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The PCome of *Caenorhabditis elegans* as a prototypic model system for parasitic nematodes: Identification of phosphorylcholine-substituted proteins

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ABSTRACT

The decoration of proteins and glycolipids with phosphorylcholine (*PCho*) has been shown in many organisms ranging from bacteria to multicellular parasites like nematodes. For bacteria this modifications is involved in invasion and persistence for pathogens. However, little is still known about the distribution of this modification on proteins, the precise epitope structures, and functions. In nematodes, the *PCho*-modification is widespread and at least on the glycosphingolipid level it represents a phylogenetic marker within the helminths. Nematode infections are still one of the most abundant diseases world-wide. *Caenorhabditis elegans* as the best characterized organism is an ideal model system for studying this type of protein modification and can therefore be regarded as a prototypic model system for parasitic nematodes. Interference with the *PCho*-decoration by targeting the glycosphingolipid biosynthesis and the choline metabolism has been shown to reduce nematode viability and fertility. Thus, the *PCho*-modification seems to play an additional important role for the development of nematodes. The development of drugs interfering with the *PCho*-substitution might, therefore, be a promising way for the development of new anthelminthic strategies.

In this study we have analyzed the PCome of *C. elegans* to identify the *PCho*-modified proteins. Furthermore, we investigated the dynamics of this modification by analyzing the different developmental stages of this nematode. Our results demonstrate highly dynamic changes of this modification during development. Furthermore, we could show that this substitution can occur on proteins with large functional diversity and subcellular localization. We could further demonstrate that the *PCho*-modification greatly depends on proper *N*-glycosylation. However, there is clear indication that there might be a high structural diversity of the *PCho*-epitopes.

1. Introduction

Phosphorylcholine (*PCho*) has been recognized as a widespread antigenic determinant in many important disease-causing parasites, such as, protozoa, and gastrointestinal and filarial nematodes. The phylum of Nematoda comprises parasitic and free-living species, some of the latter are easy to cultivate and, at least in part, genetically sequenced, thus being optimal model systems, whereas many of the former are important human parasites. Nematode infections are characterized by high morbidity but low mortality due to the ability of the parasites to establish long-lasting infections and to escape immediate elimination by the host's immune system.

PCho-bearing antigens have been found to possess immunomodulatory capacity and to interfere with key proliferative signaling pathways in B- and T-cells, dendritic cell maturation and mast cell degranulation, thus facilitating the survival of parasites within their hosts [1–9]. These effects could, therefore, contribute to the observed low antibody levels and poor lymphocyte responsiveness. Detailed data on the different types of *PCho*-carrying compounds as well as their biosynthesis, however, are limited and have only been reported in the last few years [1,10–12].

Structural analyses of nematode-derived molecules with *PCho*-epitopes have been focused, so far, on glycolipids and glycoprotein glycans. It could be shown that glycosphingolipids of the pig parasitic nematode, *Ascaris suum*, are characterized by the presence of a phosphodiester-bound *PCho*-substituent which has been assigned to C-6 of the central *N*-acetylglucosamine (GlcNAc) residue of an

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arthro-series carbohydrate core [13]. Furthermore, some glycolipid species have been found to carry phosphorylethanolamine linked to C-6 of an adjacent mannose residue in addition to *PCho* [14,15]. Comparable glycosphingolipids have been verified in different orders of parasitic Nematoda, including *Nippostrongylus brasiliensis* [16], *Litomosoides sigmodontis* [17,18], *Onchocerca volvulus* and *Setaria digitata* [18], indicating that arthro-series glycosphingolipids carrying, in part, *PCho*-substituents represent highly conserved glycolipid markers within the nematode phylum. A biosynthetic route homologous to *A. suum* glycosphingolipids was also confirmed for the free-living nematode *Caenorhabditis elegans* [14,19,20].

Analogous analyses of the *PCho*-substituted glycoprotein ES-62, an excretory/secretory (ES) product of *Acanthocheilonema viteae*, indicated that the zwitterionic substituent is linked via *N*-glycans to the polypeptide backbone [21,22]. Mass spectrometric analysis of the respective N-linked glycans revealed the presence of trimannosyl *N*-glycan variants, carrying between one and four terminal GlcNAc residues [23]. Only this type of glycans was found to be substituted with *PCho*-moieties which could be again assigned to C-6 of terminal GlcNAc residues [24,25]. Comparative studies of *N*-glycans present in extracts of *A. viteae*, *Onchocerca gibsoni* and *O. volvulus* confirmed a high conservation of such *PCho*-substituted *N*-glycans within filarial parasites [26]. For *C. elegans* two types of *PCho*-substituted *N*-glycans have been reported so far: (1) a pentamannosyl-core structure carrying up to three *PCho*-residues [27] and (2) trimannosyl-core species elongated by GlcNAc residues substituted at C-6 with *PCho* [24]. Furthermore, combinations of both types of structural motifs [11] as well as the occurrence of extended glycan structures with composition of $PCho_{1-2}dHex_{0-2}Hex_{3-5}HexNAc_{3-7}$ have been reported [10]. These data clearly demonstrate that the free-living nematode, *C. elegans*, is an excellent model organism for parasitic nematodes at the level of *PCho*-substituted glycolipids and glycoproteins.

The *C. elegans* TKO-strain which lacks all three UDP-*N*-acetyl-D-glucosamine:α-3-D-mannoside-β-1,2-*N*-acetylglucsaminyltransferases I (GnT-I; gly-12, gly-13 and gly-14) is no longer able to synthesize a variety of paucimannosidic, complex-type and fucosylated oligomannosidic *N*-glycans [28]. Nonetheless, this strain shows a normal phenotype.

Recently, we could identify the aspartylprotease ASP-6 from *C. elegans* in the axenic culture medium as the only excreted/secreted and *PCho*-modified protein of this nematode [29]. Preliminary characterizations of this protein indicated that the *PCho* moiety may be either bound via *O*-glycans or directly coupled to the polypeptide-backbone, but is obviously not linked via *N*-glycans.

A more detailed analysis of the PCome of *C. elegans* presented in this study revealed a stage-specific expression of the *PCho*-modification. Whereas in egg and dauerlarval stages the identified proteins showed a substantial diversity in function and subcellular localization, in the L4 stage most of the identified proteins were cytosolic and involved in metabolism. In adult worms, the modification seemed to play important roles in mitochondrial energy- and redox-metabolism. Thus, this work gives deeper insight into the potential functions of the *PCho*-substitution for the development of nematodes.

2. Experimental procedures

2.1. C. elegans culture

Eggs (wild-type strain N2, var. Bristol) were obtained by sodium hypochlorite treatment [30]. Synchronous populations were grown on agar plates with *Escherichia coli* as food source and harvested when 90–95% of individuals were of the desired developmental stage, as observed by light microscopy. For large scale cultivation the worms were grown on agar plates with chicken egg as food source (Baillie DL, Rosenbluth R, 1976, Worm Breeder's Gazette 2 p. 6, pers. commun.). Nematodes were cleaned by density centrifugation on sucrose.

2.2. Protein isolation

Freeze-dried *C. elegans* material (4 mg) was homogenized by ultrasonication in 120 μl 6 M urea (Sigma, Taufkirchen, Germany), 2 M thiourea (Sigma), 4% 3-3′-(cholamidopropyl)-3,3-dimethylammoniumpropylsulfat (CHAPS; Roth, Karlsruhe, Germany), 1% dithioerythitol (DTT; Fluka, Seelze, Germany), 2% Servalyte 3–10 (Serva, Heidelberg, Germany), in the presence of protease inhibitor cocktail (10%; Sigma) and centrifuged (30,000 × *g*, 1 h at 4 °C). To remove lipid contaminants the protein was precipitated with chloroform/methanol (4:1 by v/v) [31]. For isoelectric focusing the protein pellet was dissolved in 100 μl 6 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 2% Servalyte 3–10.

2.3. Two-dimensional gel electrophoresis

IEF-strips (pH 3–10; 14.5 cm) prepared according to Westermeier [32] were rehydrated for 12 h in the presence of 8 M urea, 6.5 mM DTT, 2% Servalyte 3–10, 1% Ampholine 5–7 (GE Healthcare, Freiburg, Germany) and 4% CHAPS. After removal of excess rehydration solution sample application pieces (3 mm × 5 mm) were placed onto the strips (3 mm × 18 cm) in the middle of each strip and 5 mm in front of the anode. On each sample application piece 50 μg of protein were applied. For isoelectric focusing a Multiphor II (GE Healthcare) operating in the linear mode was used. Focusing was performed at 2000 V, 1 mA, 2 W for 60 min; 2000 V, 2 mA, 2 W for 165 min; 2500 V, 2 mA, 3 W for 15 min, respectively. After focusing the IEF-strips were either stored at −80 °C or immediately equilibrated for 10 min in 2 ml equilibration stock solution (ESS; 6 M urea 0,1 mM EDTA, 0.01% bromphenol blue, 50 mM Tris–HCl pH 6.8, 30% glycerol, v/v), 15 min in 2 ml ESS I (10 ml ESS containing 200 mg SDS, 100 mg DTT) followed by 15 min in ESS II (10 ml ESS containing 200 mg SDS, 480 mg iodacetamide). Protein separation in the second dimension was performed by electrophoresis on 10% SDS-polyacrylamide gels. Analytical SDS-PAGE was carried out according to Laemmli [33]. Electrophoresis was carried out in a Protean II XI cell (Bio-Rad, Munich, Germany) with the following program: 180 min at 90 mA (600 V and 50 W limits). Gels were either stained with silver or electroblotted.

2.4. Detection of PCho-modified proteins

For Western-blotting, 2D-separated proteins were transferred to a nitrocellulose membrane by semi-dry blotting, and the membranes were incubated with an *PCho*-specific antibody (TEPC-15, Sigma, 1:1000 dilution) overnight at 4 °C in Roti-Block (Roth). Afterwards, the membranes were washed three times for 10 min with Roti-Block, incubated with horseradish peroxidase conjugated anti-mouse Ig (DakoCytomation, Hamburg, Germany, 1:3000 dilution), for 1 h at RT and again washed six times for 10 min with Roti-Block. *PCho*-modified proteins were visualized by enhanced chemiluminescence using the West Dura Substrate Kit (Perbio Science, Bonn, Germany). The corresponding spots were excised with the ExQuest™ Spot Cutter (Bio-Rad).