Molecular Cloning, Expression, and Localization of E1, an Onchocerca volvulus Antigen with Similarity to Brain Ankyrin*

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EXPERIMENTAL PROCEDURES

Study Population—After informed consent 120 residents of an area hyperendemic for onchocerciasis in Liberia/West Africa underwent physical and parasitological examinations, essentially as described (8). Microscopic examination of two skin snips obtained from the iliac crest was performed after incubation overnight in 0.9% NaCl followed by digestion with collagenase. Subjects with no evidence of onchocerciasis underwent a local Mazzotti test using a 2% diethylcarbamazine lotion. Persons with negative test results were given an oral test dose of 50 mg of diethylcarbamazine. Based on these results, individuals were classified into three groups: O. volvulus-infected individuals with various clinical symptoms of onchocerciasis (GEN), except long-standing onchodermatitis, infected individuals with chronic hyperreactive onchodermatitis (sowdah (SOW)), and individuals exposed to O. volvulus but with no detectable microfilariae in the skin and no history or present signs of onchocerciasis (PI).

Parasites and Antigen Preparation—Adult O. volvulus worms were obtained by collagenase digestion of surgically removed nodules as described (9). O. volvulus infective larvae (L3) were isolated from infected black flies as described previously (10). Live worms were frozen in liquid nitrogen. O. volvulus antigen was prepared by extraction of adult worms with 0.0625% Tris-Cl and 2% SDS containing 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 100 μg/ml 1-chloro-3-tosylamido-7-amino-2-heptanone. After centrifugation (20,000 × g, 90 min), the supernatant was frozen at −70 °C.

SDS-PAGE and Immunoblotting—Antigens were separated by SDS-PAGE in 5–15% gradient gels based on described methods (11) and were electrophoretically transferred to nitrocellulose sheets (Schleicher & Schuell, Dassel, Germany). Nitrocellulose strips were incubated with sera diluted 1:20 in PBS/BSA (5% nonfat dry milk in PBS) at 4 °C overnight, washed in PBS, incubated for 2 h at room temperature in a 1:2500 dilution of protein G horseradish peroxidase conjugate (Bio-Rad, München, Germany) in PBS/BSA, and developed using 4-chloronaphthol/H2O2.

Screening of cDNA Libraries—A λ gt11 cDNA expression library derived from adult O. volvulus was screened with a serum from a putatively immune individual reactive almost exclusively to the 90-kDa O. volvulus antigen according to described methods (12). Rabbit anti-human IgG-AP (Calbiochem, Bad Soden, Germany) was used as a second antibody, and filters were developed using the substrate system 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium. Positive clones were plaque-purified, and the EcoRI-digested cDNA inserts were subcloned into the pUC 18 vector for sequencing. A λ ZAP cDNA library prepared from adult O. volvulus was screened using (α-32P)dATP random prime (Amersham, Braunschweig, Germany) labeled probes as described (13). Filters were hybridized for 14–18 h at 42 °C, washed at 56 °C with 0.05% SSC, and exposed to x-ray film overnight. After plaque purification, inserts were subcloned into pBluescript II SK (Stratagene Cloning Systems, La Jolla, CA) for sequencing.

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Northern Blot—Approximately 10 μg of total RNA isolated from adult O. volvulus was separated on a 1% agarose gel containing formaldehyde and transferred to a nitrocellulose membrane (Schleicher & Schuell). The E1 cDNA probe used for hybridization was labeled with 32P using a random priming kit (Amersham).

Southern Blot—Southern blot analysis was performed as described (14) using O. volvulus genomic DNA (10 μg) as well as human genomic DNA (10 μg), each digested with EcoRI and HindIII (Pharmacia, Freiburg, Germany) and hybridized with the same cDNA probe as used for the Northern blot.

DNA Sequencing—The DNA sequence was bidirectionally determined (15) using α-32P-DATP and a sequencing kit (U. S. Biochemical Corp.).

Computer Analysis—Searching of the GenBank and the Protein Identification Resource Data Bank were performed using the HIBIO DNASIS software system (Pharmacia).

Expression and Purification of the Recombinant Protein—The entire coding sequence of E1 (bp 472-1855) was subcloned into the PET 20b vector (Novagen, Madison, WI) and expressed in Escherichia coli strain DH5α according to the manufacturer’s protocol. The recombinant nonfusion protein was further purified by preparative SDS-PAGE using the Prep Cell apparatus (Bio-Rad Laboratories).

Antibody Preparation—An antiserum to the E1 protein was generated by immunizing a rabbit with the purified recombinant protein in combination with complete Freund’s adjuvant. Affinity purified antibody to recombinant E1 protein was generated according to described methods (16).

Immunocytology—Onchocercal nodules surgically removed from African patients with onchocerciasis and from cattle infected with O. volvulus gibsoni were fixed in either 80% ethanol or 4% buffered formaldehyde and embedded in paraffin. Sections were incubated with 1:25 to 1:50 dilutions of the affinity purified rabbit antibody to the recombinant E1 protein or with preimmune or immune serum. Binding was detected using mouse anti-rabbit immunoglobulins and an alkaline phosphatase-anti-alkaline phosphatase complex (Dakopatts, Hamburg, Germany).

RESULTS

Detection of a 90-kDa Antigen of O. volvulus by Serum from PI—To identify O. volvulus antigens possibly relevant to protection in humans, pooled sera from GEN, SOW, and PI were used in immunoblots to screen for differences in O. volvulus antigen recognition patterns. Serum pools from PI showed recognition of a 90-kDa antigen, which was not observed using pools from GEN or SOW (Fig. 1A). Analysis using individual sera revealed preferential recognition of the 90-kDa band by the majority of the PI (28/50), a minority of SOW (6/30), and none of the GEN group (0/40) (data not shown). In the PI group studied, one serum showed almost exclusive reactivity to the 90-kDa antigen (Fig. 1B). This serum was used for screening of a cDNA expression library of adult O. volvulus.

Isolation and Characterization of E1 cDNA Clones—Approximately 100,000 recombinant phage plaques of a λ gt11 cDNA expression library of adult O. volvulus were screened with the PI serum. One of eight plaque-purified immunoreactive clones contained the largest insert of 1173 bp, which was subcloned into the M13 bacteriophage vector for sequencing. This revealed an open reading frame of 309 amino acids, starting with the first nucleotide, followed by an untranslated region and a poly(A) tail. Further analysis of a λ ZAP cDNA library prepared from adult O. volvulus using a radioactively labeled 157-bp polymerase chain reaction product of the 5’ end of the known sequence led to the identification of a full-length clone of 2043 bp, designated E1.

Northern and Southern Blot Analysis of E1—To determine the size of the message corresponding to the cDNA of E1, a radioactively labeled probe of the entire E1 cDNA was used in a Northern blot prepared from adult O. volvulus RNA. This probe identified at least three mRNAs of approximately 2.5, 6, and 7 kilobases (Fig. 2A). To examine the origin of the cDNA, the probe was used for Southern blot hybridization to genomic O. volvulus and human DNA. A single hybridization signal to a HindIII genomic fragment of 9.4 kilobases and to an EcoRI genomic fragment of 4.2 kilobases of O. volvulus DNA was detected (Fig. 2B), and no hybridization to human DNA was observed (data not shown).

Expression of the mRNA of E1 in the infective stage (L3) was detected by reverse transcriptase-polymerase chain reaction amplification of O. volvulus L3 cDNA with E1-derived primers. A signal corresponding to the expected size was observed (Fig. 2C).

Nucleotide and Deduced Amino Acid Sequence of E1—The total length of the clone is 2043 bp, including 467 bp of 5’-untranslated sequence, 1386 bp of coding sequence, and 190 bp of 3’-untranslated sequence. This corresponds to the estimated size of the smallest transcript on the Northern blot of approximately 2.5 kilobases. The site of the start of translation at nucleotide 468 is in accordance with the Kozak rule (17). The clone contains an open reading frame encoding 462 amino acids. The deduced amino acid sequence (Fig. 3) shows a potential N-linked glycosylation site at residue 220 and three potential phosphorylation sites (casein kinase II) at residues 139, 245, and 329 (18). A leucine zipper motif (Leu-Xaa4Ile-Xaa3Ile-Xaa2Ile-Xaa1-Leu) (19) is found starting at amino acid position 164. The protein contains a single cysteine residue (position 101). The encoded protein has a predicted molecular weight of 52.581, an pi of 4.6, and an overall content of acidic amino acids of 19.9%.

Comparison to Known Sequences—Comparison of the predicted amino acid sequence of E1 with Protein Data Banks revealed an identity of 34.1% to human brain ankyrin
(BRANK2) (20) (Fig. 4) and of 30.7% to human erythrocyte ankryn (ANK2) (21) over the first 223 amino acids. Over the first 103 residues, E1 is also highly similar to the predicted amino acid sequence of ankyrinG (22), which in turn is closely related to BRANK2. In this region of E1, amino acid identities of 46 and 45% to ankyrinG and BRANK2, respectively, are observed. The similarity to BRANK2 spans over residues 1318–1537 of BRANK2 comprising 126 amino acids of the C-terminal region of the spectrin-binding domain and 97 amino acids of the N-terminal region of the regulatory domain. The remaining 234 amino acids of E1 show no significant similarity to the amino acid sequence of the ankryn molecules, the E1 sequence being 64 amino acids shorter than BRANK2. However, similarities include the acidic amino acid content of 25% in the remaining 234 amino acids, compared with 24 and 20% in the corresponding regions of BRANK2 and ANK2, respectively, and a pI of 4.4 versus 4.2 in BRANK2 and 4.1 in ANK2.

The hydropathy profile of E1 is strikingly similar to that of the corresponding BRANK2 region, both sequences being very hydrophilic (not shown).

Expression of the cDNA Clone—To examine whether the cloned cDNA sequence encodes a protein reactive with serum antibody of the putatively immune individuals, the entire open reading frame was subcloned into the expression vector pET20b. This construct resulted in expression of a nonfusion protein with an apparent molecular mass of 90 kDa based on its mobility on SDS-PAGE (Fig. 5A). The highly acidic tail of E1 may account for this aberrant behavior by preventing binding of SDS, as has also been observed for recombinant human erythrocyte and brain ankryns (21, 23).

The recombinant protein is recognized by the P1 serum used for screening of the cDNA library, which reacts almost exclusively with the 90-kDa antigen (Fig. 5B). Furthermore, the rabbit antibody against the recombinant protein recognizes a 90-kDa antigen in whole O. volvulus protein extract (Fig. 5C). This strongly suggests that E1 is identical to the 90-kDa O. volvulus antigen recognized by P1 individuals. In immunoblots the recombinant E1 antigen was recognized by all of 10 P1 sera, by 4 of 6 SOW sera, and 1 of 5 GEN sera tested (not shown).

Localization—Immunohistochemical analysis of sections of adult O. volvulus and other Onchocerca species with the affinity purified E1 antibody showed a distinct staining pattern to specific structures of the hypodermis, the uterus (Fig. 6, A, B, and F) and the intestine (Fig. 6G). In the hypodermis,

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**Fig. 3. Nucleotide and derived amino acid sequence of E1.** The deduced amino acid sequence is shown in single letter code below the nucleotide sequence; both are numbered on the right. The in-frame stop codon preceding the probable initiation codon and the termination codon are marked by asterisks. The putative polyadenylation signals are underlined. The potential leucine zipper motif is boxed, and the putative N-linked glycosylation site is boxed and shaded.

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**Ankyrin-related Antigen of Onchocerca volvulus**

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was localized in the area of the basal labyrinth with the extracellular clefts (Fig. 6B), which represents the area between the hypodermal cell membrane and the basal lamina (Fig. 6H). The staining of the basal labyrinth extended over the interchordal hypodermis as well as the lateral and median chords, with exclusion of the muscles (Fig. 6, A and E). Similarly, in the uterus (Fig. 6F) and the intestine (Fig. 6G), staining was distinctly localized to the basal surface of the epithelial cells, the area of the basal labyrinth. Intense staining of the nerve ring as well as of the cytoplasm of a neuronal cell body in the median chord was observed (Fig. 6, C and D). Staining of *O. volvulus* infective larvae was also observed; however no confinement to specific structures could be made by light microscopy (not shown). A staining pattern of the same structures as described above was observed in sections of bovine *Onchocerca* parasites, such as *Onchocerca gibsoni* (Fig. 6B), *Onchocerca gutturosa*, and *Onchocerca ochengi*. In contrast, no staining was observed in sections of the nematodes *Caenorhabditis elegans*, *Loa loa*, or *Brugia malayi* using the purified antibody, but faint staining was observed using the immune serum (data not shown).

**DISCUSSION**

Here we report the cloning and characterization of an *O. volvulus* antigen selected based on its immunogenicity for putatively immune individuals. Its predicted amino acid sequence indicates that it is related to ankyrins, a group of membrane-associated proteins with diverse functions such as involvement in polarization of membrane proteins, attachment of voltage-gated ion channels to the cytoskeleton, and axon guidance (for reviews see Refs. 25 and 26). E1 is most closely related to human brain ankyrin (BRANK2); however, it lacks the membrane-binding and most of the spectrin-binding domains, which are typical for all ankyrin sequences described to date. Although the region of similarity between E1 and human and mouse ankyrins is evolutionary conserved (26, 27) and is apparently also similar in the free living nematode *C. elegans* (26, 27), no similarity in the remaining 239 amino acids of E1 to human or mouse ankyrins is observed. This is consistent with the reported high divergence between species in this part of the regulatory domain (26). An interesting but yet unresolved phenomenon shared by E1, human, and brain ankyrins is the observed discrepancy between the size of the recombinant proteins predicted by the cDNA sequences and by their migration on SDS-PAGE, with differences between 30–41%, which are attributable to regions in or adjacent to the regulatory domains (21, 23).

E1 appears to be a small transcript of the *O. volvulus* ankyrin gene. Similar small transcripts of mammalian ankyrin genes lacking the membrane-binding and large parts of the spectrin-binding domains have been observed in a number of rat and mouse tissues including brain (22, 27, 28). The current hypothesis is that these small ankyrins may be involved in functions other than that of membrane-cytoskeleton linkers (22).

We found that in *O. volvulus* the E1 protein is localized to the nerve ring, the neuronal cell bodies, and the basal labyrinth within the extracellular clefts of the hypodermis, where nerve axons are located in *O. volvulus* (30) as well as in *C. elegans*.
In mammals, brain ankyrin is not only found in the axonal membrane (32) but also in intercellular connections in brain, where it colocalizes with neuronal cell adhesion molecules (33), which bind to ankyrin (34). The leucine zipper motif in the E1 sequence may be important in binding to other proteins (35). A leucine zipper motif has also been found in the 43-kDa protein (36) suggested to arise from the small ankyrin transcripts observed in mouse skeletal muscle (28). This protein is involved in the immobilization of the acetylcholine receptor at the postsynaptic membrane of the neuromuscular junction (32). The observed localization of E1 in the neuronal cell bodies as well as in the extracellular clefts adjacent to the basal lamina raises the question of whether E1 is related to proteins involved in transynaptic signaling (37).

Both the sequence similarity to brain ankyrins and the localization within O. volvulus indicate that E1 is associated with the nervous system of the worm. In C. elegans, ankyrin is important in the development and function of the nervous system. The ankyrin gene of C. elegans is one of the axonal guidance genes whereby mutants of this unc-44 gene are characterized by the development of abnormally short chemosensory cilia, abnormal neurons, and defects in axon guidance and fasciculation (38, 39). In nematodes some of these neuronal structures are not only essential for development but are also directly exposed to the environment. Thus the chemosensory cilia represent neuronal endings accessible through the amphid openings in the head (40, 41), which are necessary for the uptake and processing of environmental signals (40, 42). Truncated cilia apparently render neurons insensitive to exogenous signals (42). For parasitic nematodes like O. volvulus, such defects could be critical, because the developmental changes undergone upon encounter with their hosts appear to depend

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**Fig. 5.** A, SDS-PAGE of recombinant E1 protein and Coomassie Brilliant Blue staining. B, immunoblot of E1 protein with the PI serum used for screening of the cDNA expression library. Lanes 1 in A and B contain vector without insert; lanes 2 in both panels contain vector plus E1 insert. The arrows indicate the positions of the recombinant E1 protein. C, immunoblot of the recombinant E1 protein (lane 1) and O. volvulus antigen (lane 2) with a rabbit antibody against E1 recombinant protein.

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**Fig. 6.** Immunohistochemical localization of the E1 protein in sections of live onchocercal worms. A, in a cross section of an adult O. volvulus female, intense labeling of the basal labyrinth of the hypodermis and the uterine epithelium is seen. I, intestine. B, distinct staining pattern of the basal labyrinth of the lateral hypodermal chord (arrow) is shown in a longitudinal section of O. gibsoni. C, anterior end of adult O. volvulus with distinct staining of the nerve ring around the esophagus. A neuronal cell body is indicated by the arrow. D, neuronal cell body (arrow) in the inner part of the hypodermal chord, intensively stained using immune serum (1:3200). E, basal labyrinth of a median chord with the pouch of the nerve axon (arrow). F and G, basal labyrinth (arrows) in the outer zone of the epithelium of the uterus (F) and intestine (G) labeled. The basal lamina remains unstained. H, schematic representation of the body wall of an adult female O. volvulus as seen by electron microscopy (24). bly, basal labyrinth; cu, cuticle; hy, hypodermis; I, intestine; mu, muscle; u, uterus. A and C–H, O. volvulus, alkaline phosphatase-anti-alkaline phosphatase. A, F, and G, with toluidin, A, ×280; C, ×670; B and E–G, ×800; A–C and E–G, affinity purified rabbit antibody to E1 protein.
on the evaluation of environmental signals (42). Interestingly, in O. volvulus these cilia are only directly exposed to the environment in the stages present in the human host (41).

It thus is conceivable that antibodies directed against proteins present in the nervous system of the worm such as E1 may have access to the parasite's neuronal structures. That antibody binding can interfere with neuronal function and development has been demonstrated in vitro and in vivo. In insect embryos antibodies raised against a single neuronal protein involved in growth cone guidance, fasciclin, can stall growth of axons (43). It will be of interest to examine if antibody to E1 has similar effects, particularly because ankyrin appears functionally related in nematodes (29, 39). Antibody binding could lead to an impairment of parasite neuronal functions and, if larval stages are affected, possibly to a developmental arrest.

Further experiments will include analysis of the E1 antibody effects on the parasite in vitro as well as of epitopes possibly relevant to protective immunity. In vivo studies may be feasible in cattle, natural mammalian hosts for Onchocerca species similar to O. volvulus (44), because antibody to E1 identified a similar protein in these parasites.

The present results raise the question of whether antibodies directed against the parasite's neuronal structures could provide protection for the host by inhibition of essential parasite functions. Neuronal proteins have not yet been associated with protection against helminths. Nevertheless, the nervous system may represent an Achilles' heel of the parasite accessible for immune-mediated intervention. The present results suggest that this could be exploited in efforts to develop a vaccine against O. volvulus.

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