Isolation and Characterization of Griffithsin, a Novel HIV-inactivating Protein, from the Red Alga *Griffithsia* sp.*

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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 EC₅₀ values ranging from 0.043 to 0.63 nM. GRFT also abeoted 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 mod-erately 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-acetylglucosamine 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-de-pendent manner. GRFT could be a potential candidate microbiocide to prevent the sexual transmission of HIV and AIDS.

Currently, more than 40 million people are infected with HIV,¹ 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 microbiocides 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 cervical-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 microbiocide development. Examples of such proteinaceous leads are cyanovirin-N (CV-N) and scytovirin isolated from aqueous extracts of the cyanobacterium, *Nostoc ellipsosporum* and *Scyttonema varium*, respectively (10, 11). Both of these antiviral proteins were discovered in extracts of cyanobacteria.

¹ The abbreviations used are: HIV, human immunodeficiency virus; HIV-1, type 1; ESI-MS, electrospray ionization mass spectrometry; MALDI-TOF MS, matrix-assisted laser desorption ionization-time of flight mass spectrometry; CNBr, cyanogen bromide; PBS, phosphate-buffered saline; BSA, bovine serum albumin; mAb, monoclonal anti-body; CV-N, cyanovirin-N; GRFT, griffithsin; ELISA, enzyme-linked immunosorbtn assay; XTT, 2,3-bis-[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt; gp, glycoprotein; HPLC, high pressure liquid chromatography; FITC, fluorescein iso-thiocyanate; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)methylgly- cine; PerCP, peridinin chlorophyll protein; LTR, long terminal repeat; sgp, sialic acid-containing glycoproteins; CV, column volumes; RT, reverse transcription.
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 activity against HIV-1-induced cytopathicity 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 detailed the isolation of a variety of metabolites, including photosynthetic pigments (12) and polysaccharides (13). *Griffithsia* sp. has 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 phycoerythrines 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-1/php 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-1Lui gp120), recombinant gp160 (HIV-1Lui gp160), and recombinant gp41 (HIV-1Lui gp41, ecdx-domain) were obtained from Advanced Biotechnologies Inc. (Columbia, MD). Fluorescein isothiocyanate (FITC)-conjugated double 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, recombinantly 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 459.1 (C1 region), 4G10 (V3 loop), IIIB-V23 (V3 loop), and 411L (V3 loop) from Dr. E. Tarczy-Hornoc. The α-glycosylated and nonglycosylated gp120 (HIV-1Lui gp120), H2L3, and HeLa CD4 LTR β-galactosidase cell lines and HIV-1 M-tropic (Ba-L and ADA) and T-tropic (IIIB) isolates were also obtained through the AIDS Research and Reference Reagent Program (National Institutes of Health).

**Collection and Classification of the Red Algae Griffithsia**—The rhodophyta *Griffithsia* sp. (*Ceramiales*) (voucher Q66D336) was collected on a rocky reef 100 meters off the eastern shore of Chatham Island, New Zealand. The red alga was collected by divers. A second collection of *Griffithsia* sp. (voucher Q66C5379) was collected in Fiordland, New Zealand, at a depth of 10 m. Both collections and identifications were made by the University of Canterbury (New Zealand) under the auspices of a contract administered by the NCI National Products Branch (Developmental Therapeutics Programs, Division of Cancer Treatment and Diagnosis, NCI, National Institutes of Health). Voucher specimens for both collections are held at the Smithsonion Institution (Washington, D. C.).

**Extraction and Isolation**—A cell-based, virally induced cytopathicity bioassay was used to guide fractionation and to track the isolation of GRFT. In brief, the lysis of mammalian cells was harvested by filtration, freeze-dried, and extracted first with H2O followed by MeOH/CHCl3 (1:1). Individual aliquots of the organic and aqueous extracts were tested for cytotoxic properties in the NCI primary anti-HIV screen (18). Only the H2O extract showed anti-HIV activity. Freeze-dried aqueous extract (10 g) was brought up in distilled, deionized H2O at a concentration of 50 mg/ml and maintained on ice. Following centrifugation, the supernatant was brought to 75% saturation with (NH4)2SO4; the protein was allowed to precipitate on ice overnight, and the solution was then centrifuged at 3,000 rpm for 50 min. The pellet was resuspended with 1% (NH4)2SO4 followed again by precipitation and centrifugation. Finally, the second pellets were saved, and the resulting supernatant was filtered (0.22-μm filter) and subjected to hydrophobic interaction chromatography. A BioCad Sprint work station (PerSeptive Biosystems) was used for the following column chromatographic steps. This protein solution was injected onto aPoros PE column (10 × 100 mm, PerSeptive Biosystems) pre-equilibrated with a starting buffer of 50 mM sodium phosphate, 1.5 M (NH4)2SO4, pH 7.5. The column was then eluted at a flow rate of 15 ml/min over the following gradients: 1) 7 column volumes (CV, equal to 7.85 ml) of the starting buffer; 2) 1.5–0 M (NH4)2SO4 over 2 CV; and 3) 0 M (NH4)2SO4 for 7 CV. The eluate was monitored for both conductivity and absorbance (280 nm). Final (NH4)2SO4 concentration in the void fraction possessing anti-HIV activity was brought to 75% saturation to precipitate the proteins on ice overnight, and the eluate was then centrifuged at 3,000 rpm for 50 min. The distilled, deionized H2O-resuspended pellets were first concentrated by a 10-kDa molecular mass limit membrane (Amicon), dialyzed against 0.02% sodium azide, and then brought up to a concentration of 25 mM Tris-HCl, pH 8.5. This solution was then injected onto a Poros HQ anion exchange column (10 × 100 mm, PerSeptive Biosystems) pre-equilibrated with a starting buffer of 25 mM Tris-HCl, pH 8.5. The column was eluted at a flow rate of 15 ml/min using the following gradients: 1) 5 CV of the starting buffer; 2) 0–1 M sodium chloride over 20 CV; and 3) 1 M sodium chloride for 5 CV. The eluate was monitored for absorbance (280 nm). Active fractions from the HQ column were concentrated and desalted by 10-kDa molecular mass limit membrane, subjected to a Bio-RP C4 reversed-phase column (4.6 × 100 mm, Covance), and eluted at a flow rate of 4 ml/min using the following gradients: 1) 10 CV of the starting buffer; 2) 1.5–0 M (NH4)2SO4 over 2 CV; and 3) 0 M (NH4)2SO4 for 7 CV. The eluate was monitored for absorbance (280 nm), and the active fraction was pooled, lyophilized, and resuspended in phosphate-buffed saline (PBS), pH 7.4. The protein solution was finally injected onto a G3000PW gel permeation column (21.5 × 600 mm, Toso Haas) and eluted with PBS, pH 7.4, at a flow rate of 5 ml/min. Molecular weight was determined by gel filtration of the protein by comparing the elution times of proteins (albumin (68 kDa), cytochrome c (12.5 kDa), and aprotinin (65 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 a Folin–Ciocalteu reagent method.

**Purification 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 was obtained by Protein G 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) 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 3% 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.

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mode using sialic 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 lysosome 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 by cyanogen bromide (CNBr) and a variety of endoproteinases (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. The cleaved peptide products were purified by reversed-phase HPLC with a 1:1 solution of hexafluoroisopropanol and 2% acetic acid.

**Antiviral Assays**—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 l-glutamine (BioWhittaker), and 50 μg/ml gentamicin (BioWhittaker) (complete medium). Exponentially growing cells were washed and resuspended in complete medium (RPMI 1640 supplemented with 10% fetal bovine serum, and 2 mM l-glutamine) containing 5 × 10<sup>5</sup> 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 (2% methyamine/4-methyl-5-sulfophenyl-2'-4'-tetrachloro-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-1 strains Ba-L and ADA were diluted in complete medium (RPMI 1640 supplemented with 10% fetal bovine serum, L-glutamate, and 2 mM -glutamine) and sources of the cell lines have been published previously (20). The carriers 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-acetylgalcosaminic acid (to act as a carrier for sialic acid). 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 μg/well of a 1 μg/ml solution in the presence or absence of 100 μM concentrations of the following sugars: glucose, mannose, galactose, fucose, xylose, N-acetylgalcosaminic, N-acetylgalactosaminic, and 100 ng/well 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. In additional ELISAs, the plate was prepared as above with glycosylated gp120, and 100 μg/ml gp120 binding to gp120 was added to the washed 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 μg/ml gp120 binding to gp120 was added to the washed wells of a 96-well plate and incubated for 1 h with serial dilutions of GRFT. 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**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 aprotinin 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 PBST and blocked with BSA. Between subsequent steps, the plate was again rinsed with PBST. The wells were first incubated with 100 ng of GRFT, followed by incubation with a 1:500 dilution of the anti-GRFT rabbit polyclonal antibody. The bound GRT 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.
HIV-1<sub>IM</sub> (Intracel, Isaquah, 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 with 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 supplemented 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 amino acid sequence without amino acid at position 31 was 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 ~13 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-amino 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 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-replicative activities of GRFT were examined by using HIV-1<sub>IM</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 inhibited potent activity against both T-tropic and M-tropic viruses (including both laboratory-adapted and primary isolates) (Table 1). In the antiviral assays, there was no evidence of direct cytotoxicity 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>IM</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 biologically 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-
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-acetylgalactosamine inhibited GRFT binding, whereas fucose, xylose, galactose, N-acetylgalactosamine, 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 mAb 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 algae *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 phycerythrin 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. 3B 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. Other experiments, which looked more directly at the glycosylation-dependent binding of GRFT to HIV-1sgp120, showed that GRFT binding was indeed glycosylation-depend-
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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

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\(^2\) J. W. Huggins, personal communication.
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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 microbical 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 microbical 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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