrial fraction from canine pancreas the protein of 22 kDa was found to be ADP-ribosylated less strongly (fig.1b, track 5). In membranes known to be active in processing

Fig.1. (a) [^{32}P]ADP-proteins separated on a 7–16% SDS-PAGE gel from intact canine pancreatic stripped rough microsomes (tracks 4,6–8) and their deoxycholate extracts (tracks 9–14) labelled by cholera toxin in the presence of no added unlabelled nucleotide (track 4), 1 mM ATP (A) (track 6), 0.5 mM GTP (G) (tracks 7,9), 0.01 mM GppNHp (Gn) (track 8), and 0.5 mM GTP plus the concentrations of NAD shown (tracks 10–14). Track 5 shows labelling in the absence of added toxin. Tracks 2 and 3 show the Coomassie blue staining of tracks 4 and 5, and track 1 molecular mass standards. (b) [^{32}P]ADP-proteins labelled in the presence of 0.5 mM GTP and cholera toxin from canine pancreatic stripped rough microsomes (tracks 1,4), canine smooth microsomes (track 5), rat liver rough microsomes (track 6), smooth microsomes (track 7), Golgi membranes (track 8), and mitochondria (track 9), and guinea-pig pancreatic rough microsomes (track 10). The self-[^{32}P]ADP-ribosylation of cholera toxin is shown in track 2, and labelling of pancreatic stripped rough microsomes with 0.5 mM GTP and pertussis toxin in track 3.

secretory proteins [11,12], i.e. rat liver rough and smooth microsomes (tracks 6,7), and in guinea-pig rough microsomes (track 10), a 22 kDa protein was labelled weakly. No labelling was detected in rat liver Golgi or mitochondrial membranes (tracks 8,9).

3.2. *ADP-ribosylation of microsomes reduces activity in co-translational processing*

The extent of co-translational processing of ^{35}S-preprolactin was reduced by prior incubation of microsomes with cholera toxin for 50 min in the presence of GTP and NAD^+, by an extent of 17%



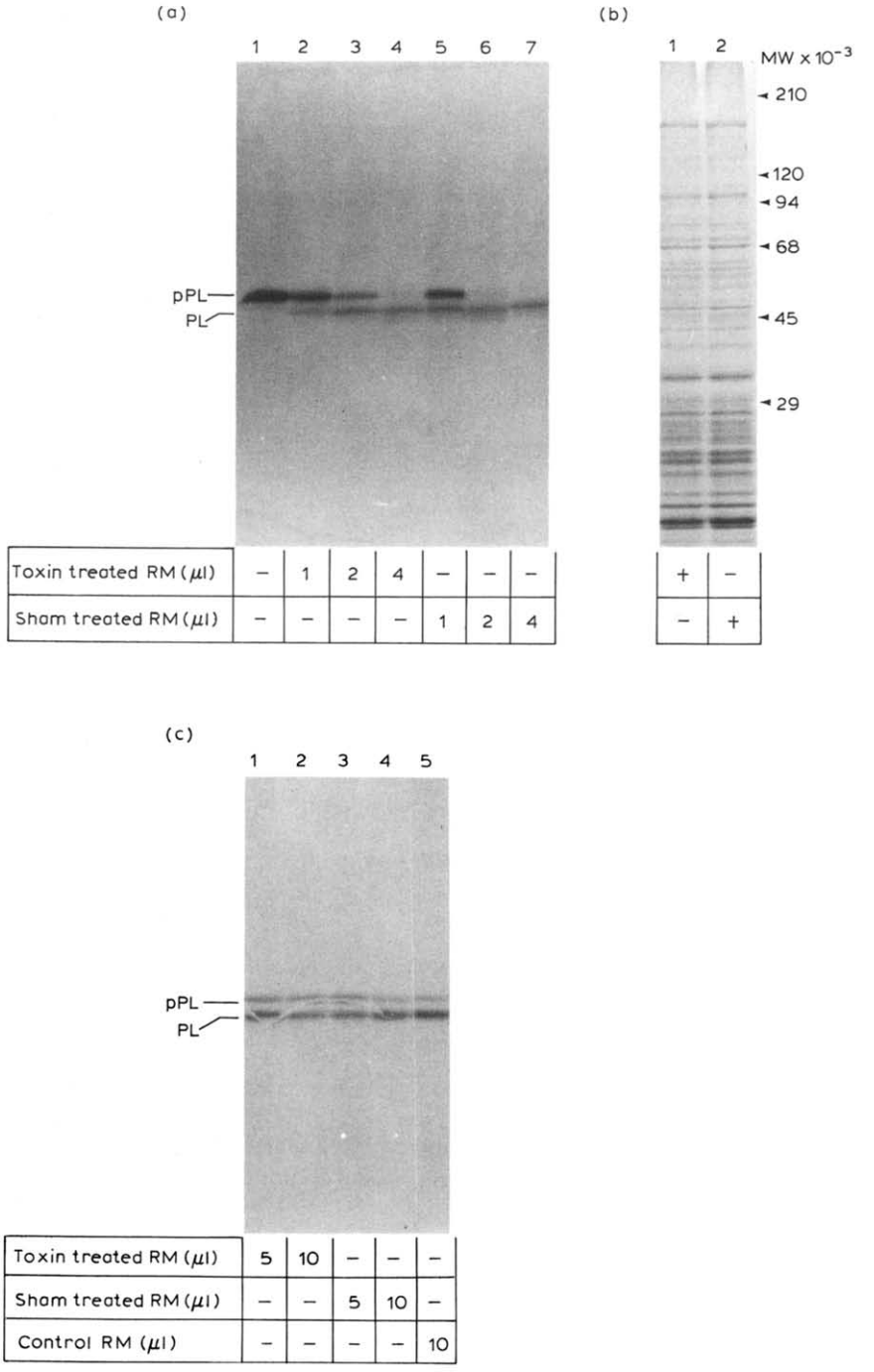


Fig.2. (a) A fluorograph of an SDS-PAGE gel (15% acrylamide) of ^{35}S-proteins translated from bovine pituitary mRNA in a rabbit reticulocyte lysate in the presence of toxin-treated (tracks 2–4) and control (tracks 5–7) pancreatic rough microsomes. (b) Coomassie blue-stained profiles of equal volumes of pancreatic microsomes used in the processing shown in (a). (c) Extent of post-translational conversion of ^{35}S-preprolactin to ^{35}S-prolactin [10] catalysed by toxin-treated and control pancreatic rough microsomes.

Table 1

Extent of processing of ^{35}S-preprolactin by toxin-treated membranes

Membranes added (μl)	Sham-treated (% ± SD)	Toxin-treated (% ± SD)[a]
	Co-translational	
1	37 ± 4	20 ± 3
2	74 ± 2	46 ± 2
4	100 ± 0	74 ± 2
	Post-translational	
5	68 ± 6	64 ± 8
10	76 ± 5	68 ± 6

[a] Mean and SD of six repeated densitometric estimations

with 1 μl of membranes (fig.2a, tracks 2,5) and about 25% with 2 and 4 μl amounts of membranes (fig.2a, tracks 3,4,6,7) (see also table 1). Comparison was made with the extent of processing obtained from equal amounts of control microsomes incubated without toxin. The protein profiles (fig.2b, tracks 1,2) and UV absorbance of the treated and control membranes were compared to ensure that equal amounts of membranes were added to the translations. The activity of signal peptidase, however, was not significantly impaired by ADP-ribosylation (fig.2c, table 1).

4. DISCUSSION

There is now increasing evidence that GTP plays a role in protein translocation at the ER, a process that would appear, at least on theoretical grounds, to require input of energy [13]. Many GTP-binding proteins, including transducin and the regulatory subunits of adenylate cyclase, possess similarities in structure and are ADP-ribosylated by microbial toxins. Most substrates are also GTPases [14]. We have shown that a highly conserved non-ribosomal 22 kDa protein exists in membranes derived from the ER of protein-secreting tissues and is ADP-ribosylated by cholera toxin under conditions similar to those described for the adenylate cyclase stimulatory subunit G_s [15] in that labelling is enhanced by GTP. In contrast to G_s, labelling of the 22 kDa protein is enhanced poorly by Gpp(NH)p. Thus, cholera toxin may ADP-ribosylate the GDP-binding rather than the GTP-binding form of this microsomal protein.

Cholera toxin induces reduction in the co-translational processing of secretory protein precursors, but does not effect signal peptidase activity of microsomal membranes, indicating that the 22 kDa GTP-binding protein may be involved in events that occur prior to cleavage of the preprotein in the lumen of the ER by signal peptidase. Further studies will be required to characterise the function of this protein in more detail.

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