

Didemnin binds to the protein palmitoyl thioesterase responsible for infantile neuronal ceroid lipofuscinosis

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ABSTRACT The marine natural product didemnin B, currently in clinical trials as an antitumor agent, has several potent biological activities apparently mediated by distinct mechanisms. Our initial investigation of didemnin B resulted in the discovery of its GTP-dependent binding of the translation elongation factor EF1 α . This finding is consistent with the protein synthesis inhibitory activity of didemnin B observed at intermediate concentrations. To begin to dissect the mechanisms involved in the cytostatic and immunosuppressive activities of didemnin B, observed at low concentrations, additional didemnin-binding proteins were sought. Here we report the purification of a 36-kDa glycosylated didemnin-binding protein from bovine brain lysate. Cloning of the human cDNA encoding this protein revealed a strong sequence similarity with palmitoyl protein thioesterase (PPT), an enzyme that removes palmitate from H-Ras and the G α s subunits of heterotrimeric GTP-binding proteins *in vitro*. Mutations in PPT have recently been shown to be responsible for infantile neuronal ceroid lipofuscinosis, which is a severe brain disorder characterized by progressive loss of brain function and early death.

Cell-permeant molecules have been used to investigate many aspects of cellular function. The study of natural products that bind intracellular proteins has extended this approach into new areas of biology. For example, natural products have facilitated the elucidation of several signal transduction pathways (e.g., brefeldin A, wortmannin, rapamycin, and FK506) (1–4).

Various natural products have also been used or are being developed as therapeutic agents. The first natural product derived from marine sources to enter clinical trials, didemnin B (ref. 5; see Fig. 1 for structure), is currently in phase II trials as an antitumor agent for treatment of breast, ovarian, cervical, myeloma, and lung cancers (6–10). Preliminary results of these clinical trials also show a partial response against non-Hodgkin lymphoma (11). The potency, specificity, and cell permeability of didemnin B led us to investigate the molecular basis for its actions on cycling cells.

Three biological activities have been reported for didemnin B that were of interest to us: its ability to inhibit protein synthesis, induce G $_1$ cell cycle arrest, and suppress a mixed lymphocyte reaction (12, 13). These activities range in effective concentrations over five orders of magnitude, suggesting they are mediated by different mechanisms. To investigate these mechanisms, we have been characterizing proteins that bind didemnin B. Using a didemnin affinity matrix, we have previously shown that a major intracellular didemnin-binding protein is the translation elongation factor 1 α (EF-1 α) (14). It was shown that didemnin B binds uniquely to the activated GTP-bound form of EF-1 α , albeit with modest affinity. Didemnin B's specificity for GTP-bound EF-1 α suggests that it recognizes the same structural determinants

required for ribosomal recognition of EF-1 α . This interaction is likely the source of didemnin's reported protein synthesis inhibition. Although it has been proposed that didemnin B's other activities are a consequence of its ability to inhibit protein synthesis (15), the effective concentrations of didemnin B's cytostatic and immunosuppressive activities are one and three orders of magnitude lower than that required for inhibition of protein synthesis, suggesting that distinct mechanisms may be involved.

To study didemnin's antiproliferative and immunosuppressive activities, affinity chromatography was used to identify additional binding proteins. In this report we describe the purification and cloning of a new didemnin-binding protein with sequence similarity to palmitoyl protein thioesterase (PPT), mutant forms of which are responsible for infantile neuronal ceroid lipofuscinosis (INCL). In light of known characteristics of this disease, models for didemnin's effect on PPT are discussed.[¶]

MATERIALS AND METHODS

Reagents. Didemnin A was a generous gift of K. Rinehart (University of Illinois). Affi-Gel-10 was purchased from Bio-Rad, and ϵ -aminocaproic acid, leupeptin, pepstatin A, phenylmethylsulfonyl fluoride, and 5'-guanylyl imidodiphosphate (GMP-PNP) were obtained from Sigma.

Synthesis of Didemnin Affinity Matrix. Didemnin A (1 mg) in 200 μ l of dichloromethane was incubated with 1.2 eq of bromotris (pyrrolidino) phosphonium hexafluorophosphate, >6 eq of triethylamine and 3 eq of fluorenylmethoxycarbonyl-protected ϵ -aminocaproic acid at 0°C for 2 min and then warmed to room temperature. After 2 h, the crude reaction mixture was purified by preparative thin-layer chromatography in dichloromethane/methanol (95:5) to provide fluorenylmethoxycarbonyl-aminocaproic acid-tethered didemnin A in a 79% yield. Proton NMR spectra were consistent with the expected product. The protecting group was removed by incubation in 20% piperidine in dimethylformamide for 20 min followed by evaporation under vacuum. Deprotected aminocaproic acid-tethered didemnin A was coupled to 6 ml of Affi-Gel-10 in dimethyl sulfoxide for 14 h at room temperature.

Purification of Didemnin-Binding Proteins. *Analytical scale.* Bovine brain (4 g) was homogenized on ice in lysis buffer (10 mM K $_2$ HPO $_4$, pH 7.1/1 mM EDTA/5 mM EGTA/10 mM MgCl $_2$ /50 mM β -glycerophosphate, pH 7.1/2 mM Na $_3$ VO $_4$ /2 mM dithiothreitol/5 mM NaF) plus protease inhibitors (10 μ g/ml pepstatin A, 10 μ g/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride) using a Tekmar tissue homogenizer

Abbreviations: EF-1 α , elongation factor 1 α ; PPT, palmitoyl protein thioesterase; INCL, infantile neuronal ceroid lipofuscinosis; GMP-PNP, 5'-guanylyl imidodiphosphate.

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¶The sequence reported in this paper has been deposited in the GenBank database (accession no. U44772).

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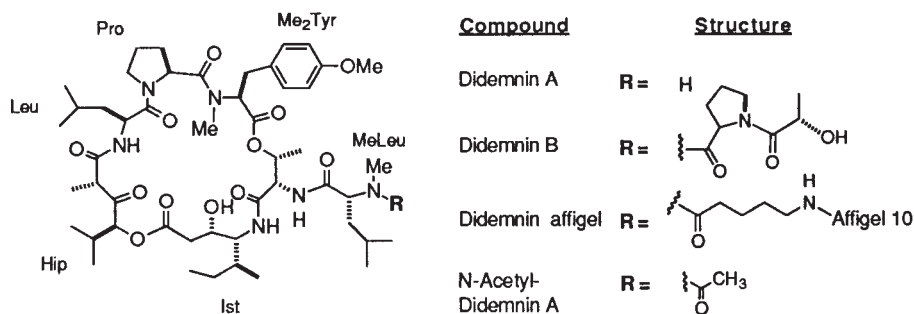


FIG. 1. Structures of didemnins and of the didemnin-based reagent used for affinity chromatography. Ist, isostatine; Hip, hydroxyisovalerylproponic acid.

before centrifugation at $100,000 \times g$ for 30 min at 4°C . The high-speed supernatant was batch adsorbed onto 0.4 ml of Sepharose SP to remove the majority of EF-1 α . The Sepharose SP eluate was incubated with 20 μl of didemnin affinity matrix for 4 h in the presence or absence of 100 μM GMP-PNP and in the presence or absence of 5 μM *N*-acetyldidemnin A (14). The matrix was washed four times with 1 ml of lysis buffer containing 0.15 M NaCl and 10 mM EDTA. Bound proteins were visualized by SDS/12.5% PAGE (16) and silver staining.

Preparative scale. To obtain enough protein for micropeptide sequence analysis, 200 g of bovine brain was homogenized, centrifuged, and adsorbed as described above before incubation with 1 ml of didemnin Affigel 10 for 90 min at 4°C . Bound proteins were washed four times with 10 ml of lysis buffer plus 0.6 M NaCl and transferred to poly(vinylidene difluoride) membrane after SDS/PAGE as described (17). Internal tryptic peptide sequence analysis was performed as described (18).

Degenerate PCR and cDNA Cloning. Fragments of the cDNA encoding the p36 didemnin B-binding protein were obtained using PCR and the degenerate oligonucleotides 1 [GGAATTCGT(G/C/T)CA(G/A/)GC(A/T/C)GA(G/A)T-A(T/C)TGGCA(T/C)GA(T/C)] and 2 [GCGGATCCTC(T/C)TG(A/C/G/T)A(G/A)(A/C/G/T)GG(T/G/A)AT(A/C/G/T)GT(T/C)TC] with 2 μg of peripheral blood leukocyte plasmid cDNA library as template (19). Reaction conditions were 2 mM MgCl_2 , 1 μM oligonucleotide primer, $1 \times$ *Taq* buffer, and 1 unit of *Taq* polymerase for 3 min at 94°C (template denaturation) followed by two initial cycles of 1-min denaturation at 94°C , 1-min annealing at 45°C , and 1.5-min extension at 72°C . Thirty more cycles were performed under the same conditions as the two initial cycles but at an annealing temperature of 55°C . These cycles were followed by a final extension at 72°C for 5 min. The resulting 249-bp PCR fragment was radioactively labeled using PCR and used to screen a human Jurkat T-cell cDNA library (20). One clone, 29B, was selected for dideoxy-nucleotide DNA sequencing (21).

mRNA Tissue Expression. mRNA blots of various human tissues (Clontech) were probed with radiolabeled clone 29B as described (20) and exposed to x-ray film (Kodak, X-Omat) using an intensifying screen at -80°C for 3 days.

RESULTS AND DISCUSSION

Purification of a 36-kDa Didemnin-Binding Protein. Because the previously identified didemnin-binding protein, EF-1 α , is abundant within cells, the identification of additional didemnin B-binding proteins required the selective removal of this protein. Taking advantage of the high isoelectric point of EF-1 α , the majority of EF-1 α was removed by using cation-exchange chromatography at pH 7.1. This precleared lysate was then analyzed for additional didemnin B-binding activities in the presence and absence of the nonhydrolyzable GTP analog GMP-PNP. As shown in Fig. 2, a major protein (36 kDa) and a minor protein (34 kDa) bind specifically to

didemnin B Affi-Gel. However, unlike the 50-kDa didemnin B-binding protein (residual EF-1 α) seen in lane 4, these new didemnin B-binding proteins do not require GTP for didemnin B binding. Nevertheless, binding of all three was blocked by excess free *N*-acetyldidemnin A (lane 5).

Cloning and Sequence Analysis of Human p36 cDNA. Purification of these new didemnin B-binding proteins was scaled up to obtain tryptic peptide sequence information. Amino-terminal sequence analysis of purified p34 and p36 showed that both proteins start at the same internal sequence, DPPAPL, suggesting they are differentially modified forms of the same gene product (data not shown). Based on internal tryptic peptide sequence information obtained from the 36-kDa protein (underlined in Fig. 3), degenerate oligonucleotide primers were synthesized and used to amplify by PCR a cDNA

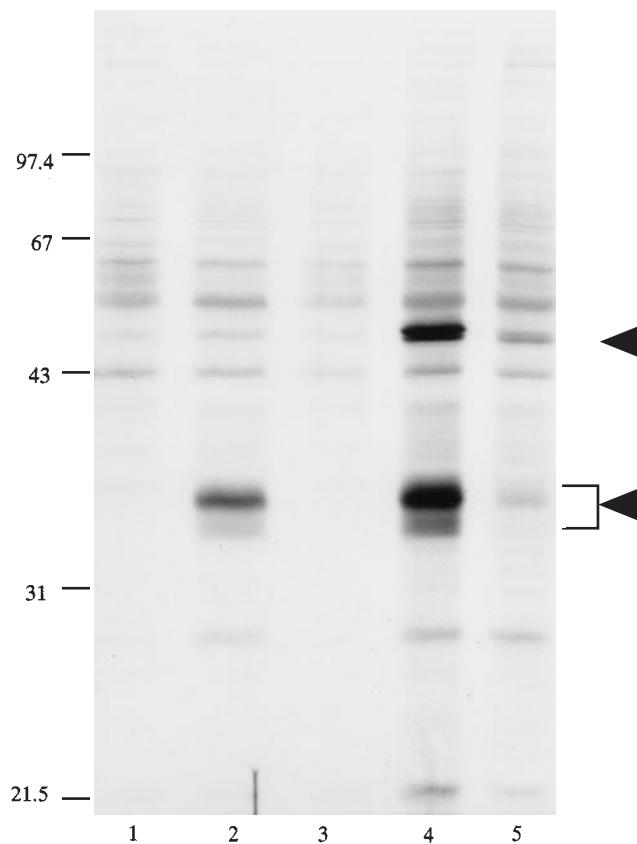


FIG. 2. Affinity chromatography of didemnin-binding proteins from bovine brain lysate. Lanes: 1, control matrix; 2, didemnin-Affi-Gel matrix; 3, control matrix with 200 μM GMP-PNP; 4, didemnin-Affi-Gel matrix with 200 μM GMP-PNP; 5, didemnin-Affi-Gel matrix with 200 μM GMP-PNP using lysate preincubated for 60 min with 5 μM *N*-acetyldidemnin A.

proteases have been reported to have esterase activity (38, 39) and that certain thioesterases have been reported to have protease activity (40). Thus, the observed correlation of lipofuscin induction and increases in endogenous or artificial serine protease inhibitors may be due to the inhibition of PPT, and it is possible that PPT also has protease activity. This hypothesis is in agreement with the results indicating that the loss of PPT correlates with INCL (26). Inconsistent with this model, however, is the finding that PPT is not inhibited by the serine protease inhibitor phenylmethylsulfonyl fluoride (24).

Although we have not yet investigated the effects of didemnin on recombinant PPT activity, its structure suggests that it may prove to be an inhibitor of that activity. Statine residues inhibit (aspartyl) proteases by structurally mimicking the tetrahedral intermediate of amide bond hydrolysis (41–44). It is possible that didemnin's structurally similar isostatine residue (Fig. 1) is playing an analogous role in the inhibition of PPT activity. In support of this hypothesis, studies of structure–activity relationships have shown that didemnin's isostatine residue is essential for its biological activity (45).

Conclusion. Didemnin B is one of the most potent cytostatic and immunosuppressive marine natural products identified to date. Although didemnin's physiological effects have been examined in detail, the molecular and cellular mechanisms by which this cyclic depsipeptide mediates its actions are unknown. As a step towards the elucidation of these mechanisms, we now report the purification from bovine brain and the molecular cloning from a human T-cell cDNA library of a specific didemnin-binding PPT. Inactivating mutations in this thioesterase have been shown to be responsible for the progressive encephalopathy INCL. Experiments are now underway to investigate the nature of didemnin interactions with human recombinant PPT expressed in Sf9 cells, including the possible effect of didemnin B on the enzymatic activity of PPT. The cell-permeant didemnin B molecule may prove to be an effective probe of PPT's cellular function and of its role in INCL.

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