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Small Collagenous Proteins Present during the Molt in *Caenorhabditis elegans*[1]

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Abstract: Immunoblotting experiments using antibodies directed against the large collagenous cuticle proteins of *Caenorhabditis elegans* revealed a class of small collagenous proteins (CP) of apparent molecular weight 38,000–52,000 present during the L4 to adult molt. These CP are smaller than most vertebrate collagens characterized to date and share many characteristics with the small collagenous products translated in vitro from RNA isolated at this molt. *C. elegans* collagen genes, collagen-coding mRNA, and collagenous in vitro products that have been characterized are also small. Detection of small CP in vivo in *C. elegans* thus lends further support to the hypothesis that such small collagenous proteins are the primary gene product precursors to the larger collagenous proteins isolated from the *C. elegans* cuticle.

Key words: anticuticle antibodies, cuticle biosynthesis, *Caenorhabditis elegans*, collagenous proteins.

The cuticle of adult *Caenorhabditis elegans* is synthesized de novo at the L4 to adult molt (4). When adult cuticles are treated with reducing agent and detergent, many large collagenous cuticle proteins (CCP) with molecular weights (M_r) of 60,000–210,000 are solubilized (5,7). However, a single predominant collagen mRNA size class (1.2–1.4 kb) has been detected at this molt (3,21) and correspondingly small (M_r of 32,000–52,000) collagenase-sensitive polypeptides are translated in vitro from these RNA preparations (21). With one minor exception, collagen-coding RNA or collagenous translation products of the large size required to represent the CCP are not found. The two *C. elegans* collagen genes sequenced so far are also small in size, and approximately 20 other genes appear to be of similar small size, as judged by restriction endonuclease mapping of cloned DNA fragments (3,13).

For these reasons, and because vertebrate collagen chains are known to be subject to extensive post-translational modifications, including interchain crosslinking (9), it is plausible that the large CCP isolated from the *C. elegans* cuticle are derived biosynthetically by covalent crosslinking of smaller primary gene product precursors. Such a model predicts that the putative precursors should be present in molting nematodes and should have relatively small M_r and other properties consistent with the observed properties of the characterized collagen genes and collagenous in vitro translation products. However, such small collagenous polypeptides have not been detected in extracts of molting nematodes after continuous or classical short-pulse labelling regimes (M. Kusch, unpubl.), perhaps because of insensitivity of the labelling methods feasible at the molt in *C. elegans* (21). As an alternative, we developed an immunoblotting procedure (11,22) capable of detecting very small amounts of the CCP. This procedure was used to screen extracts of molting nematodes for the presence of putative cuticle protein precursors.

The rabbit antiserum used in these studies was directed against the solubilized adult cuticle proteins (adult CCP). This immunogen contains all the collagenous proteins solubilized by disulfide reduction in detergent after previous extractions that remove noncuticle components from the preparation (5,6). Because the precursors should share many structural determinants with adult CCP, this antiserum appeared to be a good choice for detection of putative precursors.

These experiments show for the first time that small collagenous proteins with properties similar to the collagenous translation products of RNA isolated from molting

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nematodes (21) are indeed detectable in vivo at the molt.

Materials and Methods

Abbreviations: LO = lethargus onset. LP = lethargus peak. LL = late lethargus. YA = young adult. SS = sonication supernatant. SSS = supernatant from 12,000 *g* centrifugation of SS. SSP = 12,000 *g* pellet from SS. SDS fraction = supernatant containing proteins solubilized by boiling crude cuticles in sodium dodecyl sulfate (SDS). BME fraction = supernatant containing cuticle proteins solubilized by boiling SDS-washed cuticles in SDS and β-mercaptoethanol (BME). Small CP = small collagenous proteins. CCP = collagenous cuticle proteins (isolated directly from cuticles in BME fraction).

Nematode growth: Synchronous populations of molting nematodes were grown at 25 C from dauer as described by Cox et al. (5). A temperature-sensitive fertilization-defective mutant of *C. elegans, fer-1 (hc1ts)* I (strain BA1) was used in all experiments; growth at the restrictive temperature, 25 C, insured that cuticle preparations were substantially free of contamination by egg shells and embryonic proteins (5). The progression of the population through the L4 to adult molt was judged by the percentage of nematodes with pharyngeal pumping present at any given time during the lethargus marking the molt (4). LO populations contained about 20% nonpumping nematodes, 80% L4; LP about 75% nonpumping, 25% L4; LL about 5% nonpumping, 95% adults; young adult (YA) populations were isolated 2.5 hours after the L4 to adult molt.

To isolate protein fractions for detection of small collagenous proteins, nematodes harvested at the onset, peak, and conclusion of lethargus marking the L4 to adult molt or as very young adults were sonicated and proteins were extracted into four fractions—SS, SSP, SDS, and BME—as described below.

Protein isolation: Nematodes were sonicated and fractionated as described by Cox et al. (5) in order to obtain crude cuticles. In addition, the sonication supernatant (SS) (after pelleting of cuticles by centrifugation at 300 *g*) was further fractionated. In previous work, the SS had been discarded; it contains proteins released during sonication that do not pellet with cuticles at 300 *g* (5).

The SS was centrifuged at 12,000 *g* for 30 minutes at 4 C. This latter centrifugation separates the SS into a pellet (SSP) and supernatant (SSS), neither of which contained residual cuticle pieces visible by light microscopy. The SSP was resuspended and boiled in a solution containing 1% SDS, 10% BME (v/v), and 0.125 M tris-glycine, pH 6.8, and the fraction was then subjected to further analysis.

The crude cuticles collected by centrifugation at 300 *g* were extracted as described (5), first by boiling in SDS to solubilize muscle and other proteins still attached to cuticles after sonication (SDS fraction), and then by boiling these cleaned cuticles in SDS and BME to solubilize the collagenous cuticle proteins, or CCP (BME fraction). Protein concentrations were determined by the technique of Lowry et al. (15).

Collagenase digestion: Some SSP samples (chosen because the proteins of interest were most easily detected in this fraction) were digested with clostridial collagenase (Form III, Adv. Biofactures Corp.) at 37 C overnight. The collagenase and digestion conditions used have been documented as yielding no detectable nonspecific protease activity (17,20). Collagenase:protein ratios were routinely one unit per microgram acetone-precipitated protein suspended in collagenase digestion buffer (50 mM tris-HCl, pH 7.4, 150 mM NaCl, 5 mM *N*-ethylmaleimide, 5 mM $CaCl_2$). Undigested samples were incubated in the same buffer without collagenase.

Gel electrophoresis: Proteins were electrophoresed on 7.5% or 10% SDS-polyacrylamide gels (5,14). Aliquots of BME fractions containing 10 μg total protein or SSS, SSP, and SDS fractions containing 50 μg per lane were routinely loaded for gels to be immunoblotted.

SSP proteins were further analyzed using one-dimensional isoelectric focusing. Four percent urea–polyacrylamide slab gels (0.75 mm thick) were used; gel solutions were as described by O'Farrell (19) except that ammonium persulfate was added at 0.1% instead of 0.01% to achieve proper polymerization. A linear pH gradient of 4–

6 was established, and sample preparation and prefocusing volt-hours were as described by O'Farrell (19) for tube gels. Fifty micrograms of SSP fraction protein were electrophoresed in each lane.

In order to correlate the molecular size characteristics of the small CP seen by SDS–PAGE separation with the pI of the collagenous proteins seen by IEF separation, samples containing 50 μg SSP proteins were also prepared and electrophoresed on two-dimensional gels (19).

Proteins in all gel types were transferred to diazophenylthioether paper and immunoblotted as described below.

Antiserum preparation: Young male New Zealand White rabbits were immunized intramuscularly with 1 mg of adult CCP dissolved in 0.125 M tris-HCl, pH 6.8, 1% SDS, and 5% (v/v) BME (5) and emulsified with an equal volume of Freund's complete adjuvant. Blood was collected 14 days after immunization. Antiserum from the same rabbit was used in all experiments.

Immunoblotting: For a description of the general technique see Gershoni and Palade (11). Proteins were separated by SDS-gel electrophoresis or isoelectric focusing and then electrophoretically transferred to diazophenylthioether paper (22) prepared as suggested by the manufacturer (Bio-Rad, Inc.). The small CP could not be detected as readily when proteins were transferred to nitrocellulose instead of diazophenylthioether paper. After being blocked with a solution containing 10% (v/v) ethanolamine, 1% bovine serum albumin (Miles Pentex, Fraction V Powder), and 100 mM tris-HCl, pH 8.8, the filters were washed with wash buffer (25 mM tris-HCl, pH 7.4, 150 mM NaCl, 1% bovine serum albumin, 0.1 mM phenylmethylsulfonylfluoride, 2 mM (ethylenedinitrilo)-tetraacetic acid, and 0.1 mM dithiothreitol) for 1 hour and incubated with antiserum diluted in this buffer (1:400) for 12 hours. Filters were then washed five times (1 hour each time) in wash buffer containing 0.5% Triton X100 and 0.2% SDS and incubated for 12 hours with [^{125}I]protein A (4.25×10^5 cpm/μl) diluted in wash buffer (1:500). We prepared [^{125}I]protein A by the chloramine T method (10); specific activities were typically about 2×10^7 cpm/μg. After washing five times (10 minutes each time) with wash buffer plus detergents, the filter was exposed to XAR-5 film at −70 C for 2 hours to 1 week; sometimes intensifying screens were used.

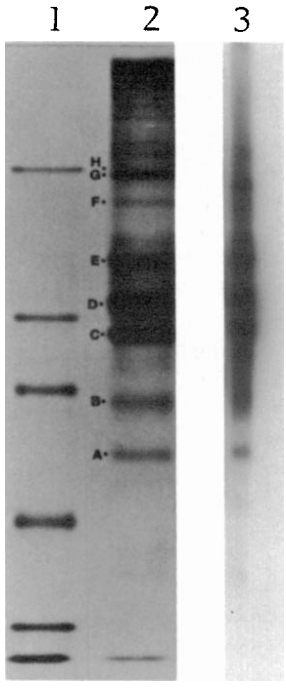

FIG. 1. Autoradiograms showing [^{35}S]-labelled (6) adult *Caenorhabditis elegans* collagenous cuticle proteins (adult CCP, lane 2) compared to the same proteins detected using immunoblotting (lane 3). Major bands are labeled A–H; proteins were separated on 7.5% SDS-polyacrylamide gels. Lane 1 shows [^{14}C]-labelled molecular weight markers with M_r of 212,000, 93,000, 66,000 43,000, and 29,000.

RESULTS

Initial antibody characterization used an immunoblotting procedure optimized for use with adult CCP and antibodies directed against them. Figure 1 compares the electrophoretic pattern of [^{35}S]-labelled CCP present in the immunogen with immunoblotting of a similar unlabelled protein mixture. The antiserum contained antibodies that bound to all the adult CCP detectable in the immunogen. Titer of the antiserum was estimated by dilution; binding to the CCP was detectable above background up to a dilution of 1:30,000. The assay was sensitive, the limit of detection being estimated as 2.5 pmoles of total CCP, based on the molar specific radioactivity of

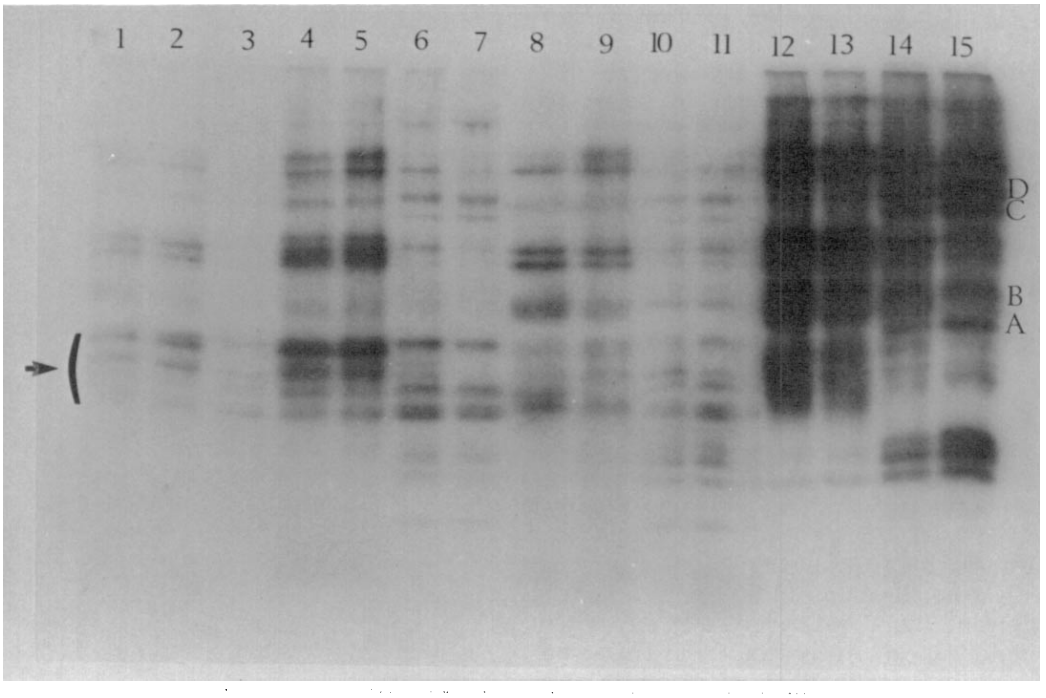

FIG. 2. Autoradiogram of immunoblot showing antibody binding to proteins isolated from molting *Caenorhabditis elegans*. SS (lanes 1–3), SSP (lanes 4–7), SDS (lanes 8–11), and BME (lanes 12–15) fractions were isolated from synchronously growing populations of BA1 nematodes at the onset (lanes 1, 4, 8, 12), peak (lanes 2, 5, 9, 13), and conclusion (lanes 3, 6, 10, 14) of the lethargus marking the L4 to adult molt, and from young adults (lanes 7, 11, 15, no SS shown). Proteins were separated on a 10% SDS-polyacrylamide gel and immunoblotted. Arrow points to the small proteins with electrophoretic mobilities similar to the translation products of RNA isolated at the L4 to adult molt. The immunoblot was exposed to film for 44 hours. Major CCP of M_r 54,000 (A), 70,000 (B), 91,000 (C), 106,000 (D).

the [^{125}I]protein A used as the detecting ligand.

Figure 2 shows an autoradiogram of an immunoblot in which each nematode fraction was tested for antibody binding after SDS-gel electrophoresis separation. The BME fractions (lanes 12–15) contained 20% of the protein (10 μg) present in the other fractions (50 μg). Antibody binding to the CCP present in each BME fraction was evident (LO, lane 12; LP, lane 13; LL, lane 14; YA, lane 15). The pattern observed in the fractions isolated early in the molt (lanes 12, 13) was similar to the pattern obtained from [^{35}S]-labelled L4 CCP (data not shown), whereas the BME fractions isolated at the conclusion of the molt or from young adults (lane 14 or 15) more closely resembled the pattern of adult CCP (Figs. 1, 2). Such patterns would be expected because the L4 cuticle is shed at the end of the lethargus. The major adult CCP are labelled A–D on the right (Fig. 2).

Antibody binding to proteins smaller than the CCP was also observed. Most important, antibody-binding species with apparent M_r between 38,000 and 52,000 were detected in every fraction (Fig. 2, arrow). The pattern of these antigenic bands changed so that the most prominent bands seen in early molt samples were between 38,000 and 52,000, whereas those appearing later were predominantly of M_r from 38,000 to 44,000, except that the 52,000 M_r band remained. This was most easily seen in the SSP fraction (lanes 4–7).

Bands containing antibody-binding proteins of lower M_r than those just described were also observed. These were most easily visualized as the three smallest bands (M_r between 31,000 and 35,000) that bind antibody in the LL and YA BME fractions (Fig. 2, lanes 14, 15). Other experiments have shown that the amount of these proteins in the cuticle increases as the adults age; therefore, these proteins appear to be

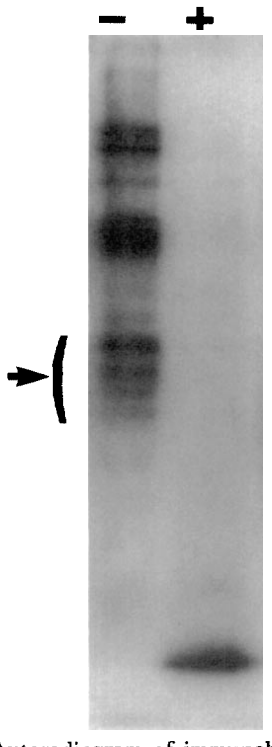

FIG. 3. Autoradiogram of immunoblot showing proteins in LP SSP fraction of *Caenorhabditis elegans* with (+) and without (−) collagenase treatment separated on a 10% SDS-polyacrylamide gel. Arrow points to collagenase-sensitive proteins (small CP) of similar mobility to collagenous polypeptides translated from RNA isolated at the L4 to adult molt. The immunoblot was exposed to film for 48 hours.

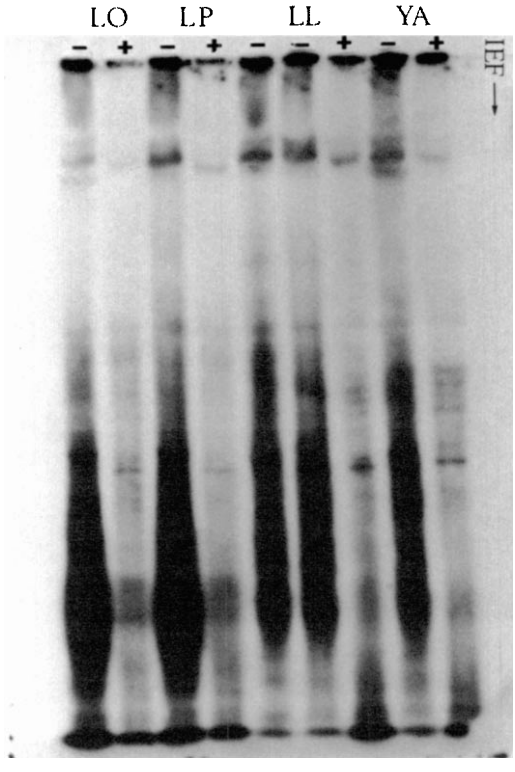

FIG. 4. Autoradiogram of immunoblot showing proteins in SSP fractions of *Caenorhabditis elegans* with (+) and without (−) collagenase treatment separated using isoelectric focusing. Electrophoresis was from top (pH 6) to bottom (pH 4). SSP fractions isolated at the onset (LO), peak (LP), and conclusion (LL) of the L4 to adult molt and also from young adults (YA) are shown. The immunoblot was exposed to film for 48 hours with an intensifying screen.

involved in cuticle maturation rather than formation (M. Kusch, J. Politz, N. Klimkow, and R. S. Edgar, unpubl.).

Figure 3 shows an autoradiogram of an immunoblot displaying the LP SSP separated by SDS-gel electrophoresis, with and without collagenase digestion; an arrow points to the small antigenic CP. Collagenase digested samples no longer contained the small antibody-binding proteins of interest (or the larger antibody-binding proteins), indicating that all of these proteins are collagenous in nature.

SSP samples were also electrophoresed on an isoelectric focusing gel containing a pH gradient between 4 and 6 (Fig. 4). The separated proteins were immunoblotted as for SDS-polyacrylamide gels. Figure 4 shows an autoradiogram of antibody binding to the proteins in the LO, LP, LL, and YA SSP fractions. As in the experiment of Figure 3, collagenous antibody-binding proteins were identified by collagenase digestion of some samples (+ lanes). Antibody binding to collagenous proteins with isoelectric points (pI) between 4 and 5 was observed. Additionally, SSP samples isolated early during the molt (LO and LP) contained collagenous proteins of more acidic pI than did the SSP samples isolated later during the molt (LL and YA). It should be pointed out that some antibody-binding bands remained after collagenase digestion (Fig. 4, + lanes). At least some of these bands probably represent antigenic peptide products of collagenase digestion (S. Politz, unpubl.). These antigenic products are evident at the dye front after SDS-gel electrophoresis of nematode extracts (e.g., at the bottom of the collagenase-digested lane in Fig. 3).

The SSP isolated at LP was analyzed by

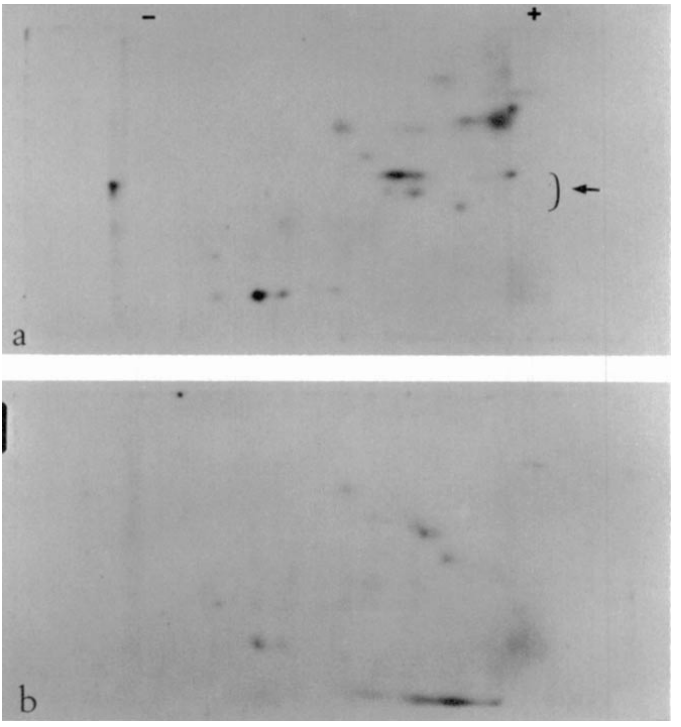

FIG. 5. Autoradiogram of immunoblot of two-dimensional O'Farrell gel showing antibody-binding proteins in LP SSP fraction of *Caenorhabditis elegans* without (a) and with (b) collagenase treatment. Isoelectric focusing was from left (pH 6) to right (pH 4); SDS-polyacrylamide electrophoresis was from top to bottom in 10% polyacrylamide. Immunoblots were exposed to film for 26 hours with an intensifying screen. Arrow points to the small collagenase-sensitive proteins with electrophoretic mobilities similar to those of the translation products of RNA isolated at the L4 to adult molt.

2D gel electrophoresis and immunoblotting. Again, collagenous proteins were identified by comparing samples with and without collagenase digestion. Five collagenase-sensitive species in the size range of the small CP were detected among the proteins present in the LP SSP (Fig. 5, arrow). The small CP detected were quite acidic (pI 4–5), as were the collagenase-sensitive proteins seen in Figure 4.

DISCUSSION

Collagens in *C. elegans* appear to be the products of a large multi-gene family consisting of 40–150 distinct collagen genes (3,13). Most, if not all, collagen gene expression appears to be for cuticle formation; the major structural components of the cuticle are collagenous, and low stringency in situ hybridization experiments have shown that virtually all collagen mRNA is localized within the hypodermis, the underlying tissue believed to be the site of cuticle synthesis (8). The relatively small size of isolated collagen genes, collagen coding mRNAs, and collagenous in vitro translation products, together with the CP described here, suggests that the large collagenous proteins isolated from the cuticle are biosynthesized from smaller primary gene products.

Using antibodies made to the adult CCP, small collagenous polypeptides were detected in *C. elegans* during the L4 to adult molt. These small CP are smaller than the large CCP isolated directly from adult cuticles (5) and are also smaller than characterized vertebrate collagen chains (150,000 M_r as newly translated chains) (1,2,17,18), with the exception of a small chain found in chondrocyte cultures (12,23).

The rabbit antiserum used in these experiments was directed against a heterogeneous immunogen. It was thus possible that the small antibody-binding bands observed in our experiments represented noncollagenous contaminants present in both immunogen and antigen mixtures. Therefore, collagenase sensitivity, rather

than antibody binding per se, independently verified the collagenous nature of the small CP in SSP fractions.

Several observed properties of the small CP are consistent with their proposed role as cuticle collagen precursors. First, they are present at the molt, a time when cuticle synthesis is maximal (4). Second, the small CP are similar in molecular size and pI to the collagenase-sensitive polypeptides that are translated in vitro from RNA isolated at the L4 to adult molt (21). Both groups of proteins are small, and in both cases the major species detected early during the L4 to adult molt are larger and somewhat more acidic than those detected later in the molt. This finding is significant because such small collagenous proteins are the only detectable collagenase-sensitive products translated from RNA isolated at this molt, with one exception (21). Third, small antibody-binding CP are collagenase-sensitive (Fig. 3), as a putative precursor role would predict. Finally, the small CP are detected in several other protein fractions besides the one containing the cuticle proteins. This occurrence would be expected of the biosynthetic precursors of the CCP because the hypodermal tissue, which appears to be the site of cuticle synthesis (8,24), is solubilized before BME extraction of cuticle proteins (5).

Although the experiments described here detected small CP that may be biosynthetic precursors to the large CCP, the limitations of antibody detection methods do not permit comparison of the number of small CP with the size of the *C. elegans* collagen gene family (3) or the number of collagenous products translated in vitro from isolated RNA (21). For example, certain CP may not be detected because they are not antigenic. Moreover, direct experimental comparison of the two-dimensional electrophoresis patterns obtained after immunoblotting versus in vitro translation has not been possible, so it is not known whether any of the collagenous spots in these two experiments exhibit the same mobilities.

Our experiments are consistent with, but do not prove, the theory that small CP are the precursors to the large CCP isolated from cuticles. Although we cannot currently rule out other explanations, the observed properties of the small CP described here make them presently the only likely candidates for biosynthetic precursors of the cuticle collagens. Because classical short pulse-labelling experiments are not feasible at the molt in *C. elegans* (21), a direct association between a specific precursor and its product will require an alternative method. Detection of the small CP may allow the identification of cuticle mutants that are missing or contain altered small CP. Such screening techniques are currently being developed.

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