Griffithsin (GRFT), a novel anti-HIV protein, was isolated from an aqueous extract of the red alga Griffithsia sp. The 121-amino acid sequence of GRFT has been determined, and biologically active GRFT was subsequently produced by expression of a corresponding DNA sequence in Escherichia coli. Both native and recombinant GRFT displayed potent antiviral activity against laboratory strains and primary isolates of T- and M-tropic HIV-1 with EC$_{50}$ values ranging from 0.043 to 0.63 nM. GRFT also abrogated cell-to-cell fusion and transmission of HIV-1 infection at similar concentrations. High concentrations (e.g. 783 nM) of GRFT were not lethal to any tested host cell types. GRFT blocked CD4-dependent glycoprotein (gp) 120 binding to receptor-expressing cells and bound to viral coat glycoproteins (gp120, gp41, and gp160) in a glycosylation-dependent manner. GRFT preferentially inhibited gp120 binding of the monoclonal antibody (mAb) 2G12, which recognizes a carbohydrate-dependent motif, and the (mAb) 48d, which binds to CD4-induced epitope. In addition, GRFT moderately interfered with the binding of gp120 to sCD4. Further data showed that the binding of GRFT to soluble gp120 was inhibited by the monosaccharides glucose, mannose, and N-acetylgalactosamine but not by galactose, xylose, fucose, N-acetylgalactosamine, or sialic acid-containing glycoproteins. Taken together these data suggest that GRFT is a new type of lectin that binds to various viral glycoproteins in a monosaccharide-dependent manner. GRFT could be a potential candidate microbicide to prevent the sexual transmission of HIV and AIDS.

Currently, more than 40 million people are infected with HIV, type 1, worldwide (1). The dominant mode of transmission of the virus is through heterosexual contact, which accounts for up to 90% of all HIV infections (2). The highly mutable nature of HIV and the daunting complexities of developing a broadly protective vaccine against the multiple clades of HIV are increasingly apparent (3–5). With no vaccine on the horizon, there is a pressing need to develop anti-HIV microbicides to prevent the sexual transmission of HIV. In recent years, the overall proportion of HIV-positive females has steadily increased. As of December 2003, women accounted for nearly 50% of all people living with HIV worldwide (1). In women, the main entry site for HIV is the cervico-vaginal mucosa, and currently the only absolute methods of protection for women are abstinence or condom used by males, neither of which may be a negotiable option for the women. For these reasons, health organizations worldwide have stated that the development of a female-controlled topical virucide for HIV is an urgent global priority (1, 2, 6).

Natural products have historically been a source for the discovery of novel therapeutic agents. It has been reported that over 60% of the anti-tumor and anti-infective agents that were approved as drugs from 1983 to 1994 owe their structural origin to compounds derived from nature (7). The NCI, National Institutes of Health, has long had a program investigating anti-HIV activity in natural product extracts (8). Our laboratory has also been particularly interested in the elucidation of protein and peptide leads that might reveal unprecedented mechanisms of anti-HIV activity and/or serve as templates for discovery and development of novel, small molecule inhibitors of HIV infection (9). Such leads are also potentially attractive for microbicide development. Examples of such proteinaceous leads are cyanovirin-N (CV-N) and scytovirin isolated from aqueous extracts of the cyanobacterium, Nostoc ellipsosporum and Scytonema varium, respectively (10, 11). Both of these antiviral proteins were discovered in extracts of cyanobacteria.
Here we report the isolation of a unique anti-HIV protein from a marine red alga. Aqueous extracts of the red alga *Griffithsia* sp. collected from the waters off New Zealand showed potent cytotoxic protective activity against HIV-1-induced cytopathy in T-lymphoblastoid cells. No presence of this activity was seen in the organic extract of this alga. Previous chemical investigations of *Griffithsia* sp. have also been studied for its production of phycoerythrin proteins (14, 15). These brightly colored pigments have shown great utility as fluorescent labels for a variety of biochemical studies. Neither the organic constituents of *Griffithsia* sp. nor the phycoerythrins have ever been reported to have antiviral activity. In this study, we describe the isolation and characterization of the novel, potent anti-HIV protein, griffithsin (GRFT), from the aqueous extract of *Griffithsia* sp. The data presented here suggest that GRFT represents another potential candidate microbicide to prevent the sexual transmission of HIV and AIDS.

**EXPERIMENTAL PROCEDURES**

**General Materials, Proteins, and Antibodies—**All solvents were HPLC-grade purchased from EM Science. Endoproteinases Lys-C, Arg-C, and Asp-N were obtained from Roche Applied Science. The origins of the CEM-SS cells and HIV-1gp have been described and were obtained from the AIDS Research and Reference Reagent Program (National Institutes of Health, Bethesda) (16). Aprotinin, bovine IgG, and α-acid glycoprotein were purchased from Sigma. The recombinant gp120 (glycosylated, HIV-1IM gp120), recombinant gp160 (HIV-1IM gp160), and recombinant gp41 (HIV-1IM gp41, ecto-domain) were obtained from Advanced Biotechnologies Inc. (Columbia, MD). Fluorescein isothiocyanate (FITC)-conjugated recombinant anti-gp120 mAb, raised against the recombinant gp120, and phycoerythrin-conjugated anti-OKT4 monoclonal antibody (mAb) were obtained from Intraceal and Ortho Diagnostics (Raritan, NJ), respectively. Peridinin chlorophyll protein (PerCP)-conjugated anti-Leu3a mAbs were obtained from Immunocytometry Systems. CV-N, recombinitely expressed in *Escherichia coli*, was prepared as described elsewhere (17). The following anti-gp120 mAbs were obtained through the AIDS Research and Reference Reagent Program (National Institutes of Health, Bethesda): HIV-1 gp120 mAb 2G12 (conformational and carbohydrate-dependent) from Dr. H. Katinger; IgG1 b12 (CD4-binding site) from Drs. D. Burton and C. Barbas; 48d (CD4-induced epitope) and 17b (CD4-induced epitope) from Dr. J. Robinson; ID6 (C1 region) from Drs. K. Ugen and D. Weiner; and 458.1 (C1 region), 4G10 (V3 loop), IIIB-V3-21 (V3 loop), and 4E10 (V4 loop) from Dr. U. A. Katinger. Full-length HIV-1 gp120 were glycosylated and nonglycosylated gp120 (HIV-1SF2 gp120), HL2/3, and aprotinin (6.5 kDa), cytochrome c (12.5 kDa), and aprotinin (6.5 kDa) by their retention time (absorbance 280 nm) and comparing the resulting calibration curve to the retention time of the active protein. Molecular mass and purity (>99%) of GRFT were confirmed by electro-spray ionization mass spectrometry (ESI-MS), and the protein concentrations were determined by amino acid analysis.

**Preparation of Anti-GRFT Polyclonal Antibodies—**A New Zealand White rabbit was immunized with 100 μg of GRFT in Freund's complete adjuvant. Booster injections of 50 μg of GRFT 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 212, the rabbit was sacrificed and bled out. The IgG fraction of the immune serum of the rabbit was isolated by protein A-Sepharose affinity chromatography (Bio-Rad) according to the manufacturer’s instructions. Reactivity of the polyclonal antibodies for GRFT was demonstrated by immunoblot and ELISA studies with 1:250 to 1:5000 of the rabbit immunoglobulin fractions.

**SDS-PAGE Analysis and Immunoblotting—**All the reagents used for SDS-PAGE were from Invitrogen. For SDS-PAGE analysis, samples were mixed with Tricine-SDS sample buffer containing 2% 2-mercaptoethanol, run on a 16% Tris-Tricine gel with Tricine-SDS running buffer, and stained by Coomassie Blue. Pre-stained molecular weight standards (SeeBlue Plus2) were from Invitrogen were used. For immunoblotting, samples were transferred to polyvinylidene difluoride membranes following SDS-PAGE, according to standard procedures. The membranes were blocked with 5% BSA in PBS and incubated for 1 h with anti-GRFT polyclonal antibodies, washed three times with PBS containing 0.05% Tween 20 (PBST), and then treated with goat anti-rabbit IgG antibodies conjugated to horseradish peroxidase (Sigma). After three washes with PBST, bound antibodies were visualized by incubating membranes in a solution of 0.05% 3,3′-diaminobenzidine and 0.003% H2O2.

**Amino Acid Analysis, N-terminal Amino Acid Sequencing, and Mass Spectrometry—**Amino acid analysis was done by using a Beckman model 6300 automated amino acid analyzer according to the manufacturer’s protocol. N-terminal amino acid sequencing was performed on Applied Biosystems model 474A and 494A sequencers according to the manufacturer’s protocols. Matrix-assisted laser desorption ionization-time of flight mass spectroscopy (MALDI-TOF MS) was done using Kratos Compact Maldi III instrument (Shimadzu) operated in a linear
mode using sinapinic acid as a matrix and trypsin as an external standard. ESI-MS was performed with a JEOL SX102 equipped with an Analytica electrospray source. The spectrometer was calibrated using a lyszyme standard (molecular weight = 14,305.2) prior to each analysis. Samples were injected into the source in a 1:1 solution of hexafluoroisopropanol and 2% acetic acid.

**Chemical and Enzymatic Cleavages of GRFT—**GRFT was subjected to digestion with cyanogen bromide (CNBr) and a variety of endoproteases (Lys-C, Arg-C, and Asp-N) per the manufacturer’s instructions. The cleaved peptide products were purified by reversed-phase HPLC using a gradient of 0.05% aqueous trifluoroacetic acid over 20 min and then increasing to 60% acetonitrile in 0.05% aqueous trifluoroacetic acid over 100 min. Amino acid sequences were determined by sequential Edman degradation using an Applied Biosystems model 474 or 494 sequencer according to the protocols of the manufacturer, and the masses of cleaved peptides were analyzed by MALDI-TOF MS.

**Assay for Anti-HIV Activity—**An XTT-tetrazolium-based assay was used to determine the anti-HIV activity of GRFT against a T-tropic laboratory strain (HIV-1gp) in CEM-SS cells as described previously (16). CEM-SS cells were maintained in RPMI 1640 media without phenol red and supplemented with 10% fetal bovine serum (BioWhittaker), 2 mM 1-glutamine (BioWhittaker), and 50 μg/ml gentamicin (BioWhittaker) (complete medium). Exponentially growing cells were washed and resuspended in complete medium with 1% fetal bovine serum and 5% of NIH-3T3 medium containing 5 × 10^5 cells was added to individual wells of a 96-well round-bottom microtiter plate containing serial dilutions of GRFT in a volume of 100 μl of medium. Stock supernatants of HIV-1gp were diluted in complete medium to yield sufficient cytopathicity (80–90% cell kill in 6 days), and a 50-μl aliquot was added to appropriate wells. Plates were incubated for 6 days at 37°C and then stained for cellular viability using a 0.4% solution of 2-thiazolyl-2'-methoxy-4-nitro-5-sulfophenyl-2'-tetrazolium-5-carboxanilide inner salt (XTT), p24 antigen production, supernatant reverse-transcriptase activity.

To test the anti-HIV activity of GRFT against HIV primary isolates in fresh human cells, monocyte-tropic HIV-1 strains Ba-L and ADA were obtained from the NIAID AIDS Research and Reference Reagent Program (National Institutes of Health, Bethesda). The low passage HIV-1RF laboratory strain (HIV-1RF) in CEM-SS cells as described previously (16). Human peripheral blood mononuclear cells and macrophages were isolated from hepatitis and HIV seronegative donors following Ficoll-Paque centrifugation as described elsewhere (20). Anti viral assays were carried out with 3-day-old phytohemagglutinin/interleukin-2 stimulated peripheral blood mononuclear cells or 6-day-cultured monocyte/macrophage. All antiviral evaluations were performed in triplicate in RPMI 1640 supplemented with 10% fetal bovine serum, l-glutamate (2 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml). HIV-1 replication in PBMC (RoJo) and monocyte/macrophage (Ba-L and ADA) cultures were determined by measurement of reverse transcriptase activity (21) in the supernatants or p24 antigen expression by ELISA (Coulter, Hialeah, FL), respectively. Anti viral data were reported as 50% inhibition concentration (EC_{50}) and assays were carried out at least in triplicate for all viruses and cells.

The cell-cell fusions were studied using methods described elsewhere (10). Uninfected CEM-SS cells (1 × 10^5 cells/50 μl) were co-cultured with chronically infected CEM/HIV-1RF cells (10^4 cells/50 μl) in the presence of various concentrations of GRFT (100 μl) or 100 μl of control medium alone in flat-bottomed 96-well microtiter plates. After 72 h of incubation, the number of syncytia was determined microscopically. Each experimental condition consisted of six replicate samples.

Attachment and additional fusion assays were performed as described previously (20) with the modifications listed below. Descriptions and sources of the cell lines have been published previously (20). The HL-60 and HeLa CD4 LTR β-galactosidase cell lines were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and l-glutamine (2 mM). HeLa CD4 LTR β-galactosidase cell lines were also supplemented with G418 (200 μg/ml) and hygromycin (100 μg/ml). Following the interaction of HIV-1gp with HeLa CD4 LTR β-galactosidase cells (attachment assay) or the co-culture of HeLa CD4 LTR β-galactosidase and HL-60 cells (fusion assay), virus replication was detected by chemiluminescence and single step gp120 assay (Gal-Screen™, Bedford, MA). Viral binding to HeLa CD4 LTR β-galactosidase cells was detected as cell-associated p24 antigen following a 1-h adsorption of virus and vigorous washing to remove unbound virus. Chicago Sky Blue, a polysulfonic acid dye inhibitor of HIV attachment and fusion, was used as a positive control for all assays (22).

**Pretreatment of HIV-1gp with GRFT—**Concentrated HIV-1gp was incubated with medium containing 194 μg of GRFT or with control medium for 60 min at 37°C. After incubation, the treated and control virus samples were diluted to yield a multiplicity of infection of 0.8 and to dilute the GRFT beyond the effective concentration. The pretreated virus samples (50 μl) were then added to individual wells of a 96-well microplate containing 5 × 10^4 CEM-SS cells (50 μl) and either 100 μl of medium alone or 194 μl of GRFT. The cultures were incubated for 7 days, and cellular viability was assessed using the XTT assay.

**Pretreatment of CEM-SS Cells with GRFT—**CEM-SS cells were incubated with medium containing 194 μg GRFT or with control medium for 60 min at 37°C. After incubation, the CEM-SS cells were washed free of GRFT using two centrifugation steps. The pretreated cells were then resuspended in culture medium (5 × 10^4 cells/50 μl) and added to individual wells of a 96-well microtiter plate containing 100 μl of medium alone or 194 μl of GRFT. A 50-μl aliquot of diluted HIV-1gp was added to appropriate wells to yield a multiplicity of infection of 0.8. The cultures were incubated for 7 days, and cellular viability was assessed using the XTT assay.

**Flow Cytometric gp120-Cell-associated CD4 Binding Assay—**For the experiments assessing the effect of GRFT on gp120 binding to cell-associated CD4, an immunoﬂuorescence flow cytometry-based gp120-binding assay was performed by using an assay format reported previously (23). The CEM-SS cell line expressing CD4, CXCR4, and CCR5 (2 × 10^5 per well) was incubated with 1% bovine serum, and 50 μl of the medium were added to appropriate wells. Plates were incubated for 6 days at 37°C and then stained for cellular viability using a 0.4% solution of 2-thiazolyl-2'-methoxy-4-nitro-5-sulfophenyl-2'-tetrazolium-5-carboxanilide inner salt (XTT), p24 antigen production, supernatant reverse-transcriptase activity.

**ELISA Protocols—**To determine the affinities of GRFT for a series of standard proteins, 100 ng each of gp160, gp120, gp41, sCD4, bovine IgG, α-acid glycoprotein, and aprtinin were subjected to an ELISA protocol as described previously (10). Briefly, the proteins were bound to a 96-well plate, which was then rinsed with PBS and blocked with BSA. Between subsequent steps, the plate was again rinsed with PBS. The plates were incubated with 0.12 μg/ml of GRFT, followed by incubation with a 1:500 dilution of the anti-GRFT rabbit polyclonal antibody preparation. The bound GRFT was determined by adding goat anti-rabbit antibodies conjugated to alkaline phosphatase (Roche Applied Science). Upon addition of the alkaline phosphatase substrate buffer, absorbance was measured at 405 nm for each well.

**Glycosylation-dependent binding of GRFT to gp120 was examined using an ELISA system as above.** Briefly, 100 μg of glycosylated or nonglycosylated gp120 (HIV-1gp, gp120) was added to the wells of a 96-well plate and incubated for 1 h with serial dilutions of GRFT. The plate was then washed and visualized using anti-GRFT polyclonal antibodies as above. In additional ELISA studies, the plate was prepared as above with glycosylated gp120, and 100 μl/well of a 1 μg/ml (78.3 nm) GRFT was added in the presence or absence of 100 mM concentrations of the following sugars: glucose, mannose, galactose, fucose, xylose, N-acetylgalcosamine, N-acetylgalactosamine, or 100 ng/mg α-acid glycoprotein (to act as a carrier for sialic acid). The plate was then washed and visualized using anti-GRFT polyclonal antibodies as above. All data points are averages of triplicate measurements at each concentration. Additional ELISA experiments were performed as above to assess more carefully the inhibition of the monosaccharides glucose, mannose, and N-acetylgalcosamine on GRFT binding to gp120. For these, 50 μg of glycosylated gp120well was incubated with serial dilutions of the monosaccharides simultaneously with 39 μg GRFT (50 μg/well). The plates were then treated identically to those described above. Additional ELISAs were performed to compare binding interactions of GRFT with sCD4, gp120, gp41, and sgp120 using an assay format reported previously (24). 96-Well assay plates (Nunc Life Technologies) were coated overnight at 4°C with 50 μl/well of a 10 μg/ml solution in PBS of mAb D7324, which is directed against the C-terminal region of gp120 (25). Coated plates were washed twice in wash buffer (PBS, 0.01% Tween 20), and nonspecific binding sites were blocked with 100 μl of 2% BSA (w/v) in PBS for 1 h at room temperature followed by two washes. Fifty nanograms (0.415 pmol/well) at 50 μl of sgp120 from
HIV-1<sub>IB</sub> (Intracel, Issaquah, WA) was incubated for 2 h at room temperature followed by three washes. PBS or 4.15 pmol/50 μl well of GRFT, CV-N, sCD4, and a variety of mAbs was added to the captured sgp120 and incubated at room temperature for 30 min, followed by three washes. To evaluate the effect of prior GRFT binding to sgp120 on the ability of CV-N, sCD4, or a variety of mAbs to bind to sgp120, 50 μl/well of serial dilutions of them were incubated with GRFT-pretreated or untreated sgp120 for 30 min at room temperature. After three washes, CV-N, sCD4, or a variety of mAbs bound to captured sgp120 was detected using an appropriate anti-IgG alkaline phosphatase conjugate (Roche Applied Science). To assess the effect of prior sgp120 binding of CV-N, sCD4, or a variety of mAbs on subsequent binding of GRFT to sgp120, 50 μl/well of serial dilutions of GRFT were incubated with CV-N, sCD4, or a variety of mAbs pretreated or control sgp120 for 30 min at room temperature. After three washes, GRFT bound to captured sgp120 was detected using a rabbit anti-GRFT polyclonal IgG, followed by three washes and incubation with a goat anti-rabbit IgG alkaline phosphatase conjugate (Roche Applied Science). Following incubations with alkaline phosphatase-conjugated anti-IgG reagents, substrate was added, and absorbance was read at 405 nm.

**Synthesis and Expression in E. coli of the Corresponding DNA Coding Sequence for GRFT**—The deduced amino acid sequence of GRFT was back-translated to a DNA sequence using an E. coli codon preference table, complemented with a termination codon and restriction sites to facilitate ligation into the expression vector, pET28c(+) (Novagen, Madison, WI). A GRFT coding sequence, coupled to codons for the N-terminal penta-His tag and thrombin recognition site, was initially synthesized as 13 overlapping, complementary oligonucleotides, which were assembled to form the full, double-stranded coding sequence. Because amino acid 31 of GRFT did not appear to be one of the 20 common amino acids, alanine was substituted in this position. The synthetic DNA was amplified conventionally by PCR using the appropriate primers and Pfu DNA polymerase (Stratagene, La Jolla, CA). Transformation of E. coli BL21(DE3) was done with the pET28c(+) construct containing the synthetic gene ligated in the correct reading frame. Induction of the clones with isopropyl 1-thio-β-D-galactoside resulted in expression of a corresponding His-tagged GRFT, which was purified by immobilized metal affinity chromatography on a nickel-nitrilotriacetic acid-agarose (Qiagen, Valencia, CA). Anti-HIV and gp120 binding activities of the natural and recombinant proteins were compared as described above.

**RESULTS**

**Isolation and Structure Determination**—The present study originated from observations of anti-HIV activity (EC<sub>50</sub> ≤ 2 μg/ml) in a crude aqueous extract of the red alga Griffithsia in the primary in vitro anti-HIV screening assay from NCI (18). Our preliminary analysis indicated that the active constituent was likely a protein that bound sgp120. Anti-HIV bioassay-guided fractionation utilizing ammonium sulfate precipitation, hydrophobic interaction chromatography, anion exchange chromatography, reversed-phase chromatography, and size exclusion chromatography yielded the homogeneous biologically active protein (see the details under “Experimental Procedures”). SDS-PAGE analysis of the purified material showed a single protein band with a relative molecular mass of ~15 kDa, which we named griffithsin (GRFT) (Fig. 1A), and subsequent immunoblotting with anti-GRFT polyclonal antibodies gave the same result (Fig. 1B). The antibodies modestly cross-reacted with higher molecular mass protein(s) at around 26 kDa in a crude aqueous extract of the red alga Griffithsia. These data may imply the possible existence of dimer form of the GRFT protein. The amino acid sequence of the purified GRFT was established by N-terminal Edman degradation of the intact protein and by N-terminal sequencing of peptide fragments cleaved by CNBr and a variety of endopeptidases (Lys-C, Arg-C, and Asp-N). The entire 121-αmino acid sequence was established except for a single amino acid at position 31, which does not match any of the 20 common amino acids (Fig. 2). ESI-MS of GRFT showed a molecular ion with m/z 12,770.05, and the calculated value for the amino acid sequence without amino acid at position 31 was m/z 12,619.00. Therefore, it was deduced that molecular mass of amino acid at position 31 was 151.05. Amino acid analysis of GRFT was also in good agreement with the deduced primary sequence but also failed to identify amino acid 31 (data not shown). These data fully supported the proposed primary amino acid sequence of GRFT. A search of the BLAST data base (26) for identification of protein sequence similarities did not reveal any homologies of greater than eight contiguous amino acids nor >30% total sequence homology between GRFT and any amino acid sequences of known proteins or transcription products of known nucleotide sequences.

**Anti-HIV Activity**—The potent cytoprotective and anti-repli- cative activities of GRFT were examined by using HIV-1<sub>IB</sub> in CEM-SS cells. GRFT displayed a concentration-dependent inhibition of virus-induced cell killing, along with concomitant decreases in supernatant RT and the HIV-1 viral core antigen, p24 (Fig. 3A). Mid-to-high picomolar concentrations of GRFT exhibited potent activity against both T-tropic and M-tropic viruses (including both laboratory-adapted and primary isolates) (Table I). In the antiviral assays, there was no evidence of direct cytoxicity from GRFT to the uninfected control cells at the highest tested concentrations of GRFT (783 nM; data not shown). Co-cultivation of uninfected and chronically infected CEM-SS with GRFT caused a concentration-dependent inhibition of cell-cell fusion (Fig. 3B). Additional binding and fusion inhibition assays using β-galactosidase indicator cells showed similar results. GRFT inhibited fusion of CD4 β-galactosidase cells with HL 2/3 cells (Fig. 3C) and also inhibited the viral HIV-1<sub>IB</sub> fusion and infection of β-galactosidase cells in a concentration-dependent manner (data not shown). GRFT was then examined for its ability to bind to and inactivate virus particles and to bind to CEM-SS cells. The infectivity of viral particles pretreated with GRFT, followed by dilution beyond effective antiviral concentrations of the protein, was essentially abolished (Fig. 3D). In contrast, when CEM-SS cells were treated with GRFT, then washed free of unbound protein, they retained susceptibility to infection by the virus (data not shown). These results indicated that GRFT behaved as a virucide.

To demonstrate feasibility of recombinant production of bio logically active GRFT, we synthesized the corresponding DNA coding sequence for GRFT (with an alanine replacing the unknown amino acid at position 31) and expressed the recombinant protein in E. coli with a penta-His tag on the N terminus. Recombinant GRFT with N-terminal penta-His tag showed equivalency to natural GRFT, both in respect to gp120-binding
characteristics as well as anti-HIV activity, even though alanine was used as a substitute for an unknown amino acid at position 31 (Table I). Thus, the recombinant production of biologically active GRFT provides a renewable source for future studies on this protein.

**Interactions between GRFT and Viral Envelope**—Because GRFT appeared to inhibit viral entry, we compared matched control and GRFT-treated sgp120 preparations in a flow cytometric sgp120/CD4-expressing cell binding assay (23) to see whether GRFT inhibits viral attachment or a subsequent fusion event. The CEM-SS cell line expresses CD4, as demonstrated by the staining of both anti-Leu3a (Fig. 4B) and anti-OKT4 mAbs (Fig. 4C). After incubation of CEM-SS cells with sgp120, the cells were stained by anti-gp120 mAb-FITC (Fig. 4A), with a concomitant decrease in the availability of the Leu3a epitope (gp120-binding site) (Fig. 4B), but with little change in OKT4 staining (non-gp120 binding epitope) (Fig. 4C), all consistent with CD4-dependent sgp120 binding to the target cells. It was first determined that GRFT did not affect the staining of cells by these three control mAbs (data not shown).

**Pretreatment of sgp120 with GRFT** substantially renewed the Leu3a epitope, indicating that GRFT blocked CD4-dependent sgp120 binding (Fig. 4B). However, overall sgp120 binding showed two peaks when GRFT-treated sgp120 was added to the cells (Fig. 4A). The decreased peak indicates inhibition of sgp120 binding to the cells, which was consistent with the recovery of the Leu3a epitope. The increased peak perhaps indicated that the GRFT-sgp120 complex nonspecifically bound to the cells.

GRFT was tested for its ability to bind HIV envelope glyco-
TABLE I

<table>
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<tr>
<th>Sample</th>
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<th>Target cell</th>
<th>Tropism</th>
<th>EC50[^b] (nm)</th>
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<td>RF</td>
<td>CEM-SS</td>
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<tr>
<td>Recombinant GRFT</td>
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<td>CEM-SS</td>
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<td>PBMC</td>
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<tr>
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<td>Ba-L</td>
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<td>M</td>
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</table>

[^a]: Target cells used in the in vitro anti-HIV assay. There was no evidence of direct cytotoxicity from GRFT to the uninfected control cells at the highest tested concentrations of GRFT (783 to 783 nM).

[^b]: Mean EC50 values were determined from concentration-response curves from eight dilutions of the test agent (triplicate wells/concentration); assays for HIV-1 RF/CEM-SS employed XTT-tetrazolium; HIV-1 RF, BA, and HIV-1 ADA were tested in human primary macrophage cultures by p24 ELISA assay. Standard errors averaged less than 10% of the respective means.

proteins. Evidence for direct interaction of GRFT with gp120, gp160, and gp41 was obtained from an ELISA experiment (Fig. 5A). There was little or no detectable interaction between GRFT and other reference proteins, including bovine IgG, α-acid glycoprotein, and aprotinin. At a very high concentration of GRFT (~5000 ng), some interaction with sCD4 was observed (data not shown). It is unlikely that this interaction was physiologically relevant because of the fact that GRFT did not affect the gp120-binding site, confirmed by availability of the Leu3a epitope in previous studies. An additional ELISA experiment showed that binding of GRFT to sgp120 is concentration- and glycosylation-dependent (Fig. 5B). In further ELISA experiments, specific monosaccharides were tested for their ability to inhibit GRFT binding to gp120. The results indicated that 100 mM concentrations of mannose, glucose, and N-acetylglucosamine inhibited GRFT binding, whereas fucose, xylose, galactose, N-acetylglactosamine, and the heavily sialylated glycoprotein α-acid glycoprotein did not (Fig. 6A). Subsequent titration experiments showed that mannose was the monosaccharide most effective at inhibiting GRFT binding to gp120 (Fig. 6B).

To undertake preliminary mapping studies to define the GRFT-binding site on the gp120, we evaluated the effect of GRFT on the reactivity of sCD4, CV-N, and a panel of mAbs with sgp120 in an ELISA. As shown in Fig. 7, these studies demonstrated that GRFT interfered strongly with subsequent recognition of sgp120 by the mAbs 48d and 2G12 (Figs. 7A and B, respectively), moderately with sCD4 and mAb IgG1b12 (Figs. 7C and D, respectively), but little or not at all with recognition of sgp120 by mAbs that recognize the C1 region or V3 loop and mAb 17b (data not shown). However, additional studies demonstrated that sCD4 and mAbs IgG1b12, 48d, and 2G12 pretreatment of sgp120 did not block subsequent binding of GRFT to sgp120 (data not shown). GRFT pretreatment of sgp120 did not block subsequent binding of CV-N to sgp120 (Fig. 7E). On the other hand, CV-N interfered strongly with subsequent recognition of sgp120 by GRFT (Fig. 7F).

DISCUSSION

Previous studies (27) have reported that certain sulfated cell wall polysaccharides from the red alga Asparagopsis armata possess anti-HIV activity. Other researchers have also reported antibacterial proteins from the red alga eucheuma serra and Galaxaura marginata (28) that strongly inhibited the growth of Vibrio sp. The red alga Griffithsia is best known for producing the fluorescent proteins of the phycocyanin class that are used as labels in a variety of biochemical and cell biology methods (14, 15). In addition, the isolation of certain matrix polysaccharides (13) and photosynthetic pigments (12) and the identification of proteins of common evolutionary interest such as the cyclophilins (29) have been published. The isolation of the anti-HIV protein GRFT from Griffithsia represents the first discovery of an antiviral protein from the Rhodophyta. GRFT is a completely novel protein with a molecular weight of 12,770, an unusual (as yet unidentified) amino acid at position 31 (151 Da), no cysteine residues among its 121 amino acids, and no homology to any of the proteins or translated nucleotide sequences in the BLAST data base (Fig. 2). Subsequent cloning of a recombinant GRFT with an Ala residue at position 31 showed that the unusual amino acid at that position could be
substituted without affecting activity and that the recombinant production of biologically active GRFT was feasible (Table I). GRFT inhibited the cytopathic effects of laboratory strains and clinical primary isolates of HIV-1 on T-lymphoblastic cells at concentrations as low as 43 pM (Table I). This concentration is lower than that reported previously (9) for most antiviral proteins from natural sources. GRFT was also shown to be equally active against both T-cell tropic (T-tropic) and macrophage-tropic (M-tropic) strains of HIV-1. This protection was mirrored by simultaneous decreases in both supernatant reverse transcriptase activity and p24 levels (Fig. 3A). GRFT at sub-nanomolar concentrations was also shown to inhibit cell-cell fusion between chronically infected and uninfected cells (Fig. 3, B and C). These protective effects were subsequently found to be mediated by the interaction between GRFT and viral components when viral but not cell pre-treatment experiments resulted in inhibition of the infectivity of HIV-1 (Fig. 3D). The spectrum of anti-HIV activity for GRFT is similar to that previously reported for the cyanobacterial proteins, CV-N (10) and scytovirin (11). The evidence that GRFT appeared to work through a virucidal mechanism led to additional studies on the specific interactions between this protein and viral components.

Initial flow cytometric experiments on GRFT-treated sgp120 indicated that GRFT did indeed inhibit CD4-dependent sgp120 binding (Fig. 4). Most interestingly, the GRFT-sgp120 complex also appeared to bind to the cells in a CD4-independent manner. Similar “nonspecific” binding of viral components to cell surfaces was also reported with gp120 pre-treated with the anti-HIV protein CV-N (30). In that case such binding to cell surfaces was speculated to be “cross-linking” through CV-N between oligosaccharides on cell surface glycoproteins with carbohydrates on the viral envelope glycoproteins. This led to the examination of the interaction of GRFT with both viral envelope glycoproteins and CD4. Initially we examined the binding of GRFT to several glycoproteins as well as some standard nonglycosylated proteins. The results clearly showed strong GRFT binding to sgp120, sgp160, and sgp41 and little or no binding to other glycoproteins such as human serum albumin and α-acid glycoprotein as well as sCD4 (Fig. 5A). These results confirmed earlier results that GRFT interacted with a viral target and indicated specificity between various glycoproteins. The results clearly showed strong GRFT binding to sgp120, sgp160, and sgp41 and little or no binding to other glycoproteins such as human serum albumin and α-acid glycoprotein as well as sCD4 (Fig. 5A). These results confirmed earlier results that GRFT interacted with a viral target and indicated specificity between various glycoproteins. The evidence that GRFT appeared to work through a virucidal mechanism led to additional studies on the specific interactions between this protein and viral components.

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Another important question about GRFT was where this protein might be binding on gp120. To address this, we performed a series of competition experiments with GRFT and various monoclonal antibodies to regions on HIV-1 gp120 (Fig. 7). GRFT moderately inhibited both the subsequent binding of the CD4-binding site-specific mAb IgG1b12 and the binding of sCD4 itself. These data indicated that GRFT bound in close proximity to a CD4-binding site on gp120. More pronounced was the inhibition of the binding of the mAbs 2G12 and 48d by pretreatment of sgp120 with GRFT. 2G12 is known to be a carbohydrate-specific antibody that binds to an epitope on the "silent face" of gp120 (33), whereas 48d is a conformationally specific antibody to gp120 that binds to a CD4-induced epitope on gp120. These results were in concordance with our previous flow cytometry studies that showed that GRFT could inhibit gp120/CD4 interactions, and our ELISA studies that showed that GRFT bound to carbohydrates present on gp120. It is interesting to note that none of the antibodies tested were able to inhibit the binding of GRFT, including 2G12. As 2G12 binds to only one specific epitope on gp120 (34), this suggests that GRFT is able to bind to more than one site on this glycoprotein. This result makes sense if GRFT is interacting in a monosaccharide-dependent manner, as we suspect. In fact, in our studies, only CV-N significantly inhibited GRFT binding. CV-N has been reported to bind to specific Man-8 and Man-9 oligosaccharides on gp120 with a 5:1 stoichiometry in respect to gp120 (35, 36) so it is likely that GRFT interacts with at least some of these same oligosaccharide binding partners.

As mentioned above, previous studies in our laboratory with anti-HIV proteins from cyanobacteria (CV-N and scytovirin) also showed carbohydrate-dependent binding by these lectins, but these cyanobacterial lectins did not show inhibition of that binding by monosaccharides (10, 11). The monosaccharide binding profile, inferred from the gp120 competition experiments with GRFT, is closer to that reported for the calcium-dependent lectin DC-SIGN, with the only significant difference being the ability of DC-SIGN to bind to fucose (37). Unlike DC-SIGN or other c-type lectins, GRFT has not shown a dependence on calcium for binding to glycoproteins (data not shown). Previous studies (30) on CV-N have shown that it does not block the binding of gp120 to DC-SIGN. The broader carbohydrate specificity of GRFT may provide the means by which the physiologically important interaction between DC-SIGN and HIV viral particles can be interrupted. In addition, GRFT may also possess a broader spectrum of antiviral activity than CV-N, which has been reported recently to also be active against other viruses, including influenza (38) and the Ebola virus (39), but inactive against certain enveloped viruses, such as the corona virus that causes severe acute respiratory syndrome.2 As the cellular entry of the severe acute respiratory syndrome virus has been reported recently to be dependent on the presence of a glycosylated envelope spike protein (S), which also binds to DC-SIGN (40), GRFT may also prove to inhibit infection by this or other viruses that present the proper oligosaccharide moieties on their surface glycoproteins.

GRFT from the red alga Griffithsia sp. and CV-N and scytovirin from the cyanobacteria Nostoc ellipsoporum and Syctonema varium, respectively, are ~10-kDa proteins with anti-HIV activity (10, 11). What is unique about all of these antiviral proteins, including GRFT, is that they all show no homology to any other primary amino acid sequences currently found in the BLAST query sets. Furthermore, despite their similar activities and molecular targets, they show no sequence homology to each other. There is little current evidence as to

Fig. 7. Effect of GRFT on binding of CV-N, sCD4, and variety of anti-gp120 mAbs to sgp120. sgp120 was coated on ELISA plates, and the ability of GRFT to compete with CV-N, sCD4, and a panel of mAbs was examined. A, mAb 48d recognizing CD4-induced epitope; B, mAb 2G12 recognizing carbohydrate-dependent conformational epitope; C, sCD4; D, mAb IgG1b12 recognizing CD4-binding site; and E and F, CV-N. A–E, captured gp120 was pretreated with GRFT prior to incubation with sCD4, various mAbs, or CV-N, and the bound sCD4, mAbs, or CV-N were detected. F, captured gp120 was incubated with GRFT after pretreatment with CV-N, and the bound GRFT was detected.

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ent since little or no binding could be visualized for the non-glycosylated sgp120 (Fig. 5B). Additional ELISAs showed that the binding of GRFT to sgp120 could be inhibited by high concentrations (100 mM) of the monosaccharides glucose, mannose, and N-acetylgalactosamine but not by xylose, fucose, galac-tose, N-acetylglucosamine, or sialic acid-containing glycoproteins (Fig. 6A). Of the inhibitions, mannose was the most effective at inhibiting GRFT/gp120 binding interactions (Fig. 6B).

Taken together, these data suggest that GRFT is a mixed specificity lectin (Man/Glc) that binds to various viral glycoproteins in a monosaccharide-dependent manner. There are several published reports on the anti-HIV activity of monosaccharide-specific plant lectins, including those with specificities similar to GRFT, such as concanavalin A (reviewed in Refs. 31 and 32). A striking difference between these lectins and GRFT is its outstanding potency. In our hands and others, the plant lectins generally provide EC50 values in whole-cell anti-HIV assays in the low micromolar range (31), whereas GRFT showed 25,000-fold more potent anti-HIV activity (~43 pm). The mechanism by which this protein achieves this unique level of potency in a monosaccharide-dependent manner represents an intriguing biophysical question that merits further study.

2 J. W. Huggins, personal communication.
why these organisms are producing these proteins and what function they may perform in their host. Speculation on this topic has so far centered on either the presence of some inherent antiviral defense mechanism in these organisms or on some structural function for these proteins in the cross-linking of polysaccharides. Both CV-N and scytovirin appear to have two potential carbohydrate binding domains (11, 41), and recent studies have shown some evidence that the same is true for the anti-HIV cyanobacterial protein MVL (42). GRFT has some structural features that may indicate the presence of four domains in its sequence separated by three of the linker sequence Gly-Gly-Ser-Gly-Gly (Fig. 2). Such an organization for this protein could explain its unusually potent activity if it indicated the possibility of multivalent binding between GRFT and oligosaccharides present on gp120. Future structural and thermodynamic studies will be necessary before any firm conclusions can be made.

GRFT itself, as well as functional derivatives or fragments thereof, provides a novel lead for further investigation of new potential therapeutic and preventative strategies against HIV infection. The use of genetically engineered microorganisms, such as

*E. coli*, for large scale production of GRFT should supply a ready source of material for further development and investigation of conventional microbial formulations and strategies for topical prophylaxis against various modes of sexual transmission of HIV infection. GRFT is a particularly attractive candidate for microbicide development because of the potent virucidal activity against M-tropic primary isolates of HIV-1, which are critically involved in sexual transmission of infection (e.g. see Refs. 43–45). A more recent strategy of microbial prophylaxis utilizes commensal bacteria such as lactobacilli to produce virucidal proteins (46–50). Such a strategy using GRFT might provide an effective and economical prophylaxis for HIV infection. Continued efforts in our laboratory will investigate this and other potential prophylactic and therapeutic uses of GRFT.

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