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Transglutaminase-Catalyzed Reaction Is Important for Molting of *Onchocerca volvulus* Third-Stage Larvae

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Highly insoluble proteins, which are probably cross-linked, are common in the cuticle and epicuticle of filarial parasites and other nematode species. We have investigated the possible involvement of transglutaminase (TGase)-catalyzed reactions in the development of *Onchocerca volvulus* fourth-stage larvae (L4) by testing the effects of TGase inhibitors on the survival of third-stage larvae (L3) and the molting of L3 to L4 in vitro. The larvae were cultured in the presence of three specific TGase inhibitors: monodansylcadaverine, cystamine, and *N*-benzyloxycarbonyl-D,L-β-(3-bromo-4,5-dihydroisoxazol-5-yl)-alanine benzylamide. None of the inhibitors reduced the viability of either L3 or L4. However, the inhibitors reduced, in a time- and dose-dependent manner, the number of L3 that molted to L4 in vitro. Molting was completely inhibited in the presence of 100 to 200 μM inhibitors. Ultrastructural examination of L3 that did not molt in the presence of monodansylcadaverine or cystamine indicated that the new L4 cuticle was synthesized, but there was an incomplete separation between the L3 cuticle and the L4 epicuticle. The product of the TGase-catalyzed reaction was localized in molting L3 to cuticle regions where the separation between the old and new cuticles occurs and in the amphids of L3 by a monoclonal antibody that reacts specifically with the isopeptide ε-(γ-glutamyl)lysine. These studies suggest that molting and successful development of L4 also depends on TGase-catalyzed reactions.

Onchocerciasis, or river blindness, is one of the leading causes of infectious blindness and severe chronic dermatitis, afflicting about 18 million people in Africa and Latin America (43). The parasite is transmitted by bites of *Simulium* black flies. Although vector control and the periodic administration of the drug ivermectin promise a drastic reduction in the burden of skin microfilariae and disease, there is also a need for alternate strategies for the control of onchocerciasis (19). The development of a vaccine against components of the infective stages of the parasite or the identification of key enzymes essential for their development that could be targeted by chemotherapeutic agents would provide new means for preventing infection and disease associated with *Onchocerca volvulus* infection. Surface and cuticular antigens and the molting process of larval stages of filarial parasites are considered potential targets for immunity or chemotherapy (8, 17, 26, 35).

The main structural components of the cuticle of *O. volvulus* and other nematodes are collagenous proteins, cross-linked by disulfide bonds and localized in the basal and inner cortical layers (6, 36). The external cortical layer and the epicuticle are highly insoluble and appear to be composed of a protein(s) cross-linked by nonreducible covalent bonds (6, 10). In some nematodes these components are referred to as cuticlin (6, 16, 34). In some of the nonreducible and insoluble cuticular proteins more than 25% of the tyrosine incorporated into the cuticle has been found as dityrosine or isotrityrosine, which resulted from oxidation of tyrosine residues from adjacent polypeptides to form a covalent bridge between the phenolic rings (11, 14, 15, 33). The highly insoluble nature of the epicuticle and cuticle of third-stage larvae (L3) and fourth-stage larvae (L4) of *O. volvulus* (28), which is also seen in other nematodes, suggested the possible presence of a biologically active transglutaminase (TGase; EC 2.3.2.13).

TGases are a family of enzymes that catalyze the posttranslational modification of proteins by introducing an isopeptide bond between internal glutamine residues and primary amines, peptide-bound lysine or polyamine, to form either ε-(γ-glutamyl)lysine or ε-(γ-glutamyl)polyamine bonds (1, 18). The covalent isopeptide cross-link is exceptionally stable and resistant to proteolysis and can be broken only after total degradation of the two peptide chains. Because TGases are widely distributed enzymes that exist in both intracellular and extracellular forms, TGase-modified proteins are evident in many mammalian systems, the fibrin network of blood clots, cell membranes, extracellular matrices, and the cornified features of the epidermis and its appendages.

The presence of putative TGases and the products of TGase-catalyzed reactions was reported in other filarial nematodes (29, 31, 41). More recently, an active *Brugia malayi* TGase with a molecular mass of 56 kDa was purified (37). Numerous ε-(γ-glutamyl)lysine isopeptide bonds were detected in adult worm extracts of *B. malayi* (29) and in sheaths of *Litomosoides carinii* microfilariae (41). Furthermore, embryos developing in utero contained very high amounts of the enzyme (29). Inhibition of enzyme activity in vitro by monodansylcadaverine (MDC) and cystamine (CS), two specific inhibitors of TGase (13, 25, 40), led to a time- and dose-dependent inhibition of microfilarial production and release by gravid female *B. malayi* and *Acanthocheilonema viteae* (29, 31), thus indicating that in these parasites, TGase-catalyzed reac-

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tions play an important role during embryogenesis, the maturation of early embryonic stages to microfilariae, and survival of the parasites.

In this report we show that TGase-catalyzed reactions also play an important role in the molting process of *O. volvulus* L3. The possible involvement of TGase in the development of L3 to L4 opens up additional putative targets for drug development.

MATERIALS AND METHODS

In vitro culturing of L3 in the presence of TGase inhibitors. *Simulium yahense* black flies were infected with *O. volvulus* microfilariae, and after 7 to 8 days L3 were harvested as described before (28, 30). L3 were set up for culture in groups of 10 larvae each in 96-well plates containing 5×10^5 bovine peripheral blood lymphocytes per ml of culture medium (1:1 NCTC 135 and Iscove's modified Dulbecco's media [IMDM] plus 20% heat-inactivated fetal calf serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 5 μg of amphotericin B [Fungizone] per ml). Using these conditions, we routinely achieved 50 to 60% successful L3 to L4 molting by day 5 in culture. L3 were cultured in vitro for 6 days at 37°C in a humidified 5% CO_2 incubator in the presence of increasing concentrations of TGase inhibitors, and the number of molting larvae was determined on day 6. Molting was manifested by shedding of the thick L3 cuticle and a marked increase in the motility of the larvae. Larval viability was assessed visually after the uptake of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) by the larvae and its reduction into the blue formazan derivative (5). Briefly, the culture medium at each point was carefully removed from each well by using finely drawn glass pipettes and was replaced with 150 μl of phosphate-buffered saline containing 0.1% MTT (Sigma Chemical Co., St. Louis, Mo.). Metabolically active larvae take up MTT and reduce it to formazan, subsequently staining themselves blue. Larvae were scored live when they stained blue uniformly along their entire length and dead when the larvae remained unstained. Three TGase inhibitors were used: MDC, a competitive pseudosubstrate that acts as an inhibitor of endogenous protein cross-linking by the enzyme and CS, an active-site inhibitor, both known to be specific for TGase (13, 25, 40), and a new synthetic inhibitor of TGase, *N*-benzyloxycarbonyl-D,L-β-(3-bromo-4,5-dihydroisoxazol-5-yl)-alanine benzylamide (kindly provided by Allen Krantz, Syntex Inc., Palo Alto, Calif.), which specifically inactivates mammalian TGases by binding to the active site of TGase (2, 4). The results shown for each inhibitor concentration are the average number of larvae that molted or the average number of larvae that were scored alive in 5 to 10 wells containing a total of 50 to 100 L3. Each experiment was repeated at least twice. The standard error between experiments never exceeded 10% and did not vary with the concentration.

Ultrastructure of larvae cultured in the presence of TGase inhibitors. Larvae that did not molt in the presence of 150 and 200 μM MDC or CS were collected and fixed overnight at 4°C with 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3), washed in the same buffer, and processed for electron microscopic examination as described before (28). For examination of the ultrastructure of larvae undergoing normal molting, L3 were cultured in vitro for 4 days and larvae from days 1, 2, 3, and 4 in culture were collected. The larvae were fixed as described above.

TGase activity in crude extracts of *O. volvulus*. Worms were homogenized on ice in prechilled lysing buffer (20 mM Tris-HCl [pH 8.5] containing 150 mM NaCl, 2 mM dithiothreitol [DTT], 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM *N*-tosyl-L-phenylalanine chloromethyl ketone, and 0.1 mM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone). The homogenate was sonicated three times (the third sonication was in the presence of 0.1% Triton X-100), and thereafter was spun at $15,000 \times g$ for 20 min. Extracts were prepared from female adult worms and from about 1,000 L3 that were cultured for 2 days in vitro. TGase activity in the crude extracts was determined by using *N*,*N*′-dimethylcasein (Sigma) as the amine acceptor and 5-(biotinamido)pentylamine (Pierce, Rockford, Ill.) as the amine donor according to the microtiter plate assay described by Slaughter et al. (38). Briefly, a microtiter plate precoated for 1 h at 37°C with 200 μl of dimethylcasein (10 mg/ml) was reacted with 200 μl of crude extracts (5 μg of adult female worm extract or extract produced from about 100 larvae at day 2 in culture) containing 1 mM 5-(biotinamido)pentylamine, 10 mM $CaCl_2$, and 10 mM DTT for 2 h at 37°C. For inhibition experiments the reaction mixture also contained ethylene glycol-bis-(β-amido-ethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), MDC, or CS. The reaction was stopped by washing the wells with 350 μl of 250 mM EDTA twice. The amount of 5-(biotinamido)pentylamine incorporated into the *N*,*N*′-dimethylcasein was determined by detection with streptavidin-alkaline phosphate and paranitrophenyl phosphate (Sigma). TGase activity is expressed as the optical density at 405 nm after stopping the reaction with 50 μl of 2 M sodium bicarbonate.

Localization of the isopeptide ε-(γ-glutamyl)lysine in larval stages of the parasite. L3 were cultured in vitro for 4 days, and larvae from days 1, 2, 3, and 4 in culture were collected. The larvae were fixed for 30 min in 0.25% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) containing 1% sucrose and were then processed for immunoelectron microscopy as described previously (27). Thin sections of embedded worms were probed with a monospecific immunoglobulin M (IgM) monoclonal antibody (kindly provided by Gerard A. Quash, Institut National de la Santé et de la Recherche Médicale, Oullins, France) which reacts with the end product of TGase-catalyzed reaction, the isopeptide ε-(γ-glutamyl) lysine (9). The sections were incubated with a second antibody, rabbit anti-mouse Ig (Accurate Chemical and Scientific Corp., Westbury, N.Y.), before interaction with 10- or 15-nm gold particles coated with protein A (Amersham Life Sciences, Arlington Heights, Ill.). A nonrelated IgM monoclonal antibody was used as a control.

Identification of putative substrate proteins for TGase activity in molting larvae. Parasite proteins that might serve as substrates for endogenous TGase during molting were identified by analyzing the incorporation of the pseudosubstrate MDC into the larval proteins by Western blotting (immunoblotting). A total of 100 L3 were cultured in the presence of 200 μM MDC or under normal culture conditions for 2 days and were then collected. Crude extracts of both molting larvae were prepared by homogenization in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer (2% SDS in 62.5 mM Tris-HCl [pH 6.8] with 5% 2-β-mercaptoethanol) and centrifugation at $12,000 \times g$. The crude extracts were separated by SDS-PAGE on a 7.5 to 20% gradient (22) and were electrophoretically transferred to nitrocellulose (42). The blot was probed with rabbit anti-MDC antibodies (kindly provided by Laszlo Lorand, Northwestern University, Chicago, Ill.). Bound antibodies were detected by ^{125}I-protein A (0.1 μCi/ml; ICN Biomedicals, Inc., Irvine, Calif.).

RESULTS

Effects of TGase inhibitors on L3 molting. The possible involvement of TGase-catalyzed reactions in the development of L4 of *O. volvulus* was studied in vitro by observing first the effects of two TGase-specific inhibitors (MDC, a competitive pseudosubstrate that acts as an inhibitor of endogenous protein cross-linking by the enzyme, and CS, an active-site inhibitor) on the viability of molting L3 and the ability of L3 to molt to L4. Parasite viability was assessed visually after the uptake of MTT by the larvae. L3 were cultured in the presence of increasing concentrations of MDC or CS, and the number of molting larvae was determined on day 6. Both inhibitors reduced, in a dose-dependent manner, the molting ability of L3 in vitro (Fig. 1). In comparison with the typical 50 to 60% molting under normal culture conditions, 50 to 100% of molting was inhibited by 50 to 300 μM MDC and CS. Inhibition of 50% was observed with 50 μM CS and 100 μM MDC. CS or MDC at 150 μM completely inhibited molting. This inhibition was not due to a lethal effect of the inhibitors on L3. In parallel experiments larvae were treated with MTT on different days during culturing in the presence of various concentrations of the inhibitors, and their viabilities were assessed after 24 h. As shown in Fig. 1, L3 that were cultured in the highest concentration of CS (300 μM) were still viable. With MDC, only concentrations greater than 200 μM had any significant effect on L3 viability. The viability curves in Fig. 1 represent the data from larvae cultured in the presence of the drugs for 3 days. We have chosen to present the results for day 3 in culture because this is the critical time during molting that can indicate that the larvae are viable and ready for molting. Larvae usually complete their L3 to L4 molt by day 5 (28). Similar results were also obtained on successive days; the larvae were motile and viable during all days in culture (data not shown). Since the effects of the TGase inhibitors on molting were not due to lethality, we concluded that the effects were specific to the molting process. A third TGase inhibitor, *N*-benzyloxycarbonyl-D,L-β-(3-bromo-4,5-dihydroisoxazol-5-yl)-alanine benzylamide, which was developed to specifically inactivate mammalian TGases by binding to the active site (4), was also tested in our molting assay. The inhibitor completely inhibited the molting of *O. volvulus* L3 to L4 at 100 and 200 μM (95 and 98%, respectively) (data not shown).

To investigate the period during which the molting process is primarily affected by the inhibitors, two of the inhibitors were added at different days during the molting process. Culture medium containing 150 μM MDC or CS was added to the larvae on day 0 or on days 1, 2, 3, and 4 by replacing the normal

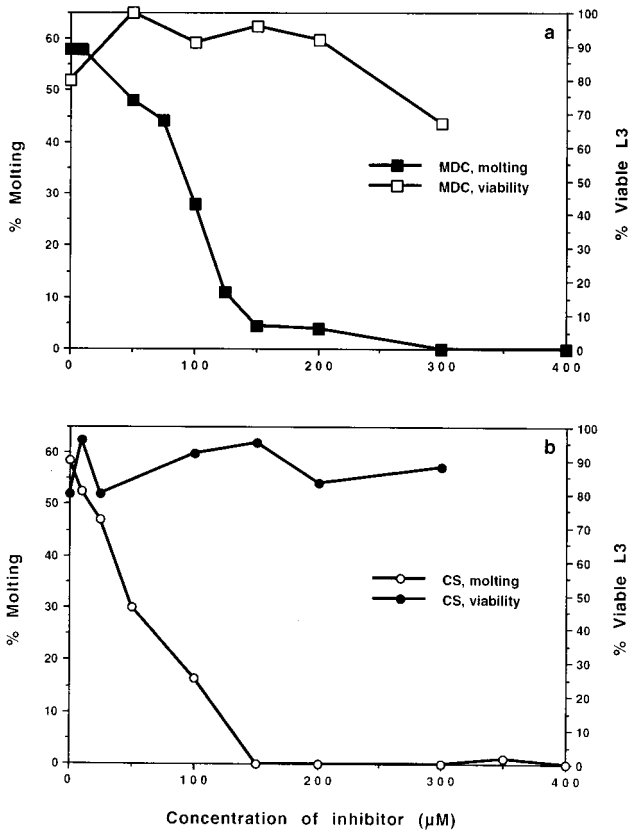

FIG. 1. Molting and viability of *O. volvulus* L3 in the presence of TGase inhibitors. A total of 50 to 100 L3 were cultured in the presence of increasing concentrations of MDC (a) or CS (b), and the molting rate was determined on day 6. A total of 50 to 60% L3 molted by day 5 under normal control culture conditions. Larval viability after 3 days in culture in the presence of the drugs was assessed visually after the uptake of MTT by the larvae and its reduction into the blue formazan derivative. Each experiment was repeated at least twice, and the standard error between experiments never exceeded 10%.

culture medium, and the larvae were then allowed to remain in culture until day 6, when the number of molting larvae was determined. Both inhibitors induced inhibition of molting but at different time points during the molting process (Fig. 2). The presence of the competitive pseudosubstrate MDC during the first 24 h of the molting process was essential for the complete inhibition of molting. If the inhibitor was added after 1 or 2 days, 21 and 37% of larvae molted, respectively (63 and 35% inhibition, respectively), in comparison with 57% molting under normal conditions. When the inhibitor was added after 3 or 4 days, there was no significant effect on molting. By contrast, the TGase active-site inhibitor CS was completely inhibitory, even when it was added on day 2 in culture. When the inhibitor was added on day 3 or 4, only a partial inhibition was observed; 37 and 42% of the larvae molted, respectively, in comparison with 57% molting in normal cultures. This effect on days 3 and 4 could be due to the inhibitory effect of CS on a subpopulation of larvae which are slower in their molting process than the majority of the larvae and which molt only after 5 days in culture. In conclusion, the effects of MDC and CS on molting are mostly critical during the first 2 days in culture.

Ultrastructure of larvae that did not molt in the presence of TGase inhibitors. *O. volvulus* larvae that did not molt in cultures containing the MDC or CS inhibitors continuously were collected and processed for electron microscopy. As shown in Fig. 3d, e, and f, the larvae that did not molt in the presence of MDC had initiated the molting process but never completed it as it happens under normal conditions (Fig. 3c). The larvae had a visible L4 epicuticle and cuticle, in addition to the outer L3 epicuticle and cuticle, indicating that the new L4 cuticle had begun to be synthesized (Fig. 3d). In some larvae, irregular separations between the L4 epicuticle and the L3 cuticle were observed (Fig. 3e and f), similar to those seen in normal cultures on day 1 (Fig. 3a). However, we could not find any larvae in which the separation between the cuticles was complete, as seen in normal cultures on days 2 and 3 (Fig. 3b and c, respectively). The cuticular ultrastructures of the larvae that did not molt in the presence of CS were similar to the ones cultured in the presence of MDC (data not shown).

TGase activity in crude extracts of *O. volvulus*. Extracts of L3 after 2 days in culture and extracts of female adult worms were tested in a microtiter assay for the presence of endogenous TGase activity by using *N*,*N*′-dimethylcasein as an amine acceptor and 5-(biotinamido)pentylamine as an amine donor. As shown in Fig. 4, we detected TGase activity in extracts of both larvae from 2 days in culture and female adult worms, which were inhibited by 50 mM EGTA by 81.6 and 97.4%, respectively. TGase activity is Ca^{2+} dependent and can be inhibited by EDTA or EGTA (1, 18). Because we did not have enough material to do more inhibition assays with the larval extracts, we tested the effects of MDC and CS only on the TGase activity in female adult worm extracts. In the presence of 20 and 200 μM MDC, TGase activity was inhibited by 88.7 and 86.9%, respectively. In the presence of 20 and 200 μM CS, TGase activity in the female adult worm extracts was inhibited by 68.1 and 79%, respectively.

Localization of the isopeptide ε-(γ-glutamyl)lysine in larval stages of the parasite. Immunoelectron microscopy staining of L3 during molting with a monoclonal antibody that is directed against the isopeptide ε-(γ-glutamyl)lysine (9) permitted the localization of the isopeptide produced by the action of an endogenous TGase in the intermediate stages of *O. volvulus* molting larvae. Examination of thin sections of larvae on days 1 to 3 in culture revealed the newly formed epicuticle and cuticle of L4 in areas beneath the basal layer of the old L3

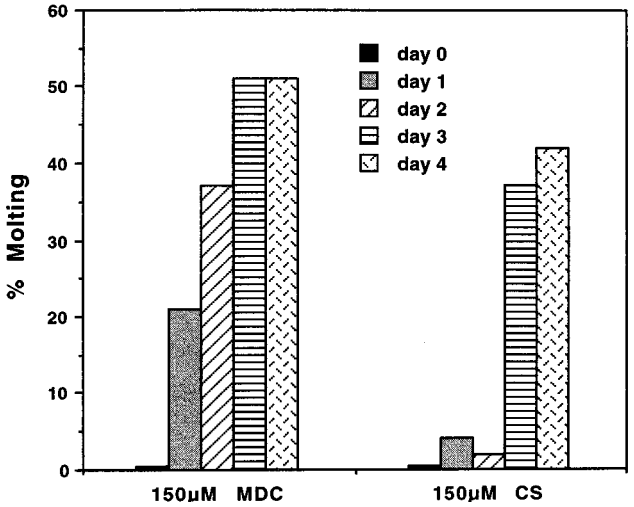

FIG. 2. Molting of *O. volvulus* L3 in the presence of TGase inhibitors that were added at different days during molting. The inhibitors MDC or CS were added at different days during the molting process of 50 L3 and were kept in culture until day 6, when the number of molting larvae was determined. A total of 57% of the larvae molted under normal culture conditions without inhibitors.

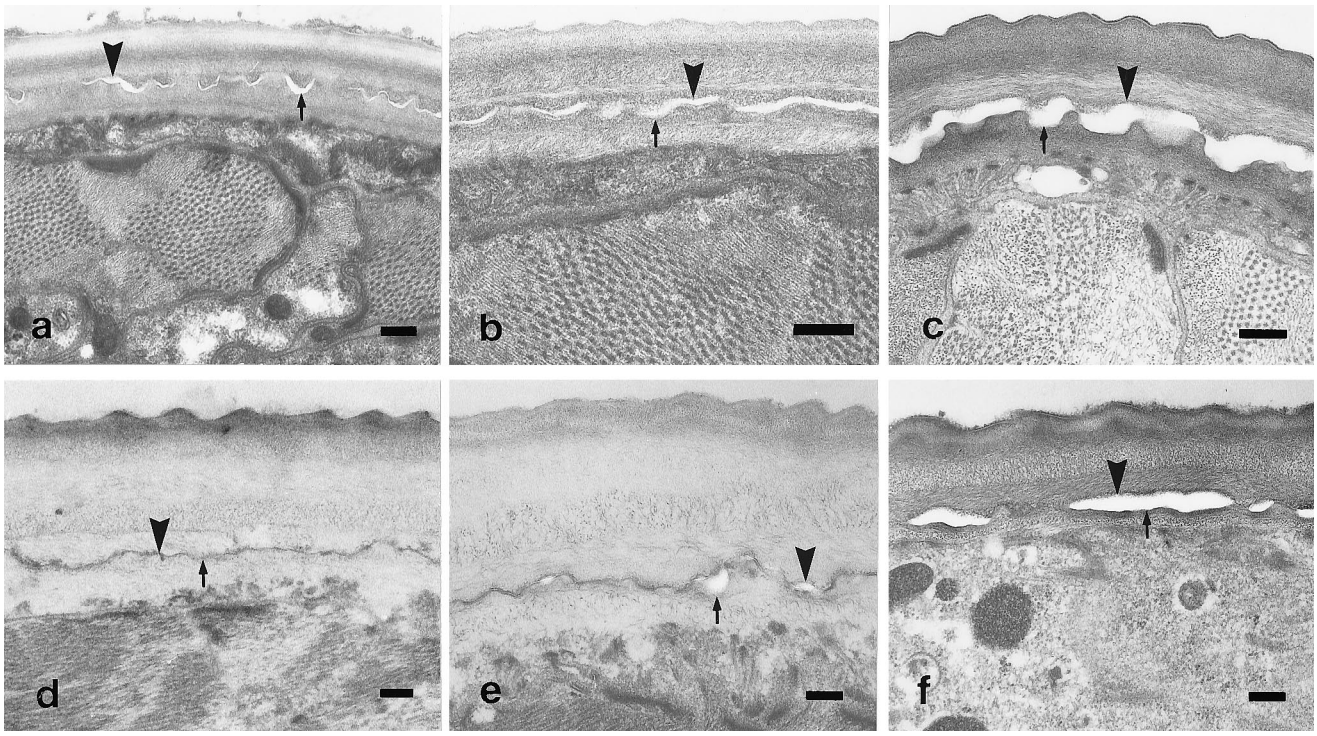

FIG. 3. Ultrastructures of larvae during normal molting and larvae that did not molt in the presence of the MDC inhibitor. L3 during normal molting were collected on days 1, 2, and 3, and L3 that did not molt in the presence of 150 and 200 μM MDC were collected on day 6. Thin sections of larvae during the normal molting process for larvae on day 1 (a), day 2 (b), and day 3 (c) and thin sections of three different worms that did not molt in the presence of MDC (d, e, and f) are presented (bars, 0.25 μm). Separations between the L4 epicuticle and the L3 cuticle are marked by arrows and arrowheads, respectively.

cuticle (Fig. 5a, arrowhead) and the separation between the two cuticles below the old basal layer of L3 (Fig. 5b, arrowhead), as seen during normal molting (Fig. 3a and b). The monoclonal antibody recognized the isopeptide mostly in areas around the region where the separation between the cuticles takes place, in some areas of the cuticle of the newly formed L4, and in the lower part of the old cuticle, where the separation occurs (Fig. 5a and b). Interestingly, the density of staining is higher in the larvae after 1 day in culture, just before the separation between the cuticles starts, than in larvae 2 to 3 days

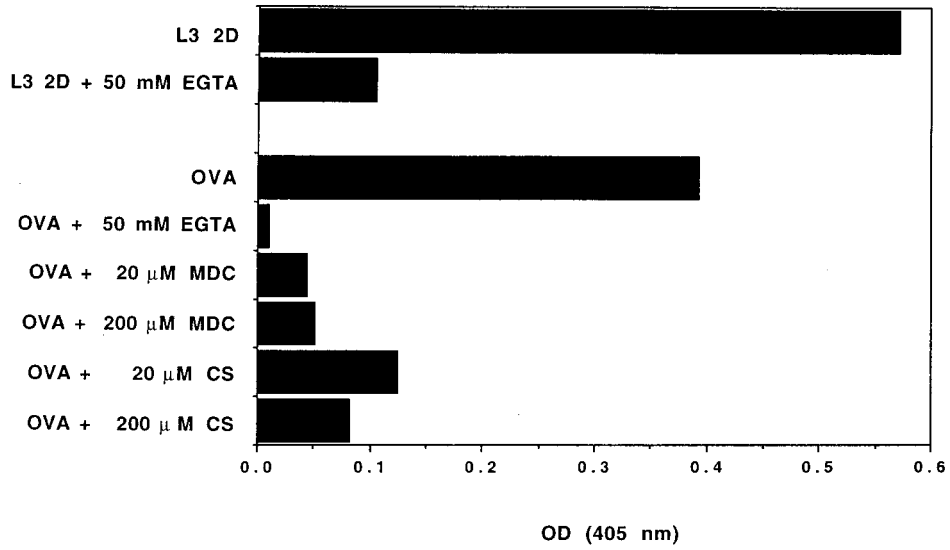

FIG. 4. TGase activity in *O. volvulus* worm extracts. Extracts equivalent to 100 L3 2 days in culture (L3 2D) or 5 μg of female adult worm extracts (OVA) were tested in a microtiter plate assay as described in Materials and Methods. For specific inhibition, the reaction mixture also contained 50 mM EGTA, 20 or 200 μM MDC, and 20 or 200 μM CS. The amount of 5-(biotinamido)pentylamine that was incorporated into the *N,N′*-dimethylcasein by the parasite endogenous TGase was determined by staining with streptavidin-alkaline phosphate and paranitrophenyl phosphate. TGase activity is expressed as the optical density (OD) at 405 nm after stopping the reaction with 50 μl of 2 M sodium bicarbonate.

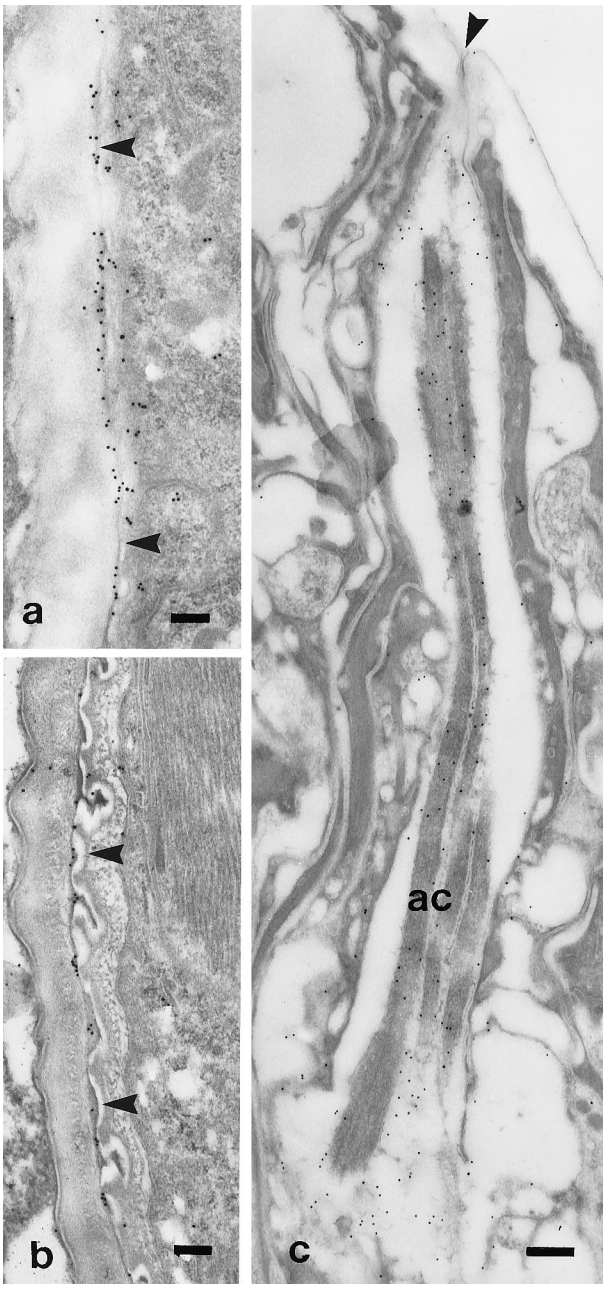

FIG. 5. Ultrastructural localization by immunoelectron microscopy of the isopeptide ε-(γ-glutamyl)lysine product of the TGase-catalyzed reaction in *O. volvulus*. Thin sections of *O. volvulus* L3 during molting in vitro after 1 and 2 days in culture (a, b) and a longitudinal section of the head region of *O. volvulus* L3 (c) were first incubated with a monoclonal antibody directed against the isopeptide ε-(γ-glutamyl)lysine. The sections were then reacted with rabbit anti-mouse Ig and protein A coupled to 10- or 15-nm gold particles for indirect antigen localization (bars, 0.25 μm). Note the area of the L4 epicuticle and cuticle (a; arrowhead), where the separation between cuticles takes place, and the partial separation between the basal layer of the L3 cuticle and the L4 epicuticle (b; arrowhead). The amphidial opening (arrowhead) and amphidial cilia (ac) are marked in panel c.

in culture, when the separation between the cuticles is almost complete. A control IgM monoclonal antibody did not cross-react with any proteins in the larvae (data not shown). When longitudinal cross sections in the head region of L3 were probed with the isopeptide-specific monoclonal antibody, we found that the antibodies also bound to proteins in the amphids of the larvae (Fig. 5c), near the amphidial opening and the amphidial cilia.

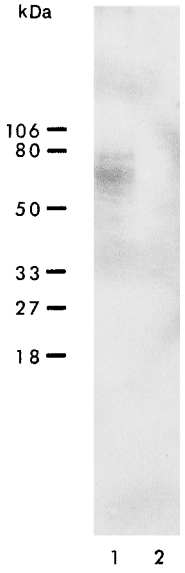

FIG. 6. Identification of putative parasite substrate proteins for endogenous larval TGase. Protein extracts of molting L3 in the presence of 200 μM MDC (lane 1, 100 larvae per lane) or during normal culture conditions (lane 2, 100 larvae per lane) were subjected to electrophoresis and were transferred to nitrocellulose. The Western blot was probed with rabbit anti-MDC antibodies and ^{125}I-protein A. Molecular mass markers are indicated on the left.

Identification of endogenous substrates for larval TGase. Preliminary studies that were directed toward the identification of the substrate proteins to be cross-linked by the TGase-catalyzed reaction in larvae took advantage of the incorporation of the exogenous MDC into larval proteins during the molting process. Larvae were cultured with 200 μM MDC for 2 days and were then collected, and the proteins were extracted by homogenization as described in Materials and Methods. Proteins bound to MDC were identified by Western blot analysis with rabbit anti-MDC antibodies. As shown in Fig. 6, two major protein bands were detected: a single band of 80 kDa and a band of 68 to 74 kDa (lane 1). Protein extracts of larvae that were cultured without MDC did not cross-react with these antibodies (lane 2).

DISCUSSION

In the present study we have shown that TGase appears to be an important enzyme for the molting process of L3 to L4 and, consequently, for the successful development of *O. volvulus* L4. Our conclusion is based on results from four different independent experiments. First, we have shown the inhibitory effects of three specific TGase inhibitors on the molting of L3 to L4: MDC, a known high-affinity pseudosubstrate for TGase; CS, which binds to the active site of TGase (13, 25, 40); and a new synthetic inhibitor of TGase, *N*-benzyloxycarbonyl-D,L-β-(3-bromo-4,5-dihydroisoxazol-5-yl)-alanine benzylamide, which was developed by Syntex Inc. for potential use in chemotherapy and which as CS specifically inactivates TGases by binding to the active site of the enzyme (2, 4). These structurally unrelated inhibitors, which interact with two different steps of the TGase-catalyzed reaction, are all inhibitory and exert the same effect on L3 during molting. Because these inhibitors were not lethal to the larvae, it was suggested that the inhibitory effect was specific to

the molting process and, therefore, indirectly indicated that a specific TGase that catalyzes cross-linking is present and active in the larvae during molting. Both inhibitors, MDC, which competes with the endogenous larval proteins that serve as a substrate(s) for TGase-catalyzed cross-linking of proteins, and CS, the active-site inhibitor, had to be present during the first 24 to 48 h, the beginning of the molting process, to exert a complete inhibitory effect (Fig. 2). After the third day in culture, when complete separation of the cuticle usually occurs (Fig. 3c), a complete inhibitory effect was not observed. These findings implied that the effect of the inhibitors was specific to some of the first changes that occur in the cuticle during the molting process: development of the new L4 epicuticle and cuticle and the beginning of the separation between the cuticles (Fig. 3a and 5a). This conclusion is supported by the ultrastructural studies (Fig. 3d to f). Larvae that did not molt in the presence of the inhibitors were found to be at the different stages of development that precede complete separation between the cuticles. Some larvae had visible L4 epicuticles and cuticles, and in some we observed irregular separations between the L4 epicuticle and the L3 cuticle. However, we never observed within unmolted larvae a complete separation between the cuticles (Fig. 3b and c). This indicated that when we generated inhibition of TGase-catalyzed reactions we indirectly prevented the complete separation between the cuticles and consequently ecdysis, shedding of the old cuticle. Second, the product of a TGase-catalyzed reaction, the isopeptide ε-(γ-glutamyl)-lysine, was found to be mostly localized in the lower part of the L3 cuticle and the L4 epicuticle and cuticle at the start of the molting process around the sites where the separation between the old and new cuticles occurs (Fig. 5a and b). This underscores the presence of products produced by the action of TGase activity and indirectly indicates that an active TGase is also present in the cuticles during the molting process. In preliminary immunogold labeling experiments we were able to localize a putative parasite TGase with a cross-reacting monoclonal antibody raised against a guinea pig liver TGase. Interestingly, this monoclonal antibody reacted in molting larvae with a protein present in the same areas where the monoclonal antibody against the isopeptide ε-(γ-glutamyl)lysine bound (data not shown). Third, the activity of an endogenous parasite TGase, which was inhibited by EGTA, was detected in extracts of molting larvae on day 2 in culture and in extracts of adult female worms. TGase activity in adult worm extracts was also inhibited by 200 μM MDC and CS (86.9 and 79%, respectively). The incomplete inhibition is probably due to competition with endogenous substrates or other proteins present in the worm extracts. In studies with the *B. malayi* TGase (37), it was demonstrated that 50% of the purified enzyme activity was inhibited by 500 μM CS. We have not been able to characterize the enzyme biochemically because of the difficulty in obtaining sufficient material for a proper analysis. Lastly, we have shown as well that during molting MDC was cross-linked to endogenous larval proteins, which were detected with anti-MDC antibodies (Fig. 6), thus implying the presence of an endogenous TGase in molting larvae which catalyzed the cross-linking of MDC to parasite proteins. On the basis of the results presented above, we conclude that an endogenous larval TGase is active during the molting process and that inhibition of the TGase-catalyzed reactions apparently prevents complete separation of the cuticles and, consequently, ecdysis.

Two observations were unexpected; first, the development of the new epicuticle and cuticle of L4 apparently was not inhibited, as might have been anticipated if a TGase would have been responsible for cross-linking epicuticular and cuticular proteins during cuticle formation in the developing L4; second, the ε-(γ-glutamyl)lysine products of the TGase-catalyzed reaction were also localized in the amphids of the larvae. Experimentally, our observations clearly indicated that the specific stage during the molting process when the cuticles are separated was somehow affected and consequently caused incomplete molting. The question is how TGase regulates or controls the molting process if its inhibition prevents complete molting. It appears most likely that a complete and normal development of the L4 epicuticle and cuticle, which might not be evident only by ultrastructural analysis, is necessary before complete separation between the cuticles can occur. It is possible that TGase in the larval stages of *O. volvulus* is responsible for cross-linking proteins that are an important part in the assembly of the L4 epicuticle and cuticle and that must evolve during the molting process and precede complete separation of the cuticles. Therefore, failure of those proteins to be cross-linked in the presence of inhibitors might have prevented the normal development of the L4 epicuticle and cuticle and, consequently, the separation between the L3 and L4 cuticles to complete the molt. In addition, it could be that proteins in the amphids are responsible for regulating the molting process, and when their complete posttranslational modification is inhibited, the molting process is incomplete. It was suggested that the amphids, which are nerve cells with a secretory activity, may, under neurosecretory control, allow products to get to the surface of molting larvae. At present, the role of the amphids during the molting process of nematodes is not clear. The only study that found some direct relationship between amphids and molting was described by Delves et al. (7) in *Dirofilaria immitis*. They found that in molting larvae a fuchsinophilic substance in the amphids was associated with the pre-ecdyzed stages of L3; once the ecdysis started the staining disappeared. The significance of TGase activity and the products of the TGase-catalyzed reaction in the amphids and their relationship to molting need more study.

The challenge will be to identify the specific components in the epicuticle, cuticle, or amphids that provide the substrate(s) for this reaction. Interestingly, one of the known substrates for TGase in mammalian systems was shown to be collagen (3, 20, 21). Collagens have also been shown to be one of the major protein groups that are significant for the development of larval cuticles (6, 10, 34, 36). The amphids consist of invaginated channels that are covered with a cuticular layer which is structurally similar to the external parts of the cortical layer of the cuticle of *O. volvulus* L3 (39). Our preliminary results have identified an 80-kDa protein and proteins of 68 to 74 kDa that are potential substrates for an endogenous TGase in molting larvae (Fig. 6). Analysis of these proteins would indicate if they are collagen-like proteins. Interestingly, collagen-like proteins in nematodes have molecular masses of 30 to 120 kDa on reducing gels (12, 36).

It is likely that TGase-catalyzed reactions are important to the molting process of not only *O. volvulus* but other nematodes as well. This is supported by the studies with the synthetic TGase inhibitor *N*-benzyloxycarbonyl-D,L-β-(3-bromo-4,5-dihydroisoxazol-5-yl)-alanine benzylamide, which was tested against helminth infections and which was found to be inhibitory (4). This inhibitor was able to reduce in vitro (100 μM drug for 7 days) the ability of L4 of *Nippostronglus brasiliensis* to molt to adult stages and also affected the viability and mobility of *N. brasiliensis*. In addition, the inhibitor reduced the number of parasites that developed in a mixed infection with *Nematospiroides dubius* and *Hymenolepsis nana* in mice. The same inhibitor at 100 μM completely inhibited the molting of *O. volvulus* L3 in our study. Recently, an active TGase from an adult *B. malayi* worm was biochemically purified and characterized and its partial amino acid sequence was determined (37). In previous studies (29, 31), it was reported that TGase inhibitors were lethal to *B. malayi* L3 and, in *B. malayi* and

A. viteae, also affected other stages of development, microfilaria production, and microfilaria release by gravid female worms. In addition, an indirect support for the importance of TGase-catalyzed reactions for the molting of *Onchocerca* larvae could be derived from the report by Lok et al. (24), showing that synthetic retinoids can inhibit the L3 to L4 molt of *O. lienalis* in vitro. It has been shown in mammalian systems that the levels of keratinocyte TGase are specifically suppressed by retinoic acid (23, 32). It is possible that the presence of synthetic retinoids in the study of Lok et al. (24) also reduced the levels of TGase in the molting larvae and thus caused, indirectly, arrested molting.

In conclusion, TGase-catalyzed posttranslational modification of proteins seems to be important to the successful development of *O. volvulus* L4, because inhibition of TGase activity prevents the complete separation between L3 and L4 cuticles, and therefore ecdysis. The identification of TGase as an essential enzyme for the molting process of *O. volvulus* L3 and the development of L4 opens up important new avenues for drug development. Other important areas of study include the molting process in nematodes, the role of amphids during development, and the regulation of epicuticle and cuticle formation during molting.

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