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Chrysoptin Is a Potent Glycoprotein IIb/IIIa Fibrinogen Receptor Antagonist Present in Salivary Gland Extracts of the Deerfly*

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Salivary gland lysates of the deerfly (genus *Chrysops*) contain chrysoptin, an inhibitor of ADP-induced platelet aggregation, which presumably assists the fly in obtaining a blood meal. Chrysoptin has now been isolated, and its cDNA has been cloned and expressed. Chrysoptin was purified to homogeneity using anion exchange and hydrophobic interaction chromatography and found to be a protein with a molecular mass of 65 kDa as determined by gel electrophoresis. N-terminal amino acid sequencing allowed for the synthesis of degenerate oligonucleotides that led to cloning, from salivary gland specific mRNA, of the cDNA encoding this platelet inhibitor. No RGD sites are present in the predicted sequence. A search of GenBank™ did not reveal significant sequence homology between chrysoptin and other proteins. The molecular mass predicted from the cDNA was 59 kDa. Predicted glycosylation and phosphorylation sites may account for this difference in molecular mass, as recombinant chrysoptin expressed in Sf21 cells had a molecular mass of 65 kDa, matching that of the natural protein. Chrysoptin functions by inhibiting the binding of fibrinogen to the fibrinogen/glycoprotein IIb/IIIa receptor on platelets with an IC_{50} of 95 pmol. These results reveal that insect salivary glands are a source of fibrinogen receptor antagonists.

To facilitate blood feeding, hematophagous arthropods produce a number of bioactive substances that overcome the hemostatic mechanisms of the host. Arthropods trigger primary hemostasis by damaging blood vessels while probing the skin. As platelet aggregation is fundamental to hemostasis, some hematophagous arthropods appear to have evolved the ability to secrete platelet inhibitors in their saliva. Previous reports of antiplatelet activity in arthropod saliva include findings of prostacyclin activity in ticks (1, 2); apyrase activity in ticks (3), mosquitoes (4), blood-sucking bugs (5), and tse-tse flies (6); and a nitrosylheme protein from *Rhodnius prolixus* that has the capacity to release nitric oxide (7). Other than the protein from *Rhodnius*, these antiplatelet activities have not been well characterized, and it is not clear whether these or other activities account for the platelet inhibitory activity found in salivary gland lysates.

Deerflies (genus *Chrysops*), also referred to as "greenheads" because of their brilliant green eyes, frequent the coast of the northeastern United States for several weeks in midsummer. Because these flies inflict painful bites that sometimes bleed, we reasoned that deerfly salivary gland extract (DFE)[1] might contain an inhibitor of platelet aggregation. We reported previously that DFE inhibits ADP-, thrombin-, and collagen-induced aggregation of human platelets (8). DFE differs from the antiplatelet activities found in other arthropods in two respects. First, it does not alter cAMP levels in platelets, suggesting that it does not contain prostaglandin-like activity. Second, it does not contain apyrase activity. We report the isolation and characterization of chrysoptin, the protein in DFE responsible for inhibition of platelet aggregation. We also report the cloning and expression of the cDNA encoding chrysoptin. Chrysoptin acts by inhibiting the binding of fibrinogen to the glycoprotein IIb/IIIa receptor.

EXPERIMENTAL PROCEDURES

Deerflies—Deerflies were obtained from traps along saltwater marshes in northeastern Massachusetts using a modified Dustbuster (BioQuip), allowing for the collection of flies in a live state. Flies were brought to the laboratory and dissected within 8 h.

Salivary Gland Homogenates—Deerflies were cooled to 4 °C to decrease their activity. Under a dissecting microscope, their salivary glands were removed and placed in phosphate-buffered saline (PBS). Glands were then transferred to vials containing either 50 μl of PBS for protein purification or 500 μl of Trizol for mRNA purification. Vials containing 50 glands in PBS or 150 glands in Trizol were stored at −80 °C until use.

Preparation of Human Platelets—Venous blood was obtained from volunteers who had not ingested caffeine-containing beverages for at least 12 h or cyclooxygenase inhibitors for at least 10 days. The blood was anticoagulated with 0.1 volume of 110 mM sodium citrate and used within 1 h. Platelet-rich plasma (PRP) was prepared by centrifuging the blood at 120 × *g* for 10 min at room temperature. The upper layer (the PRP) was removed by a large-mouthed pipette and kept at room temperature. The lower layer was centrifuged at 1500 × *g* for 15 min, and the upper layer thus obtained, platelet-poor plasma, was removed and used to set the 100% baseline during the aggregation assay.

Platelet Aggregation Assays—Assays were performed using a standard nephelometric technique (9). Aggregations were monitored in a platelet aggregation profiler, model PAP-4 (Bio/Data Corp., Hatboro, PA) in which 200-μl aliquots of PRP and platelet-poor plasma were incubated at 37 °C and stirred at 1000 rpm for 2 min. Fractions of salivary gland extract or recombinant wild type protein from HPLC were incubated with PRP for 30 s, prior to the addition of ADP (Bio/Data) as agonist to a final concentration of 18 μM. Other agonists included the addition of collagen (50 μl of a 1.9 mg/ml solution) and thrombin (5 μl of an 8.9 mg/ml solution) as recommended by the supplier (Hematologic Technologies, Essex Junction, VT).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF169229.

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[1] The abbreviations used are: DFE, deerfly salivary gland extract; AE, anion exchange; bp, base pair(s); HI, hydrophobic interaction; HPLC, high pressure liquid chromatography; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PRP, platelet-rich plasma; ss, single-stranded; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-glycine.

Fibrinogen Binding: Competitive Binding Assay—Fibrinogen was iodinated using Iodobeads (Pierce) as described previously (10). ^{125}I-Fibrinogen was used at a concentration of 1.9 μM, approximately three times its K_d of 0.68 μM, to ensure 99% saturation of the receptors under the experimental conditions used (11). ^{125}I-Fibrinogen was incubated with varying concentrations, as determined by amino acid analysis, of chrysoptin purified from salivary glands. The incubation was carried out in a buffer containing 10 mM HEPES, pH 7.4, 150 mM NaCl buffer for 10 min at 25 °C. Two hundred microliters of gel-filtered platelets activated with 10 μM ADP were added, and the mixture (350 μl) was incubated at 25 °C for an additional 60 min. Platelets and bound fibrinogen were separated from unbound fibrinogen by centrifugation through an oil mixture consisting of one part no. 556 fluid AC and two parts no. 550 fluid AE (Dow Corning, Saginaw, MI), and the platelet pellet was counted using a Cap RIA 16 gamma counter (Capintec, Inc., Ramsey, NJ). Results were expressed as the ratio of the number of fibrinogen molecules bound in the presence of chrysoptin (B) to the number bound in the absence of inhibitor (B_o).

Enzyme Assays—Apyrase and adenylate kinase activities of salivary gland extracts were measured by the methods of Battastini *et al.* (12) and Hess and Derr (13) as described (8).

Purification of Chrysoptin from Salivary Glands—One thousand frozen glands in PBS were thawed on ice, vortexed for 10 s, and transferred to a 10-ml centrifuge tube with 10 μl of proteinase inhibitor mixture consisting of 0.1 mM Na-*p*-tosyl-L-arginine-methylester-HCl and 1 μg/ml each of antipain, leupeptin, pepstatin A, chymostatin, and aprotinin. The combined glands, in about 5 ml of PBS, were mixed and passed twice through a 21 gauge needle. The extracts were centrifuged at 12,000 × *g* for 2 min at 4 °C, and the supernatant was dialyzed against the anion exchange HPLC starting buffer, 20 mM Tris-HCl, pH 8.0, using a Slide-A-Lyzer 10K dialysis cassette (Pierce). The dialyzed material was aliquoted in 1-ml fractions and stored at −20 °C. All HPLC analyses were carried out at room temperature on a Smart system and monitored at 214 nm and 280 nm using a multiwavelength detector (Amersham Pharmacia Biotech).

Anion Exchange (AE)-HPLC—Dialyzed salivary gland extracts were filtered through a 0.4 μm Tuffryn filter (Gelman Sciences, Ann Arbor, MI). The extracts were injected onto an anion exchange column (Mono Q PC 1.6/5, Amersham Pharmacia Biotech), equilibrated with Buffer A (20 mM Tris-HCl, pH 8.0), and eluted under the following linear step gradient conditions, where Buffer B was 20 mM Tris-HCl, pH 8.0, 1 M NaCl: 100% Buffer A for 5 min, 50% Buffer B at 15 min, and 100% Buffer B at 20 min, maintained until 30 min, and 100% Buffer A at 35 min. The flow rate was 100 μl/min, and fractions were collected at 1-min intervals. Eluted fractions were tested for their ability to prevent platelet aggregation. Active fractions were combined and concentrated using a low protein binding Omega Macrosep concentrator 30K (Gelman Sciences), centrifuged at 5000 × *g*, and stored at −20 °C. For the recombinant protein, serum-free medium containing chrysoptin was concentrated using the Macrosep concentrator 30K, filtered through a 0.4 μm sterile filter, and purified by HPLC as described above.

Hydrophobic Interaction (HI)-HPLC—Active fractions from AE-HPLC were mixed to a 1:1 ratio with 4 M $(NH_4)_2SO_4$ and filtered through a 0.4 μm sterile filter. HI-HPLC was performed on a Phenyl Superose PC 1.6/5 column (Amersham Pharmacia Biotech) using 50 mM NaH_2PO_4, pH 7.4 (Buffer A) and 50 mM NaH_2PO_4, 2 M $(NH_4)_2SO_4$, pH 7.4 (Buffer B) at a flow rate of 50 μl/min. Following equilibration in Buffer A, samples from AE-HPLC were injected and fractionated. Fractions were collected 10 min after sample loading under the following gradient conditions: 0–100% Buffer B in 5 min, maintained 100% Buffer B until 10 min, 100–36.8% Buffer B at 35 min, maintained for 6 min, 36.8–0% Buffer B at 56 min, and 100% Buffer A for an additional 10 min. Fractions were tested for their ability to inhibit platelet aggregation induced by 18 μM ADP. Active fractions were concentrated using the Microsep concentrator 30K and stored at −20 °C for further use.

Size Exclusion HPLC—Size exclusion HPLC was used to estimate the molecular mass of the purified proteins and, in separate experiments, to evaluate the possible interaction between chrysoptin and fibrinogen under native conditions. A Superose 12 PC 3.2/30 (Amersham Pharmacia Biotech) column was used with a buffer consisting of 50 mM sodium phosphate (pH 7.0), 150 mM NaCl, at a flow rate of 50 μl/min. Twenty-five microliters of 12.5 μg of standard proteins was injected. Standard markers were purchased from Amersham Pharmacia Biotech and consisted of blue dextran (2000 kDa), bovine serum albumin (68 kDa), and ribonuclease A (13.5 kDa).

Polyacrylamide Gel Electrophoresis—Tricine SDS-polyacrylamide gel electrophoresis on 10–20% gradient gels were run using precast Tricine gels (Bio-Rad) and stained with Coomassie Brilliant Blue R250 or with the Silver Stain Plus kit (Bio-Rad) following the instructions of the manufacturer. Prestained molecular mass markers were obtained from Bio-Rad, and nonstained markers were obtained from Amersham Pharmacia Biotech.

Amino Acid Analysis and Sequencing—Amino acid analysis of AE/HI-isolated chrysoptin was performed on an Applied Biosystems 420/120 instrument following hydrolysis with HCl. N-terminal amino acid sequencing was performed on an Applied Biosystems 470A instrument at the Harvard Microchemistry Facility and yielded the first 40 amino acid residues of chrysoptin.

Preparation of ss cDNA from Deerfly Salivary Glands—RNA was isolated from 500 dissected salivary glands using the Trizol reagent method (Life Technologies, Inc.). The glands were homogenized by passage through a 25 gauge needle, and RNA was separated by chloroform extraction and isopropanol precipitation. Poly(A)+ RNA was isolated from 15 μg of total RNA using the Oligotex mRNA kit (Qiagen, Valencia, CA). The mRNA was converted into ss cDNA with reverse transcriptase (Roche Molecular Biochemicals) in conjunction with an oligo $CCCGGGT_{20}$ primer. The ss cDNA was purified by binding to a Qiagen spin column and eluting with 50 μl of Tris-EDTA, pH 8.0, buffer.

Cloning of Chrysoptin cDNA by PCR—Fig. 6*B* illustrates schematically the strategy used to clone the chrysoptin cDNA. In this figure, nucleotide 144 corresponds to the first amino acid in chrysoptin and is the reference point for the oligonucleotide primers described. One hundred nanograms of ss cDNA were used in each of the PCRs. Degenerate 24-mer PCR primers were made based on the N-terminal sequence of chrysoptin. The forward primer was called A8-1 (GCC AGC AGC GAY GAY ACC AGC GAG) and encoded amino acids 1–8. Four reverse (antisense) primers were made: A5-1 and A6-1 (TGR AAR TCR TTR ATR TGR ACR ATG and TGG AAR TCG TTR ATG TGG ACG ATG, respectively), both encoding amino acids 20–13, and B5-1 and B6-1 (TCR TCR GTY TGY TCR AAR CGR GCG and TCR TCG GTC TGY TCG AAG CGA GCG, respectively), both encoding amino acids 28–21. PCR conditions were 94 °C preheating for 5 min followed by 35 cycles of 94 °C denaturation for 1 min, 55 °C annealing for 1 min, 72 °C extension for 1 min, and postextension at 75 °C for 7 min.

In the first PCR, using primer A8-1 with primers A5-1 and B5-1 and *Taq* polymerase (Roche Molecular Biochemicals), bands of 60 and 84 bp were found on 1.5% agarose gels consistent with the sizes predicted from the N-terminal amino acid sequence. These products were purified from the gel, cloned into pCR-TOPO cloning vector (Invitrogen, Carlsbad, CA), and sequenced.

In a second PCR, A8-1 and oligo $CCCGGGT_{20}$ were used as forward and reverse primers, respectively, on the ss cDNA as template in the presence of *Taq* plus long polymerase (Stratagene, La Jolla, CA) at the following conditions: 94 °C preheating for 5 min; 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 3 min; and postextension at 72 °C for 7 min. After treating the reaction with *Taq* polymerase at 72 °C for 20 min to add a dA tail to the ends, the PCR products were run on a 1% agarose gel, and the 1700-bp band was excised, cloned into pCR-TOPO, and sequenced.

In a third PCR, directed at obtaining the 5′ end of the cDNA, oligonucleotides $CCCGGGT_{20}$ and B5-1 were used as forward and reverse primers, respectively, on the ss cDNA that was previously dA-tailed, under the following conditions: 94 °C preheating for 5 min; 35 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min; and 72 °C postextension for 20 min. The PCR products were run on a 1.5% agarose gel, and the band migrating at 250 bp was purified, cloned, and sequenced.

A fourth and final PCR was carried out to obtain the full-length chrysoptin cDNA as a single fragment. Based on the DNA sequence at the 5′ end, a forward primer with an *Eco*RI adapter, GGAAT TCT GAG TCG CGA TTT GAA ACT GTT, was made. This primer and the reverse primer, oligo $CCCGGGT_{20}$, were used on the ss cDNA template in the presence of plaque-forming unit DNA polymerase (Stratagene) under the following conditions: 94 °C preheating for 5 min; 30 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 3 min, and 72 °C postextension for 7 min. The reaction was run on a 1% agarose gel, and the band migrating at 1850 bp was excised from the gel and cloned into a pCR-blunt vector (Invitrogen). Plasmid preparations were made from two of the clones, and the DNA was sequenced in both directions by manual sequencing using T7 Sequenase (Amersham Pharmacia Biotech) as well as automated dideoxy sequencing using a Li-Cor 4000L device. The coding sequences were identical between the two clones, although several differences were noted in the 3′-untranslated region (data not shown). The sequence has been submitted to GenBank™ and has the accession number AF169229.

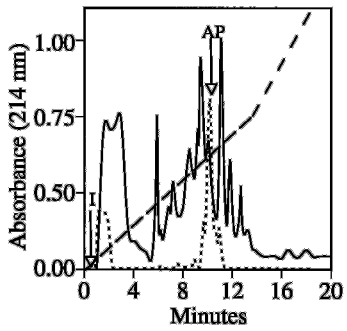

FIG. 1. **Anion exchange HPLC elution profile of the deerfly salivary gland extract.** ——, chromatogram of the crude deerfly salivary gland extract. - - - - -, rechromatography of the active fractions. – – – –, chromatography conducted as described under "Experimental Procedures" using a NaCl gradient in the elution buffer. *AP,* active peak; *I,* injection.

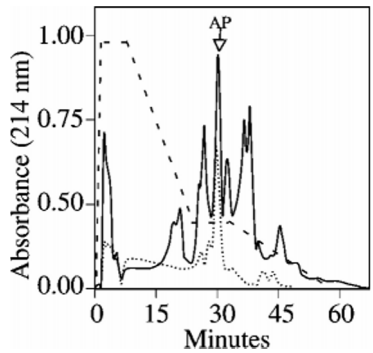

FIG. 2. **Hydrophobic interaction HPLC elution profile of the deerfly salivary gland extract.** ——, profile of the total extract. - - - - -, chromatogram of the active fractions from anion exchange HPLC. – – – –, chromatography conducted as described under "Experimental Procedures" using an $(NH_4)_2SO_4$ gradient in the elution buffer. *AP,* active peak; *I,* injection.

Expression of Chrysoptin in Sf21 Cells—Chrysoptin cDNA made in the final PCR was excised from pCR-blunt vector with *Eco*RI and cloned into the *Eco*RI site of pBacPAK9 vector (CLONTECH, Palo Alto, CA). Pure plasmid DNA was made and transfected into Sf21 cells as described in the CLONTECH protocol. Seventy-two hours after transfection, the medium containing the chrysoptin recombinant virus was harvested and used to infect fresh Sf21 cells. For the production of recombinant protein, fresh Sf21 cells were grown in serum-free medium and infected with the recombinant virus for 48 h. The medium was harvested and concentrated, and the protein was purified by AE/HI-HPLC.

Generation of Chrysoptin Mutants—Several alanine substitution mutations were introduced into the chrysoptin coding sequence using the Quick Change mutagenesis kit (Stratagene). Sequencing of the resultant DNAs was performed to confirm the mutations. These mutants were expressed in Sf21 cells and tested for functional activity. Activity was noted in the culture supernatants. As a result, HPLC purification of the mutants was not performed.

RESULTS

Isolation and N-terminal Sequence of Chrysoptin—An HPLC approach to the isolation of chrysoptin was undertaken. A variety of separation modalities were evaluated, including anion exchange, reverse phase, and hydrophobic interaction chromatography. Biological activity was determined by inhibition of ADP-induced platelet aggregation and was not retained following reverse phase HPLC. This observation may have resulted from the relatively harsh effects of acetonitrile and trifluoroacetic acid on the tertiary structure of chrysoptin. The milder buffer conditions encountered with AE and HI resulted in the retention of biological activity following chromatography. Thus, AE followed by HI was selected for the isolation of the active principle.

AE-HPLC was used to initially separate salivary gland extracts for the native protein and culture medium for the recombinant protein. Fractions 9 and 10, eluting at 9 and 10 min, respectively (Fig. 1), were the only ones found to inhibit ADP-induced platelet aggregation. Because multiple components could be hidden within this peak, the active fractions were applied to a HI column and yielded a single active peak (Fig. 2). Size exclusion HPLC revealed that the activity migrated with a native molecular mass of approximately 65 kDa (Fig. 3), similar to the results on gel electrophoresis (Figs. 4 and 5). Gel electrophoresis further revealed that the combined HPLC approaches resulted in preparation of an essentially homogenous single active component. This protein was named chrysoptin, consistent with the genus name, *Chrysops*. N-terminal amino acid sequencing revealed the following 40 residues: AS(S)D(D)SREFPLSIVHINDFHARFEQTDELG(G)E (K/C)KPTAK(K/C)V. Residues in parentheses were not definitive, and for the last two such residues, the amino acid following the slash is predicted from the cDNA sequence.

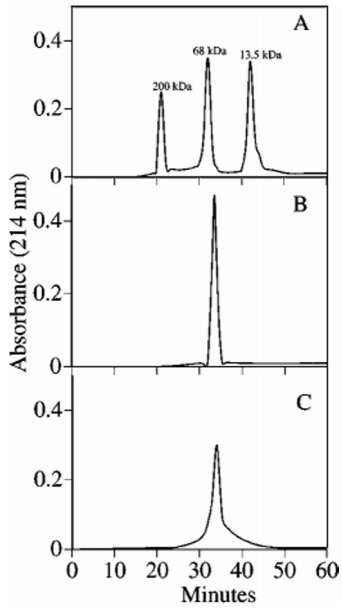

FIG. 3. **Size exclusion HPLC elution profiles of purified proteins.** Chromatography was conducted as described under "Experimental Procedures." In *A,* molecular mass standards from *left* to *right* are as follows: 200, 68, and 13.5 kDa. *B,* chrysoptin purified from DFE. *C,* recombinant chrysoptin purified from the supernatant of Sf21 cells grown in serum-free medium.

Cloning of the cDNA Encoding Chrysoptin—The N-terminal sequence of purified natural chrysoptin was used as a guide to the synthesis of degenerate and oligo-dT oligonucleotides for use in a series of PCRs that yielded the full-length cDNA encoding chrysoptin (Fig. 6*B*). The approach used is detailed under "Experimental Procedures" and in Fig. 6*B*. Forward and reverse degenerate oligonucleotide primers that corresponded to sequences within the N-terminal 40 residues were synthesized and used in two PCRs to amplify a small portion near the 5′ end of the chrysoptin cDNA. Sequencing of the two amplified bands yielded sequence information that corresponded to the amino acid residues between the degenerate primers. New primers with the exact nucleotide sequence could have been synthesized and used to amplify the cDNA. However, use of the already-synthesized degenerate forward primer was successful, in conjunction with the oligo-dT primer, in the amplification of the 1700 nucleotides corresponding to the major (3′) portion of the chrysoptin cDNA. The dA-tailed cDNA was used in another PCR with the oligo-dT primer and a reverse degenerate primer near the N terminus of chrysoptin. The 250-bp

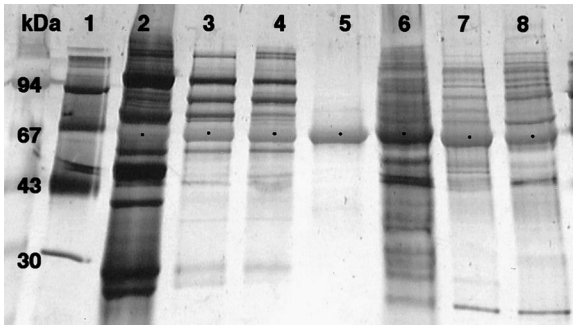

FIG. 4. **Polyacrylamide gel electrophoresis of total deerfly salivary gland and recombinant proteins identified by silver staining.** *Lane 1,* deerfly salivary gland crude extract; *lanes 2* and *3,* active fractions 9 and 10 after AE-HPLC of gland extract; *lane 4,* natural chrysoptin purified following HI-HPLC; *lane 5,* crude recombinant extracts; *lanes 6* and *7,* active fractions 9 and 10 of the recombinant protein after AE-HPLC. Molecular mass standards were as indicated (*MW*).

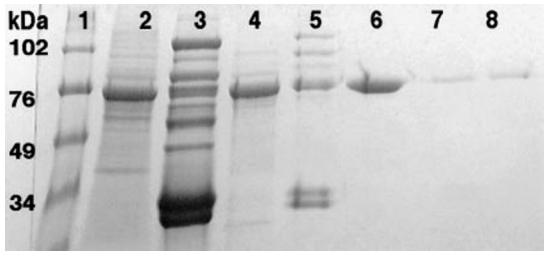

FIG. 5. **Purification of natural and recombinant chrysoptin from HPLC as identified by SDS-polyacrylamide gel electrophoresis identified by Coomassie Blue staining.** *Lane 1,* prestained low range of molecular mass standard; *lane 2,* supernatant medium of Sf21 cells expressing recombinant chrysoptin; *lane 3,* total gland extracts; *lanes 4* and *5,* active fractions of recombinant and natural fly proteins after AE-HPLC; *lane 6,* active recombinant protein peak on HI-HPLC; *lanes 7* and *8,* HI-HPLC peaks of wild type fly protein active fractions 9 and 10 from AE-HPLC.

band obtained was sequenced, and an exact forward primer near the 5′ end was synthesized, with the addition of an *Eco*RI linker, and used with the oligo-dT primer in the presence of a high fidelity polymerase to amplify salivary gland ss cDNA. An 1850-bp PCR product for the cDNA encoding the complete coding portion of chrysoptin was obtained and sequenced in both directions by both automatic and manual sequencing methods to ensure the fidelity of resulting sequence. The two techniques yielded identical sequences.

PCR cloning resulted in two cDNAs that differed only at the 3′ end, one cDNA being longer by 27 bases, indicating that there may be more than one allele of chrysoptin among the deerfly population that was collected.

Comparison of the deduced amino acid sequence to that of proteins in GenBank™ did not reveal any homology (Fig. 6*A*). The initiating methionine codon (ATG) is at 59 bp in the cDNA. An open reading frame of 1662 bp continues to the stop codon (TAA) at bp position 1721. From the amino acid and DNA sequence analysis, it can be deduced that the protein is secreted with a signal peptide of 25 amino acids encoded between nucleotides 59 and 144. Two putative polyadenylation signals (AATTTA) starting at positions 1759 and 1792 bp were identified. The commonly occurring AATAAA poly(A) signal, present between bp 1802 and 1807, may also serve as a polyadenylation signal, although it is close to the 3′ end. Five putative *N*-linked glycosylation sites are present between bp 374 and 382, 617 and 625, 671 and 679, 941 and 949, and 1274 and 1282. In contrast to other fibrinogen receptor antagonists, no RGD sites are found in the cDNA, although an RGN site is present at bp 1268–1276.

Expression of Recombinant Chrysoptin in Baculovirus—Expression of chrysoptin was undertaken in order to confirm, by measuring biological activity, that the correct protein and corresponding cDNA had been isolated from deerfly salivary gland extracts. The natural material can only be obtained during the short summer season of the deerfly, as this insect has not been reared in the laboratory. The availability of recombinant protein would eliminate the difficulties in obtaining limited quantities of natural material for further studies, such as the structure-function relationship of chrysoptin. Furthermore, mutations in the chrysoptin sequence, induced to determine functional domains, can only be done on recombinant material.

It was reasonable to express the chrysoptin in baculovirus, as it is an insect protein. It was expressed using full-length cDNA including its signal peptide sequence. Recombinant chrysoptin was isolated from Sf21 culture supernatants, purified using HPLC, and examined by gel electrophoresis (Fig. 5). Inhibition of ADP-induced platelet aggregation by recombinant chrysoptin is demonstrated in Fig. 7*A*. Recombinant chrysoptin was also effective at inhibiting collagen and thrombin-induced platelet aggregation (not shown).

Platelet Inhibitory Activity of Chrysoptin Mutants—Several alanine mutations were introduced into the coding sequence of chrysoptin to determine the functional relevance of the RGN and putative *N*-linked glycosylation sites. Mutation of either the Arg or Asn residue of the RGN sequence did not diminish activity, as measured by inhibition of platelet aggregation. Activity of the mutant containing the AGN sequence is shown in Fig. 7*B*. Likewise, individual mutations of the other putative *N*-linked glycosylation sites did not diminish activity. In contrast, converting all of the asparagines in the putative glycosylation sites to alanines led to an inactive molecule. This latter result is consistent with our observation that expression of chrysoptin in *Escherichia coli* yielded an inactive protein, although other possibilities exist, as noted under "Discussion." Note that the Asn of the RGN tripeptide is also one of the putative *N*-linked glycosylation sites.

Inhibition of Fibrinogen Binding by Chrysoptin—Because the binding of fibrinogen to its receptor on the platelet surface is essential for aggregation responses to all agonists, the effect of chrysoptin on fibrinogen binding was examined. Purified natural chrysoptin inhibited ^{125}I-fibrinogen binding to activated platelets in a concentration-dependent manner with an IC_{50} of 95 pmol (Fig. 8). It is likely, but not proven, that all of the platelet inhibitory activity in DFE is accounted for by chrysoptin.

Chrysoptin and Fibrinogen Do Not Interact with Each Other under Native Conditions—The results of the above experiment suggest that chrysoptin interacts directly with the fibrinogen receptor rather than the fibrinogen molecule itself. To further address the possibility that chrysoptin interacts directly with fibrinogen, these two entities were examined via gel filtration both separately and after co-incubation in the same citrate buffer used for aggregation experiments (Fig. 9). The observation that the fibrinogen peak in Fig. 9*C* does not shift to the left and that the chrysoptin peak does not diminish in size suggests that chrysoptin and fibrinogen do not interact under conditions that allow chrysoptin to interact with platelets. This result is consistent with chrysoptin interacting directly with the fibrinogen receptor.

Apyrase and Adenylate Kinase Assay—Because apyrase activity has been found in arthropod salivary gland extracts from ticks (3), mosquitoes (4), blood-sucking bugs (5), and tse-tse flies (6), chrysoptin was examined for such activity. Although the assay was sensitive enough to detect quantities of apyrase below the threshold for antiplatelet activity, negligible apyrase

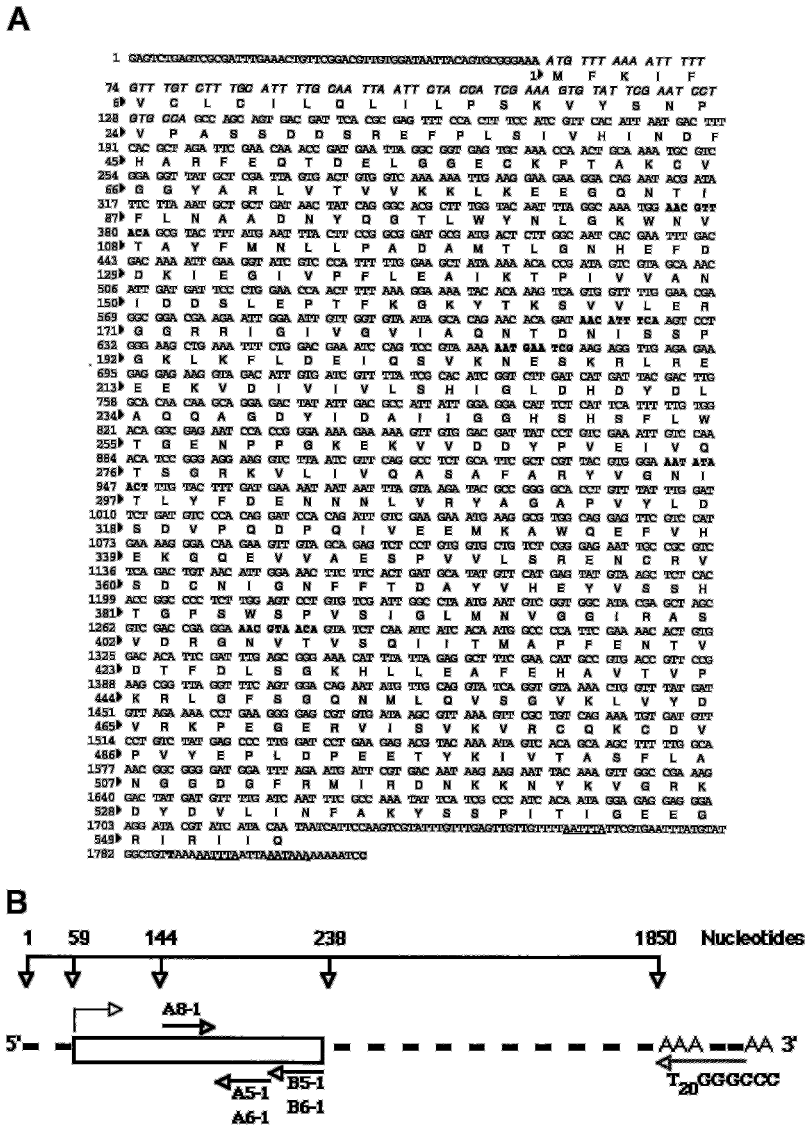

FIG. 6. *A*, complete nucleotides and predicted amino acid sequence of chrysoptin. The initiating codon starts at base 59 in the sequence. The 25-amino acid leader peptide is in *italics*. The putative glycosylation sites are in *boldface*. The poly(A) sites are *underlined*. The 3′ end nucleotides in the two clones are indicated in *boldface*. This sequence has the GenBank™ accession number AF169229. *B*, cloning strategy for obtaining the full-length cDNA encoding chrysoptin. Primers are as noted under "Experimental Procedures." Nucleotide 59 is the adenine of the initiating ATG.

activity was detected in aliquots corresponding to twice the IC_{50} of chrysoptin. In addition, aliquots of chrysoptin did not contain adenylate kinase activity (data not shown).

DISCUSSION

Platelets have an important role in the pathophysiology of unstable angina pectoris, acute myocardial infarction, transient ischemic attack, and stroke, which together make up the most common causes of mortality in the U.S. During the past 15 years, an array of agents directed at various steps of platelet activation and aggregation have been developed and tested in basic and clinical trials. These agents include RGD peptides and derivatives, monoclonal antibodies to the IIb/IIIa receptor, von Willebrand factor antagonists, serotonin receptor antagonists, thromboxane A2 receptor antagonists, prostacyclin and analogues, synthetic thrombin inhibitors, apyrases, and ticlopidine and clopidogrel (14, 15). Many of these antiplatelet agents have shown promise in basic and clinical trials, and several are now approved for clinical use, but their utility has often been hampered by systemic side effects, including hypotension and prolongation of bleeding times (16, 17). Consequently, there is a need for the development of more potent and more selective antiplatelet agents.

The data presented here demonstrate that salivary gland lysates of the deerfly contain chrysoptin, a potent inhibitor of platelet aggregation. Chrysoptin is encoded by a cDNA with a conventional leader sequence and at least five potential glycosylation sites. The cDNA encodes a protein with a molecular mass of 58 kDa that is apparently posttranslationally modified to a mature protein of 65 kDa. It appears that glycosylation is necessary for activity, although which of the five putative sites are critical is not known. Alanine substitution mutants introduced individually at the five putative *N*-linked glycosylation sites maintained the capacity to inhibit ADP-induced platelet aggregation, but a chrysoptin in which all five sites were mutated was inactive, consistent with the lack of expression of a functional molecule in *E. coli*. Caution must be exercised in interpreting these results, as additional possibilities exist. For example, it is possible that the expression in *E. coli* of an inactive protein could be due to improper folding of chrysoptin, which contains six cysteine residues, rather than a lack of glycosylation. It is also possible that appropriate glycosylation is needed in order for chrysoptin to fold properly, as has been reported for other proteins (18). It would be reasonable to evaluate the role of glycosylation in receptor blockade by chrysoptin by expressing the protein in the presence of glycosylation inhibitors or by deglycosylation of the recombinant protein. Activity of the reduced protein could also be determined to examine the contribution of the disulfide bonds to function. No RGD sites are present in the cDNA, although an RGN site is present. Substitution of either the Arg or Asn with alanine did

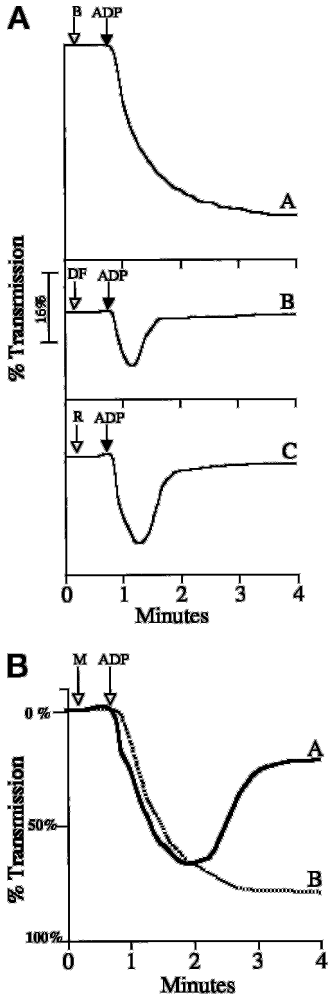

FIG. 7. ***In vitro* inhibition of ADP-induced aggregation of normal human platelet-rich plasma by the native deerfly and recombinant proteins (*A*) and mutant chrysoptins (*B*).** *A,* the *open arrow* indicates the addition of the native deerfly protein (*DF*), recombinant protein (*R*), or buffer (*B*). The *filled arrow* indicates the addition of ADP. *Curve A* shows the normal aggregation curve of PRP in absence of the inhibitor, *curve B* shows the addition of purified native fly protein, and *curve C* shows the addition of the purified recombinant protein. *B, arrow M* indicates the addition of mutant chrysoptins, and *arrow ADP* indicates addition of ADP. *Curve A* demonstrates functional activity of the mutant in which the RGN site has been converted to AGN. *Curve B* demonstrates the absence of activity of the mutant in which all five potential *N*-linked glycosylation sites have been mutated to Ala.

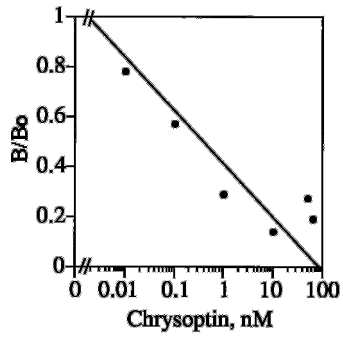

FIG. 8. **Effect of chrysoptin on ^{125}I-fibrinogen binding to human platelets activated with 10 μM ADP.** Results are expressed as the ratio of the number of fibrinogen molecules bound in the presence of chrysoptin (*B*) to the number bound in the absence of inhibitor (B_o) These results are representative of three experiments.

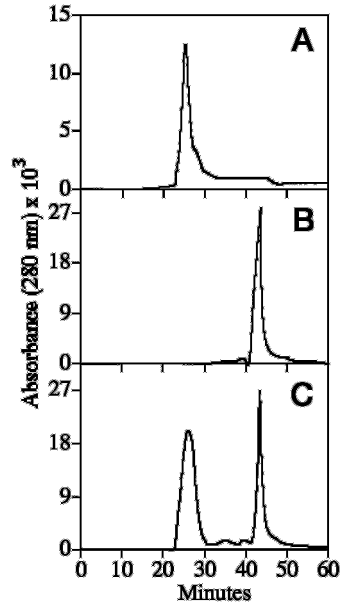

FIG. 9. **Chrysoptin and fibrinogen do not interact under native conditions.** Fibrinogen (4.1 μg) (*A*) and HPLC-purified recombinant chrysoptin (2.7 μg) (*B*) in citrate buffer were analyzed by size exclusion chromatography. To determine whether these two entities interacted with each other under native conditions, 6.0 μg of fibrinogen were incubated with 1.2 μg of HPLC-purified recombinant chrysoptin for 10 min at 37 °C and separated (*C*).

not diminish activity. This result indicates that neither a conventional RGD nor related RGN or AGN sites are needed for activity of recombinant chrysoptin. Note in addition that this particular Asn also belongs to one of the putative glycosylation sites that was mutated without loss of function.

There does not appear to be a relationship between chrysoptin and the disintegrins, a family of integrin inhibitory proteins from viper venoms (19). Both chrysoptin and the disintegrins are potent platelet inhibitors and inhibit fibrinogen binding. However, chrysoptin has a molecular size of approximately 65 kDa, whereas the disintegrins range in size from 5.4 to 9 kDa. In addition, chrysoptin (IC_{50} = 95 pmol) is 300 times more active than trigramin, a representative disintegrin (IC_{50} = 30 nmol), at blocking fibrinogen binding (20). Explanations for the potency of chrysoptin include the possibility that it binds to multiple sites on the fibrinogen receptor or that it may alter the conformation of the receptor, thereby preventing fibrinogen binding to either RGD or non-RGD sites, such as the γ chain dodecapeptide (21). Studies investigating how the inhibition of fibrinogen binding by chrysoptin is mediated should help to elucidate the normal mechanisms of platelet function.

Chrysoptin is one of the most potent inhibitor of fibrinogen binding known. Although this study is the first to report the isolation of a platelet aggregation inhibitor from an arthropod, other potent antihemostatic activities have been described in arthropods. These include vasodilators from the sand fly *Lutzomyia longipalpis* (22) and the black fly *Simulium vittatum* (23) and inhibitors of blood coagulation factor Xa from the soft tick *Ornithodora moubata* (24) and the black fly *Simulium vittatum* (25). An overriding concept in these studies is that an organism (such as an arthropod) can be examined for a particular function (such as blood-feeding), leading to the discovery of novel proteins and genes. Such proteins and genes will provide new tools with which to study hematologic and cardiovascular pharmacology and may lead to new therapeutic agents.

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