Direct Molecular Cloning and Expression of Two Distinct Abrin A-chains*

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The protein toxin abrin, which possesses an N-glycosylase activity toward eukaryotic 28 S rRNA, may have a potential in the deliberate eradication of certain cells. Here we report, by polymerase chain reaction technique, the isolation of genomic DNA sequences encoding two distinct abrin A-chains. A third sequence which encoded a part of a third type of A-chain was also isolated. The deduced amino acid sequences of the two full-length A-chains were about 84% similar. Addition of mRNA encoding the full-length A-chains to reticulocyte lysate strongly inhibited protein synthesis in the lysates, and a corresponding glycosylase activity on rRNA was observed. Addition of the same mRNA to toxin-resistant wheat germ extracts led to synthesis of the expected 30-kDa protein which could be precipitated with antibodies specific for abrin.

The seeds of the plant Abrus precatorius contain several proteins which are among the most poisonous compounds known. The most potent of these toxins, abrin, is a heterodimer consisting of one A-chain and one B-chain, each of approximately 30 kDa (for review, see Ref. 1). The B-chain binds the toxin to cells, whereas the A-chain, after endocytosis and transport probably to the trans-Golgi compartment, is able to penetrate the lipid bilayer and enter the cytosol. Once in the cytosol the A-chain inactivates protein synthesis by an N-glycosylase activity on eukaryotic 28 S rRNA (2). Abridin, which is believed to be a member of a growing list of proteins with this particular catalytic activity. The group includes the A-chains of other plant toxins such as ricin and modeccin (2), and single chain ribosome-inactivating proteins from plants such as gelonin and trichosantin (3), as well as the A fragments of bacterial toxins such as Shigella dysenteria type 1 (Shiga) toxin and Escherichia coli Shiga-like toxin (4). A comparison of the primary sequences obtained for these N-glycosylases shows considerable similarity among proteins of this group (5-7), indicating that the prokaryotic and eukaryotic A-chains have evolved from a common ancestral gene (5).

Several isoforms of abrin and the closely related Abrus agglutinin have been isolated in various laboratories (9, 13). Furthermore, the amino acid content of the different abrin toxins and Abrus agglutinins obtained from the same plant varies slightly (13), suggesting that these proteins are encoded by several distinct but related genes. The amino acid sequence based on sequencing of overlapping peptides of the A-chain of one of these abrins has been reported (14).

The current interest in abrin and similar plant toxins is partly due to the potential these molecules may have in various therapies. Perhaps the most promising approach applying toxins in therapy is the use of immunotoxins, where the native toxins or toxin A-chains are linked to antibodies directed toward specific cells (15). There are many problems inherent to the use of toxins for therapy, and the possibility of generating toxin A-chains by recombinant techniques may for several reasons be of great advantage in such applications. Here we report on the direct molecular cloning of two distinct abrin A-chain genes, as well as the expression of the N-glycosylase activity from these genes.

MATERIALS AND METHODS

Isolation of Genomic DNA—DNA was isolated according to published methods (16) from leaves of A. precatorius from a plant grown from seeds obtained from Dr. S. Olsnes, Oslo, Norway. Briefly, approximately 1 g of A. precatorius leaves was frozen and ground to a powder in liquid N2. 10-20 ml of extraction buffer containing 400 mM NaCl, 40 mM EDTA, 100 mM Tris/Cl, pH 8.0, 2% SDS, 10 mM dithiothreitol, and 0.1 mg/ml proteinase K was added, and the mixture was incubated with shaking at room temperature for 2 h and subsequently at 50°C for 30 min. 50 units of RNase T1 was added, and the mixture was incubated at 37°C for 15 min. DNA was extracted with phenol/chloroform (1:1) and precipitated with isopropanol.

Oligonucleotide Primers—On the basis of the published amino acid sequence of the A-chain of abrin (14) three different PCR primers were prepared.

Primer Astart encodes the first 10 NH2-terminal amino acids of the A-chain. It has a 5'-in-frame start codon in an optimal Kozak sequence (17) and two suitable restriction sites, KpnI and NcoI, for subcloning.

5' GCGTGACATGGAGAATAGCCAATAAAAATTTTTCCAGAAGG

3' KpnI Ncol

Primer Astop encodes the last 9 COOH-terminal amino acids of the abrin A-chain and has a KpnI restriction site for subcloning.

5' TACCAATACACATATTGGAGAATAGCCAATAAAAATTTTTCCAGAAGG

3' KpnI

Both primers Astart and Astop are constructed as guessmers in the 5' end and as mixtures of several oligonucleotides in the 3' end. Primer A146-151 is complementary to the internal amino acids 146-151 in native abrin A-chain and carries a KpnI restriction site 5' in the sequence.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) X54873 and X54872.

1 The abbreviations used are: PCR, polymerase chain reaction; kb, kilobase(s); kbp, kilobase pair(s).
PCR—0.5 μg of genomic DNA was used in a 100-μl PCR mixture with 1 × PCR buffer (10 mM Tris/HC1, pH 8.5, 50 mM KCl, 1.5 mM MgCl2, 0.001% gelatin), 200 μM dNTPs, 100 pmol of each of the primers of either the pair Astart/Astop or A146-151/Astop, and 2.5 units of Taq DNA polymerase (18). Taq DNA polymerase was from Perkin-Elmer Cetus, and dNTPs were from Pharmacia LKB Biotechnology Inc. PCR was performed for 30 cycles of denaturation at 94 °C for 1 min, annealing for 1 min at 45, 50, or 55 °C, and primer extension for 3 min at 72 °C. 5 μl of the PCR mixture was analyzed on 1.5% agarose gels in 1 × Tris borate buffer.

Subcloning and Sequencing—DNA fragments of 0.7 and 0.3 kbp from PCR reactions were isolated from the agarose gels, digested with KpnI, and ligated to the in vitro transcription vector pGEM7Zf(+) (Promega Biotech). The ligation mixtures were used to transform E. coli DH5α cells made competent by Bethesda Research Laboratories. Clones carrying the 0.3-kbp fragment were directly sequenced by using magnetic beads as solid support in solid phase sequencing (19). The 0.7-kbp fragments were sequenced by the same methods after subcloning applying a unique StuI site located in the middle of these fragments. All inserts were sequenced at least twice on both strands.

In Vitro Transcription and Translation—mRNA was transcribed from pGEM7Zf(+) derivatives with the 0.7-kbp abrin A-chain genes downstream from the T7 RNA polymerase promoter according to published methods (20). mRNA was translated in nuclease-treated reticulocyte lysate or wheat germ extract as previously described (21). Translation products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography (21). Reticulocyte lysates which had not been treated with nuclease were incubated with purified abrin toxin or with toxin-encoding mRNA derived from the pGA7.2 or pGA7.3 plasmids. rRNA was isolated, exposed to aniline purified abrin toxin or with toxin-encoding mRNA derived from the pGA7.2 or pGA7.3 plasmids. rRNA was isolated, exposed to aniline and electrophoresed as previously described (21).

Southern Blot—Approximately 10 μg of genomic DNA was cut by various restriction enzymes and applied to each lane on a 0.8% agarose gel in Tris borate buffer. The gels were blotted onto Hybond N (Amersham Corp.) by capillary blotting overnight, and the blots were processed as described by the manufacturer. The blots were probed by end-labeled synthetic oligonucleotide probes or by plasmids carrying toxin genes labeled by nick translation (22).

RESULTS

Genomic clones for the related toxin ricin have earlier been obtained in several laboratories (16, 23). This work revealed that ricin belonged to a multigene family, and similar to other plant lectin genes the ricin gene contained no introns. Our cloning strategy as presented here was based on the assumption that a similar situation existed also for abrin and its A-chains. Thus, by the application of degenerate primers based on the published amino acid sequence for one abrin A-chain and gradually increasing the stringency of hybridization, we were able to PCR reactions to amplify specific genomic sequences of the expected size. As shown in Fig. 1, the main product after amplification of genomic Abrus DNA with primers Astart/Astop annealed at 50 °C was a fragment of approximately 0.75 kbp, whereas the fragment obtained with the A146-151/Astop primers at this temperature was about 0.35 kbp. Also at lower annealing temperatures in the PCR reactions the same bands were visible, but in particular the product obtained with the A146-151 primer contained a smear of bands in the 0.3-0.4-kbp range (data not shown). The amplified DNA fragments were subsequently subcloned and sequenced to verify their similarity with the published abrin sequence, as well as with other related toxins.

Analysis of cloned DNA fragments from the PCR reaction using the Astart/A stop primers revealed two types of cloned DNA of the expected size (0.75 kbp) covering the entire abrin A-chain. Random sampling and sequencing of clones containing the 0.75-kbp fragment obtained revealed that the two
Cloning and Expression of Abrin A-chains

The amino acid sequences deduced from the indicated clones are aligned with the previously published abrin A-chain sequence (denoted ABRINA) (14). Amino acids derived from the amplification primers are underlined. The second cysteine deduced from the clone pGA7.7 is marked with an asterisk.

All three types of proteins encoded by these sequences contain one potential glycosylation site (amino acid 200 in Fig. 1), but glycosylation of abrin A-chains isolated from A. precatorius has not been reported (1).

We and others recently showed that the N-glycosylase activity of ricin A-chain could be expressed and assayed in cell-free systems (21, 24). To establish that functional proteins were encoded by the full-length pGA7.3 and pGA7.2 clones, both were expressed and tested for functional glycosylase activity as earlier described for ricin (21). As shown in Fig. 3A the transcription of both genes generated mRNA which after addition to reticulocyte lysates strongly inhibited the translational activity of the lysates. Thus, addition of the order of 0.1 fmol of mRNA derived from these clones inhibited the incorporation of [35S]methionine in the lysates to about 50%. This is similar to the amount of ricin A-chain encoding mRNA which is necessary to inhibit the reticulocyte lysates to the same degree (data not shown (10)), indicating that the RNAs encoding abrin and ricin have about equal potencies in inhibiting the translational activity in reticulocyte lysates. Generally mRNA derived from pGA7.2 was slightly more potent than mRNA derived from pGA7.3 in inhibiting the incorporation in the reticulocyte lysates (see below). The inhibition of the reticulocyte lysates could be counteracted by antibodies specific for abrin, whereas antiserum toward ricin had no activity in this assay, indicating that the observed inhibition was due to generation of functional abrin A-chain protein (data not shown).

The N-glycosylase activity characteristic of abrin A-chain removes one particular adenine base from the sugar-phosphate backbone of 28 S rRNA (2). This renders the backbone susceptible to cleavage by anilin, and the cleavage products can easily be analyzed by agarose gel electrophoresis of rRNA.
pGA7.3 clones to wheat germ extract, which is relatively resistant to the glycosylase activity, generated polypeptides of the expected size, i.e. approximately 30 kDa. The polypeptides could be precipitated with antisera specific for abrin but not by antisera toward the related, but immunologically distinct, ricin toxin (Fig. 4). The results obtained also show that the amounts of protein generated from pGA7.2 tended to be slightly greater than the amounts generated from pGA7.3, indicating that the pGA7.2-derived mRNA was translated with somewhat higher efficiency than the pGA7.3-derived mRNA (Fig. 4, lane 4 versus lane 3).

Southern blot analysis after digestion of A. precatorius DNA with EcoRI indicated that A-chain-specific probes hybridized to at least three bands (5.2, 3.7, and 1.2 kbp) under relatively stringent conditions (4 x SSC, 50 °C), indicating that there are at least three different sequences encoding abrin A-chain-related proteins in the Abrus genome (data not shown).

DISCUSSION

The most important finding presented here is that abrin A-chain genes indeed can be isolated directly from genomic A. precatorius DNA by a simple PCR reaction. Thus, also the tides could be precipitated with antisera specific for abrin but with somewhat higher efficiency than the pGA7.3-derived tinct, ricin toxin (Fig. 4).

The relationship between this A-chain and the A-chain deduced from the pGA7.2 clone is unclear. Previously the amino acid composition of at least two different abrin A-chains as well as the A-chain amino acid composition of Abrus agglutinin have been published (9–13). The amino acid composition deduced from the pGA7.2 sequence has about equal similarity to the reported amino acid composition of all these proteins including the A-chain of abrin-a. Thus, the data on amino acid composition is inconclusive in assigning which particular type of abrin A-chain the pGA7.2 sequence encodes. In the case of ricin the corresponding ricinus agglutinin A-chain has been reported to be about 100-fold less active compared with ricin A-chain in inhibition of reticulocyte lysate (25). However, in our case, the mRNA derived both from pGA7.3 and pGA7.2 appeared to be about equally effective in inhibiting the translational activity of reticulocyte lysates.

The molecular cloning of the related toxic lectin ricin revealed that ricin is synthesized as a prepro-protein which is processed to yield the mature A- and B-subunits (16, 23). The high degree of similarity between the ricin and the abrin A-chains, as well as the earlier reported similarity between the NH2-terminal end of the B-chains of ricin and abrin (8), makes it conceivable that abrin also is synthesized as a prepro-protein. Furthermore, the recent cloning of the gene for the related single chain ribosome-inactivating protein, trichosan, revealed that this gene also encodes a prepro-protein with an NH2-terminal signal sequence as well as a COOH-terminal extension of 19 amino acids which is removed from the mature protein (26). The expression in cell-free systems of these proteins may facilitate experiments to deduce which amino acids and domains within the A-chains are important for function. Furthermore, the possibility of generating recombinant abrin A-chain will facilitate an exploration of this protein for therapeutic purposes. The fact that abrin A-chain is immunologically different from ricin A-chain (25) indicates that after systemic administration of an immunotoxin containing one of these toxin A-chains and the subsequent appearance of neutralizing antibodies in the patient, the treatment may be beneficially continued with an immunotoxin containing as active principle another toxin A-chain.

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REFERENCES


FIG. 4. Immunoprecipitation of abrin A-chains expressed in wheat germ lysates. Toxin encoding mRNA was added to wheat germ lysates containing [35S]methionine and incubated for 45 min at 30 °C. An aliquot of the lysates was diluted in phosphate-buffered saline containing 1 mg/ml bovine serum albumin. 1 µg of rabbit anti-abric or anti-ricin sera was added and incubated for 1 h. The precipitates were collected by adding Sepharose-Protein A (Pharmacia), washing two times in buffer containing 0.5 M NaCl, solubilized in 2% sodium dodecyl sulfate by boiling, and applied to a 15% acrylamide gel. The gel was dried and subjected to autoradiography. Lane 1, 14C molecular weight markers (myosin, phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, and lysozyme); lane 2, precipitate obtained with anti-abric serum or anti-ricin sera was added and incubated for 1 h. The precipitates were collected by adding Sepharose-Protein A (Pharmacia), washing two times in buffer containing 0.5 M NaCl, solubilized in 2% sodium dodecyl sulfate by boiling, and applied to a 15% acrylamide gel. The gel was dried and subjected to autoradiography. Lane 1, 14C molecular weight markers (myosin, phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, and lysozyme); lane 2, precipitate obtained with anti-abric serum or anti-ricin sera was added and incubated for 1 h. The precipitates were collected by adding Sepharose-Protein A (Pharmacia), washing two times in buffer containing 0.5 M NaCl, solubilized in 2% sodium dodecyl sulfate by boiling, and applied to a 15% acrylamide gel. The gel was dried and subjected to autoradiography. Lane 1, 14C molecular weight markers (myosin, phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, and lysozyme); lane 2, precipitate obtained with anti-abric serum or anti-ricin sera was added and incubated for 1 h.

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Cloning and Expression of Abrin A-chains