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Cloning and Expression of Catalytic Subunit of MLIII, the Ribosome-Inactivating Protein from *Viscum album*

A. G. Tonevitsky[1]*, I. I. Agapov[1], I. B. Pevzner[1], N. V. Malyuchenko[1], M. M. Moisenovich[1], U. Pfueller[2], and M. P. Kirpichnikov[1]

[1]Faculty of Biology, Lomonosov Moscow State University, Moscow 119899, Russia; fax: (7-095) 196-0522; E-mail: tonevits@genetika.ru
[2]Institute of Phytochemistry, University of Witten/Herdecke, Stockumer Str. 10, D-58448, Witten, Germany; E-mail: uwep@uni-wh.de

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Abstract—We have cloned the gene encoding a precursor of mistletoe (*Viscum album*) toxin MLIII. Analyses of nucleotide and deduced amino acid sequences of this gene revealed significant differences between MLI and MLIII preprotoxin genes. Immunochemical properties of recombinant A-subunit expressed in *Escherichia coli* and renatured were investigated using a panel of monoclonal antibodies raised against three mistletoe toxins (MLI, MLII, and MLIII). Ribosome-inactivating activity of recombinant MLIII A-subunit was detected in cell-free lysate of rabbit reticulocytes.

Key words: mistletoe toxin, recombinant MLIII A-subunit, monoclonal antibodies

Toxic lectins of mistletoe (*Viscum album*), namely MLI (viscumin), MLII, and MLIII, are members of the type II ribosome-inactivating protein family (RIPII) [1]. RIPIIs are heterodimeric glycoproteins composed of two subunits linked with a disulfide bond. A-Subunit of these toxins expresses N-glucosidase activity and can modify the eucaryotic 28S rRNA of major 60S ribosomal subunit, which results in arrest of protein synthesis in the cell. Viscumin (MLI) acts by the same mechanism as ricin [2]. B-Subunit of the toxins is a lectin specific to ether galactose (MLI), or N-acetylgalactosamine (MLIII), or both sugars (MLII) [3].

MLs (MLI, MLII, and MLIII) are found in all mistletoe extracts. The ratio between MLs changes in mistletoe leaves in a season-dependent manner [4]. MLI, MLII, and MLIII differ in molecular weight. The molecular weight of purified A-chain of MLI (MLIA) is 29 kD, and that of MLIB is 34 kD. At high concentrations, MLI forms a dimer with molecular weight of 115 kD. Molecular weights of MLII- and MLIII A-chains are 27 and 25 kD and those for B-chains are 32 and 30 kD, respectively [5]. But the N-terminal amino acid sequences of all three mistletoe lectins are highly homologous [6].

Besides the above differences, three mistletoe lectins appear functionally distinctive. Among them, MLIII is the most toxic against various cell types, both normal and tumor, whereas MLI and MLII are less toxic [6, 7].

Aqueous mistletoe leaf extracts containing toxic MLs are widely used in some European countries as immunomodulators in antitumor therapy because they possess anti-mutagenic activity and can evoke apoptosis [8].

RIPIIs are used as convenient models for investigations into intracellular protein transport [9, 10]. On their way to the ribosome, RIPIIs are involved in various transports, such as receptor-mediated endocytosis, endosomal compartmentation, retrograde transfer into Golgi apparatus and endoplasmic reticulum, and, finally, translocation of active enzymatic constituent from an intracellular compartment into the cytosol [11]. Enzymatic subunits of plant toxins are used for conjugation with target mole-

Abbreviations: RIPII) ribosome-inactivating protein type II; MLI, -II, -III) mistletoe lectin I (viscumin), II, III; MLIA or rMLIA) natural or recombinant viscumin A-subunit; rMLgA) recombinant MLIII A-subunit; RTA) natural ricin A-subunit; ABA) natural abrin A-subunit; EMLA) A-subunit of European mistletoe lectin I; KMLA1, -2, -3) A-subunit of Korean mistletoe lectin 1, 2, 3; IMLA1, -2) A-subunit of Indian mistletoe lectin 1 or 2; PCR) polymerase chain reaction; ELISA) enzyme-linked immunosorbent assay; mAB) monoclonal antibodies; PBS) phosphate-buffered saline; BSA) bovine serum albumin; OD) optical density.

* To whom correspondence should be addressed.

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cules to develop directed therapeutics allowing selective elimination of target cells [12].

Development of a recombinant toxin offers great opportunities for investigation into the properties of RIPIIs and for their practical use. For instance, recombinant toxins are widely used in gene engineering of immunotoxins, which have a decided advantage over protein conjugates in their lower molecular weight, absence of glycosylation, and facility for chemical donation with signaling sequences allowing alteration in intracellular transport and enhancement in reactivity of immunotoxins [13]. The membrane-penetrating capability of toxins makes possible their application as vehicles for delivery of various peptides into the cytoplasm in the development of next-generation vaccines [14, 15].

We previously produced a panel of monoclonal antibodies raised against three native ML isoforms (MLI, MLII, and MLIII) [16] that allow immunochemical detection of both whole toxins and their individual subunits in pharmacological mistletoe extracts used for cancer therapy. The success of this therapy depends on the percentages of MLI, MLII, and MLIII in the extracts used. At present, clinical trials of the recombinant MLI are being carried out [17]. In the present study, we have cloned the MLIII precursor gene (using DNA isolated from the mistletoe leaves) and expressed the A-subunit of this toxic lectin. We have also determined the nucleotide sequence of the cloned gene and its corresponding amino acid sequence in comparison with that of viscumin (MLI) and identified the main differences in primary protein structure between the A-chains of MLI and MLIII. We have studied the secondary structure and properties of renatured recombinant MLIII A-chain using a panel of mABs raised against the native subunits of three ML isoforms (MLs I, II, and III). In cell-free lysate of rabbit reticulocytes, the recombinant MLIIIA was found active towards the eucaryotic ribosomes resulting in inhibition of endogenous globin synthesis.

MATERIALS AND METHODS

Chemicals, bacterial strains, plasmids, and modifying enzymes. Oligonucleotides were synthesized by Sintol (Russia). DNA-modifying enzymes were purchased from Fermentas (Lithuania). Equipment and chemicals for the isolation of plasmid DNA and purification of PCR products were from QIAGEN GmbH (Germany). All the chemicals used were of analytical grade and were purchased from Sigma (Germany).

The cells of *Escherichia coli* strain LE392 (*hsdR574*(r_{K-} m_{K+}) *supE44 supF58 lacY1 galK2 galT22 metB1 trpR55*) were purchased from Promega (USA). The cells of *E. coli*, strain BL21 [B F^- *dcm ompT hsd*S_B (r_{B-} m_{B-}) *gal*λ] (DE3), were purchased from Stratagene (USA). The plasmid pUC19 (Fermentas) was used for the cloning and sequencing of the complete gene encoding a toxic mistletoe lectin, and the plasmid pET11cjoe (Novagen, USA) was used for expression of the enzymatic subunit of the lectin.

Toxins and their subunits. The toxic lectins MLI, MLII, and MLIII were purified from leaves of mistletoe (*Viscum album*) as previously described [16]. The native A-subunit of viscumin was purified by affinity chromatography on immobilized antibodies specific to the toxin subunits as previously described [18]. Ricin from Palma Christi (*Ricinus communis*) and its subunits were purified according to the previously described protocols [19, 20]. The purity of prepared specimens was determined using electrophoresis and ELISA.

The active recombinant MLI A-subunit (rMLIA) prepared previously [18] was used in comparative studies.

Cloning of the mistletoe lectin gene. The total genome DNA was extracted from dry mistletoe leaves gathered from a single host tree of Scotch pine (*Pinus silvestris*) in Germany as described elsewhere [21] and additionally purified with phenol.

On the reported MLI gene sequence ([22], accession No. A58957) the following primers containing the *Eco*RI and *Bam*HI restriction sites were synthesized for the cloning of a fragment of the entire lectin gene into the plasmid pUC19:

Forward-ML: 5′ AAA**GAATTC**ATGAATGCGGTTATGGACTCAAGAA 3′
*Eco*RI

Reverse-ML: 5′ AAA**GGATCC**TCATGGCACGGGAAGCCACATTTG 3′
*Bam*HI

Another pair of primers containing the *Nde*I and *Bam*HI restriction sites was used for the cloning of a fragment of the lectin A-subunit gene into the expression plasmid pET11cjoe:

Forward-ML13: 5′ AAAAGCTAGC**CATATG**TACGAGAGGCTAAGACTCAGA 3′
*Nde*I

Reverse-ML13: 5′ AAAAA**GGATCC***TCATTA*GGAGGAAGATGGCCGGTCC 3′
*Bam*HI Stop Stop

A nucleotide sequence of the mistletoe lectin gene was amplified by PCR using the total mistletoe DNA as a template and *Taq* DNA-polymerase. The final reaction volume was 30 μl. The reaction medium contained: 67 mM Tris-HCl, pH 8.8, 16.6 mM of $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.01% Tween-20, 0.2 mM of each deoxynucleoside triphosphate, 10 pM of each synthetic oligonucleotide primer (forward-ML and reverse-ML), 0.5 U of *Taq* DNA-polymerase, and 20 ng of total *V. album* DNA. The reaction was carried out on a GeneAmpв PCR system 2400 thermocycler (Perkin-Elmer, USA) as follows: primary denaturation at 94°C for

4 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and elongation at 72°C for 1 min.

PCR products were separated electrophoretically in 1% agarose gel. A DNA fraction containing the product with expected length of ~1700 bp was recovered from gel slices with the QIAquick spin kit. Both the PCR product and the plasmid pUC19 were digested with the *EcoR*I and *BamH*I endonucleases and ligated with the T4 DNA-ligase. Identity of the inserted polynucleotide fragment was assessed by restriction analysis. The strain *E. coli* LE392 was used for transformation with the vector thus obtained. Sequencing of prepared recombinant DNA products was performed at MWG-Biotech AG (Germany). The nucleotide sequence of the mistletoe lectin gene fragment encoding the A-chain was amplified by PCR using the plasmid pMLg2 (pUC19 vector carrying a sequence of the entire gene encoding the ML preproprotein) as a template and the High Fidelity PCR Enzyme Mix. PCR was carried out in 30 µl of the standard reaction mixture using an amplification protocol adopted for the complete gene sequence on the MC-16 Tercic amplifier (DNA Technology, Russia).

PCR products were separated electrophoretically in 1% agarose gel. A DNA fraction containing the product with expected length of ~760 bp was recovered from gel slices with the QIAquick spin kit. Both the PCR product and the plasmid pET11cjoe were digested with the *Nde*I and *BamH*I endonucleases and ligated with the T4 DNA-ligase. Identity of the inserted polynucleotide fragment was assessed by restriction analysis. The strain *E. coli* BL21(DE3) was used for transformation with the vector thus obtained.

The nucleotide sequence of the gene fragment encoding the recombinant ML A-subunit was determined at the All-Russia Research Institute of Agricultural Biotechnology, Russian Academy of Agricultural Sciences. Deduced amino acid sequence of the recombinant protein was compared with that of previously cloned A-chains of MLs from European mistletoe (EMLA, CAA03513), Korean mistletoe (KMLA1, AAM46932; KMLA2, AAM46933; and KMLA3, AAM46934), and Indian mistletoe (IMLA1, AAL87006; and IMLA2, AAL87005), as well as with A-chain amino acid sequences of ricin (RTA, P02879) and abrin (ABA, P11140) using the ClustalX (v. 1.81) software.

Expression of recombinant proteins. Transformed *E. coli* cells were grown under continuous agitation at 37°C in 5 ml of Luria–Bertani medium containing 50 µg/ml of ampicillin. Culture growth was monitored by optical density at 600 nm. Induction of product expression was initiated in the culture at the OD_{600} = 0.6-1.0 with 1 mM isopropyl β-D-thiogalactoside (IPTG). Four hours after the initiation the cells were centrifuged at 4000*g* for 10 min. Bacterial clones expressing recombinant proteins with expected molecular weight of about 30 kD were selected using SDS-PAGE as described by Laemmli [23] in 15% polyacrylamide gel under reducing conditions.

Immunoblotting. To confirm specificity of expressed proteins in *E. coli* cell lysates, immunoblotting was performed with the monoclonal antibodies TA7 raised against denatured A-chains of MLs [24]. Proteins were blotted onto a 45-µm nitrocellulose membrane (Schleicher & Schuell, Germany; Ref. No. 401099) according to a semidry blotting protocol. Following the protein transfer verification by membrane staining with Ponceau S dye, the membrane was incubated in 4% dry defatted milk solution in PBS containing 0.05% Tween 20, washed, and incubated with the TA7 mABs (10 µg/ml). Then the membrane was washed and incubated with goat anti-mouse IgG antiserum conjugated with horseradish peroxidase (IMTEK, Russia). The membrane was stained in PBS solution containing 3 mg of diaminobenzidine, 10 mg 4-chloro-1-naphthol, 32% ethanol, and 0.05% H_2O_2.

Isolation and purification of recombinant proteins. To isolate inclusion bodies, a pellet of *E. coli* cells producing either the previously cloned viscumin A-subunit (rMLIA) [18] or newly obtained rMLgA was subjected to the standard purification protocol [25]. The pellet from 100 ml of *E. coli* culture was suspended in 20 ml of STET buffer (50 mM Tris-HCl containing 8% sucrose, 50 mM EDTA, and 1.5% Triton X-100) and ultrasonicated. The suspension was centrifuged 12,000*g* for 30 min at 4°C. The above procedure was repeated thrice. Then the pellet was washed with PBS and frozen. Upon inclusion body purification, both supernatants and pellets were analyzed by SDS-PAGE in 15% polyacrylamide gel. The yield of protein was determined quantitatively in comparison to viscumin samples of known concentrations placed onto the same gel. The pellet containing 4 mg of recombinant A-subunit was dissolved in 1 ml of buffer containing 7 M guanidine hydrochloride, 50 mM Tris-HCl, pH 8.0, and 2% 2-mercaptoethanol followed by incubation at room temperature for 1 h and centrifuged at 12,000*g* for 15 min. For renaturation, a 750-µl aliquot of the denatured protein was added dropwise into PBS, pH 7.4, under agitation up to 130-fold dilution, until the final protein concentration was 15 µg/ml. The solution was kept overnight at 4°C. Aggregates were removed by centrifugation at 12,000*g* at 4°C for 30 min. The supernatant was dialyzed against PBS, pH 7.4. The recombinant proteins rMLIA and rMLgA were concentrated, and contents of the recombinant A-subunit was determined by ELISA with mABs specific to A-subunits of MLI, MLII, and MLIII.

ELISA. Immunochemical properties of the produced recombinant proteins were studied using sandwich-ELISA. In this assay the following previously raised and characterized monoclonal antibodies were used: MNA4, MNA9 [26, 27], H8 [28], and H11 [26, 28] specific to the native A-subunits of toxic mistletoe lectins and TA7 [24] specific to their denatured A-subunits.

Sandwich-ELISA was carried out as follows. The primary antibodies (10 μg/ml, PBS solution) were placed into the wells of a 96-well immunological plate, 100 μl per well. Following the washes with PBS containing 25 mM of lactose and 0.05% Tween 20, the plate was incubated with 0.1% BSA and washed. Then the natural mistletoe toxins at varied concentration and varied dilutions of recombinant proteins were added into the wells and incubated at 37°C for 1 h followed by washing. Then 100 μl of biotin-labeled secondary antibodies (1 μg/ml) were placed into each well and incubated at 37°C for 1 h. After the washing, the plate was incubated with the streptavidin–peroxidase conjugate (IMTEK) followed by washing and development with *o*-phenylenediamine (Sigma, USA) dissolved in buffer containing 0.1 M of sodium citrate, 0.05 M sodium phosphate, and 0.05% H_2O_2, pH 5.0. The colored reaction products were detected at 492 nm on the vertical Multiscan spectrophotometer (Labsystems, Finland).

In ELISA with mABs TA7, the native MLs or recombinant proteins were sorbed onto the plate, washed, incubated with biotin-labeled TA7 antibodies and developed using the streptavidin–peroxidase conjugate.

Protein synthesis in a cell-free system. Activity of recombinant proteins was tested from the inhibition of endogenous globin synthesis (incorporation of ^{3}H-labeled leucine into the protein) in a cell-free system based on rabbit reticulocyte lysate. The final volume of reaction mixture was 20 μl including 18 μl of the cell-free system and 2 μl of the protein solution tested. The reaction mixture contained: 24 mM Hepes, pH 7.6, 12 mM creatine phosphate, 1 mM ATP, 0.2 mM GTP, 0.03 mM of each amino acid, 1.2 mM of dithiothreitol, 0.05 mM potassium acetate, 1.2 mM $MgCl_2$, 9 μl reticulocyte lysate, 1.2 μl ^{3}H-labeled leucine solution (1 mCi/ml), and 2 μl of either PBS solution of recombinant protein with known concentration or PBS only (control). The mixture was incubated at 30°C for 1 h, then an aliquot (5 μl) was diluted in 50 μl of water, and proteins were precipitated with 1 ml of 10% trichloroacetic acid on a boiling water bath for 2 min. The specimens thus obtained were filtered through Glasfaser-Vorfilter GF92 filters (Schleicher & Schuell, Ref. No. 451004). The filters were air-dried, and their radioactivity was measured on the Liquid Scintillation System 6891 DELTA 300 (Tracor Analytic, USA). The sample containing no toxin was taken as 100% label incorporation, and the sample with 2 μl of the ricin A-chain (10 μg/ml, PBS solution) was taken as 0%.

RESULTS

1. Cloning and sequence analysis of the mistletoe lectin gene. The primers, whose sequences were deduced from the reported MLI gene sequence [22], were synthesized for amplification of the entire preproprotein gene and its fragment encoding A-subunit from the total mistletoe DNA template. The recombinant product of cloned entire *rMLg* gene appreciably differed from the preproprotein MLI gene in its nucleotide sequence, whose homology was 79%. Multiple nucleotide substitutions were found all along the sequence. The greatest difference was in the area encoding a linker peptide between protein A- and B-chains, in which two fragments composed of seven and eight nucleotides were present in the *rMLg* gene but absent in the gene encoding the MLI preproprotein.

A deduced amino acid sequence of an individually amplified fragment of the lectin rMLgA gene has 89% identity in conservative amino acid residues with native MLI A-subunit, and B-chain has 86% identical amino acids with MLI B-subunit. The identity of the entire protein is 88.1%, and its resemblance is 91.5%. The amino acid sequence of rMLgA has 35.2% identity and 46.3% resemblance with the ricin A-chain (RTA), and 42.4% identity and 53.4% resemblance with the abrin A-chain (ABA). All of the amino acid residues involved in enzymatic activity of the ribosome-inactivating protein A-subunits (marked by rectangles on Fig. 1) and common conservative amino acids of RIPs are present in rMLgA. However, it is worth noting that the recombinant rMLgA protein compared with MLI A-subunit (EMLA) contains 25 substituted amino acid residues. As compared with EMLA, it has six additional negatively charged amino acid residues (aspartic and glutamic acids in positions 102, 144, 192, 197, 224, and 263) with substitution R102D of a positively charged amino acid by a negatively charged one. Moreover, rMLgA has two additional positively charged amino acid residues (arginines 106 and 262). These changes influence both the isoelectric point of the protein and its overall charge at neutral pH. An important change is apparently the substitution of Asn112 in EMLA by Thr in rMLgA. This position corresponds to the site of glucosylation comprising the NxS/T sequence in EMLA, and because of the substitution N112T this site is absent in rMLgA. Most of the other substitutions are conservative changes of nonpolar aliphatic or polar uncharged amino acids.

2. Expression of A-subunit from the mistletoe lectin gene. Bacterial clones containing (as judged from restriction analysis) plasmids carrying the expected MLgA-encoding fragment were used for expression. Expression of the recombinant A-subunits in *E. coli* cells was assessed by SDS-PAGE in 15% polyacrylamide gel. The maximum expression was achieved 3-4 h after induction with IPTG. The clones expressing proteins with molecular weight about 30 kD were chosen for further studies. Recombinant proteins are preferably harbored within inclusion bodies, and their production was about 10% of the total protein (Fig. 2a). The identity of the recombinant product was confirmed by immunoblotting with TA7 antibodies specific to denatured ML A-subunits (Fig. 2b).

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CLUSTAL X (1.81) multiple sequence alignment

rMLgA     ----YERLRLRVTHQTTGDEYFRFITLLRDYVSSGS-FSNEIPLLRQSTIPVSDAQRFVL
EMLA      ----YERLRLRVTHQTTGDEYFRFITLLRDYVSSGS-FSNEIPLLRQSTIPVSDAQRFVL
KMLA1     ----YERLRLRVTHQTTGDEYFRFITLLRDYVSSGS-FSNEIPLLRQSTIPVSDAQRFVL
KMLA2     ----YERLRLRVTHQTTGDQYFKFITLLRDHVSSGS-LSNQIPLLRQSTVPVSDTQRFVL
KMLA3     ----YERLRLRVTHQTTGDEYFRFIKLLRDSVSSGS-FSNDIPLLPPS-IPVSSAQRFVL
IMLA1     ----YERLRLRVTHQTTGEEYFRFITLLRDYVSSGS-FSNEIPLLRQSTIPVSDAQRFVL
IMLA2     ----YERLRLRVTHQTTGDEYFRFITLLRDYVSSGS-FSNEIPLLRQSTIPVSDAQRFVL
ABA       ----QDRPIKFSTEGATSQSYKQFIEALRERLRGG--LIHDIPVLPDP-TTLQERNRYIT
RTA       IFPKQYPIINFTTAGATVQSYTNFIRAVRGRLTTGADVRHEIPVLPNR-VGLPINQRFIL
                     *  :* :.* .**  :*  :  *  . ::**:*      :   :*::

rMLgA     VELTNQGG---DSITAAIDVTNLYVVAYQAGDQSYFLR---DAPDGAERHLFTGTT-RSS
EMLA      VELTNQGG---DSITAAIDVTNLYVVAYQAGDQSYFLR---DAPRGAETHLFTGTT-RSS
KMLA1     VELTNQGG---DSITAAIDVTNLYVVAYQAGDQSYFLR---DAPDGAERHLFTGTT-RSS
KMLA2     VELSNQGG---DSITAAIDVTNLYVVAYQAGNQSYFLR---DAPRGAETYLFTGTT-RSS
KMLA3     VELTNQLGKWEDSITAAIDVTNLYVVAYQAGDQSYFLR---DAPDGAERHLFTGTT-RSS
IMLA1     VELTNEGG---DSITAAIDVTNLYVVAYQAGDQSYFLR---DAPRGAETHLFTGTT-RSS
IMLA2     VELTNEGG---DSITAAIDVTNLYVVAYQAGDQSYFLR---DAPDGAERHLFTGTT-RSS
ABA       VELSNSDT---ESIEVGIDVTNAYVVAYRAGTQSYFLR---DAPSSASDYLFTGTD-QHS
RTA       VELSNHAE---LSVTLALDVTNAYVVGYRAGNSAYFFHPDNQEDAEAITHLFTDVQNRYT
          ***:*       *:  .:**** ***.*:** .:**::   :    *  :***..  : :

rMLgA     LPFTGSYTDLERYAG-HRDQIPLGIEELIQSVSALRY---PGGSTRAQARSIIVLIQMIS
EMLA      LPFNGSYPDLERYAG-HRDQIPLGIDQLIQSVTALRF---PGGSTRTQARSILILIQMIS
KMLA1     LPFTGSYTDLERFAG-HRDQIPLGREELIQSVSALRF---PGSNTRAQARSFIILIQMIS
KMLA2     LPFNGSYPDLERYAG-HRDQIPLGIDQLIQSVSALRF---PGSNTRAQARSFIILIQMIS
KMLA3     LPFNGSYADLERYAG-HRDRIPLGREPLIRSVSALDY---PGGSTRAQASSIIIVIQMIS
IMLA1     LPFNGSYPDLERYAG-HRDQIPLGIDQLIQSVTALRF---PGGNTRTQARSILILIQMIS
IMLA2     LPFTGSYTDLERYAG-HRDQIPLGIEELIQSVSALRY---PGGSTRAQARSIIILIQMIS
ABA       LPFYGTYGDLERWAHQSRQQIPLGLQALTHGISFFRS---GGNDNEEKARTLIVIIQMVA
RTA       FAFGGNYDRLEQLAGNLRENIELGNGPLEEAISALYYYSTGGTQLPTLARSFIICIQMIS
          :.* *.*  **: *   *:.* **   * ..:: :      * .    * :::: ***::

rMLgA     EAARFNPIFWRVRQDINSGESFLPDMYMLELETSWGQQSTQVQQSTEDVFNNPFRLA-IS
EMLA      EAARFNPILWRARQYINSGASFLPDVYMLELETSWGQQSTQVQHSTDGVFNNPIRLA-IP
KMLA1     EAARFNPILWRARQYISSGGSFLPDTYILQLETSWGQQSTQVQHSTDGVFNNPIRLT-IS
KMLA2     EAARFNPILWRARQYISSGGSFLPDTYILQLETSWGQQSTQVQHSTDGVFNNPIRLT-IS
KMLA3     EAARFNPILWRARQYINSGVSYLPDVYMLELEASWGQQSTQVQQSTDGVFNNPIRLG-IS
IMLA1     EAARFNPILWRARQYINSGASFLPDVYMLELETSWGQQSTQVQQSTEGVFNNPIRLA-IP
IMLA2     EAARFNPIFWRVRQDINSGESFLPDMYMLELETSWGQQSTQVQQSTDGVFNNPFRLA-IS
ABA       EAARFRYISNRVRVSIQTGTAFQPDAAMISLENNWDNLSRGVQESVQDTFPNQVTLTNIR
RTA       EAARFQYIEGEMRTRIRYNRRSAPDPSVITLENSWGRLSTAIQESNQGAFASPIQLQ-RR
          *****. *  . *  *  .    **  :: ** .*.. *  :*.* :..* . . *

rMLgA     TGNFVTLSNVRDVIASIAIMLFVCRDRPSSS-
EMLA      PGNFVTLTNVRDVIASLAIMLFVCGERPSSS-
KMLA1     TGVFVTLSNVRDVIASLAIMLFVCEDRPSSS-
KMLA2     TGVFVTLSNVRDVIASLAIMLFVCED------
KMLA3     TGNFVWLSNVRDVIASLGIMVFVCRD------
IMLA1     PGNFVTLTNVRDVIASLAIMLFVCGERPSSS-
IMLA2     TGNFVTLSNVRDVIASLAIMLFVCGE------
ABA       NEPVIVDSLSHPTVAVLALMLFVCNPPN----
RTA       NGSKFSVYDVSILIPIIALMVYRCAPPPSSQF
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Fig. 1. Multiple alignment of amino acid sequences of plant type II ribosome-inactivating protein A-chains: viscumin from European mistletoe (EMLA, CAA03513), Korean mistletoe lectins (KMLA1, AAM46932; KMLA2, AAM46933; and KMLA3, AAM46934), Indian mistletoe lectins (IMLA1, AAL87006; and IMLA2, AAL87005), ricin (RTA, P02879), and abrin (ABA, P11140) with deduced amino acid sequence of the recombinant protein rMLgA. Amino acid changes in rMLgA compared with EMLA are given in bold.

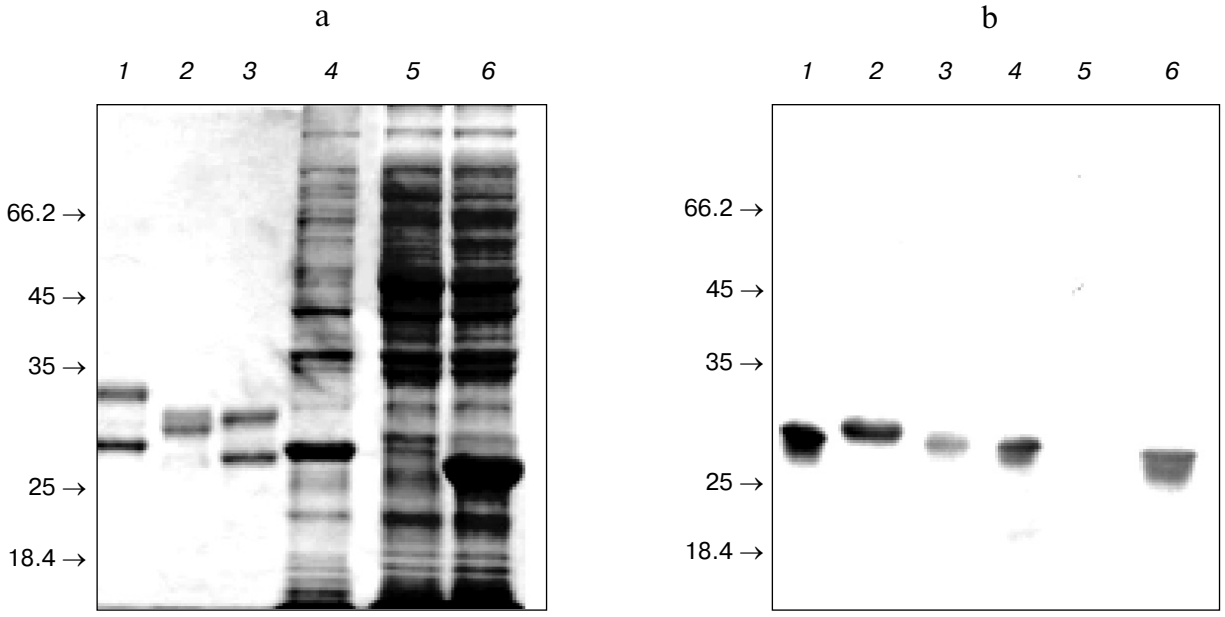

Fig. 2. Immunoblotting of natural and recombinant toxic mistletoe lectins with TA7 antibodies: a) proteins blotted onto a nitrocellulose membrane and stained with Ponceau S; b) proteins developed with TA7 antibodies. Lanes: *1*) MLI; *2*) MLII; *3*) MLIII; *4*) lysate of *E. coli* BL21(DE3) cells transformed with a plasmid carrying the rMLgA-encoding polynucleotide sequence; *5*) lysate of the initial *E. coli* BL21(DE3) cells; *6*) lysate of *E. coli* BL21(DE3) cells transformed with a plasmid carrying the rMLIA-encoding polynucleotide sequence.

The inclusion bodies containing recombinant protein were washed from other cellular components with STET buffer. To quantify the protein yield by electrophoresis, viscumin samples with known concentration were placed onto the same gel. The purified inclusion bodies were dissolved in a denaturing buffer containing 7 M guanidine hydrochloride. Denatured recombinant proteins were then folded by gradual 130-fold dilution so that the final protein concentration was 15 μg/ml. The yield of renatured protein was about 10%.

3. Immunochemical properties of recombinant proteins. The antibodies TA7 were found to be specific to denatured A-subunits of all three toxic mistletoe lectins MLI, MLII, and MLIII (Fig. 2b). Epitope analysis has shown that the antibodies TA7 recognize the linear epitope FTGTT corresponding to amino acids 101-105 in the MLA sequence [24]. This epitope is present in both recombinant proteins rMLIA and rMLgA. The antibodies TA7 were used for the first immunochemical evaluation of recombinant proteins after renaturation. We have demonstrated that antibodies TA7 uniformly interacted with recombinant proteins. Afterwards, these antibodies were used for equation of recombinant A-subunit concentrations for ELISA with other mABs and for the testing of protein activities in a cell-free protein-synthesizing system.

The mABs MNA4 and MNA9 recognize different non-overlapping fragments of the MLI A-subunit, and the sandwich MNA9–MNA4 allows determination of MLA in individual form or in the whole toxin [26, 27]. The sandwich-ELISA with the use of MNA9–MNA4-biotin system was proposed for the determination of MLI in mistletoe leaf extracts [16].

Interestingly, the previously obtained recombinant protein rMLIA was recognized in this system, whereas newly prepared rMLgA was not (Fig. 3a). So we anticipated that this new expressed protein was not a derivative of the viscumin A-subunit. The primers used for the cloning of viscumin A-subunit from the total mistletoe DNA template could also border the sequences of MLII and/or MLIII A-subunits. The recombinant proteins were assayed in ELISA with mABs specific to MLII and MLIII: MNA4 (specific to both the MLI and MLII A-subunits), H8 (specific to the MLIII A-subunit), and H11 (specific to both the MLII and MLIII A-subunits) [26, 28].

Using the sandwich-ELISA with MNA4–H11-biotin system specific to MLII, we have demonstrated that the recombinant products rMLIA and rMLgA are not derivatives of the MLII A-subunit (Fig. 3b).

The recombinant protein rMLgA was only detected in the system H8–H11-biotin (Fig. 3c), thus carrying inference that the A-subunit encoded by the cloned gene has antigen determinants of toxic lectin MLIII.

4. Toxic properties of recombinant ML A-subunits. Ribosome-inactivating enzymatic activity of the recombinant A-subunits was tested from the inhibition of protein biosynthesis in a cell-free system containing rabbit

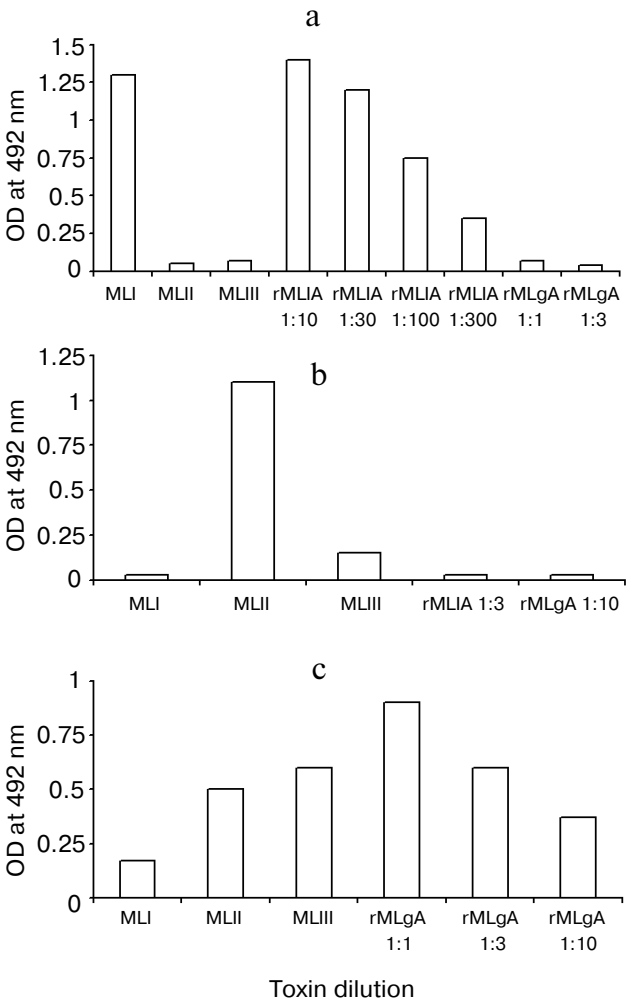

Fig. 3. Interaction of renatured recombinant proteins rMLIA and rMLgA in sandwich-ELISA with MNA9–MNA4 (a), MNA4–H11 (b), and H8–H11 (c) mAB pairs. The concentration of the native toxins is 100 ng/ml.

reticulocyte lysate. The natural viscumin A-subunit and active recombinant rMLIA A-subunit prepared earlier [18] and renatured from inclusion bodies in the same way as rMLgA were taken as controls.

The recombinant rMLgA protein was shown to be active in the cell-free protein-synthesizing system, inducing a 60% decrease in ^{3}H-labeled leucine incorporation into the synthesized globin. However, the natural (MLIA) and recombinant (rMLIA) viscumin A-subunits taken at the same concentration (800 ng/ml) were more active (resulting in decrease of the label incorporation by 81 and 89%, respectively) (Fig. 4).

DISCUSSION

Cloning and sequencing of the mistletoe lectin gene. In this study, using the mistletoe DNA, we have cloned the gene encoding a toxic lectin that significantly differs in its nucleotide and corresponding amino acid sequences from the gene encoding viscumin (MLI). The primary sequence analysis of the recombinant A-subunit (rMLgA) revealed some differences in its amino acid sequence from the natural MLI A-subunit. These differences include numbers of negatively and positively charged amino acid residues resulting in different isoelectric points (pI = 6.11 and 5.26) and overall protein charges at neutral pH (−1.87 and −5.86) of MLIA and rMLgA, respectively. The absence of the glycosylation site in rMLgA is consistent with our data on staining of polyacrylamide gels with Schiff's reagent after electrophoresis of toxins, proving that MLIII A-subunit is not glycosylated (data not shown) and enabling to conclude that the recombinant A-subunit is a derivative of MLIII.

Three genes for A- and B-chains have been cloned earlier from mistletoe gathered in Korea [29], and nucleotide and translated amino acid sequences for these

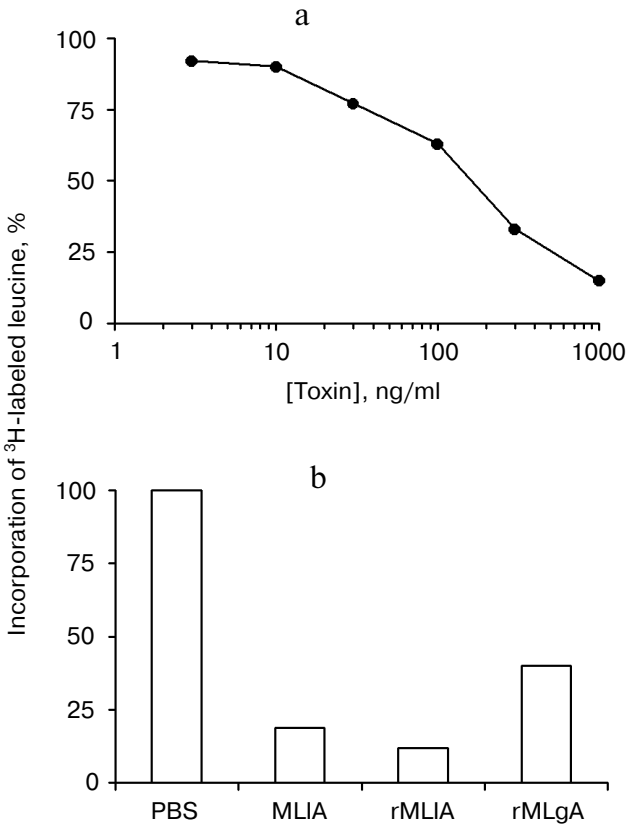

Fig. 4. Ribosome-inactivating activities of the natural MLI A-subunit and recombinant proteins rMLIA and rMLgA determined from the inhibition of ^{3}H-labeled leucine incorporation into globin molecules synthesized in a cell-free system containing rabbit reticulocyte lysate: a) dependence of the activity of native viscumin A-chain on its concentration; b) comparison of the native and recombinant protein activities at the protein concentration of 800 ng/ml. The sample containing no toxin was taken as 100% ^{3}H-labeled leucine incorporation, and the sample with 10 μg/ml of the ricin A-chain was taken as 0%.

genes have been compared with known sequence of viscumin (MLI) cloned from European mistletoe [22]. It turned out that the N-glycosylation site present in the viscumin A-chain was absent in one of the genes in its region corresponding to A-subunit (KMLA1). A comparison of amino acid sequences for rMLgA and KMLA1 demonstrates that the protein produced in our study differs by 21 amino acids, despite the lack of glycosylation site in both proteins.

Immunochemical properties of the produced and purified A-chain, such as the interaction with anti-MLIII antibodies and absence of any reaction with antibodies specific to the mistletoe lectins MLI and MLII, are evidence that the cloned gene does encode the toxic mistletoe lectin MLIII. A comparison of amino acid sequences of MLI and MLIII A-subunits shows the epitope specificity of monoclonal antibodies raised against MLIII in our previous study. With this aim, we predicted possible immunogenic sites in viscumin from its tertiary structure [30] using the DSSP software for calculation of the exposition extent for amino acid residues [31] as well as with consideration of the temperature B-factor values, secondary structure, and variability of amino acid sequences in a protein homologous series [32]. The following regions were considered to be the most immunogenic, which contained: a) exposed amino acid residues (more than 70% of the residue area); b) elements of secondary structure (preferably β-sheets); c) sites mobile in B-factors. Based on the prediction and on the amino acid substitutions in the given regions, we conclude that the key amino acid residues interacting with anti-MLIII monoclonal antibody are Asp94, Thr112, Gln208, and Glu211 in the MLIII A-chain.

We demonstrated earlier the importance of the inter-subunit region of the toxin for its transport inside the cell and dissociation in endoplasmic reticulum [27]. A model system has been used, in which a cytotoxic activity of viscumin has been determined on hybridoma MNA5 cells synthesizing monoclonal antibodies against isolated viscumin A-subunit. The resistance of the hybridoma to the toxin is indicative of viscumin dissociation into subunits inside the target cell with antigen–antibody complex formation hindering the A-chain translocation through the membrane into the cytoplasm. Analysis of amino acid composition of the A-subunit domain that immediately comes into contact with B-subunit demonstrates that this MLIIIA region contains amino acid residue Glu211, which can influence intracellular dissociation of the toxin as well as that of individual forms of A-subunit present in pharmacological mistletoe extracts [33]. Moreover, the lack of interaction between the monoclonal antibody MNA5 specific to the inter-subunit region of isolated MLI and MLIII A-subunits obtained in this study (data not shown) is evidence for possible involvement of Asp211-Gly212 of MLIA in the structure of epitope MNA5.

The nature of ML heterogeneity is still under discussion. The first point of view is that MLII and MLIII are isoforms of the major toxic mistletoe lectin MLI, resulting from its post-translation modification [34]. Alternatively, many known type II RIPs are characterized by multigenicity. In particular, a small family comprised by eight genes (some of which are inactive) encodes ricin and agglutinin of ricin [35]. Despite a high degree of homology (93% for A-subunits and 81% for B-subunits), these proteins are encoded by distinct genes. Note that the homology of MLs B-subunits is also lower than the homology of A-subunits. MLI has an affinity to galactose, MLIII to N-acetylgalactosamine, and MLII is nearly equally specific to both the above sugars [3]. This is indicative of differences in structure and amino acid sequence of carbohydrate-binding centers between B-subunits of MLs. The catalytic ML subunits are also non-identical, since the monoclonal antibodies H11 interacting with MLIII do not interact with MLI (Fig. 3c), and vice versa, the monoclonal antibodies TA5 interacting with the MLI A-subunit do not interact with MLIII [26]. The natural MLIII A-subunit does not contain oligosaccharides, that is the antigen determinant recognized by anti-MLIII antibodies is only composed by amino acid residues. Thus, the above data are indicative of significant differences between ML toxins (including A- and B-subunits) in their primary structure, possibly because several distinct genes are responsible for their biosynthesis. Our data are in agreement with the data reported by Park et al. [29] on the cloning of enzymatic subunit of viscumin, which prove the existence of three genes responsible for the production of toxin A-subunits.

Toxic properties of the recombinant mistletoe lectin A-subunits. In our study we have investigated the enzymatic properties of A-subunits of the recombinant MLIII (rMLgA) and MLI (rMLIA), and natural MLI (MLIA) considered as specific N-glycosidases affecting eukaryotic ribosomes and resulting in inhibition of endogenous globin synthesis in a cell-free system containing rabbit reticulocyte lysate. The recombinant rMLgA compared with both natural and recombinant A-subunits of viscumin taken at the same concentration caused less profound decrease in incorporation of the ^{3}H-labeled leucine into the protein synthesized (Fig. 4). This difference in activity may reflect heterogeneity of rMLgA specimen we have prepared, in which some portion of improperly folded molecules can exist, which are detected in ELISA, but have no activity in the cell-free protein-synthesizing system.

The abovementioned differences between A-subunits of MLI and MLIII in number of charged amino acid residues and in overall charge of the protein molecule at pH 7.4 in the renaturation buffer can influence the folding efficiency of these denatured proteins.

Thus, in this study, using the total mistletoe DNA, we have cloned the entire gene encoding the prepropro-

tein of toxic MLIII and differing from MLI gene in its nucleotide and corresponding amino acid sequences. Also, we have expressed the enzymatic A-subunit from this gene. The recombinant A-subunit is active in a cell-free protein-synthesizing system resulting in inhibition of the incorporation of leucine labeled with tritium into endogenously synthesized globin.

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