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Enzymatic deglycosylation of the dendrotoxin-binding protein

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The neuronal membrane protein which binds the K^+-channel ligands dendrotoxin, mast cell degranulating peptide, and β-bungarotoxin was purified from rat brain membranes. When analysed on 10% SDS gel electrophoresis, the purified protein contained two peptides: the toxin-binding subunit of apparent M_r 90000 and another peptide of M_r 38000. Neuraminidase treatment reduced the M_r of the toxin-binding subunit to 70000. Glycopeptidase F gave a further reduction to M_r 65000. In contrast, the peptide of M_r 38000 showed no change in M_r upon treatment with neuraminidase and/or glycopeptidase F. It is concluded that the toxin-binding subunit of the dendrotoxin-binding protein, a presumptive K^+ channel, is a sialated membrane protein with a peptide core of, at most, M_r 65000.

Deglycosylation; K^+ channel; Dendrotoxin; Neuraminidase; Glycopeptidase F

1. INTRODUCTION

The dendrotoxins (DTX) and β-bungarotoxins (β-BTX) from snake venom and mast cell degranulating peptide (MCD) from bee venom have been identified as potent and specific blockers of a class of neuronal voltage-dependent K^+ channels [1–4]. These K^+ channels seem to play an important role in brain physiology as DTX, MCD and β-BTX are highly epileptogenic [5]. In addition, MCD induces long-term potentiation in the hippocampus [6]. Specific binding sites have been found for DTX, MCD and β-BTX in brain membranes [7–9]. These binding sites were located on the same high-M_r membrane protein [10–12] here referred to as DMB protein (from D for DTX, M for MCD and B for β-BTX). The DMB protein was recently purified from rat brain [13]. When analysed on 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) the apparently pure preparation contained two peptides of M_r 80000 and 38000 [13,14]. Previous crosslinking studies with radiolabeled DTX or MCD had also indicated an M_r of 76000–80000 for the toxin-binding subunit of DMB protein [11]. Here, the extent of glycosylation of the subunits of this presumptive K^+ channel is investigated.

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2. MATERIALS AND METHODS

2.1. *Materials*

DMB protein was prepared as described in [13]. Neuraminidase (*Arthrobacter ureafasciens*) and glycopeptidase F (*Flavobacterium meningosepticum*) were from Boehringer, Mannheim. The catalytic subunit of cAMP-dependent protein kinase was from Sigma.

2.2. *Phosphorylation*

DMB protein (300 ng) was phosphorylated by incubation in 52 μl of 12 mM Na-Hepes, 9 mM Tris-Cl (pH 7.2), 9 mM $MgCl_2$, 70 mM KCl, 0.6 mM EDTA, 6.3% (v/v) glycerol, 0.10% (w/v) Triton X-100, 0.02% (w/v) soybean phospholipid, 3 mg/ml dithiothreitol containing 70 μM of [γ-^{32}P]ATP (8–12 cpm/fmol), and 150 ng of the catalytic subunit of cAMP-dependent protein kinase. Incubation was for 12 min at 30°C.

2.3. *Deglycosylation*

Aliquots of phosphorylated DMB protein (46 ng in 8 μl) were either heated in the presence of SDS (0.18%, w/v, final concentration) to 95°C (denatured) or left untreated (native). After temperature adjustment to 37°C neuraminidase (5–13 mU in 5 μl of 0.2 M Na-acetate, pH 5.0) or glycopeptidase F (1 U in 8 μl of 12 mM K^+-phosphate, 70 mM EDTA, pH 7.2) were

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added. Incubation temperature for both enzyme reactions was 37°C. Deglycosylation was stopped with 15 μl Laemmli sample buffer and the reaction mixture analysed on 10% SDS-PAGE [15].

3. RESULTS AND DISCUSSION

DMB protein was phosphorylated with the catalytic subunit of cAMP-dependent protein kinase and then enzymatically deglycosylated with neuraminidase and/or glycopeptidase F. The catalytic subunit of cAMP-dependent protein kinase phosphorylates stoichiometrically and specifically the toxin-binding subunit of DMB protein [16]. The subunit of M_r 38000 is also phosphorylated to a small extent after prolonged incubation with the kinase and [γ-^{32}P]ATP. Phosphorylation is used here to visualize the subunits of DMB protein.

Phosphorylated DMB protein was denatured by heat and SDS and then treated with neuraminidase. This reduced the M_r of the toxin-binding subunit to 70000 which indicates the presence of sialic acid residues. Subsequent incubation with glycopeptidase F, which cleaves *N*-glycans between asparagine and the carbohydrate chain [17], gave a further reduction to M_r 65000 (fig.1, denatured). When prior neuraminidase treatment was omitted, glycopeptidase F had no effect. In contrast, when native phosphorylated DMB protein was treated with only glycopeptidase F the enzyme did reduce the M_r of the toxin-binding subunit to 65000, although inefficiently (fig.1, native). No intermediates between M_r 90000 and 65000 or between M_r 70000 and 65000 were observed, which indicates the presence of only one sugar chain [17]. Both enzymes had no effect on the subunit of M_r 38000 of DMB protein. Also the toxin-binding subunit could not be reduced below M_r 65000, even on prolonged (up to 4.5 h at 37°C) incubation with the enzymes. This makes the presence of proteases unlikely. It is therefore concluded that the toxin-binding subunit of DMB protein is a sialated and *N*-glycosylated glycoprotein. The M_r of its peptide core is 65000 or less if the protein contains glycopeptidase F-resistant sugar chains. During the course of this work it was observed that the apparent M_r of the toxin-binding subunit, as determined with SDS-PAGE, changes with the percentage of acrylamide of the gel. In 8% SDS-PAGE the toxin-binding subunit has an apparent M_r of around 80000 whereas in 10% SDS-PAGE the apparent M_r is 90000. The apparent M_r of the other peptide (M_r 38000) which is present in the purified DMB protein preparation did not change with the percentage of acrylamide. Similar results were observed in earlier crosslinking experiments with iodinated toxins and brain membranes: A crosslinking study which used 8% SDS-PAGE obtained an M_r of 76000–80000 [11] whereas a study which used 10% SDS-PAGE obtained an M_r of 95000 [18] for the toxin-binding subunit. In a crosslinking study by Dolly et al. [19] an M_r of 65000 was found for the (glycosylated) toxin-binding subunit of DMB protein of rat brain. The authors used 8% SDS-PAGE but, as the buffer system was not specified, they might not have used the Laemmli buffer system.

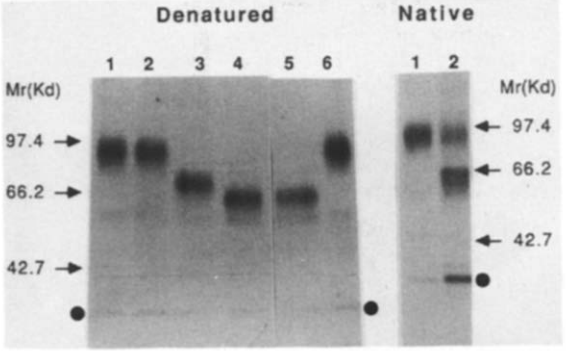

Fig.1. Enzymatic deglycosylation of DMB protein. DMB protein was phosphorylated as described in section 2.2. (Denatured) After phosphorylation DMB protein was heat/SDS-denatured and then treated with the indicated enzymes. Tracks: (1,6) phosphorylated DMB protein (control); (2) glycopeptidase F for 3 h; (3) neuraminidase for 90 min; (4) neuraminidase for 90 min and then glycopeptidase F for 50 min; (5) like track 4 but glycopeptidase F for 3 h. (Native) Tracks: (1) phosphorylated DMB protein (control); (2) phosphorylated DMB protein treated with glycopeptidase F for 4.5 h. An autoradiogram of a 10% SDS-PAGE is shown. Dots mark the position of the subunit of M_r 38000.

Recently, families of cDNA clones have been isolated from rat and mouse brain which are assumed to code for neuronal voltage-dependent K^+ channels [20,21]. The peptides derived from these clones have M_r between 56000 and 72000. The M_r of the deglycosylated toxin-binding subunit of DMB protein falls within this range. As DMB protein is a likely candidate for a voltage-dependent brain K^+ channel, a relationship to the above cDNA clones cannot be excluded.

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