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Short Communication

First identification of a phosphorylcholine-substituted protein from *Caenorhabditis elegans*: isolation and characterization of the aspartyl protease ASP-6

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Abstract

Caenorhabditis elegans is a widely accepted model system for parasitic nematodes, drug screening and developmental studies. Similar to parasitic worms, *C. elegans* expresses glycosphingolipids and glycoproteins carrying, in part, phosphorylcholine (*P*Cho) substitutions, which might play important roles in nematode development, fertility and, at least in the case of parasites, survival within the host. With the exception of a major secretory/excretory product from *Acanthocheilonema viteae* (ES-62), no protein carrying this epitope has been studied in detail yet. Here we report on the identification, characterization and localization of the aspartyl protease ASP-6 of *C. elegans*, which is excreted by the nematode in a *P*Cho-substituted form. Within the worm, most prominent expression of the protein is observed in the intestine, while muscle and epithelial cells express *asp-6* to a lesser extent. In animals harboring an ASP-6::GFP fusion protein, diffuse fluorescence throughout the body cavity of adult worms indicates that the chimeric protein is secreted.

Keywords: mass spectrometry; nematodes; posttranslational modification; protease.

Antigens carrying phosphorylcholine (*P*Cho) moieties have been recognized as structural entities in prokaryotic and eukaryotic pathogens, including gastrointestinal and filarial nematodes, which are able to establish long-lasting infections. Within the host, *P*Cho-bearing molecules were shown to interfere with key proliferative signaling pathways in B- and T-cells, thus contributing to the low antibody levels and poor lymphocyte responsiveness observed (Harnett and Harnett, 2001; Goodridge et al., 2003; Harnett et al., 2004; Marshall et al., 2005).

Structural analyses of nematode-derived antigens with *P*Cho-epitopes have so far focused mainly on glycosphingolipids and glycoprotein glycans. As shown initially for the pig parasitic nematode *Ascaris suum*, the respective glycolipids are characterized by the presence of a phosphodiester-bound *P*Cho substituent, which has been assigned to C-6 of the central GlcNAc residue of an arthro-series carbohydrate core (Lochnit et al., 1998; Friedl et al., 2003; Griffitts et al., 2005). Comparable glycosphingolipids have been verified in different orders of parasitic Nematoda, including *Litomosoides sigmodontis* (Baumeister et al., 1994; Wuhrer et al., 2000), *Onchocerca volvulus* and *Setaria digitata* (Wuhrer et al., 2000), indicating that arthro-series glycosphingolipids carrying, in part, *P*Cho 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* (Gerdt et al., 1997, 1999; Griffitts et al., 2005).

Studies on the attachment of *P*Cho to proteins have mainly been focused on ES-62, an excretory/secretory product from *Acanthocheilonema viteae*, indicating that the zwitterionic substituent was linked via N-glycans to the protein backbone (Harnett et al., 1993, 1994). Mass spectrometric analysis of these N-glycans revealed *inter alia* the presence of trimannosyl core variants, with or without fucose, carrying between one and four terminal *N*-acetylglucosamine residues (Haslam et al., 1997). Only this type of glycan was found to be substituted with *P*Cho. Recently, Haslam and co-workers provided evidence that *P*Cho-substituents were also located at C-6 of GlcNAc (Haslam et al., 2002; Haslam and Dell, 2003). Similar to glycosphingolipids, comparative studies of respective sugar chains from of *A. viteae*, *Onchocerca gibsoni* and *O. volvulus* confirmed high conservation of such *P*Cho-substituted N-glycans within filarial parasites (Haslam et al., 1999). For *C. elegans*, however, two different types of N-linked *P*Cho-epitopes have been reported so far: (1) a pentamannosyl-core structure carrying up to three *P*Cho-residues (Cipollo et al., 2002); and (2) a trimannosyl-core species elongated by *N*-acetylglucosamine residues substituted at C-6 with *P*Cho (Haslam et al., 2002). Combinations of both types of structural motifs have also been reported (Cipollo et al., 2005). Data on the enzymes and biosynthetic pathways involved in the formation of *P*Cho-substituted glycolipids and proteins, however, are still limited (Lochnit et al., 2000, 2005; Cipollo et al., 2004, 2005). Nevertheless, such enzymes might be attractive targets for the development of new anthelmintics.

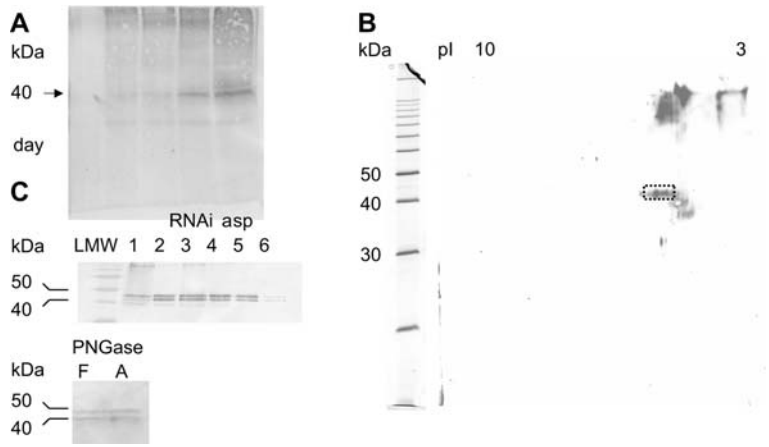

Figure 1 Western blot analyses of an excreted protein from *C. elegans* recognized by the *P*Cho-specific antibody TEPC-15. (A) Detection of the protein in culture supernatants of *C. elegans*. Worms (*C. elegans* strain N2, var. Bristol) were cultivated on agar plates with *E. coli* as food source (Sulston and Hodgkin, 1988) and separated from bacterial contaminants by sucrose density centrifugation. Eggs were obtained by sodium hypochlorite treatment (Sulston and Hodgkin, 1988) and maintained in axenic, chemically defined medium (Vanfleteren, 1978). The resulting synchronized populations were grown at 20°C. Aliquots were collected from culture supernatants at the time intervals indicated and analyzed by SDS-PAGE (Laemmli, 1970) in 12% polyacrylamide slab gels in conjunction with Western blotting using TEPC-15 and an alkaline phosphatase-coupled secondary antibody. (B) Two-dimensional gel electrophoresis of proteins recovered from the supernatant of axenic *C. elegans* cultures. Two-dimensional gel electrophoresis and electroblotting were carried out using published protocols (Eckerskorn, 1999; Görg and Westermeier, 1999). *P*Cho-positive proteins were visualized by Western blot analysis using TEPC-15. Protein spots corresponding to those boxed by dotted lines were excised from the Coomassie Brilliant Blue-stained gel and subjected to trypsin digestion and mass spectrometric analysis. (C, upper panel) Identification of the excreted protein as ASP-6 by RNA interference. Synchronized larvae 1 (L1) were transferred to 6-well plates containing nematode growth medium agar (with 25 μg/ml carbenicillin, 1 mM IPTG) together with transformed *E. coli* strains induced overnight [*asp-1*, Y39B6B.g, V-12I20 (lane 1); *asp-2*, T18H9.2,V-6B15 (lane 2); *asp-3*, H22K11.1, X-3H19 (lane 3); *asp-4*, R12H7.2, X-6O01 (lane 4); *asp-5*, F21F8.3, V-5N12 (lane 5); and *asp-6*, F21F8.7, V-5N20 (lane 6); MRC Geneservice, Cambridge, UK] (Fraser et al., 2000). Worms were cultivated for 72 h at 17°C. L4 were transferred to new plates (10 larvae/well) and cultivated for another 72 h at 37°C in the presence of the corresponding bacteria before the progeny was harvested and assayed for TEPC-15 reactivity by SDS-PAGE and Western blotting. (C, lower panel) Western blot analysis of ASP-6 after digestion with PNGase F or PNGase A. Protein aliquots were incubated with these enzymes as described elsewhere (Kurokawa et al., 2002) and analyzed by SDS-PAGE and Western blotting using TEPC-15.

Parasitic nematodes release enzymes and metabolic products that, together with cuticular material, are collectively termed excretory/secretory (ES) antigens. These liberated enzymes have been ascribed numerous functions, such as penetration of host tissue barriers, anticoagulation, extracorporal digestion, proteolytic cleavage of surface-bound immunoglobulin, and inactivation of complement and cytotoxic mediators expressed by host leucocytes (Knox and Kennedy, 1988; Knox and Jones, 1990; Becker et al., 1995; Brown et al., 1999; Tort et al., 1999; Geldhof et al., 2000). Furthermore, proteases were reported to trigger Th2-type immune responses (Finkelman and Urban, 1992). Intriguingly, *C. elegans* extracts also exhibit strong proteolytic activity at acidic pH, which is almost completely inhibited by pepstatin (Sarkis et al., 1988). Using pepstatin affinity chromatography, five aspartyl proteases (ASP) were isolated (Geier et al., 1999), whereas at the cDNA level, more than 12 putative aspartyl proteases were identified that display high homology to a variety of enzymes from mammalian parasites (Tcherepanova et al., 2000) (http://www.wormbase.org). Since aspartyl proteases are important for the survival of mammalian parasites, the corresponding enzymes in *C. elegans* are also excellent targets for anti-parasitic drug development.

Cultivating *C. elegans* in axenic medium, we detected a *P*Cho-positive protein with an apparent molecular weight of 40 kDa in culture supernatants by Western blot analysis using the *P*Cho-specific antibody TEPC-15. Recognized protein species could be resolved into two bands when optimal electrophoretic conditions were employed. After synchronizing the cultures by starting with eggs obtained by sodium hypochlorite treatment of adult worms, the protein was detectable after 24 h and its amount increased with cultivation time (Figure 1A). To identify this protein, the culture medium was dialyzed against water overnight to remove low-molecular-weight compounds. Further purification was achieved by strong anion exchange chromatography on Sepharose Q (data not shown). The protein fraction obtained was desalted by acetone precipitation, separated by 2-D gel electrophoresis after converting sulfhydryl residues into the acetamido form, and electroblotted onto polyvinylidene difluoride (PVDF) membranes or stained with Coomassie Brilliant Blue. The *P*Cho-modified protein at 40 kDa was visualized by Western blot analysis using TEPC-15 antibodies, which revealed two major isoelectric forms (Figure 1B). Corresponding Coomassie Blue-stained spots were excised and the protein was in-gel digested with trypsin. The resulting peptides were subjected to nano-liquid chromatography/electrospray ionization-ion trap-mass spectrometry (nano-LC-ESI-IT-MS) to identify the protein by peptide mass fingerprinting and sequence information obtained by tandem mass spectrometry (ESI-

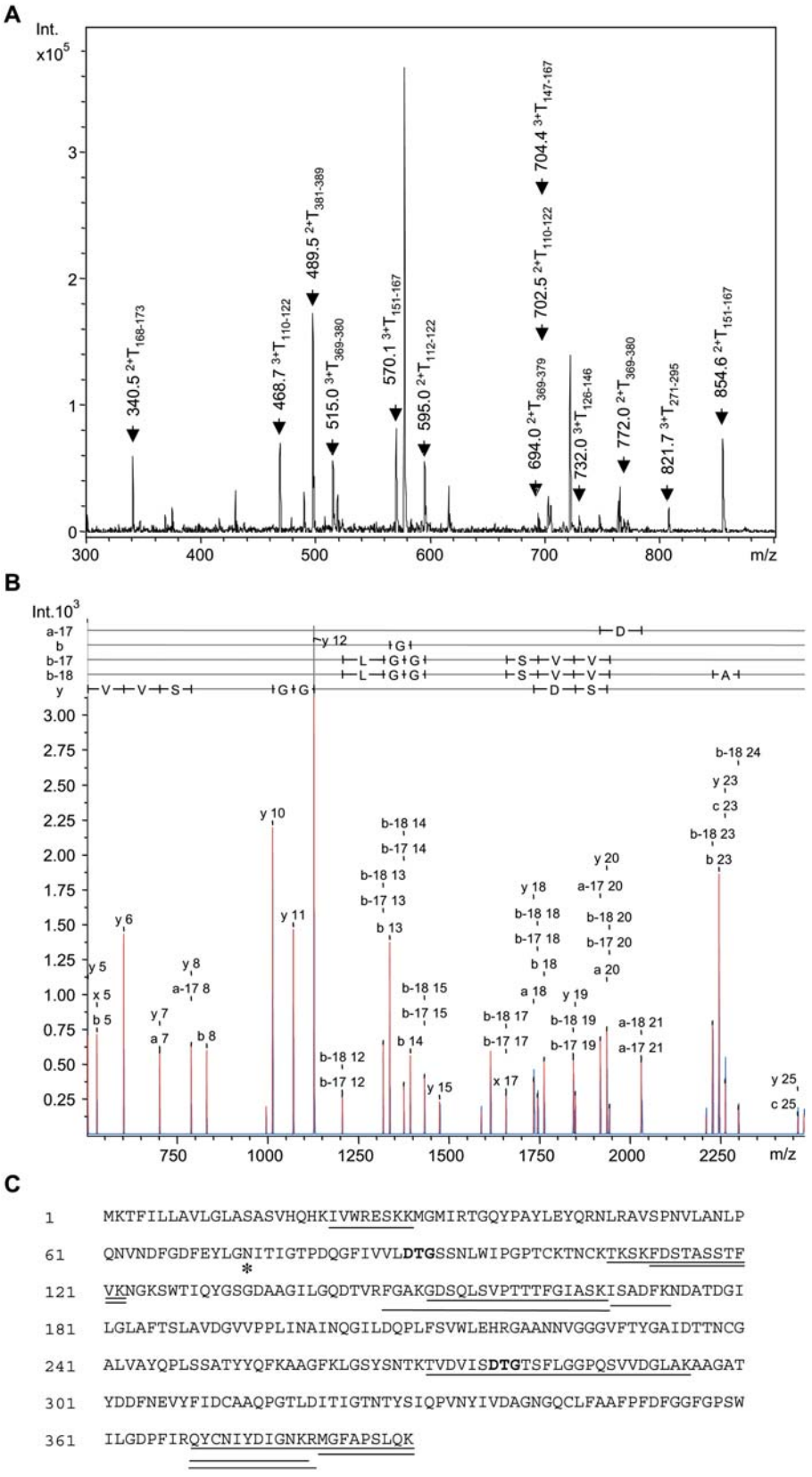

Figure 2 Mass spectrometric identification of ASP-6.
Nano-LC-ESI-IT-MS analysis was performed as described elsewhere (Grabitzki et al., 2005), except that a voltage of 1200–2500 V was applied for electrospraying. (A) Summary spectrum of all protonated tryptic peptides registered. Identified peptides are marked. (B) Assigned MS/MS spectrum of the tryptic peptide (*m/z* 821.7 $[M+3H]^{3+}$) after deconvolution. (C) Amino acid sequence of the identified *C. elegans* protein ASP-6. Conserved catalytic motifs of aspartyl proteases are shown in bold. The potential N-glycosylation site is indicated by an asterisk. Peptides identified by peptide mass fingerprinting and nano-LC-ESI-IT-MS/MS are underlined.

IT-MS/MS). Peptide masses were deduced by manual deconvolution of multiply charged ions (Figure 2A). Using the monoisotopic, protonated peptide masses ($[M+H]^+$) obtained at *m/z* 599.0, 616.0, 680.0, 978.0, 994.0, 1037.0, 1045.0, 1153.0, 1189.0, 1387.0, 1404.0, 1404.1, 1493.0, 1526.0, 1529.0, 1543.0, 1700.4, 1708.0, 1708.2, 1708.3, 2111.2, 2162.0, 2162.2, 2194.0, 2408.5, 2420.8, 2444.2, 2462.2 and 2463.1, a database search within the taxonomy category of *C. elegans* was performed using the ProFound software program (Version 4.10.8, Proteometrics; www.proteometrics.com) within the molecular mass range 30–50 kDa and pI range 5.0–10.0 with a mass tolerance of 1.00 Da. The search revealed the 41.84-kDa putative aspartyl protease ASP-6 (Figure 2C) with a Z-value of 2.22. The assignment was further confirmed by tandem mass spectrometry of the tryptic peptides. As shown in Figure 2B, fragment ions of the peptide at *m/z* 821.7 ($[M+3H]^{3+}$) confirmed the predicted amino acid sequence. Similar results could be achieved with other peptides assigned, thus confirming the identity of the protein (data not shown).

To test for the presence of proteolytic activity in the axenic culture medium or in total worm homogenate, an assay was used employing fluorescein isothiocyanate (FITC)-casein as substrate. Approximately 75% of the measured proteolytic activity could be inhibited by pepstatin, an inhibitor of aspartyl proteases. Attempts to purify the *P*Cho-modified form of ASP-6 from the axenic culture medium or from total worm homogenate by pepstatin affinity chromatography, however, failed and resulted exclusively in the isolation of several unmodified proteins with pepstatin-sensitive proteolytic activity (data not shown).

RNA interference (RNAi) experiments targeting the different *asp* genes revealed no visible phenotypes (Kamath and Ahringer, 2003). The harvested worms were washed with 50% aqueous ethanol and the proteins recovered were analyzed by SDS-PAGE and Western blotting using *P*Cho-specific TEPC-15 antibodies (Figure 1C). Only in the case of the *asp-6* RNAi experiment (Figure 1C, lane 6) was a significant reduction in TEPC-15 reactivity observed, thus confirming the identity of the secreted polypeptide.

Lectin analyses of the *P*Cho-substituted ASP-6 protein provided evidence for the presence of GlcNAc residues due to the binding of wheat germ agglutinin (WGA), whereas concanavalin A (ConA) did not show any reaction, indicating the absence of oligomannosyl, hybrid-type and/or diantennary N-glycans. Treatment of the protein with peptide-N-glycosidase F (PNGase F) abolished the binding of WGA (data not shown). Intriguingly, treatment with PNGase F or PNGase A affected neither recognition by TEPC-15 antibodies nor the electrophoretic mobility of the protein (Figure 1C), thus ruling out an N-glycan-based *P*Cho-linkage. The fact that no shift in electrophoretic mobility was observed after PNGase F and PNGase A treatment might be due to the presence of only short-chained glycans.

Two green fluorescent protein (GFP) reporter fusions were utilized to investigate the spatiotemporal expression pattern of the *asp-6* gene, as well as the localization of the ASP-6 protease. First, the promoter of the *asp-6* gene was used to drive GFP expression in *C. elegans* (p_{asp-6}GFP). Second, GFP was fused to the carboxy-terminus of the ASP-6 protein and the fusion was driven by the *asp-6* promoter (p_{asp-6}ASP-6::GFP). Transgenic animals harboring GFP under the control of the *asp-6* promoter show fluorescence in various tissues at the gravid-adult stage, while expression is not detectable during earlier developmental stages (Figure 3). Expressing tissues include the intestine, muscle cells, pharynx and hypodermal cells. Animals carrying the full-length ASP-6::GFP fusion show diffuse fluorescence in the pseudocoelom, indicating that the fluorescent chimera is secreted. This observation is further supported by the marked decrease in fluorescence yield by the full-length ASP-6::GFP fusion, which is typical of extracellular GFP.

It is documented in the literature that *C. elegans* extracts or tissues exhibit strong proteolytic activity at acidic pH. Several proteases account for this feature: a cathepsin D activity, which can be inhibited by pepstatin, two thiol proteases, cathepsins Ce1 and Ce2, which are inhibited by leupeptin, a thiol-independent leupeptin-insensitive protease, cathepsin Ce3 (Sarkis et al., 1988), different serine and metallo proteases (Gimenez-Pardo et al., 1999), as well as a non-lysosomal cathepsin E, which is analogous to a secreted protease from *O. volvulus* (Jolodar and Miller, 1998). In addition, a number of aspartyl proteases (ASP) have been either individually isolated from *C. elegans* (Geier et al., 1999) or postulated on the basis of genomic data (Jolodar and Miller, 1998; Yan et al., 1999). Only the aspartyl proteases ASP-1, ASP-2, ASP-5 and ASP-6 contain the conserved N-glycosylation site, presupposed to be necessary for lysosomal targeting, and four conserved cysteine residues, whereas ASP-3 and ASP-4 lack this N-glycosylation site, thus suggesting a different function and/or localization of these two proteins. Recent studies revealed that ASP-3 and ASP-4 are involved in neurodegeneration in *C. elegans* (Syntichaki et al., 2002). Expression analysis of the enzymes fused with GFP further demonstrated predominant localization in the intestine, but also, to a much lesser extent, in muscle cells, hypodermis and neurons. ASP-1 displays high homology with enzymes from mammals and invertebrates (Tcherepanova et al., 2000), as well as an aspartyl protease precursor from the human enteric parasite *Strongyloides stercoralis* (Gallego et al., 1998). Furthermore, it has been shown that ASP-1 is exclusively expressed in the lysosomes of intestinal cells in late embryonic and early larval stages. ASP-2 and ASP-5 are homologous to an enzyme termed Sjasp from *Schistosoma japonicum* (Becker et al., 1995), ASP-3 and ASP-6 are related to an aspartyl protease from *Aedes aegypti*, and ASP-4 is similar to an enzyme from *Ancylostoma caninum* (Harrop et al., 1996).

It remains unclear whether the aspartyl protease identified in this study has specific substrates or functions other than unspecific lysosomal protein degradation. The fact that we could not isolate the *P*Cho-modified ASP-6 protein by affinity chromatography led to the assumption that the modified ASP-6 may represent the excretory form of the protease, which is possibly devoid of pepstatin-binding activity, whereas the non-*P*Cho-modified form may represent the active lysosomal protease. Thus,

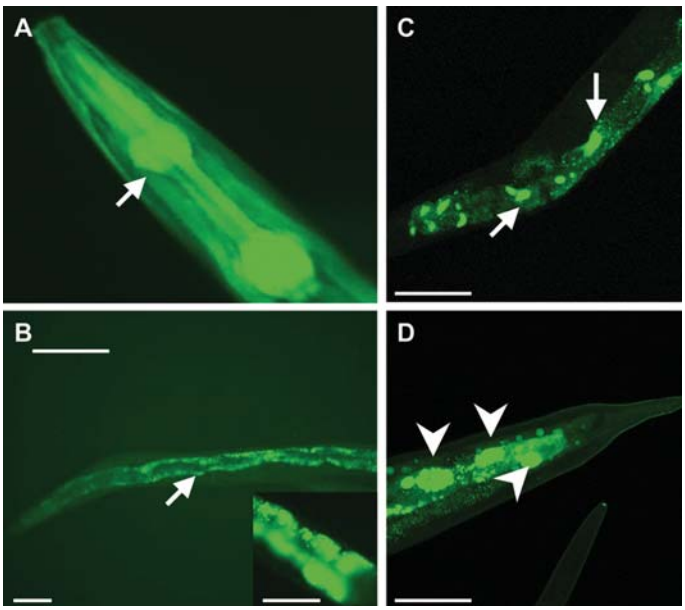

Figure 3 Expression and localization of ASP-6.
The expression pattern of ASP-6 was investigated by a polymerase chain reaction (PCR)-based approach using either translational or transcriptional GFP reporter fusions (Hobert, 2002). Asp-6 was amplified from the cosmid F21F8 and GFP from the plasmid PD95.75. For amplification of asp-6 (PCR 1) or GFP (PCR 2), primers A (5′-ATC GAA TGA GAA AGC AGC AAA-3′) and B (5′-AGT CGA CCT GCA GGC ATG CAA GCT ACC TGA AAA TAA ATA TTT CAG AAA AA-3′, translational fusion; 5′-AGT CGA CCT GCA GGC ATG CAA GCT ATA CCT GAA AAT AAA TAT-3′, transcriptional fusion) or primers C (5′-AGC TTG CAT GCC TGC AGG TCG ACT-3′) and D (5′-AAG GGC CCG TAC GGC CGA CTA GTA GG-3′) were used, respectively. For the fusion PCR (PCR 3), primers A* (5′-GGG CCC AAC TGT TTG TCC CTC ACA ACG-3′) and D* (5′-GGG CCC GGA AAC AGT TAT GTT TGG TAT ATT GGG-3′) were employed. Products from PCR 1 and PCR 2 were directly used for PCR 3. The PCR 3 product was excised from the agarose gel, purified and injected into the gonads of wild-type (N2) animals, together with plasmid pRF4 carrying the *rol-6*(*su1006*) dominant co-transformation marker. Injections were carried out at a concentration of 50 ng/ml DNA. Injected animals were recovered and allowed to lay eggs for 3 days at 25°C. F1 progeny were screened for the roller phenotype induced by the *rol-6*(*su1006*) dominant allele. F1 rollers were recovered and examined for GFP expression. A total of 111 F1 transgenic animals were obtained and examined. (A) Expression of full-length $p_{asp\text{-}6}$ASP-6::GFP in the pharynx, the feeding organ of the nematode (indicated by the arrow). (B) Expression in the intestine (indicated by the arrow). Diffuse expression is also evident through the body cavity. Detailed intestinal expression is shown on the bottom right corner of panel (B). (C,D) Animals carrying GFP under control of the *asp-6* promoter. Strong expression is observed in intestinal and epithelial cells (arrows and arrowheads, respectively). White scale bars represent 100 μm.

the released form could be regarded as a 'shuttle molecule' for the export of *P*Cho-modified antigens. For parasitic nematodes, such a mechanism might be a way to deliver permanently immunomodulatory components to the host, as could be demonstrated in the case of the excretory/secretory product ES-62 of *A. viteae* (Harnett and Harnett, 2000, 2001). In this context, it might also be speculated as to whether the *P*Cho-modification could – at least for some proteins – have the function of an export signal. Intriguingly, the secreted form of ASP-6 was not recognized by ConA, whereas it clearly reacted with WGA. This might be an indication of advanced trimming of oligomannosidic oligosaccharides and the presence of truncated glycans on the secreted form. It might be further speculated that the released protein represents the proportion of newly synthesized enzyme that has not been phosphorylated and targeted to the lysosomes, but has been trimmed and secreted instead. Since recognition of the *P*Cho-epitope by *P*Cho-specific antibody was not affected by PNGase F and PNGase A treatment, we conclude that *P*Cho-epitopes are not linked via N-glycan to the protein moiety in this case. The precise assignment of the type of linkage between *P*Cho-moieties and the polypeptide backbone, however, awaits further studies.

Acknowledgments

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