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A Potent Novel Anti-HIV Protein from the Cultured Cyanobacterium *Scytonema varium*†

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ABSTRACT: A new anti-HIV protein, scytovirin, was isolated from aqueous extracts of the cultured cyanobacterium *Scytonema varium*. The protein displayed potent anticytopathic activity against laboratory strains and primary isolates of HIV-1 with EC_{50} values ranging from 0.3 to 22 nM. Scytovirin binds to viral coat proteins gp120, gp160, and gp41 but not to cellular receptor CD4 or other tested proteins. This unique protein consists of a single 95-amino acid chain with significant internal sequence duplication and 10 cysteines forming five intrachain disulfide bonds.

More than 40 million people in the world today are infected with human immunodeficiency virus (HIV).[1] Current therapeutic regimens have focused on reverse transcriptase and protease inhibitors, but the emergence of mutant drug resistant strains has created the need for more effective and less toxic anti-HIV agents. Natural products have traditionally been a source for the discovery of novel therapeutics. Approximately 60% of the anticancer and anti-infective agents that were approved as drugs from 1983 to 1994 have their origins as natural products (*1*). In recent years, there has been an interest in cyanobacteria as a new source of bioactive and structurally diverse secondary metabolites that may fill a role as new lead compounds for drug development.

Two trends in the biological activity of cyanobacterial extracts that have been noted are interference with the assembly of actin and tubulin protein polymers in eukaryotic cells and the production of metabolites which target mammalian ion channels. Natural product toxins found in freshwater and seawater are also attributed to cyanobacteria. These include the neurotoxins responsible for red tide, paralytic shellfish poisoning and the cyclic peptide hepatotoxin, nodularin, which inhibits protein phosphatases (*2*). Although toxins have been the dominant metabolites isolated from cyanobacteria, new screening programs have resulted in the discovery to date of 424 compounds which include enzyme inhibitors, herbicides, antimycotics, antifeedants, and antimalarial and immunosuppressive agents (*3*).

As part of a program investigating anti-HIV activity in natural product extracts, we undertook the investigation of the aqueous extract of laboratory cultures of the terrestrial cyanobacterium *Scytonema varium*. The crude extract potently protected T-lymphoblastoid cells from HIV-1-induced cell killing in a XTT−tetrazolium anti-HIV assay (*4*). Further studies suggested that the active component might be proteinaceous, and indeed, cyanobacteria are known to produce biologically active amino acid-derived natural products, including cyanovirin-N, an anti-HIV protein isolated from the cyanobacterium *Nostoc ellipsosporum* (*5*). Sulfoglycolipids, glycolipids, and polysaccharides isolated from cyanobacterial species have also shown anti-HIV activity (*6*). Although the ecological function of these

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[1] Abbreviations: HIV, human immunodeficiency virus; EC_{50}, effective concentration of the drug that results in 50% protection of cells from HIV-induced killing; rgp120, recombinant, glycosylated HIV-1_{IIIB} gp120; rgp160, recombinant, glycosylated HIV-1_{IIIB} gp160; rgp41, recombinant, glycosylated HIV-1_{HxB2} gp41; HPLC, high-performance liquid chromatography; HSA, human serum albumin; BSA, bovine serum albumin; LC−MS, liquid chromatography−mass spectrometry; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; PEC, *S*-(*β*-4-pyridylethyl)cysteine; ESI-MS, electrospray ionization mass spectrometry; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; TPBS, 0.05% Tween 20 in PBS; XTT, 2,3-bis(2-methyl-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide inner salt; PBMC, peripheral blood mononuclear cell; *β*-gal, *β*-galactose; DMEM, Dulbecco's minimal essential medium; *m*/*z*, mass-to-charge ratio; UDA, *Urtica dioica* agglutinin; WGA, wheat germ agglutinin; THP, thermal hysteresis protein.

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secondary metabolites is for the most part unknown, studies have shown that they may play a role in deterring predation by other species (*3*). A role beyond this is hard to postulate because the observed biological activity may not have an apparent role in the life history of the organism (i.e., hepatotoxins).

We describe herein the isolation and characterization of the novel anti-HIV protein, scytovirin. This protein was found to be potently active against laboratory and clinical isolates of HIV and was shown to target HIV envelope glycoproteins gp120 and gp41. Scytovirin was proven to bind to target glycoproteins through a carbohydrate-dependent mechanism, but was not inhibited by monosaccharides. We have isolated, to the best of our knowledge, the first reported protein from a *Scytonema* sp. and the second reported anti-HIV protein from a cyanobacterium.

EXPERIMENTAL PROCEDURES

General Methods and Materials. All solvents were HPLC grade and were purchased from EM Science. Endoproteinases Arg-C and Glu-C were obtained from Roche Molecular Biochemicals (Indianapolis, IN). The monomeric sugars, wheat germ agglutinin, human and bovine serum albumins (HSA and BSA, respectively), aprotinin, bovine IgG, α-acid glycoprotein, and Sephadex G-100 were purchased from Sigma Corp. Oligosaccharides were purchased from Glyko, Inc. (Novato, CA). The rgp120 (recombinant, glycosylated, HIV-1_{IIIB} gp120), rgp160 (recombinant, HIV-1_{IIIB} gp160), and rgp41 (recombinant, HIV-1_{HxB2} gp41, ecto domain) were obtained from Advanced Biotechnologies Inc. (Columbia, MD). The sCD4, glycosylated and nonglycosylated gp120 (HIV-1_{SF2} gp120), HL2/3 and HeLa CD4 LTR β-gal cell lines, and HIV-1 M-tropic (Ba-L and ADA) and T-tropic (IIIB) isolates were obtained from the National Institute of Allergy and Infectious Diseases AIDS Research and Reference Program (National Institutes of Health). The origins of the CEM-SS cells and HIV_{RF} have been described previously (*4*).

All HPLC separations were obtained using a Rainin SD-1 system with a Knauer variable-wavelength detector monitored at 210 nm and a Rainin Dynamax C_{18} 300 Å column (1 cm × 25 cm) unless otherwise stated. Electrospray ionization mass spectra were recorded on a Hewlett-Packard HP1100 integrated LC−MS system equipped with an ion spray interface. Samples were introduced into the mass spectrometer at a flow rate of 0.2 mL/min with the following instrumental conditions: nebulizer pressure (N_2) of 25 psig, drying gas flow (N_2) of 10 L/min, drying gas temperature of 350 °C, capillary voltage of 4000 V, fragmentor voltage of 80 V, and mass range of 250−1600 amu. SDS−PAGE was performed as previously described (*7*) on a Novex apparatus using a 14% polyacrylamide resolving gel (precast, Novex). Gels were run at a constant current of 25 mA/gel for 60 min at room temperature. The GenBank nonredundant database, BLASTP, was used to search for N-terminal amino acid sequence similarity (*8*).

Collection and Culturing. *S. varium* Kutzing ex Bornet et Flahault (Nostocales, Scytonemataceae), designated strain HG-24-1, was obtained from the culture collection at the Department of Chemistry, University of Hawaii at Manoa. A clonal culture was prepared by repeated subculture on solidified medium. The cyanobacterium was grown in 20 L glass bottles containing inorganic medium A_3M_7 (*9*). Prior to autoclaving, the pH of the medium was adjusted to 7.0 with NaOH. Cultures were illuminated continuously at an incident intensity of 25 μmol of photons PAR (photosynthetically active radiation) m^{-2} s^{-1} from banks of cool-white fluorescent tubes. Aeration was provided at a rate of 5 L/min with a mixture of 0.5% CO_2 in air. After incubation at 24 ± 1 °C for a period of 25 days, the biomass was harvested by filtration and freeze-dried. The yield of lyophilized cells from 60 L of culture was 0.35 g/L.

Extraction and Isolation. Anti-HIV bioassay-guided fractionation was used to track the isolation of scytovirin; 423 g of lyophilized cells was prepared and extracted as previously described (*9*). A 10 g portion of the aqueous extract (99 g total) was subjected to vacuum liquid chromatography on Bakerbond wide pore C_4 media, eluting with a stepwise gradient of 0 to 100% methanol. A 1.0 g portion of the 2:1 (v/v) water/methanol fraction (3.5 g total) was loaded on a Sephadex G-100 (5.5 cm × 19 cm) column and eluted with phosphate buffer (25 mM, pH 7) containing 0.4 M NaCl and 0.02% NaN_3. Active fractions were pooled and filtered through an Amicon Centriprep 10 000 Da molecular mass cutoff filter to desalt and concentrate the material. The resulting 83 mg was purified using reversed-phase HPLC eluting with a gradient of 0 to 60% acetonitrile in 0.05% aqueous TFA in 40 min at a flow rate of 3 mL/min, followed by isocratic elution for 15 min with 60% acetonitrile in 0.05% aqueous TFA, yielding 3.6 mg of scytovirin.

Structure Determination. Disulfide bonds were reduced and alkylated as previously described (*10*). The derivatized peptide was purified by reversed-phase HPLC using a gradient elution of 0.05% aqueous TFA for 40 min and then increasing to 60% acetonitrile in 0.05% aqueous TFA over the course of 45 min. The *S*-(β-4-pyridylethyl)cysteine (PEC) derivative (250 μg) was subjected to endoproteinase Arg-C and endoproteinase Glu-C digestion per the manufacturer's instructions at an enzyme:substrate ratio of 1:20. The cleaved peptide products were purified by reversed-phase HPLC using a gradient of 0.05% aqueous TFA for 20 min and then increasing to 60% acetonitrile in 0.05% aqueous TFA over the course of 100 min. Amino acid sequences were determined by sequential Edman degradation using an Applied Biosystems model 494 sequencer according to the protocols of the manufacturer.

Disulfide Determination. To a 1.0 mg sample of native, nonreduced scytovirin were added 60 μL of 100 mM ammonium bicarbonate (pH 8.0), 6 μL of acetonitrile, and 6 μL of a 40 μM solution of trypsin in H_2O. The mixture was incubated at 37 °C for 16 h and then separated by reversed-phase HPLC using a C_3 column (Zorbax) eluting with a linear gradient from 0 to 100% acetonitrile in H_2O with 5% (v/v) CH_3COOH in the mobile phase. Peaks corresponding to peptide fragments were analyzed by ESI-MS, and evaluated using peptide recognition software to detect all possible disulfide-linked peptide fragments (*11*).

Antibody Production. A New Zealand white rabbit was immunized with 100 μg of scytovirin in Freund's complete adjuvant. Booster injections of 50 μg of scytovirin in Freund's incomplete adjuvant were administered on days 13, 29, 51, 64, 100, and 195. On days 7, 21, 42, 63, 78, and 112, 10 mL of blood was removed from the rabbit. On day

112, the rabbit was sacrificed and bled out. The IgG fraction of the immune sera of the rabbit was isolated by protein A−Sepharose affinity chromatography (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. The reactivity of the polyclonal antibodies for scytovirin was demonstrated by ELISA studies with 1:100 to 1:1000 dilutions of the rabbit immunoglobulin fractions.

Chitin Binding Assay. Scytovirin (100 μg) and wheat germ agglutinin (100 μg) each were dissolved in 900 μL of phosphate-buffered saline (pH 8.0) and applied to chitin microcolumns (8 mm × 10 mm). The samples were recycled three times over the columns and eluted five times with 1 mL of phosphate-buffered saline and once with 1.0 mL of 0.1 M acetic acid. Samples were desalted and concentrated by reversed-phase HPLC using a gradient of 0 to 60% acetonitrile in 0.05% aqueous TFA over 45 min at a flow rate of 3 mL/min, before being analyzed by SDS−PAGE.

ELISA Protocols. To determine the affinities of scytovirin for a series of standard proteins, 100 ng each of gp160, gp120, gp41, sCD4, bovine IgG, α-acid glycoprotein, aprotinin, human serum albumin (HSA), and bovine serum albumin (BSA) were subjected to an ELISA protocol as previously described (*12*). Briefly, the proteins were bound to a 96-well plate, which was then rinsed with PBS containing 0.05% Tween 20 (TPBS) and blocked with BSA. Between subsequent steps, the plate was again rinsed with TPBS. The wells were incubated with 100 ng of scytovirin, followed by incubation with a 1:500 dilution of the anti-scytovirin rabbit polyclonal antibody preparation. The amount of bound scytovirin was determined by adding goat anti-rabbit antibodies conjugated to alkaline phosphatase (Roche Molecular Biochemicals). Upon addition of the alkaline phosphatase substrate buffer, absorbance was measured at 405 nm for each well.

Glycosylation-dependent binding of scytovirin to gp120 was examined using an ELISA as described above, with glycosylated and nonglycosylated gp120 (HIV-1_{SF2} gp120) added to the 96-well plate and incubated with eight serial dilutions of scytovirin at a high concentration of 100 ng/mL.

To study the effect of monomeric and complex sugars on scytovirin and gp120 binding, ELISA plates were treated as described above with the following modifications. The 96-well plates were first incubated with 100 ng of gp120 and then treated with a preincubated (1 h) 1:1 (v/v) mixture of scytovirin and sugar to yield a final concentration of 0.005 mM scytovirin and 500 mM sugar per well. The monomeric sugars *N*-acetylgalactosamine, fucose, xylose, *N*-acetylglucosamine, mannose, glucose, and galactose were tested as well as complex oligosaccharides, mannose 7, mannose 8, mannose 9, a hybrid-type N-linked oligosaccharide, and an A3 complex-type N-linked oligosaccharide.

Anti-HIV Assays. An XTT−tetrazolium-based assay was used to determine the anti-HIV activity of scytovirin on acute HIV-1 infection in CEM-SS cells as previously described (*4*). The effects of scytovirin on pretreatment of CEM-SS cells and HIV-1_{RF}, delayed addition to HIV-1_{RF}-infected cells, and cell−cell fusion were studied using methods described elsewhere (*13*).

Antiviral assays used to study the activities of laboratory strains and primary isolates of virus have been published previously (*14*). The low-passage HIV-1 pediatric isolate ROJO was derived as previously described (*15*). Peripheral blood mononuclear cells (PBMC) and macrophages were isolated from hepatitis and HIV sero-negative donors following Ficoll-Hypaque centrifugation as described elsewhere (*16*).

Attachment and additional fusion assays were performed as previously described (*15*) with the modifications listed below. Descriptions and sources of the cell lines have been previously published (*15*). The HL2/3 and HeLa CD4 LTR β-gal cell lines were maintained in DMEM with 10% fetal bovine serum, penicillin (100 units/mL), streptomycin (100 μg/mL), and L-glutamine (2 mM). HeLa CD4 LTR β-gal cell lines were also supplemented with G418 (200 μg/mL) and hygromycin (100 μg/mL). Following the interaction of HIV-1_{IIIB} with HeLa CD4 LTR β-gal cells (attachment assay) or the coculture of HeLa CD4 LTR β-gal and HL2/3 cells (fusion assay), virus replication was detected by chemiluminescence using a single-step lysis and detection method (Tropix Gal-screen, Bedford, MA). Viral binding to HeLa CD4 LTR β-gal cells was detected as cell-associated p24 antigen following a 1 h adsorption of virus and vigorous washing for removal of unbound virus. Chicago Sky Blue, a polysulfonic acid dye inhibitor of HIV attachment and fusion, was used as a positive control for all assays (*17*).

RESULTS

Isolation and Structure Determination. Anti-HIV bioassay-guided fractionation of the aqueous extract of *S. varium* resulted in the isolation of scytovirin. The aqueous extract was subjected to reversed-phase vacuum liquid chromatography, size exclusion chromatography, ultrafiltration, and reversed-phase HPLC to yield the homogeneous protein. The calculated total yield of scytovirin was 0.03% of the dry mass of the cyanobacterial material that was used.

SDS−PAGE analysis showed a single protein band with a relative molecular mass of ~9 kDa, and ESI-MS analysis of this protein provided a molecular mass of 9712.8 Da. Reduction and alkylation of scytovirin with 4-vinylpyridine generated the *S*-(β-4-pyridylethyl)cysteine (PEC) derivative which resulted in an ESI-MS molecular mass of 10 774.3 Da. This was consistent with the presence of 10 disulfide-linked cysteines. Amino acid analysis of scytovirin indicated that it contained two glutamine or glutamic acid and five arginine residues; therefore, the alkylated derivative of scytovirin was digested separately with endoproteinases Arg-C and Glu-C to yield fragments for N-terminal amino acid sequencing. The primary amino acid sequence of the resulting 11-peptide fragments, along with the intact *S*-(β-4-pyridylethyl)cysteine (PEC) derivative, was determined by automated sequential Edman degradation and analyzed by ESI-MS to provide the entire primary sequence of scytovirin (Figure 1).

Six fragments were obtained from the endoproteinase Arg-C digest. The fragment consisting of residues 1−19 gave a molecular ion at *m*/*z* 2096.0 (calcd *m*/*z* 2096.2), residues 20−30 *m*/*z* 1405.3 (calcd *m*/*z* 1405.6), residues 31−43 *m*/*z* 1530.4 (calcd *m*/*z* 1530.7), residues 44−67 *m*/*z* 2686.8 (calcd *m*/*z* 2686.9), residues 68−78 *m*/*z* 1378.2 (calcd *m*/*z* 1378.6), and residues 79−95 *m*/*z* 1765.7 (calcd *m*/*z* 1766.0). These data fully supported the deduced amino acid sequence of scytovirin.

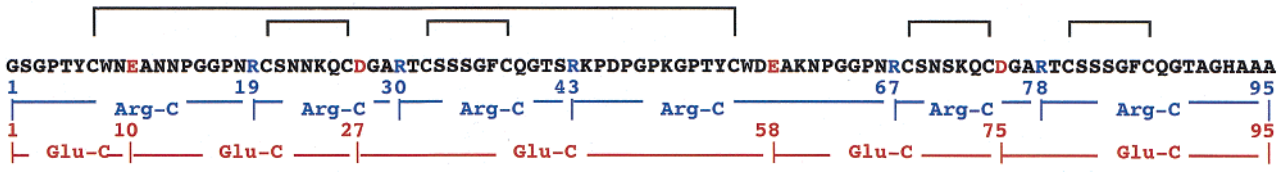

FIGURE 1: Primary amino acid sequence of scytovirin. The protein was sequenced by a combination of N-terminal Edman degradation and ESI-MS of overlapping peptide fragments generated by endoproteinase digestions. Selected peptides isolated by reversed-phase C_{18} HPLC from digests with endoproteinase Arg-C and Glu-C are shown. Residue numbers are indicated in blue for Arg-C and red for Glu-C. Disulfide cross-links were identified by ESI-MS analysis of peptide fragments generated by tryptic digestion of scytovirin, and are marked with solid lines above the sequence.

Endoproteinase Glu-C cleaved peptide bonds C-terminally at glutamic acid and aspartic acid, producing five fragments that also supported the proposed amino acid sequence. Fragments at *m*/*z* 1217.8, 1998.9, 3591.9, 1986.2, and 2050.0 corresponded to residues 1−10, 11−27, 28−58, 59−75, and 76−95, respectively, and provided overlapping confirmation of the amino acid sequence (Figure 1). Therefore, it was deduced that scytovirin was a 95-amino acid protein, with a molecular mass of 9713 Da, containing five intrachain disulfide bonds.

Disulfide Bond Pattern. To establish the locations of these intramolecular bonds, an aliquot of the nonreduced protein was treated with trypsin and the resulting peptides were analyzed by ESI-MS. Peptide recognition software (*11*) was used to identify the theoretical disulfide-bonded fragments. Two disulfide links were unambiguously defined by the presence of the fragments at *m*/*z* 1318 and 1553. The program gave the single match of the Cys32−Cys38 disulfide for *m*/*z* 1318. Similarly, the fragment at *m*/*z* 1553 was in agreement with the Cys80−Cys86 disulfide. Two possible matches for *m*/*z* 2511 were proposed, with the first involving two disulfide links between the fragments consisting of amino acids 20−30 and 31−43. The second possibility consisted of one disulfide link between amino acids 20−24 and 25−43. The first option was not viable because we had already deduced a disulfide bond between Cys32 and Cys38; therefore, it was not possible to have two bonds between these fragments. Thus, the third disulfide bond was established as being between Cys20 and Cys26.

A fragment at *m*/*z* 2719 again gave two possible matches, one of which was two disulfide links between amino acids 68−78 and 79−95 and the other one disulfide link between amino acids 68−72 and 73−95. As a Cys80−Cys86 bond had already been assigned, it was not possible to have two disulfide links between the fragments, so a Cys68−Cys74 bond was proposed. By the process of elimination, the fifth bond was assigned to the Cys7−Cys55 disulfide. This deduction was supported by a fragment at *m*/*z* 3851 that corresponded to links between amino acids 51−67 and 1−19, and an additional fragment at *m*/*z* 3158 that linked amino acids 51−60 and 1−19. Thus, the disulfide linkage pattern was identified as follows: Cys20−Cys26, Cys32−Cys38, Cys68−Cys74, Cys80−Cys86, and Cys7−Cys55 (Figure 1).

Sequence Similarities. A search of the BLAST database (*8*) for identification of protein sequence similarities indicated the sequence was 55% identical to that of a domain within a much larger cloned polypeptide from the multicellular green alga *Volvox carteri* (*18*) (Figure 2A). This polypeptide consists of three repeats of a 48-amino acid chitin-binding domain separated by an extensin-like module from a cysteine protease domain.

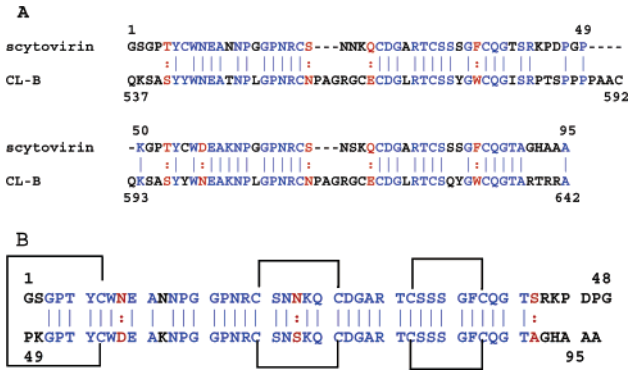

FIGURE 2: Sequence similarities. (A) Aligned amino acid sequences of scytovirin and the similar region of a cloned polypeptide (CL-B) from *V. carteri*. Numbers to the top and bottom of the sequences indicate amino acid residue numbers. Identical residues between scytovirin and *Volvox* are indicated in blue, conserved changes in red, and gaps by dashes. (B) Sequence homology between the two domains of scytovirin indicated in blue and conserved changes in red. Disulfide cross-links are marked with solid lines above the sequence.

A conidiospore surface protein from *Trichoderma harzianum* (*19*), precursor proteins of the stinging nettle (*Urtica dioica*) agglutinin (UDA) isolectins (*20*), and cloned antifreeze proteins from *Dendroides canadensis* (*21*) and *Tenebrio molitor* (*22*) also exhibited lower scoring matches to scytovirin (sequences that are 31, 33, 27, and 28% identical, respectively). The stinging nettle proteins consist of a signal peptide with two chitin-binding domains, a hinge region, and a carboxyl-terminal chitinase domain. The thermal hysteresis (antifreeze) proteins which showed sequence identity are similar in size (9 kDa), Cys-, Thr-, and Ser-rich, and fully disulfide bonded and contain repeated sequences of 12 or 13 amino acids.

Binding Studies. Because of the primary sequence similarity between scytovirin and the inner conserved core region of chitin-binding proteins, we investigated the ability of scytovirin to bind to a chitin substrate. Microcolumns packed with chitin were loaded with scytovirin or wheat germ agglutinin (WGA) as a positive control, and eluted at neutral and low pH. SDS−PAGE of the resulting fractions showed that scytovirin was present in the initial fraction that was recycled through the column, while WGA was present only in the fraction eluted with low-pH buffer (data not shown). These results indicated that although scytovirin contains a primary structural motif like the lectins and chitin-binding proteins, it does not exhibit strong binding affinity toward chitin.

Viral Envelope Molecular Target Interactions. In an effort to identify potential molecular targets for scytovirin, the protein was tested for its ability to bind glycoproteins and

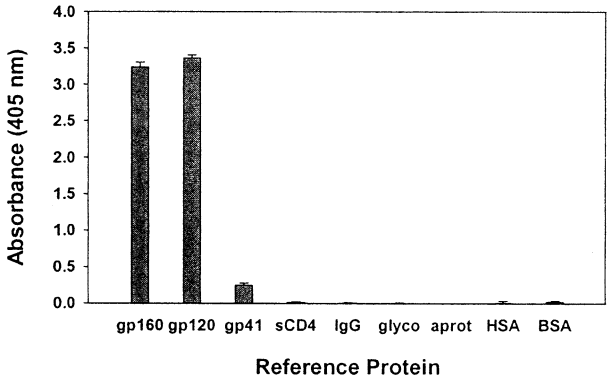

FIGURE 3: ELISA study of the binding of scytovirin to reference proteins. Proteins were bound to a 96-well plate, and the plate was incubated with scytovirin. Bound scytovirin was visualized with anti-scytovirin polyclonal antibodies as indicated by the absorbance at 405 nm. gp160, recombinant, HIV-1_{IIIB} gp160; gp120, recombinant, glycosylated, HIV-1_{IIIB} gp120; gp41, recombinant, HIV-1_{HxB2} gp41, ecto domain; sCD4, soluble CD4; glyco, α-acid glycoprotein; aprot, aprotinin; HSA, human serum albumin; and BSA, bovine serum albumin.

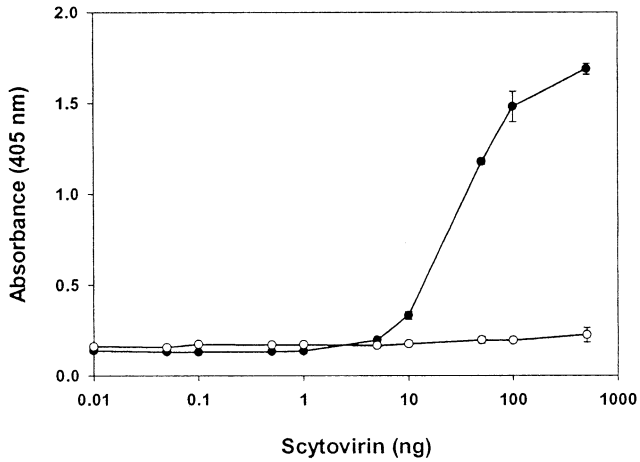

FIGURE 4: ELISA study of the concentration-dependent binding of scytovirin to gp120. Glycosylated (●) or nonglycosylated (○) gp120 was bound to an ELISA plate and then treated with scytovirin. The amount of bound scytovirin was determined by the absorbance at 405 nm as described in Experimental Procedures.

the cellular receptor sCD4. Evidence for direct interaction of scytovirin with gp160, gp120, and, to a lesser degree, gp41 was obtained from an ELISA experiment in which scytovirin was bound to gp160-, gp120-, and gp41-treated wells but not bound to wells containing sCD4 or other reference proteins, including bovine IgG, α-acid glycoprotein, aprotinin, human serum albumin, and bovine serum albumin (Figure 3). An additional ELISA experiment determined that binding of scytovirin to gp120 is glycosylation-dependent (Figure 4).

Further ELISA binding experiments in the presence of monomeric sugars showed that scytovirin was not inhibited from binding to gp120 by *N*-acetylgalactosamine, fucose, xylose, *N*-acetylglucosamine, mannose, glucose, and galactose. However, when the protein was tested against higher-order oligosaccharides, scytovirin−gp120 binding was inhibited by oligomannose 8 and oligomannose 9 but not by oligomannose 7, a hybrid-type N-linked oligosaccharide, or an A3 complex-type N-linked oligosaccharide.

Anti-HIV Activity. Scytovirin exhibited comparable activity against the T-tropic laboratory strain HIV-1_{RF} in CEM-SS

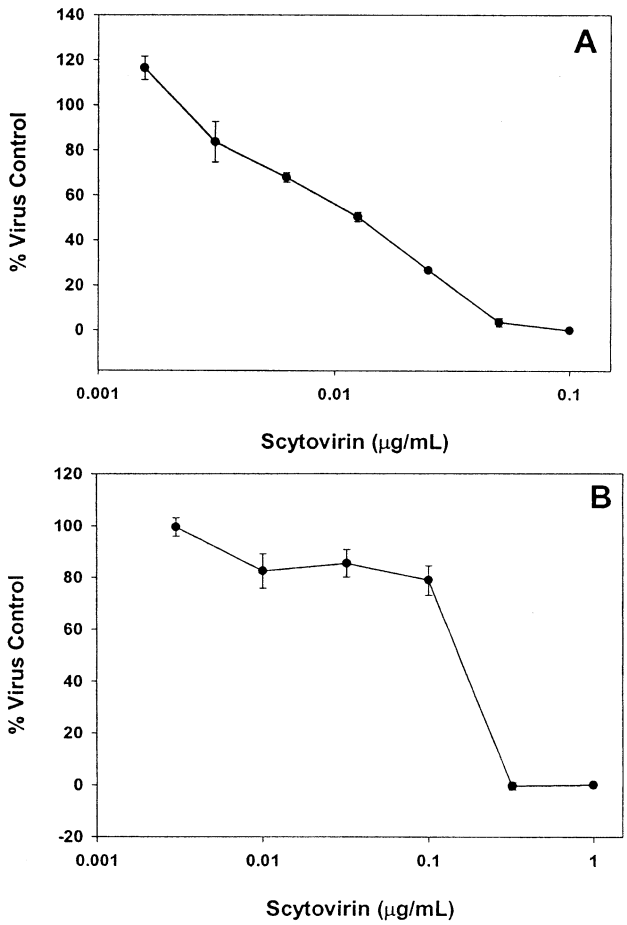

FIGURE 5: Effects of scytovirin on virus−cell interactions. (A) Effect of scytovirin on syncytium formation between uninfected and chronically infected CEM-SS cells in a cocultivation assay. (B) Effect of scytovirin on the fusion of CD4 β-gal cells with HIV-1 Env-expressing HL2/3 cells.

cells, and against the primary isolate ROJO in PBMCs, with EC_{50} values of 0.3 and 7 nM, respectively. Scytovirin was also active against the M-tropic primary isolates Ba-L and ADA in macrophages, albeit less potent with EC_{50} values of 22 and 17 nM, respectively. Delayed addition experiments showed that scytovirin had to be present within the first 8 h of virus infection for antiviral activity. Pretreated uninfected CEM-SS cells retained normal susceptibility to HIV infection after the removal of scytovirin. Likewise, cell-free virus retained normal infectivity after pretreatment and removal of scytovirin.

Cocultivation of uninfected and chronically infected CEM-SS cells with scytovirin caused a concentration-dependent inhibition of cell−cell fusion (Figure 5). Additional binding and fusion inhibition assays using β-gal indicator cells gave similar results. Scytovirin inhibited fusion of CD4 β-gal cells with HL 2/3 cells (Figure 5) and also inhibited the cell-free HIV-1_{IIIB} fusion and infection of β-gal cells in a concentration-dependent manner.

DISCUSSION

Scytovirin is a novel anti-HIV protein isolated from the cultured cyanobacterium *S. varium*. It is a 95-amino acid protein, with a molecular mass of 9713 Da, containing five intrachain disulfide bonds. The amino acid sequence of scytovirin was deduced by a combination of endoproteinase

digestions, N-terminal Edman degradation, and ESI-MS characterization.

Scytovirin shows strong internal sequence duplication. When amino acids 1−48 and 49−95 are aligned, 36 residues (75%) are directly homologous and three (6%) represent conservative amino acid changes (Figure 2B). The bonds formed by the C20−C26 and C32−C38 disulfides correspond closely to those defined by the C68−C74 and C80−C86 disulfide links. These homologous regions, with two disulfide bridges linking cysteines located at six-residue intervals, suggest the presence of two functional domains that are linked by the C7−C55 bond.

The deduced amino acid sequence of scytovirin is strongly similar (55%) to that of the chitin-binding domain of a cloned polypeptide from the multicellular green alga *V. carteri*. Of the 658 amino acids in the *Volvox*-cloned polypeptide, the chitin-binding domain consists of a common structural motif of 30−43 amino acids with glycines and cysteines at conserved positions. Although a large number of the cysteines are conserved in scytovirin, the protein has a disulfide bonding pattern different from that of the chitin-binding domain of *Volvox*.

Scytovirin shows lower levels of sequence similarity when compared to precursor proteins to *Urtica diocia* (UDA) agglutinin isolectins. Although the sequence match includes the chitin-binding region, scytovirin again has a different disulfide bonding pattern. The similarity to the conidiospore surface protein and the thermal hysteresis proteins (THPs) is due mainly to the conserved cysteines spaced at six-residue intervals. As there are no published data other than the sequence for the conidiospore protein, the function and importance of this spacing are unknown. The 12-amino acid repeat found in the THP proteins follows this six-residue cysteine spacing and, along with other key residues, is thought to be important for the structural integrity and function of the antifreeze proteins.

Although scytovirin has a lectin-like primary structure, it does not appear to belong to the chitin-binding class of proteins. It does not bind chitin, and does not have the same disulfide bonding pattern as the chitin-binding domains determined thus far. Proteins with lectin properties are capable of cross-linking carbohydrate-containing polymers due to the presence of multiple chitin-binding domains. Since the envelope glycoprotein of HIV is heavily glycosylated, HIV infectivity and virus-cell fusion may be inhibited by lectins that are specific for the sugars (i.e., GlcNAc and mannose) present in the gp120 molecule. It has been shown that the D-mannose-specific lectin, concanavalin A (*23*), does block HIV infectivity and virus-cell fusion, and the GlcNAc-specific lectins, MHL (*24*) and UDA-1 (*25*), are inhibitors of HIV-induced cytopathicity.

Unlike the lectins described above, scytovirin did not show specificity for D-mannose or *N*-acetylglucosamine, sugars associated with antiviral activity (*25*). However, when the protein was tested against larger oligosaccharides, scytovirin−gp120 binding was inhibited by oligomannose 8 and oligomannose 9; this result is similar to that recently described for cyanovirin-N (*26*), suggesting that scytovirin may interact preferentially with sites on gp120 comprising high-mannose oligosaccharides.

Scytovirin displayed strong activity against laboratory-adapted strains and primary isolates of HIV-1. It appeared to be equally potent against T-tropic laboratory strains and primary isolates of HIV-1, but 300-fold less effective against M-tropic strains. Delayed addition experiments showed that scytovirin had to be present within the first 8 h of virus infection for antiviral activity. Pretreatment and removal of scytovirin caused a normal susceptibility to HIV infection in uninfected CEM-SS cells, and a normal infectivity in cell-free virus. This reversibility of viral inhibition contrasts markedly with the effects of the cyanobacterial protein, cyanovirin-N, which potently inactivates the virus on contact (*5*).

The pretreatment and delayed addition studies of scytovirin suggest that it must be continually present early in the viral life cycle to be maximally protective. ELISA studies indicated that scytovirin binds specifically to glycosylated gp120 and gp160 and, to a lesser degree, gp41, but not to the cellular receptor CD4 or to other reference proteins. All of these studies together suggest that scytovirin is an inhibitor of HIV binding and/or fusion. In future studies, it will be interesting to see if scytovirin shares with cyanovirin-N any of the remarkable structural features of that potent anti-HIV molecule (*27*).

Previous studies on polysaccharides have indicated the presence of structures similar to high-mannose oligosaccharides in cyanobacterial heterocyst and spore envelopes (*28*). Since two cyanobacterial proteins, cyanovirin-N and scytovirin, which interact with high-mannose oligosaccharides have been identified, perhaps there is a functional role for proteins with similar carbohydrate binding properties in cyanobacteria. The exact role of such proteins in the life cycle of cyanobacteria is yet to be determined.

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