Studies on the Site and Mechanism of Attachment of Phosphorylcholine to a Filarial Nematode Secreted Glycoprotein*

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We have recently shown that the immunomodulatory substance phosphorylcholine (PC) is covalently attached to ES-62, a major secreted protein of the filarial nematode parasite Acanthocheilonema viteae, via an N-linked glycan. Linkage of PC to N-glycans is previously unreported, and hence we have investigated the biochemical changes underlying it. PC addition was found by pulse-chase experiments to be a fairly early event during intracellular transport, occurring within 40–60 min of protein synthesis. Biosynthetic labeling/immunoprecipitation experiments revealed that addition of PC to ES-62 was blocked by (i) brefeldin A, an inhibitor of trafficking of newly synthesized proteins from the endoplasmic reticulum (ER) to the Golgi, (ii) 1-deoxynojirymycin, an inhibitor of glucosidase activity in the ER, and (iii) 1-deoxymannojirimycin, an inhibitor of mannosidase I in the cis Golgi. Swainsonine, an inhibitor of mannosidase II in the medial Golgi, did not affect PC addition. Taken together these data indicate that PC attachment is a post-ER event which is dependent on generation of an appropriate substrate during oligosaccharide processing. Furthermore, they strongly support that PC addition takes place in the medial Golgi and that the substrate for addition is the 3-linked branch of Man\textsubscript{5}GlcNAc\textsubscript{3} or Man\textsubscript{5}GlcNAc\textsubscript{3}.

Filarial nematodes are arthropod-transmitted parasites of vertebrates. Of the eight species that infect humans, three, Wuchereria bancrofti, Brugia malayi, and Onchocerca volvulus, are of major medical importance. The former two parasites inhabit the lymphatics, where they are associated with a number of pathological conditions, the most important of which is elephantiasis. O. volvulus resides in subcutaneous tissues, where it is relatively benign. However, larval forms (microfilariae) released by adult female worms migrate continuously through the skin and may cause chronic debilitating lesions. Furthermore, the microfilariae may invade the eye, invoking a number of pathological changes, and ultimately causing blindness. It is currently estimated that there are almost 150 million people infected with these parasites in the tropics, and a further 1,000 million at risk (1).

Filarial nematodes release a number of proteins into their environment. A rather unusual characteristic of many of these proteins is that they contain phosphorylcholine (PC)\textsuperscript{1} in apparent covalent association (reviewed in Ref. 2). The function of this PC on secreted proteins has yet to be unequivocally established, but it is likely that it plays a role in interfering with the ability of the host immune system to respond to the worm. It has recently been shown, for example, that PC-containing antigens of B. malayi can inhibit phytohemagglutinin-induced proliferation of human T-cells (3), and ES-62, a major PC-containing secreted protein of the rodent filarial parasite Acanthocheilonema viteae (4), is able to inhibit polyclonal murine B-cell proliferation induced by ligation of the antigen receptors (5). In both cases, the observed effect is confirmed as being due to PC, as it can be mimicked by PC bound to bovine serum albumin.

PC is also found on a number of nonsecreted proteins of filarial nematodes, where its function is unknown. It appears to be particularly abundant within the uterus and digestive tract (6), but the significance of this is uncertain. PC is a component of teichoic acids in the cell wall (7) and lipoteichoic acids in the cell membrane (8) of certain species of Gram-positive bacteria, where it appears to play a crucial role in the maintenance of normal cell shape and physiology (9). Whether related roles exist with respect to filarial nematodes remains to be established. Certainly, the mechanism of attachment of PC is similar in both groups of organisms, in that it involves linkage to carbohydrate (10–11). The same appears to be true of PC found in fungi (12).

Recently we have observed that attachment of PC to ES-62 is via a glycan of the N-type. This conclusion is based on the findings that PC and glycan groups are both lost following exposure of ES-62 to N-glycosidase F (11) and absent following culture of A. viteae in the presence of tunicamycin (13). The attachment of PC to an N-type glycan is a previously unreported finding, and hence we are interested in elucidating the biosynthetic mechanisms involved. Such information could be of therapeutic value as synthesis of PC-glycans may represent an urgently required (particularly with respect to O. volvulus) novel target for chemotherapy in filarial infections.

This article describes three approaches to gaining information on how attachment of PC to an N-type glycan is achieved. They each rely on the fact that newly synthesized glycoproteins, which are destined for secretion, follow a fixed route through the cell prior to exit (reviewed in Ref. 14). The three approaches are as follows. (i) Pulse-chase time course experiments in combination with immunoprecipitation analysis are used to investigate how soon after synthesis of ES-62 PC is attached. This should indicate whether addition is an early or late event during intracellular trafficking. (ii) Brefeldin A, an

\[\text{ES-62} \rightarrow \text{Golgi} \rightarrow \text{Secreted} \]

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\[\text{1 The abbreviations used are: PC, phosphorylcholine; dNM, 1-deoxynojirymycin; ER, endoplasmic reticulum; dMM, 1-deoxymannojirimycin; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.}\]
inhibitor of intracellular trafficking from the ER to the Golgi, is employed (reviewed in Ref. 15). Use of this reagent may pinpoint addition of PC to one of these two subcellular locations. (iii) Inhibitors of oligosaccharide processing are used (reviewed in Ref. 16). N-Type glycans of newly synthesized glycoproteins undergo a series of trimming/addition steps during trafficking. Inhibitors of oligosaccharide processing are available that block the action of individual processing enzymes, each of which has a characteristic glycansubstrate specificity and subcellular location.

In combination, these three approaches should help establish when, where, and to what PC is added during trafficking of ES-62.

**EXPERIMENTAL PROCEDURES**

**The Parasite—**Jirds (*Meriones libycus*) were infected by subcutaneous injection of *A. viteae* 3rd stage larvae recovered from infected ticks (*Ornithodoros moubata*) (17). Adult parasites were recovered following direct visual examination of the skin and underlying body surfaces of jirds. The worms were transferred to Petri dishes containing RPMI complete medium (RPMI 1640 with added glucose (1% w/v), glutamic acid (2 mM), penicillin (100 units/ml), streptomycin (100 μg/ml) and built-in pH (7.2) (with 20 mM Hepes). Time Course of Synthesis of ES-62—Groups of three adult female *A. viteae* were cultured overnight at 37 °C in 10 ml of methionine-free RPMI complete medium, in an atmosphere of 5% CO₂, 95% air. The medium was removed and replaced with 2 ml of the same containing 0.74 MBq [³⁵S]methionine (37 TBq/mmol; Amersham Int., Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA, UK). After 15 min, the labeling medium was removed and replaced with 10 ml of RPMI complete medium. A chase was then allowed to proceed for defined time periods, before worms were employed for preparation of parasite whole worm extracts ("cell extract"). One group of female worms was also cultured as above for preparation of secreted proteins ("secreted extract"). 15 min after pulsing, the medium was removed and replaced with 5 ml of RPMI complete medium. This was repeated at various time points and then all recovered media was employed for secreted extract preparation.

**Preparation of Parasite Whole Worm ("Cell") Extract—**This was essentially undertaken as described previously (18). Briefly, worms were extensively homogenized in 1 ml of PBS, pH 7.2, in the presence of the proteinase inhibitors N⁶-p-tosyl-L-lysine chloromethyl ketone (50 μg/ml), Nα-L-phenylmethyl sulfonyl fluoride (1 μM), inhibitors were obtained from Sigma, Poole, Dorset, UK. The samples were then centrifuged (20,000 × g for 25 min), and the supernatant was retained. Worm extracts (cell extract) were stored at −20 °C.

**Preparation of Secreted Extract—**Spend culture medium was filtered using a 0.22-μm membrane (Sigma) to remove microorganisms released by adult female worms. It was then concentrated using Centriprep tubes with a 30-kDa cut-off membrane (Amicon Ltd., Upper Mill, Stonehouse, Gloucestershire GL10 2BJ, UK). A further concentration step (to 100–150 μl) was then undertaken using Centricron microconcentrators, again with a 30-kDa membrane. These were also employed to "wash" the samples with PBS, pH 7.2. The final volume of all of the samples was adjusted to 200 μl, and they were stored at −20 °C.

**Measurement of Trichloroacetic Acid-perspicilable Radioactivity—**This was undertaken essentially as described previously (18). Briefly, two 10-μl aliquots of radiolabeled secreted extract subjected to trichloroacetic acid precipitation using 10% (w/v) trichloroacetic acid precipitation and in some cases immunoprecipitation/SDS-PAGE. In some experiments, [L-³⁵S]methionine was replaced with [methyl-²H]choline chloride (3.7 MBq; 2.78 TBq/mmol) and homocholine-free medium, replaced with choline-free. In these experiments, the preincubation period was increased to 16 h, to optimize the incorporation of [³H]choline into ES-62, and the experiments were terminated after a 60-min chase period for preparation of parasite cell extracts for analysis by SDS-PAGE.

**Culture of Worms with Inhibitors of N-Linked Oligosaccharide Processing—**Adult female *A. viteae* were divided into groups of three, placed in test tubes containing 5 ml of methionine-free RPMI complete medium, and one of the following then added: 1-deoxynynojirimycin hydrochloride (dMM), 1-deoxynorjirimycin (dNM), swainsonine (Boehringer Mannheim, UK). dMM and dNM were employed at a concentration of 1 mM and swainsonine and 10 μM. A control (no reagent) was also used to which no worm addition of PC to one of these two subcellular locations.

**Determination of radioactivity was undertaken using an LKB Wallac 1217 Rackbeta liquid scintillation counter.**

SDS-PAGE/Fluorography—Samples were resolved by SDS-PAGE, using a Bio-Rad electrophoresis cell according to the manufacturer’s instructions. 10% (w/v) acrylamide gels were employed, and 2-mercaptoethanol was used for reduction of samples. 14C-Labeled molecular weight markers (Amersham Corp.) were also run to allow molecular weight estimations. Gels were treated with the fluorographic reagent AMPLIFY (Amersham Corp.) before exposure to pre-shielded x-ray film (Hyperfilm-MP, Amersham Corp.) and storage at −70 °C.

Immunoprecipitation—Radiolabeled sample (10 μl; 1–5,000 dpm) was added to 5 μl of either rabbit antiserum raised against purified ES-62 (anti-ES-62 (4)), 5 μl of normal rabbit serum, or 5 μl of TEPC 15 (1 mg/ml, Sigma), a mouse IgA myeloma protein designated as having specificity for PC based on the finding that PC inhibits its binding to PC-containing molecules such as pneumococcus C polysaccharide (related compounds, glycerophosphorylcholine, phosphocholine, choline, are all inferior to PC in this respect, suggesting that PC represents the true specificity) (19). Protein samples were incubated with gentle agitation for 1 h at 37 °C. An appropriate concentration of either goat-anti-rabbit IgG serum or goat anti-mouse IgA and goat anti-mouse Ig (equal volumes) was added, and samples were incubated with gentle agitation for 1 h at 37 °C and then left overnight at 4 °C to allow precipitates to form. Precipitates were washed (3 × ) with ice-cold 10 mM Tris-HCl, 50 mM NaCl, 0.1% (v/v) Nonidet P-40, pH 8.3, and then subjected to analysis by SDS-PAGE/fluorography.

**Culture of Worms with Brefeldin A—**Three adult female *A. viteae* were cultured in a test tube containing 5 ml of methionine-free RPMI complete medium and brefeldin A at a concentration of 1 μg/ml. A control (no brefeldin A) was also set up. Worms were incubated for 2 h at 37 °C. The medium in each case was removed and replaced with the same but containing 2.0 MBq of [L-³⁵S]methionine. Following 1, 20 ml of RPMI complete medium containing brefeldin A (1 μg/ml) was added. Following overnight incubation the medium was removed and secreted, and cell extracts were prepared as described above. These were then subjected to trichloroacetic acid precipitation and in some cases immunoprecipitation/SDS-PAGE. In some experiments, [L-³⁵S]methionine was replaced with [methyl-²H]choline chloride (3.7 MBq; 2.78 TBq/mmol, Amersham) and methionine-free medium, replaced with choline-free. In these experiments, the preincubation period was increased to 16 h, to optimize the incorporation of [³H]choline into ES-62, and the experiments were terminated after a 60-min chase period for preparation of parasite cell extracts for analysis by SDS-PAGE.

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Radiolabeled sample (10 μl) was then undertaken using Centricron microconcentrators, or no enzyme as a control as described previously (20). 100-ng aliquots of radiolabeled secreted extract were subjected to digestion using Endoglycosidase H (Boehringer Mannheim Biochemical) and rabbit antiserum raised against purified glycolipid-containing molecules such as pneumococcus C polysaccharide (related compounds, glycerophosphorylcholine, phosphocholine, choline, are all inferior to PC in this respect, suggesting that PC represents the true specificity) (19). Loss of both radiolabels from the protein was confirmed by analysis of one-tenth volume of each reaction (1 μl of original radiolabeled samples) by SDS-PAGE. The remainder of each sample was made up to 200 μl and individually passed through a column (20 × 1 cm) containing Bio-Gel P-2 gel according to the manufacturer’s instructions. The lectin is digoxigenin-labeled; the indicator system is an anti-digoxigenin antibody linked to alkaline phosphatase, with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate being employed as substrate.

**Gel Filtration of Radiolabeled ES-62 following Exposure to N-Glycosidase F—**10 μl of either [H]glucosamine or [H]choline-labeled ES-62 was subjected to digestion using N-glycosidase F as described previously (11). Loss of both radiolabels from the protein was confirmed by analysis of one-tenth volume of each reaction (1 μl of original radiolabeled samples) by SDS-PAGE. The remainder of each sample was made up to 200 μl and individually passed through a column (20 × 1 cm) containing Bio-Gel P-2 gel according to the manufacturer’s instructions (Bio-Rad). A series of 300-μl fractions were collected and examined for radioactivity using an LKB Wallac 1217 Rackbeta liquid scintillation counter.
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RESULTS

Phosphorylcholine Is in Covalent Association with an N-Type Glycan Attached to ES-62—It has previously been demonstrated that PC is removed from ES-62 by N-glycosidase F (11) and absent from ES-62 produced by worms cultured in tunicamycin (13). The logical conclusion from these data is that PC is covalently attached to ES-62 via an N-type glycan. This is further supported by (i) the observation that the [3H]choline-labeled products of ES-62 exposed to N-glycosidase F migrated within the area occupied by released [3H]glucosamine-labeled N-type glycans during gel filtration (Fig. 1); and (ii) radiolabeled choline cannot be displaced from ES-62 by incubation in high concentrations of nonradioactive choline or phosphorylcholine, and likewise radiolabeled choline does not interact with nonradioabeled ES-62 (results not shown).

Pulse-Chase Analysis of Time of Addition of PC—The aim of this approach was to elucidate when PC is attached to ES-62 during its synthesis, processing, and transport prior to secretion. Groups of adult worms were pulsed with [35S]methionine during its synthesis, processing, and transport prior to secretion. The secreted extracts indicated that radiolabeled protein first appeared in the culture medium within 2–3 h of the initiation of the chase period and that secretion of labeled protein has virtually ceased by 5 h (Fig. 2). Examination of the radiolabeled material by SDS-PAGE/fluorography indicated that, as previously established for A. viteae ES (4), it is almost entirely ES-62 (not shown). We therefore subjected cell extracts obtained from worms subjected to chase for various periods to immunoprecipitation using anti-ES-62 or anti-PC (TEPC 15). Analysis of the immunoprecipitates by SDS-PAGE/fluorography indicated that the labeled protein could be detected faintly as a sharp band at the beginning of the chase period (Fig. 3A, zero time point). Synthesis therefore occurs rapidly under these in vitro conditions, a result consistent with the rate of synthesis observed for the surface/ES product, GP29, from the related parasite, B. malayi (21). ES-62 was more clearly detected, and as a more diffuse band, after 20 min of the chase period (Fig. 3A). The more diffuse appearance is likely to be due to glycosylation, although the presence of large amounts of immunoglobulin heavy chain (molecular mass 50–65 kDa) in the immunoprecipitates may interfere with the normal migration of ES-62 and hence cause it to appear more diffuse. Radiolabeled ES-62 can no longer be detected in cell extracts after 2–3 h (Fig. 3A), a result consistent with its appearance in the medium at this time (Fig. 2). The specificity of the immunoprecipitation reaction is shown in Fig. 3B; replacement of anti-ES-62 with normal rabbit serum results in loss of detection of the major band of molecular mass 62 kDa seen at the 60-min time point in Fig. 3A. PC attachment to ES-62 was clearly detected after 60 min of chase, as judged by immunoprecipitation employing TEPC 15 (Fig. 3C). Employment of a control antibody to demonstrate TEPC 15 specificity is not necessary as the antibody does not bind ES-62 shown by biosynthetic radiolabeling to lack PC (see Figs. 7B and Fig. 8). The increase in intensity of ES-62 demonstrated between the 40- and 60-min time points in Fig. 3A may also be consistent with the majority of pulse-labeled ES-62 undergoing PC attachment at this point, as the monoclonal anti-ES-62 reagent contains a strong element of anti-PC activity (4). Taken together, these results suggest that PC is attached to ES-62 within 40–60 min of synthesis of the protein.

Effect of Brefeldin A on Addition of PC to ES-62—Preliminary experiments (results not shown) indicated that parasites...
cultured in the presence of [35S]methionine and brefeldin A at a concentration of 1 μg/ml demonstrated an almost total block on secretion (<5% of control) but a normal level of protein biosynthesis (>95% of control) as measured by trichloroacetic acid-precipitable radioactivity in secreted and cell extracts, respectively. Immunoprecipitation analysis employing anti-ES-62 revealed as expected the presence of ES-62 in secreted samples prepared from control worms (Fig. 4, track c) but not brefeldin-A treated (Fig. 4, track d) samples. The antiserum was also employed to examine a cell extract prepared from control and brefeldin A-treated worms. ES-62 was recognized in both extracts but was clearly more abundant in the brefeldin A-treated extract (Fig. 4, track b). This observation is again consistent with retention of ES-62 in brefeldin A-treated worms. The retained ES-62 was found to largely lack PC, however, as demonstrated when [3H]choline was employed to biosynthetically label ES-62 in control and brefeldin A-treated cell extracts (Fig. 5). The amount of radiolabeled protein as a whole in the extract prepared from worms exposed to brefeldin A (track b) is in fact drastically reduced relative to the control (track a). Clearly, therefore, brefeldin A causes major inhibition of attachment of PC to parasite proteins including ES-62.

**Effect of Inhibitors of Oligosaccharide Processing on Addition of PC to ES-62**—The aim of this approach was to disrupt processing of N-linked oligosaccharides in an attempt to prevent PC addition. Three different inhibitors of oligosaccharide processing were employed. For interruption of glucosidase I and II activities, the inhibitor dMM was used. For interruption of mannosidase I and mannosidase II activities, respectively, dMM and swainsonine were employed (for a detailed review of the properties of all three reagents, see Ref. 16). Adult *A. viteae* were cultured in the presence of inhibitors and [35S]methionine. Worms in all cases remained healthy (as judged by motility) throughout the culture period, although it was observed by measurement of trichloroacetic acid-precipitable radioactivity in the culture medium (not shown) that dMM virtually abolished protein secretion. Similar effects have been noted by other workers with respect to this reagent (reviewed in Ref. 16).

Adult *A. viteae* were cultured in the presence of [3H]choline or [3H]glucosamine plus dNM, and cell extracts were prepared and examined by SDS-PAGE/fluorography. This clearly indicated that exposure to dNM prevented attachment of PC to ES-62, as the parasite product can be detected when [3H]glucosamine (A) or [3H]choline (B) in the absence (lane a) or presence (lane b) of dNM, and cell extracts were prepared and analyzed by SDS-PAGE/fluorography. The distances migrated by radiolabeled molecular weight standards are shown on the left.

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blocks removal of two such mannose residues, it would be expected that ES-62 prepared from worms exposed to swainsonine should show greater interaction with the lectin than control protein. As shown in Fig. 9 (cf. lanes B and D), much greater binding is indeed observed. (ii) N-Type glycans lose their sensitivity to endoglycosidase H, following processing from a Man$_5$ to a Man$_4$ structure (24). We have previously shown that PC-containing N-glycans are not cleaved from ES-62 by this enzyme (20), and the same is true of the glycan showing weak lectin binding (Fig. 9, lane C). If removal of Man$_{4–5}$ is prevented by swainsonine, however, it might be predicted that glycan cleavage would take place. Again, as shown in Fig. 9, this is in fact the case as exposure of swainsonine-treated ES-62 to endoglycosidase H virtually abolishes lectin binding (Fig. 9, lane A). The same is also true of binding of TEPC 15 (result not shown).

**DISCUSSION**

This article describes three approaches to examining addition of PC to ES-62, a major PC-containing secreted product of the rodent filarial nematode, *A. vitiae*. The first approach was designed to investigate when PC is attached to ES-62, during intracellular transport. It was found that if worms were pulsed with $[^{35}S]$methionine for 15 min, a further 2–3 h was required before labeled ES-62 was detected in the culture medium (Fig. 2). This represents a similar turnover rate to the major surface glycoprotein of related *Brugia* species, Gp29 (21), but is more rapid than the *Brugia* “ladder” protein, Gp15/400 (25). With respect to detecting radiolabeled ES-62 within whole worm (cell) extracts, this was possible by immunoprecipitation using anti-ES-62. A faint sharp band was observed on completion of the pulse period and a stronger diffuse band after a further 20 min (Fig. 3A). PC was detected on ES-62 by this enzyme (20), and the same is true of the glycan showing weak lectin binding (Fig. 9, lane C). If removal of Man$_{4–5}$ is prevented by swainsonine, however, it might be predicted that glycan cleavage would take place. Again, as shown in Fig. 9, this is in fact the case as exposure of swainsonine-treated ES-62 to endoglycosidase H virtually abolishes lectin binding (Fig. 9, lane A). The same is also true of binding of TEPC 15 (result not shown).
labeled cell extracts, by SDS-PAGE/immunoprecipitation employing anti-ES-62, in both control and brefeldin A-treated extracts (particularly the latter). When $^3$H-choline-labeled cell extracts were examined by SDS-PAGE, however, the intensity of the band detected in brefeldin A-treated extracts was considerably reduced relative to the control. Thus, brefeldin A inhibits addition of PC to ES-62. The primary effect of brefeldin A is considered to be blockage of transfer of protein-containing vesicles from the ER to the Golgi (28, 29), but it can also cause Golgi disintegration (15). Both of these previously described observations would favor the Golgi as the site of PC addition to ES-62.

The final approach adopted was utilization of inhibitors of oligosaccharide processing. One of the reagents employed, dNM, was found to block both secretion (Fig. 7) and PC addition (Fig. 6), properties common to brefeldin A (Figs. 4 and 5). Since dNM has been previously shown to interfere with transport of some proteins from the ER to the Golgi (22), it was considered that our results might reflect dNM acting in a similar manner to brefeldin A, i.e., blocking transport. However, it was observed that several molecules could be labeled with $^3$H-choline in the cell extract but that dNM unlike brefeldin A was only able to prevent this with respect to some of them. Thus if PC attachment is simply a consequence of being in the correct cellular compartment, then clearly only some of the proteins that can be radiolabeled must be prevented from leaving the ER by dNM treatment. However, we also had to consider that dNM will block oligosaccharide processing at a very early stage. This raised the alternative possibility that the ability of dNM to prevent PC attachment might be due to failure to create a critical substrate. Furthermore, studies with dMM were strongly supportive of this idea in that treatment of worms with this inhibitor led to the secretion of an ES product which lacked PC. The steps in oligosaccharide processing which dMM blocks (30) are shown in Fig. 10. By inhibiting mannosidase I in the cis Golgi, it prevents the removal of three mannose residues. Clearly, therefore, it can be argued that the removal of one or more of these sugars is crucial for the addition of PC to ES-62. If this does not take place then PC is not attached. As the processing steps which dMM inhibit occur in the cis Golgi, this indeed confirms that PC is added to the N-glycan, post-ER.

The failure of dNM to prevent addition of PC to some of the other molecules in the cell extract may suggest that their mode of attachment of PC is not based on use of an N-type glycan. This is consistent with some preliminary data we have obtained that intriguingly suggests that PC may be more frequently attached to nonsecreted molecules of filarial nematodes via O-type glycans. This is currently being explored further. Maizels and colleagues (31) have previously produced some preliminary data consistent with PC being attached to molecules containing O-glycans.

Addition of PC is not prevented by exposure of A. viteae to swainsonine, the inhibitor of mannosidase II in the medial Golgi. This result can be explained by the oligosaccharide processing steps, which swainsonine inhibits, not being required for PC addition to take place. Fig. 10 shows that two mannose residues are removed from the 6-linked branch of the N-glycan by mannosidase II. The fact that blockage of this step has not prevented PC addition might suggest that PC is added to the 3-linked branch of the glycan. Also, based on its resistance to endoglycosidase H (20) and to the finding that N-glycans of swainsonine-treated ES-62 are susceptible (Fig. 9, track a), the substrate oligosaccharide would appear to be subject to processing by mannosidase II. Clearly, if this is the case, PC cannot be bound to the two mannose residues that this enzyme targets.

If the N-glycan to which PC is attached is indeed trimmed by mannosidase II, then it must also be subject to the action of GlcNAc transferase I. This is because addition of GlcNac by this enzyme results in generation of the appropriate substrate for mannosidase II. Whether the addition of the GlcNAc is a prerequisite for PC addition is uncertain. If PC addition is an earlier event, then it would appear that it does not inhibit GlcNAc attachment. Conversely, we have no evidence of further oligosaccharide processing beyond removal of Man$_{3-5}$ in that we have been unable to biosynthetically label ES-62 with $^3$H-galactose (result not shown) or find any evidence of complex glycans by lectin binding (20). This raises the question as to whether further processing is blocked by PC addition and leads us to consider whether transfer of GlcNAc, rather than removal of Man$_{3-5}$, may be the crucial event for PC addition. Certainly our data are consistent with the glycan of ES-62, to which PC is attached initially, resembling the Man$_{3-8}$ structure which results after the action of GlcNac transferase I and then subsequently being converted into Man$_{6-8}$ by the action of mannosidase II (Fig. 10). However, since PC addition has been shown to be independent of the action of mannosidase II, attachment could with equal likelihood occur following removal of Man$_{3-5}$. Regardless of which (if either) model is correct, the data obtained using dMM and swainsonine, allied to the lack of evidence for formation of complex glycans, strongly support the site of PC addition as being the
medial Golgi.

In summary, the data we have obtained provide the first information on the site and mechanism of synthesis of filarial PC-glycans, previously unstudied structures that may play an important role in parasite survival. Furthermore, in addition to being of purely scientific interest, such data in combination with structural analysis could ultimately be of therapeutic value in that it may provide ideas for designing inhibitors of PC attachment for use as drugs. One idea, for example, would be to design a reagent which resembles the glycan substrate for PC addition in order that it may act as a competitive inhibitor. The prevention of PC addition resulting from exposure of worms to reagents such as dNM and dMM confirms that such an approach is a feasible proposition.

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