Biochemistry (Moscow), Vol. 70, No. 3, 2005, pp. 306-315. Translated from Biokhimiya, Vol. 70, No. 3, 2005, pp. 378-389.

Cloning and Expression of Mistletoe Lectin III B-Subunit

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

[1]Faculty of Biology, Lomonosov Moscow State University, 119899 Moscow, 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

Received March 3, 2004
Revision received April 5, 2004

Abstract—Aqueous extracts of mistletoe (*Viscum album* L.) contain toxic proteins (lectins) MLI (viscumin), MLII, and MLIII. We previously cloned the gene encoding MLIII precursor. In the present study, a gene fragment encoding the carbohydrate-binding subunit of mistletoe toxic lectin MLIII was cloned and expressed in *Escherichia coli* cells. The structure and immunochemical properties of recombinant MLIII B-subunit were investigated using a panel of monoclonal antibodies against ML-toxins. Sugar-binding activity of recombinant MLIII B-subunit was determined by ELISA. Amino acid sequence analysis of the cloned MLIII compared with known mistletoe toxins and other ribosome-inactivating type II proteins (ricin, abrin *a*, and nigrin *b* B-subunits) revealed essential features of the recombinant MLIIIB primary structure that could determine sugar specificity of the lectin as well as immunomodulating and anti-tumor properties of mistletoe extracts.

Key words: mistletoe toxic lectin, recombinant MLIII B-subunit, monoclonal antibody

A parasite of coniferous and deciduous trees and frutescent plants, European mistletoe (*Viscum album* L.) is widely used in medicine. Toxic mistletoe lectins are found in all mistletoe extracts and, together with viscotoxins and other substances of non-protein nature, are their main constituents. At present, aqueous extracts from mistletoe leaves are used as immunomodulating [1] and anti-tumor agents [2] in clinical practice in Europe.

Abbreviations: ABB) abrin *a* subunit B; GalNAc) N-acetylgalactosamine; Gnd-HCl) guanidine hydrochloride; IPTG) isopropyl-β-D-thiogalactoside; MLI, MLII, and MLIII) European mistletoe (*Viscum album*) lectins I (viscumin), II, and III; NK) natural killers; MLIB and MLIIIB) native B-subunits of MLI and MLIII; mAB) monoclonal antibody; RIPI and RIPII) ribosome-inactivating protein type I and II; rMLIA and rMLIB) recombinant MLI subunits A and B; rMLIIIA and rMLIIIB) recombinant MLIII subunits A and B; RTB) ricin subunit B; SNAV) nigrin *b* (*Sambucus nigra* agglutinin V); SNAVB) nigrin *b* subunit B; KML-1, KML-2, and KML-3) lectins 1, 2, and 3 from Korean mistletoe *Viscum album* subsp. *coloratum*; KMLB1, KMLB2, and KMLB3) B-subunits of KML-1, KML-2, and KML-3; VCA) lectin from Korean mistletoe *Viscum album* subsp. *coloratum* (*Viscum album* subsp. *coloratum* agglutinin); VCAB) VCA subunit B; DTT) dithiothreitol.

* To whom correspondence should be addressed.

Three known isoforms of toxic mistletoe lectins—MLI (viscumin), MLII, and MLIII [3]—belong to the ribosome-inactivating protein type II (RIPII) family [4]. Like all RIPII members, mistletoe toxins are glycoprotein heterodimers composed of two subunits bound together by disulfide bonds. The toxin subunit A possesses highly specific N-glycosidase activity and cleaves the adenine residue at position 4324 of eucaryotic 28S rRNA of major ribosomal 60S subunit [5], which results in arrest of protein synthesis in the cell. The B-subunit is a lectin binding carbohydrates on the cell surface and inducing cell agglutination *in vitro* [6]. B-Subunit can also facilitate internalization of the enzymatic subunit of the toxin, so that RIPII proteins are more cytotoxic in comparison to the RIPI proteins composed of a single active subunit [7].

Despite high homology of their amino acid sequences [8, 9], toxic mistletoe lectins are different in sugar specificity determined from the inhibition of hemagglutination by various mono- and disaccharides. MLI is more specific to galactose, whereas MLIII predominantly binds GalNAc, and MLII has equal affinity to both sugars [6, 10, 11]. These toxins are also different in molecular weights of their A- and B-chains, which are, respectively, 29 and 34 kD for MLI, 27 and 32 kD for MLII, and 25 and 30 kD for MLIII. In concentrated solutions, MLI forms noncovalent heterotetramer AB-

0006-2979/05/7003-0306

BA with molecular weight of 115 kD, whereas MLII, MLIII, ricin, and abrin *a* remain heterodimeric [6].

Moreover, the toxins are different in their biological activity. Buessing et al. [2] found that MLIII is most active in induction of apoptotic death of cultured human lymphocytes. This data is consistent with the reports of Dietrich et al. [8] and Kopp et al. [12], who have demonstrated that MLIII is the most potent (followed by MLII and MLI) inhibitor of the growth of leukemic Molt4 cells. Hajto and coworkers [1] found that the sugar-binding MLI subunit B stimulates the activity of natural killers (NK-cells) *in vivo*, whereas the A-chain has no activity at the same concentration because of inability to bind with carbohydrate-containing cell receptors.

The production of recombinant sugar-binding subunits of toxic mistletoe lectins seems to be useful in studies on possible therapeutic effect of isolated B-chain and on the effect of glycosylation on protein structure and function. Eck and coworkers [13] expressed recombinant MLI subunit B and studied both its lectin activity and biological activity of the heterodimer formed from co-renaturation of recombinant MLI subunits A and B. The recombinant heterodimer had the same biochemical and biological properties as the native toxin MLI. The binding properties of rMLIB and native MLIB were found different towards β-D-lactose and N-acetylgalactosamine and similar towards asialofetuin.

In this study, we have cloned a preprolectin gene fragment encoding the B-subunit of mistletoe (*V. album* L.) toxin MLIII and expressed the rMLIIIB protein in *Escherichia coli* cells. The deduced amino acid sequence of MLIII B-chain was compared with amino acid sequences of B-chains of viscumin and other mistletoe lectins, ricin, abrin *a*, and nigrin *b*. We found some differences in primary structure between MLIB and MLIIIB proteins. Using ELISA, we demonstrated that the renatured recombinant protein rMLIIIB interacts with specific monoclonal antibodies and expresses sugar-binding activity.

MATERIALS AND METHODS

***Escherichia coli* strains, plasmids, enzymes, and chemicals.** Oligonucleotides were synthesized by Sintol (Russia). DNA-modifying enzymes were purchased from Fermentas (Lithuania). The kits QIAprep and QIAquick (QIAGEN GmbH, Germany) were used for isolation of plasmid DNA and purification of PCR (polymerase chain reaction) products. The cells of *E. coli*, strain BL21 (*E. coli* B F^- dcm ompT $hsdS_B$ (r_B^- m_B^-) gal λ) (DE3), were purchased from Stratagene (USA). The plasmid pET11cjoe was kindly provided by A. Patel (MRC Virology Unit, Institute of Virology, Glasgow, UK) and used for the cloning and expression of the sugar-binding subunit of MLIII. All chemicals were purchased from Sigma (USA).

Toxins and their subunits. The toxic mistletoe lectins MLI, MLII, and MLIII were purified from leaves of mistletoe (*Viscum album* L.) as previously described [3]. The purity of prepared specimens was determined using SDS-PAGE and ELISA.

Cloning and sequencing of the gene fragment encoding the mistletoe lectin MLIII subunit B. From the reported sequence of previously cloned full-length gene encoding a precursor of the toxic mistletoe lectin MLIII [9], the following oligonucleotide primers containing the *Nde*I (forward primer) and *Bam*HI (reverse primer) restriction sites (given in bold) were synthesized for the cloning of a fragment of the MLIIIB-encoding preprolectin gene into the expression vector pET11cjoe: forward-ML3B: 5′AAAAGCTAGC**CATATG**GACGATGTTACCTGCACTG 3′; reverse-ML3B: 5′AAAAA**GGATCC***TTA-TCA*TGGCACGGGAAGCCACATTT 3′. This DNA fragment was amplified from the plasmid pUC19 carrying the full-length gene encoding the mistletoe preprolectin MLIII (rMLg2) by PCR with *Taq* DNA-polymerase. Reaction was carried out in a standard reaction mixture (30 μl, final volume) containing 1.5 mM $MgCl_2$, the primers forward-ML3B and reverse-ML3B (10 pM each), and 2 ng of rMLg2 plasmid DNA template on a MasterCycler 5370 thermocycler (Eppendorf, Germany). The amplification protocol was as follows: primary denaturation at 94°C for 4 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 58°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 ~800 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 *Bam*HI endonucleases and ligated with the T4 DNA-ligase. The strain *E. coli* BL21 (DE3) was used for transformation with the vector thus obtained. Restriction analysis of plasmids isolated from the ampicillin-resistant clones confirmed the presence of inserted DNA fragment of expected length.

A nucleotide sequence of the preprolectin MLIII gene fragment encoding B-chain was determined by MWG-Biotech AG (Germany). The deduced amino acid sequence of the recombinant protein was compared with the primary sequences of B-subunits of the following RIPII proteins: toxic lectins from European mistletoe *V. album*: MLI (A58957) and MLIII (P81830); lectins from Korean mistletoe *V. album* subsp. *coloratum*: VCA (AAL40417), KML-1 (AAM46935), KML-2 (AAM46936), and KML-3 (AAM46937); as well as ricin (X02388), abrin *a* (M98344), and nigrin *b* (P33183) using the ClustalX (v.1.81) software. A phylogenetic tree of known lectin B-chains from European and Korean mistletoes was constructed on the basis of their amino acid sequences according to the neighbor-joining method described by Saitou and Nei [14].

Expression of the recombinant protein. Transformed *E. coli* cells carrying a plasmid with the inserted preprolectin MLIII gene fragment encoding B-subunit were grown on a shaker at 37°C in 5 ml of Luria–Bertani medium containing 50 μg/ml of ampicillin. Induction of protein synthesis was initiated in the culture at A_{600} = 0.6-1.0 with 1 mM (final concentration) of isopropyl β-D-thiogalactoside (IPTG). Four hours after additional initiation at 28°C, the cells were harvested by centrifugation at 4000*g* for 10 min. The cells were lysed in a standard sample buffer containing 2-mercaptoethanol, and the protein expressed was analyzed by 12.5% SDS-PAGE. Bacterial clones expressing the recombinant protein with expected molecular weight of about 30 kD were selected.

Immunoblotting. To confirm specificity of expressed proteins in *E. coli* cell lysates after electrophoretic separation, immunoblotting was performed with polyclonal mouse antiserum against the denatured toxin MLIII and with monoclonal antibody TA7 raised against denatured A-chains of all three mistletoe toxins [15]. Proteins were blotted onto a 0.45-μm nitrocellulose membrane (Schleicher & Schuell, Germany; Ref. No. 401099) according to a semidry blotting protocol. The membrane with adsorbed proteins was incubated overnight in 4% dry defatted milk solution in PBS containing 0.05% Tween 20, washed, and then incubated for 1 h with either anti-MLIII antiserum (1 : 1000) or TA7 mABs (10 μg/ml) at 37°C. Then the membrane was washed and incubated with goat anti-mouse IgG antiserum conjugated with horseradish peroxidase (IMTEK, Russia). Membrane was stained in 15 ml of PBS solution containing 3 mg of 3,3′-diaminobenzidine, 10 mg of 4-chloro-1-naphthol, 32% ethanol, and 0.06% H_2O_2.

Isolation, purification, and renaturation of the recombinant protein. The recombinant protein rMLIIIB was found in inclusion bodies, which were isolated by sequential lysis of the cells with lysozyme, DNase I, RNase A, and Triton X-100 followed by washing of the protein with STET buffer (50 mM Tris-HCl, pH 8.0, containing 8% sucrose, 50 mM EDTA, and 5% Triton X-100). The protein concentration and homogeneity was determined by SDS-PAGE. The purified recombinant protein rMLIIIB was dissolved in buffer containing 7 M of Gnd-HCl, 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, the denatured rMLIIIB protein was added dropwise into PBS, pH 7.4, up to 130-fold dilution (the final protein concentration was 15 μg/ml) and incubated for 16 h at 4°C. As an internal control of renaturation, the native MLIII toxin was denatured in the buffer with Gnd-HCl and then renatured under the same conditions as used for rMLIIIB. Protein aggregates were removed by centrifugation, and antigenic epitopes of MLIII subunits A and B and sugar-binding activity of MLIII chain B were assayed in remaining dissolved protein by ELISA.

ELISA. The concentration of renatured proteins was determined using enzyme immunoassay (ELISA) with polyclonal anti-MLIII serum. Lectins were sorbed onto immunological plates and incubated with anti-MLIII serum and then with the secondary goat anti-mouse IgG antibodies conjugated with horseradish peroxidase. Immunochemical properties of rMLIIIB were studied using sandwich-ELISA with a pair of previously raised anti-MLIII mABs, E12 [16] and MTC12 [17]. Specificity of given antibodies to the MLIII subunit B was confirmed by ELISA in the presence of dithiothreitol (DTT). In this case, we used the previously described pair of mABs, H8 and H11 [18], specific to the MLIII subunit A. Previous screening allowed selection of glycosylated immunoglobulins 3F12 carrying sugar residues specific to the mistletoe toxins MLI, MLII, and MLIII. We tested the carbohydrate-binding activity of rMLIIIB by ELISA with glycosylated immunoglobulins 3F12 and anti-MLIIIB mAB MTC12. ELISA was conducted in Costar 96-well plates (Corning, USA) according to the standard protocol [9].

RESULTS

Cloning and sequencing of the toxic mistletoe lectin III subunit B. Based on the previously cloned full-length sequence of the MLIII preprolectin gene [9], we have constructed oligonucleotide primers for the amplification of the preprolectin gene fragment encoding the B-subunit. The resulting recombinant product rMLIIIB was compared by deduced amino acid sequence with the B-subunit primary sequences of toxic lectins from European mistletoe *V. album* (MLIB and MLIIIB), Korean mistletoe *V. album* subsp. *coloratum* (VCAB, KML1B, KML2B, and KML3B) (Fig. 1), as well as with B-subunits of ricin (RTB), which is specific both to galactose and N-acetylgalactosamine [19, 20], abrin *a* (ABB), which is preferably specific to galactose [6, 21], and nigrin *b* (SNAVB) specific to N-acetylgalactosamine [22] (Fig. 2).

The recombinant MLIII subunit B is highly homologous to other toxic mistletoe lectins (table). Using the neighbor-joining method [14], we have constructed a phylogenetic tree for the B-subunits of toxic lectins isolated from European and Korean mistletoe (Fig. 3). The Korean mistletoe lectin VCA is phylogenetically closer to the European mistletoe lectin MLIII, whereas MLI is more closely related to KML1, KML2, and KML3.

The recombinant protein we have obtained differs in its amino acid sequence determined by the method of Edman degradation from the B-subunit of European mistletoe lectin MLIII (MLIIIB) by three amino acid residues: Asn173 (in rMLIIIB) → Asp; Ala201 → Thr; and Ser255 → Pro; the two last substituted residues in the MLIIIB primary structure are identical to those in MLIB. The VCA subunit B also closely resembles rMLIIIB with 19 substitutions. The amino acid sequence of rMLIIIB

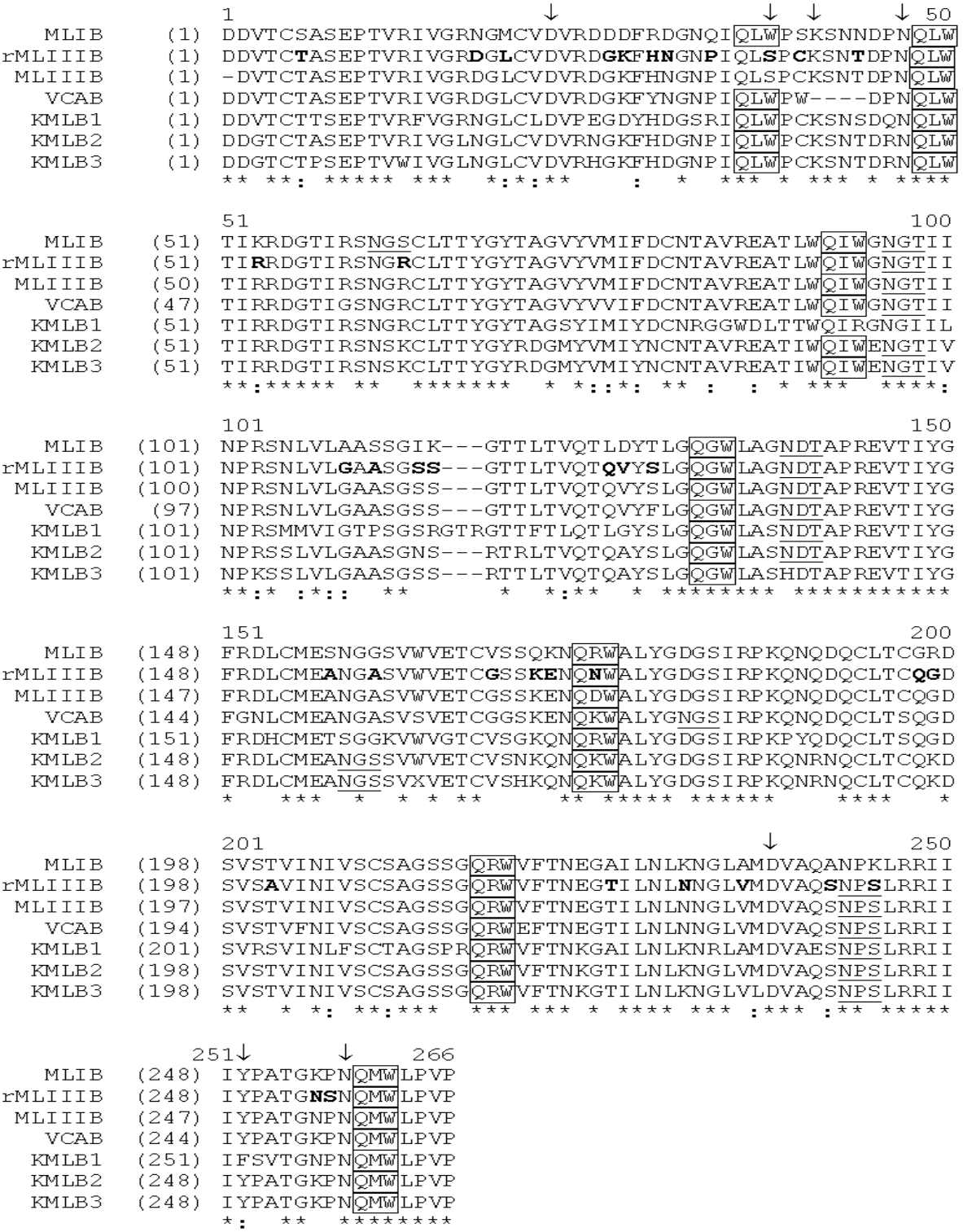

Fig. 1. Multiple alignment of B-subunit amino acid sequences of toxic lectins from European mistletoe *V. album*: MLI (MLIB, A58957) and MLIII (MLIIIB, P81830), recombinant MLIII (rMLIIIB), and lectins from Korean mistletoe *V. album* subsp. *coloratum*: VCA (VCAB, AAL40417), KML-1 (KMLB1, AAM46935), KML-2 (KMLB2, AAM46936), and KML-3 (KMLB3, AAM46937). Identical amino acid residues are marked by asterisks, resemblance by colons, possible sites of glycosylation (N-x-T/S) are underlined, repeated sequences Q-x-W are highlighted by rectangles, conservative residues in sugar-binding sites of proteins are marked by arrows. Amino acid residues in the rMLIIIB sequence that are different from those in MLIB are bolded.

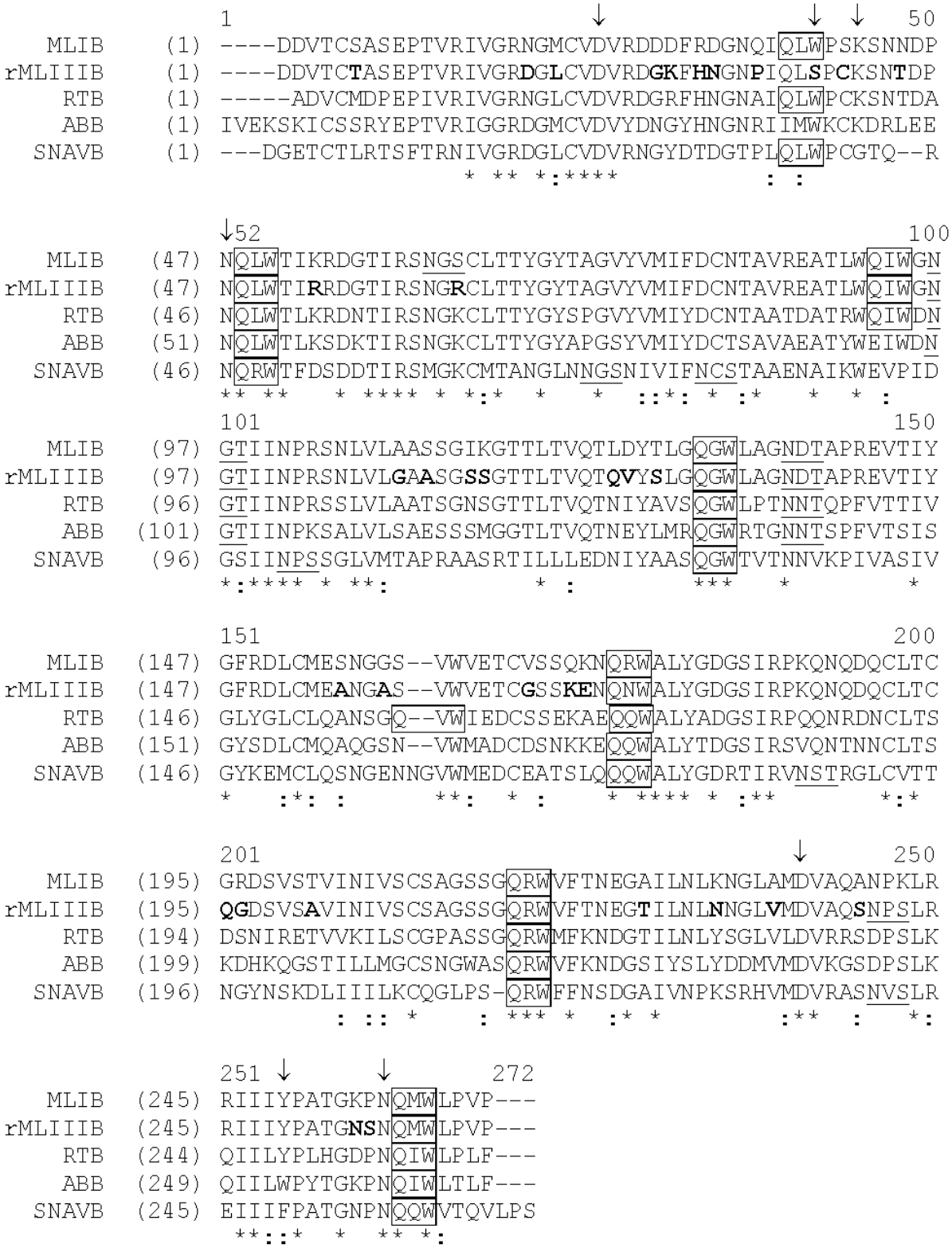

Fig. 2. Multiple alignment of B-subunit amino acid sequences of ribosome-inactivating type II proteins: European mistletoe *V. album* lectins MLI (MLIB, A58957) and recombinant MLIII (rMLIIIB), ricin (RTB, CAA26230), abrin *a* (ABB, AAA32624), and nigrin *b* (SNAVB, 2210286A). Notations are the same as in Fig. 1.

Identity and resemblance of B-subunit amino acid sequences of the toxic lectins MLI (MLIB, A58957) and MLIII (MLIIIB, P81830) from European mistletoe *V. album* L., recombinant MLIII (rMLIIIB), and Korean mistletoe *V. album* subsp. *coloratum* lectins VCA (VCAB, AAL40417), KML-1 (KMLB1, AAM46935), KML-2 (KMLB2, AAM46936), and KML-3 (KMLB3, AAM46937)

Identity, % / Resemblance, %	MLIB	rMLIIIB	MLIIIB	VCAB	KMLB1	KMLB2	KMLB3
MLIB		86.3	86.7	82.9	71.8	81.4	79.5
rMLIIIB	89.7		98.5	91.6	73.3	86.3	84.8
MLIIIB	90.1	98.5		92.0	73.3	86.7	85.2
VCAB	86.7	92.4	92.8		68.0	81.4	80.2
KMLB1	80.5	79.7	79.7	75.2		71.8	70.7
KMLB2	86.7	88.6	89.0	84.4	78.2		96.2
KMLB3	85.6	87.8	88.2	84.0	77.8	97.0	

compared with that of MLIB has 36 substitutions of amino acid residues, most of which are not conservative and can influence both the physical properties and sugar specificity of the protein.

We have found two significant substitutions of alternatively charged amino acid residues: Asp28 in MLIB is substituted by Lys in rMLIIIB, and Lys170 in MLIB by Glu in rMLIIIB. Moreover, in six positions basic amino acid residues are substituted by polar or aliphatic ones: Lys115 and Lys242 → Ser; Arg173, Lys229, and Lys254 → Asn; Arg196 → Gly, and three additional positively charged amino acids appear: Lys28, Arg63, and Lys169. We have also found three substitutions of acidic amino acid residues by polar or aliphatic ones: Asp27 → Gly; Asp31 → Asn; and Asp125 → Val; and appearance of two additional negatively charged residues: Asp18 and Glu170. The majority of these substitutions are also present in the primary structures of MLIIIB and VCAB. Because of these differences, rMLIIIB compared with MLIB contains two less negatively charged and five less positively charged amino acid residues, so the obtained recombinant protein is characterized by lower p*I* (5.43 versus 7.63 of the native MLIB) and the charge at neutral pH (−2.23 and +0.69, respectively).

Amino acid substitutions at positions 27 and 38 can influence the structure and properties of the N-terminal carbohydrate-binding site of rMLIIIB. Gly27 instead of Asp in MLIB is observed in B-chain structures of all mistletoe lectins analyzed, as well as of RTB and SNAVB, whereas the substitution Trp38 → Ser was found in MLIIIB only. The latter substitution of the conservative aromatic amino acid residue in the N-terminal carbohydrate-binding site could lead to decreased affinity of MLIIIB to sugars because of the lack of stacking interaction of aromatic amino acid with the hydrophobic plane of a binding sugar residue. Amino acid substitutions at positions 239, 242, and 254 are essential for the C-terminal sugar-binding site. The presence of Ser239 and Asn254 in rMLIIIB allows positioning of GalNAc in this site.

The amino acid residues Gln34 and Ile114 are involved in hydrophobic interaction between B-subunits of two MLI molecules, when the heterotetramer is formed [23], so their substitution by Pro and Ser, respectively, makes MLIII and other mistletoe toxins unable to form the tetramer.

The substitution of Lys242 in MLIB by Ser in rMLIIIB transmutes Asn240 in MLIII into a possible glycosylation site comprising the sequence N-x-T/S. In MLIB, the glycosylation site is in position Asn61. The substitution of Ser63 in MLIB by Arg in rMLIIIB withdraws glycosylation at this site. The glycosylation site Asn240 is present in structures of other mistletoe lectins, which also do not undergo glycosylation in position Asn61. The overall number of possible glycosylation sites

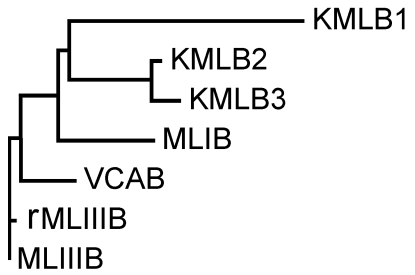

Fig. 3. Phylogenetic tree for B-subunits of the toxic lectins MLI (MLIB, A58957) and MLIII (MLIIIB, P81830) from European mistletoe *V. album*, recombinant MLIII (rMLIIIB), and Korean mistletoe *V. album* subsp. *coloratum* lectins VCA (VCAB, AAL40417), KML-1 (KMLB1, AAM46935), KML-2 (KMLB2, AAM46936), and KML-3 (KMLB3, AAM46937).

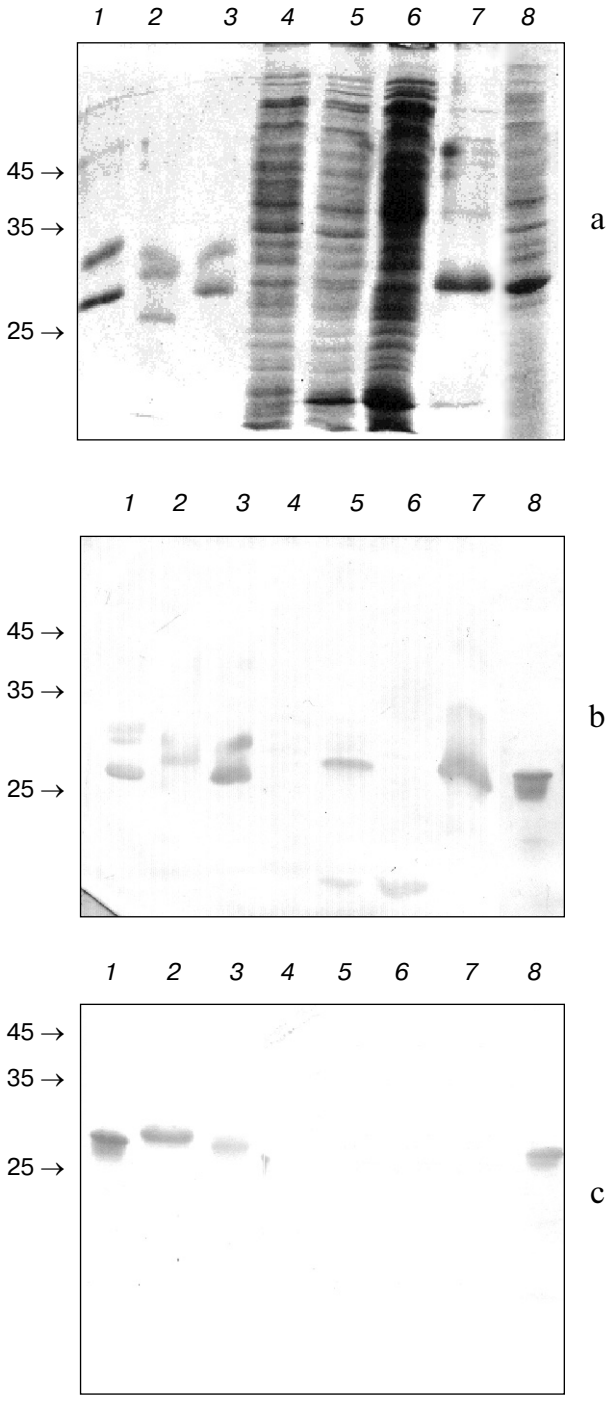

Fig. 4. SDS-PAGE in 12.5% polyacrylamide gel (a) and immunoblotting of native mistletoe lectins and recombinant protein rMLIIIB, which was expressed in *E. coli* cells, with anti-MLIII serum (b) and monoclonal antibody TA7 (c). Lanes: *1*) MLI; *2*) MLII; *3*) MLIII; *4*) lysate of cells producing rMLIIIB, no induction; *5*) lysate of cells producing rMLIIIB, induction with IPTG; *6*) supernatant of the lysate of cells producing rMLIIIB, induction with IPTG; *7*) purified rMLIIIB; *8*) lysate of cells producing rMLIIIA, induction with IPTG. On the left, molecular weights (kD) of ovalbumin, lactate dehydrogenase, and restriction endonuclease Bsp98I.

is three in MLIB, rMLIIIB, MLIIIB, KMLB1, and KMLB3 whereas in VCAB and KMLB2 it is four. Zimmermann and Pfueller [24] demonstrated that B-subunits of the native toxic lectins MLI and MLIII have only two glycan components.

The amino acid sequence of rMLIIIB, as well as B-subunits of the Korean mistletoe lectins KML-1, KML-2, and KML-3, contains ten cysteine residues, whereas their number is nine in MLIB and eight in VCAB. Additional cysteine derives from the substitution Ser40 → Cys, so MLIIIB, KMLB1, KMLB2, and KMLB3 can form another disulfide bond with Cys21 in the N-terminal domain of the protein, which is also characteristic of B-subunits of other RIPII.

The recombinant rMLIIIB contains 53.6% conservative amino acid residues identical to those of RTB and resembles RTB by 79.0% in its primary structure; these identity and resemblance values of rMLIIIB are 53.2 and 67.3% with ABB and 43.1 and 56.5% with SNAVB, respectively.

Expression of recombinant B-subunit of toxic mistletoe lectin III. Bacterial clones carrying plasmids with inserted gene encoding the mistletoe lectin subunit B were chosen for expression analysis. Expression of recombinant proteins in *E. coli* cells was determined by 12.5% SDS-PAGE (Fig. 4a). Maximum production of the protein was achieved 4 h after induction with IPTG. Clones expressing recombinant protein with molecular weight of about 30 kD were selected for further experiments. Specificity of produced recombinant protein was confirmed by immunoblotting. Since mABs recognizing the denatured B-subunit of MLIII in immunoblotting have not yet been raised, we applied two-step immunological assay: the first step with the use of polyclonal anti-MLIII serum against denatured toxin suggested the recombinant protein to be a derivative of mistletoe toxins (Fig. 4b); and the second step with monoclonal antibody TA7 against denatured A-chains of three toxic mistletoe lectins proved that the produced protein is not the A-chain of MLIII (Fig. 4c).

The recombinant MLIII subunit B is found in inclusion bodies of *E. coli* cells and constitutes ~3% of the total protein. The inclusion bodies containing the recombinant B-subunit were purified from other cell components by lysis of cells with detergents followed by washing of the recombinant protein with STET buffer. For the assessment of protein yield and purity, we conducted SDS-PAGE with given concentrations of native mistletoe toxin MLIII.

Immunochemical properties and sugar-binding activity of the recombinant B-subunit of toxic mistletoe lectin III. The prepared recombinant protein rMLIIIB was dissolved in a denaturing buffer containing Gnd-HCl. Protein folding was conducted by gradual 130-fold dilution of rMLIIIB in PBS, so that the final protein concentration was 15 μg/ml. To follow the folding process, the native toxin MLIII was also denatured in the buffer con-

taining Gnd-HCl and afterwards renatured under the same conditions. The primary immunochemical detection of renatured proteins and alignment of their concentrations for further immunoassays with mABs was performed using polyclonal anti-MLIII serum (data not shown).

We studied immunochemical properties of the renatured proteins using sandwich-ELISA with the pair of mABs, E12 [16] and MTC12 [17], previously raised against MLIII. As shown previously, mAB E12 interacts with B-subunit of MLIII [16]. The specificity of mAB MTC12 to ML subunits was not clear. We studied it by ELISA in the presence of dithiothreitol (Fig. 5). Taken in combinations H8–E12-biotin and MTC12–H11-biotin, the mABs H8 and H11, which are specific to the MLIII subunit A [18], could only recognize the entire native mistletoe toxin MLIII in sandwich-ELISA. In these mAB systems the signal decreased by 70-75% in the presence of reagent splitting the disulfide bond between A- and B-subunits of the protein. In sandwich-ELISA the mAB pair MTC12–E12-biotin interacted equally with native and DTT-treated MLIII. Thus, the mAB MTC12 was found to be specific to the B-subunit of toxic mistletoe lectin MLIII.

We studied the renatured proteins for antigen epitopes using sandwich-ELISA with the anti-MLIIIB mAB pair E12–MTC12-biotin. The folding of antigen epitopes in rMLIIIB was found to be successful under the given renaturation conditions. The antigenic activity of recombinant protein was 20% of that of native MLIII (the control, taken as 100%), and the activity of MLIII after denaturation–folding was 83% of control (Fig. 6a).

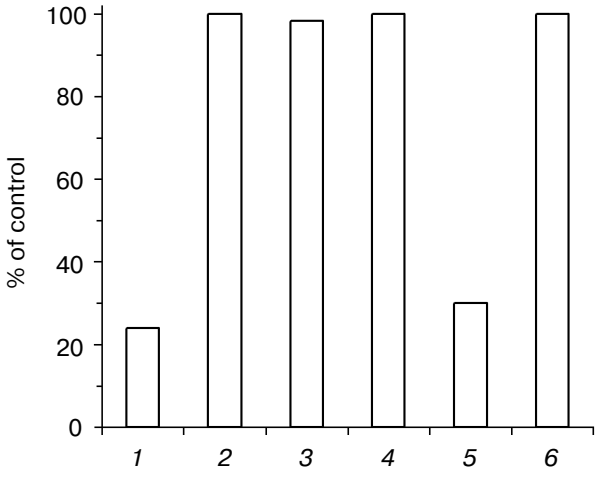

Fig. 5. Subunit specificity of the anti-MLIII monoclonal antibody MTC12 (the interaction with native MLIII in sandwich-ELISA in the absence of DTT was taken as 100%): H8/MLIII + DTT/E12-biotin (*1*), H8/MLIII/E12-biotin (*2*), MTC12/MLIII + DTT/E12-biotin (*3*), MTC12/MLIII/E12-biotin (*4*), MTC12/MLIII + DTT/H11-biotin (*5*), MTC12/MLIII/H11-biotin (*6*).

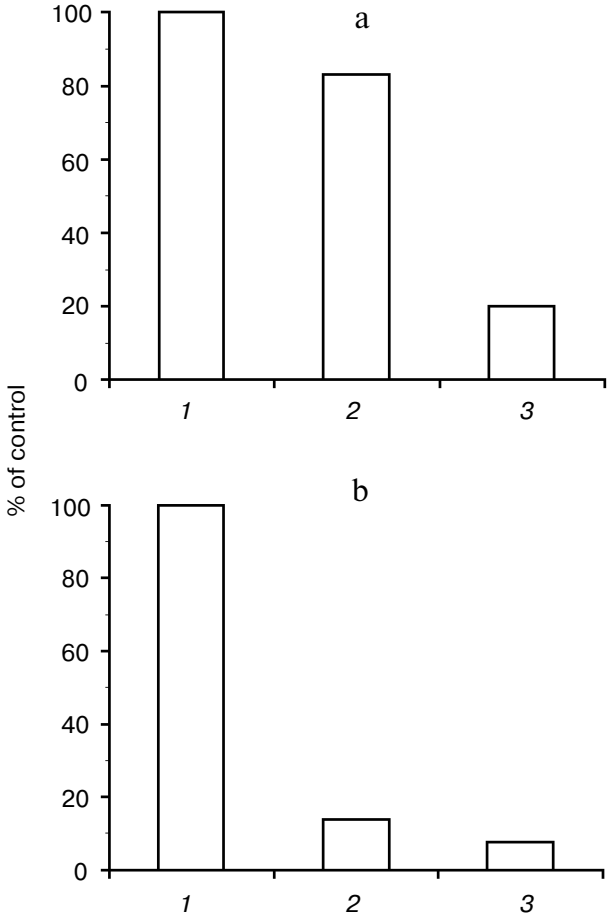

Fig. 6. Interaction of native MLIII (*1*), denatured MLIII (dMLIII) (*2*), and renatured recombinant MLIII subunit B (rMLIIIB) (*3*) in sandwich-ELISA with anti-MLIIIB mABs (E12 and MTC12) and glycosylated immunoglobulins (3F12) in systems E12–MTC12-biotin (a) and 3F12–MTC12-biotin (b). Interaction with the native MLIII in sandwich-ELISA (control) is taken as 100%.

To analyze the carbohydrate-binding activity of the renatured proteins, the glycosylated immunoglobulins 3F12 carrying sugars interacting with lectins were sorbed onto a plate, and the anti-MLIIIB mAB MTC12 was used for lectin detection. Activity of the renatured proteins was significantly lower in the system 3F12–MTC12-biotin compared with the system E12–MTC12-biotin, even when the MLIII folded after denaturation was used, which retained only 13.8% of the native MLIII activity. The activity of rMLIIIB was 7.5% of control (Fig. 6b).

DISCUSSION

Cloning and sequence analysis of the toxic mistletoe lectin III subunit B. In this study, we have cloned the mistletoe preprolectin III gene fragment encoding the B-

subunit. The toxic mistletoe lectins MLI and MLIII are highly homologous by amino acid sequence, but different in affinity to carbohydrate residues of galactose and N-acetylgalactosamine [6, 10, 11]. A comparative analysis of amino acids comprising the carbohydrate-binding sites of B-subunits of these proteins can explain the influence of differences in primary structure on the protein specificity to carbohydrates. The differences we have found are probably evidence that these toxic lectins are encoded by distinct genes rather than are products of post-translational modifications of a common precursor.

We found previously that the mAB TB33 raised against MLI B-chain does not recognize B-subunits of MLII and MLIII [25]. Catalytic subunits of toxic mistletoe lectins are also different because the mAB H11 specific to MLIII chain A does not react with MLI [26], and the mAB TA5 specific to MLIA does not interact with MLIII [17]. All those observations suggest significant differences in primary structures of both A- and B-chains of toxic mistletoe lectins, which is indicative of several genes encoding these *V. album* toxins. Soler et al. [27] proved that two distinct genes encode A-subunits of ML toxins in European mistletoe. Our data are consistent with the study of Park et al. [28], who found three genes encoding KML-1, -2, and -3 in Korean mistletoe.

As one can judge by the phylogenetic tree for amino acid sequences of mistletoe lectin subunits B created in our study (Fig. 2), the European mistletoe lectins MLI and MLIII are disposed rather distantly, and in evolutionary aspect this difference comes from the presence of different genes encoding these proteins rather than from post-translational modifications of the proteins. At least two groups of toxins can be assigned among lectins of Korean mistletoe, one of which is highly homologous to MLIII from European mistletoe (lectin VCA), and the other to MLI (lectins KML-1, KML-2, and KML-3). Toxins KML-2 and KML-3 are highly homologous and comprise a separate group, wherein their identity by amino acid sequence is 98.2% [28]. The sequence of the European mistletoe lectin MLII is not yet known; nonetheless, one can expect that its determination would substantially complete the picture and facilitate understanding of the origin and evolution of toxic mistletoe lectins.

Immunochemical properties and carbohydrate-binding activity of recombinant B-subunit of toxic mistletoe lectin III. In accordance with the data of immunochemical analysis of the recombinant MLIII B-subunit properties, only 20% of renatured protein is active in the reaction with monoclonal antibodies E12 and MTC12. Yet lesser extent of correct folding demonstrates rMLIIIB in the case of carbohydrate-binding activity. This fact can be explained by more complex structure of lectin carbohydrate-binding sites formed by several β-sheets [23]. The confirmation of this suggestion is that our internal experimental control, the native MLIII toxin denatured in the buffer with Gnd-HCl and renatured again, possessed low lectin activity as well. We analyzed the carbohydrate-binding activity of the proteins using glycosylated immunoglobulins, which, owing to their size, can make a steric obstacle in the interaction of specific mAB with MLIIIB epitopes. In particular, we found that the native MLIII was not active in the 3F12–E12-biotin sandwich-ELISA system (data not shown). Nonetheless, a fifth part of the recombinant rMLIIIB protein was successfully renatured and epitopes were properly folded, because the mAB E12 and MTC12 used in the assay did not recognize the denatured MLIII in immunoblotting or ELISA (data not shown).

Mechanisms responsible for the pharmacological effect of the toxic mistletoe lectins have not been adequately studied. A supposition has been put forward that anti-tumor activity of ML-toxins is due to their immunomodulating activity rather than cytotoxicity [29]. Some investigators assert that viscumin is the major effective component in mistletoe extracts; however, the contribution of MLII and MLIII, as well as other viscumin-like components in the effect of the extracts cannot be excluded. The found differences in cytotoxic effect of ML-toxins [8, 12] might result from different carbohydrate specificity of the protein subunits B as well as from the peculiarities of intracellular transport of their enzymatic subunits. We demonstrated previously that individual chains of toxin, which are found in mistletoe extract, can alter its properties [30]. Thus, further studies of the isolated recombinant MLIII subunit B regarded as an agent for anti-tumor and immunomodulating therapy are required.

This study was supported in part according to the bipartite agreement between the Federal Ministry of Science and Technologies of Russia and the Ministry of Education and Scientific Researches of Germany (project RUS 01/237).

REFERENCES

1. Hajto, T., Hostanska, K., and Gabius, H.-J. (1989) *Cancer Res.*, **49**, 4803-4808.
2. Buessing, A., Suzart, K., Bergmann, J., Pfuller, U., Schietzel, M., and Schweizer, K. (1996) *Cancer Lett.*, **99**, 59-72.
3. Eifler, R., Pfueller, K., Goeckeritz, W., and Pfueller, U. (1993) in *Lectins: Biology*, *Biochemistry*, *Clinical Biochemistry* (Basu, J., Kundu, M., and Chakrabarti, P., eds.) Vol. 9, Wiley Eastern Ltd., India, pp. 144-151.
4. Barbieri, L., Battelli, M. G., and Stirpe, F. (1993) *Biochim. Biophys. Acta*, **1154**, 237-282.
5. Endo, Y., Tsurugi, K., and Franz, H. (1988) *FEBS Lett.*, **2**, 378-380.
6. Franz, H., Ziska, P., and Kindt, A. (1981) *Biochem. J.*, **195**, 481-484.
7. Stirpe, F., Barbieri, L., Batelli, M. G., Soria, M., and Lappi, D. A. (1992) *Biotechnology*, **10**, 405-424.

8. Dietrich, J. B., Ribereau-Gayon, G., Jung, M. L., Franz, H., Beck, J. P., and Anton, R. (1992) *Anti-Cancer Drugs*, **3**, 507-511.
9. Tonevitsky, A. G., Agapov, I. I., Pevzner, I. B., Maluchenko, N. V., Moisenovich, M. M., Pfueller, U., and Kirpichnikov, M. P. (2004) *Biochemistry (Moscow)*, **69**, 642-650.
10. Ziska, P., Gelbin, M., and Franz, H. (1993) in *Lectins: Biology*, *Biochemistry*, *Clinical Biochemistry* (Van Driessche, E., Franz, H., and Beeckmans, S., eds.) Vol. 8, Textop, Hellerup, Denmark, pp. 10-13.
11. Wu, A. M., Chin, L.-K., Franz, H., Pfuller, U., and Herp, A. (1992) *Biochim. Biophys. Acta*, **1117**, 232-234.
12. Kopp, J., Korner, I.-J., Pfueller, U., Gockeritz, W., Eifler, R., Pfuller, K., and Franz, H. (1993) in *Lectins: Biology*, *Biochemistry*, *Clinical Biochemistry* (Van Driessche, E., Franz, H., and Beeckmans, S., eds.) Vol. 8, Textop, Hellerup, Denmark, pp. 41-47.
13. Eck, J., Langer, M., Moeckel, B., Witthohn, K., Zinke, H., and Lentzen, H. (1999) *Eur. J. Biochem.*, **265**, 788-797.
14. Saitou, N., and Nei, S. (1987) *Mol. Biol. Evol.*, **4**, 406-425.
15. Agapov, I. I., Tonevitsky, A. G., Maluchenko, N. V., Moisenovich, M. M., Bulah, Y. A., and Kirpichnikov, M. P. (1999) *FEBS Lett.*, **464**, 63-66.
16. Moisenovich, M. M., Egorova, S. G., Chelnokova, O. V., Demina, I. A., Polosukhina, E. R., Kozlovskaya, N. V., Popova, E. N., Fattakhova, G. V., Solopova, O. N., and Agapov, I. I. (2000) *Russ. J. Immunol.*, **5**, 376-384.
17. Tonevitsky, A. G., Agapov, I., Temiakov, D., Moysenovich, M. M., Maluchenko, N. V., Solopova, O. N., Würzner, G., and Pfueller, U. (1999) *Arzneim.-Forsch./Drug Res.*, **49**, 970-975.
18. Fattakhova, G. V., Agapov, I. I., Solopova, O. N., Moisenovich, M. M., and Tonevitsky, A. G. (2001) *Biotekhnologiya*, **3**, 59-70.
19. Rutenber, E., and Robertus, J. D. (1991) *Proteins*, **10**, 260-269.
20. Hatakeyama, T., Yamasaki, N., and Funatsu, G. (1986) *J. Biochem.*, **100**, 781-788.
21. Wu, A. M., Wu, J. H., Herp, A., Chow, L.-P., and Lin, J.-Y. (2001) *Life Sci.*, **69**, 2027-2038.
22. Van Damme, E. J. M., Barre, A., Rouge, P., van Leuven, F., and Peumans, W. J. (1996) *Eur. J. Biochem.*, **237**, 505-513.
23. Niwa, H., Tonevitsky, A. G., Agapov, I. I., Saward, S., Pfüller, U., and Palmer, R. A. (2003) *Eur. J. Biochem.*, **270**, 2739-2749.
24. Zimmermann, R., and Pfueller, U. (1998) in *COST 98 "Effect of Antinutrients on the Nutritional Value of Legume Diets"* (Bardocz, S., Pfueller, U., and Pustzai, A., eds.) Vol. 5, European Communities, Brussels, pp. 55-62.
25. Tonevitsky, A. G., Rakhmanova, V. A., Agapov, I. I., Shamshiev, A. T., Usacheva, E. A., Prokof'ev, S. A., Denisenko, O. N., Alekseev, Yu. O., and Pfueller, U. (1995) *Immunol. Lett.*, **44**, 31-34.
26. Tonevitsky, A. G., Agapov, I. I., Pevzner, I. B., Maluchenko, N. V., Moisenovich, M. M., Yurkova, M., and Pfueller, U. (2004) *Arzneim.-Forsch./Drug Res.*, **54**, 242-249.
27. Soler, M. H., Stoeva, S., Schwamborn, C., Wilhelm, S., Stiefel, T., and Voelter, W. (1996) *FEBS Lett.*, **399**, 153-157.
28. Park, C. H., Lee, D. W., Kang, T. B., Lee, K. H., Yoon, T. J., Kim, J. B., Do, M. S., and Song, S. K. (2001) *Mol. Cells*, **12**, 215-220.
29. Pfueller, U. (1996) in *Proc. II Scientific Workshop of COST 98*, *Budapest* (Bardocz, S., Gelencser, E., and Pusztai, A., eds.) Vol. 11, Office for Official Publications of the European Communities, Luxemburg, p. 46.
30. Tonevitsky, A. G., Marx, U., Agapov, I., and Moisenovich, M. (2002) *Arzneim.-Forsch./Drug Res.*, **52**, 67-71.