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Isolation from Opossum Serum of a Metalloproteinase Inhibitor Homologous to Human α1B-Glycoprotein[†,‡]

Joseph J. Catanese and Lawrence F. Kress*
Molecular and Cellular Biology Department, Roswell Park Cancer Institute, Buffalo, New York 14263
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ABSTRACT: Fractionation of opossum (*Didelphis virginiana*) serum with $(NH_4)_2SO_4$, followed by chromatography on DEAE-Sepharose, phenyl-Sepharose, and Mono Q HR 5/5, has resulted in the isolation in homogeneous condition of a metalloproteinase inhibitor designated oprin (opossum proteinase inhibitor). Oprin is a single-chain glycoprotein (26% carbohydrate) with an estimated M_r = 52000, pI = 3.5, and E(1%/1 cm) = 11. Oprin inhibited snake venom metalloproteinases, but showed no activity on venom serine proteinases or on bacterial metalloproteinases. Incubation of *Crotalus atrox* α-proteinase (EC 3.4.24.1) with oprin, and analysis of the reaction products by chromatography on Mono Q HR 5/5 and by electrophoresis under nondenaturing conditions, indicated formation of an inactive enzyme/inhibitor complex. The complex dissociated during SDS/polyacrylamide gel electrophoresis. An opossum liver cDNA library was immunoscreened, and clones containing cDNA encoding for part of the open reading frame for oprin were isolated. The cDNA inserts contained nucleotide sequences corresponding to two internal amino acid sequences of oprin which had been separately determined by protein sequence analysis. Protein database screening using a 211 amino acid sequence deduced from one of the cDNA inserts showed no significant homology to known proteinase inhibitors. There was, however, a 36% identity with human α1B-glycoprotein, a plasma protein of unknown function related to the immunoglobulin supergene family. In addition, the amino-terminal sequence of oprin showed 46% identity with human α1B-glycoprotein in a 26 amino acid residue overlap. Comparisons of sequence, molecular weight, and disulfide content of the two proteins suggest that oprin contains 4 of the 5 domains found in human α1B-G. The presence of oprin in opossum serum may partially account for the resistance of this marsupial to those localized effects of rattlesnake envenomation which are caused by venom metalloproteinases.

The major manifestations of rattlesnake envenomation in most mammalian victims are localized hemorrhage, tissue necrosis, edema, and systemic coagulation defects, all of which result in part from the direct or indirect action of metalloproteinases and serine proteinases in the venom, (Ohsaka, 1979). However, some mammals exhibit a resistance or decreased sensitivity to the localized and lethal effects of rattlesnake and other poisonous snake venoms (Ovadia & Kochva, 1977; De Wit, 1982; Tomihara et al., 1987), and this phenomenon has been studied extensively in the North American opossum (*Didelphis virginiana*).

Opossums resisted the lethal effects of rattlesnake venom and showed little or no localized hemorrhage, edema, or tissue

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necrosis following injections of venom in amounts 5–60 times the dose known to be lethal to susceptible mammals (Kilmon, 1976; Werner & Vick, 1977). Studies in vitro have shown that the proteolytic and hemorrhagic activity of *Crotalus atrox* (western diamondback rattlesnake) venom were blocked in the presence of opossum serum (Huang & Perez, 1980), and the lethality of venom injected into mice was reduced when the venom had been mixed with opossum serum (Werner & Faith, 1978). The mechanism of neutralization of venom proteolytic activity by opossum serum was not determined in these studies.

A primary natural defense mechanism against the influx of venom metalloproteinases and any endogenous proteinases which might be activated by them is the victim's plasma proteinase inhibitor system (Heimburger, 1975). This system typically functions by rapid or progressive formation of stoichiometric complexes between the inhibitor and the proteinase involved, and the complexes are then quickly cleared from the circulation (Imber & Pizzo, 1981). The fact that hemorrhage following snakebite is caused by metalloproteinases in the venom (Bjarnason & Tu, 1978; Kurecki & Kress, 1985b) suggested that serum from animals resistant to the hemorrhagic effects of venom might contain metalloproteinase inhibitor(s) unlike those present in human or other mammalian sera.

This report describes the purification and partial characterization of oprin (opossum proteinase inhibitor), a component of opossum serum which inactivates venom metalloproteinases and some venom hemorrhagic toxins. Evidence is presented that the inactivation occurs via inhibitor/enzyme complex formation and that the sequence of oprin shows homology to human α1B-glycoprotein.[1]

Experimental Procedures

Materials. Opossum (*Didelphis virginiana*) serum was obtained from Research Biogenics, Inc., Elgin, TX; opossum liver was from Cocalico Biologicals, Reamstown, PA; lyophilized snake venoms were from Miami Serpentarium (*C. atrox*, *Crotalus adamanteus*, *Crotalus basiliscus*) or Sigma (*Bitis arietans*, *Vipera russelli*); DEAE-Sepharose, phenyl-Sepharose, Mono Q HR 5/5, and calibration standards for isoelectric focusing were from Pharmacia; molecular weight standards and electrophoresis grade purity reagents for PAGE[2] were from Bio-Rad; bovine trypsin, α-chymotrypsin, and porcine elastase were from Worthington; clostridiopeptidase A and thermolysin were from Boehringer Mannheim. *Pseudomonas aeruginosa* protease and elastase were kindly provided by Dr. K. Morihara, Kyoto, Japan. *C. atrox* proteinases were prepared as follows: α-proteinase and HT-b according to Kruzel and Kress (1985); β- and γ-proteinases by slight modifications of Pfleiderer and Sumyk (1961); HT-a according to Bjarnason and Tu (1978). *C. adamanteus* proteinase II and proteinase H were prepared as described (Kurecki et al., 1978; Kurecki & Kress, 1985b). BAEE, BTEE, and Congo red elastin were from Sigma; casein (Hammarsten) was from BDH Chemicals; and hide powder azure was obtained from Calbiochem or prepared in this laboratory (Rinderknecht et al., 1968). Dialysis tubing (Spectrapor) from Fisher was used according to manufacturer's instructions.

Assay of Venom Proteolytic Activity. Crude *C. atrox* venom (10 mg) was dissolved in 0.05 M Tris-HCl–2 mM $CaCl_2$, pH 8.0, to give a concentration of approximately 5 A_{280} units[3] per milliliter and stored in 1.0-mL aliquots at −20 °C until use. This was the standard venom proteinase preparation used to assay the inhibitory activity of opossum serum and of column fractions. Proteolytic activity on hide powder azure was measured by incubating the *C. atrox* proteinase preparation (1.5 μg) in 65 μL of 0.05 M Tris-HCl–2 mM $CaCl_2$, pH 8.0, for 5 min. Aliquots of 50 μL were then added to 3.95 mL of buffer containing 15 mg of hide powder azure, reacted for 1 h at 37 °C, and filtered (Whatman No. 1), and A_{595} was read against a substrate blank. The reaction was linear with enzyme concentration over the range 0.1–0.3 A_{595}. The venom proteinase preparation showed no loss of activity after 6 months storage and several freeze/thaw cycles. Under the assay conditions used, approximately 94% of the proteolytic activity was due to *C. atrox* metalloproteinases, as judged by the decrease in substrate digestion by EDTA-treated venom.

One unit of proteolytic activity gave an increase in absorbance at 595 nm of 1.0 per minute. The specific activity (units of proteolytic activity per A_{280} unit) of the crude venom preparation was approximately 150.

Opossum Serum Inhibitory Activity. The *C. atrox* proteinase preparation was preincubated alone or with inhibitor material (opossum serum or column fractions) for 5 min at 25 °C, and aliquots were assayed for residual proteolytic activity as described above. Oprin specific activity was defined as units of proteolytic activity inhibited per A_{280} unit of inhibitor material. Similarly, the opossum α_1-proteinase inhibitor and α_1-antichymotrypsin activity of column fractions was determined by incubating the fractions with trypsin or chymotrypsin and assaying for residual activity on BAEE (Schwert & Takenaka, 1955) or BTEE (Hummel, 1959), respectively.

Other Proteolytic Enzyme Assays. The inhibitory activity of oprin on the following proteolytic enzymes was determined according to the published procedures using the substrates indicated: *C. atrox* α-, β-, and γ-proteinases (Kruzel & Kress, 1985) and *C. adamanteus* proteinase II (Kurecki et al., 1978) (hide powder azure); porcine elastase (Shotton, 1970) (Congo red elastin); *P. aeruginosa* protease and elastase (Kurecki & Kress, 1985a) (casein and FAGLA, respectively); clostridiopeptidase A (Wünsch & Heidrich, 1963) (PZ-Pro-Leu-Gly-Pro-Arg); thermolysin (Feder, 1968) (FAGLA); carboxypeptidases A and B (Folk & Schirmir, 1963; Folk et al., 1960) (hippuryl-L-Phe and hippuryl-L-Arg)

Determination of Hemorrhagic Activity and Inhibition of Hemorrhagic Activity. Hemorrhagic activity was determined as previously described (Bjarnason & Tu, 1978). The crude venoms or purified venom hemorrhagic factor to be tested was dissolved in 0.9% saline, and aliquots (100 μL) were injected subcutaneously into the backs of Swiss white mice (HalCR). The animals were sacrificed after 5 h and the hemorrhagic responses photographed. The amounts of venom or hemor-

[1] Nomenclature: The name oprin ("opossum proteinase inhibitor") was initially coined to distinguish this particular inhibitor from other inhibitors which were purified from opossum serum in this laboratory. In view of the homology between oprin and human α1B-G and the fact that α1B-G has been reported in many other mammalian sera (Stratil et al., 1988; Patterson et al., 1991), the name opossum α1B-glycoprotein is more appropriate. In this paper, the terms oprin and opossum α1B-glycoprotein are used interchangeably.

[2] Abbreviations: SDS, sodium dodecyl sulfate; PTH, phenylthiohydantoin; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; BTEE, benzoyl-L-tyrosine ethyl ester; BAEE, benzoyl-L-arginine ethyl ester; FAGLA, 3-(2-furylacryloyl)-Gly-Leu amide; PAGE, polyacrylamide gel electrophoresis; HT-a, *Crotalus atrox* hemorrhagic toxin a; HT-b, *C. atrox* hemorrhagic toxin b; oprin, opossum proteinase inhibitor; α1B-G, α1B-glycoprotein.

[3] An A_{280} unit is that amount of protein which if dissolved in 1 mL and read in a 1-cm light path at 280 nm will give an absorbance of 1.

rhagic factor used in these assays caused development of a 1–1.5-cm hemorrhagic spot under the assay conditions. No attempts were made to quantitate this activity. Inhibition of hemorrhagic activity was determined by preincubating the venoms or hemorrhagic factors with varying amounts of opossum serum, column fractions, or pure oprin, and assaying aliquots for partial or complete inhibition of the hemorrhagic response.

Physical and Chemical Properties of Oprin. Molecular weight was estimated by vertical slab SDS/PAGE (Weber & Osborn, 1969) using 6% and 7.5% polyacrylamide concentrations. Samples were also run using 12.5% gels (Laemmli, 1970). Gels were stained overnight in 0.125% Coomassie Blue R-250 and then diffusion destained in water–methanol–acetic acid (5:4:1). Molecular weights were then estimated from the relative electrophoretic mobilities of the inhibitor compared to standards of known molecular weight. Following SDS/PAGE, glycoproteins were visualized by use of the periodic acid–Schiff reagent (Segrest & Jackson, 1972). The isoelectric point of oprin was determined by electrofocusing using an LKB Multiphor and thin-layer polyacrylamide gels prepared according to the manufacturer's instructions (Winter et al., 1977). Amino acid analyses (Moore & Stein, 1963) were performed on a Dionex D-400 analyzer on samples that had been hydrolyzed for 24, 48 and 72 h. Half-cystine was determined as cysteic acid (Hirs, 1956) using performic acid oxidized samples hydrolyzed for 24 h. Oprin samples were dialyzed against H_2O, and tryptophan was determined spectrophotometrically in the presence of 0.1 M NaOH (Goodwin & Morton, 1946). Sulfhydryl content was determined using Ellmann's reagent (Riddles et al., 1983) on native and denatured oprin. *N*-Acetylneuraminic acid content was determined using the thiobarbituric acid method (Cassidy et al., 1966), and total neutral sugar was determined by the phenol–sulfuric acid method (Hirs, 1967). $E(1\%/1\ cm)$ for oprin was determined on samples which had been dialyzed against H_2O for 72 h, lyophilized for 48 h in tared vials, and weighed immediately on a Cahn Model 4100 Electrobalance. No correction was made for possible moisture gain.

Temperature stability was determined by exposing solutions of oprin (3.3 mg/mL in 0.02 M CHES, pH 9.5) for 15 min to temperatures ranging from 40 to 70 °C, cooling to 25 °C, and assaying for oprin activity. Control activity was that of an oprin solution kept at 25 °C. pH stability was determined by incubating oprin solutions (3.3 mg/mL) for 72 h at 4 °C in the following buffers: 0.05 M sodium phosphate, pH 11.5; 0.05 M CAPS, pH 10.5; 0.05 M CHES, pH 9.5; 0.05 M TAPS, pH 8.5; 0.05 M HEPES, pH 7.5; 0.05 M Bis-Tris, pH 6.5; 0.05 M succinate, pH 5.5; 0.05 M acetate, pH 4.5; 0.05 M formate, pH 3.5; and 0.05 M sodium phosphate, pH 2.5. All solutions were then adjusted to pH 8.0 by dialysis (48 h) against 0.05 M Tris-HCl, pH 8.0, and assayed for oprin activity. Control activity was that of an oprin solution kept at pH 9.5.

Amino-Terminal and Internal Peptide Sequence Determination. The inhibitor (1 nmol) was dialyzed for 96 h against H_2O, lyophilized, and redissolved in H_2O. Aliquots (330 pmol) were sequenced on an Applied Biosystems 470A gas-phase sequencer equipped with an automated on-line HPLC analysis system (Applied Biosystems Model 120A PTH analyzer) using the standard 03RPTH program. Residues were detected as the PTH derivatives by gradient elution from an octadecylsilyl column (C18 PTH) using tetrahydrofuran–sodium acetate and acetonitrile–DMPTU according to the manufacturer's instructions. No contaminating sequences were detected, and a repetitive yield of 92% was calculated on the basis of PTH-Leu recovered in cycles 1 and 10.

Oprin (360 μg) was incubated with trypsin (sequencing grade, Boehringer Mannheim) at a 1:20 (w/w) ratio or with endoproteinase Glu-C (sequencing grade, Boehringer Mannheim) at a 1:5 (w/w) ratio in 0.05 M Tris-HCl, pH 8.0, containing 0.002 M $CaCl_2$ for 1 h at room temperature. Oprin samples without proteinase were included as amino-terminal sequence controls. Electrophoresis and electrophoretic transfer were performed according to Moos et al. (1988). Incubations were applied to a 15% SDS slab gel at 60 μg of oprin per lane and electrophoresed at 20 mA for the first 30 min, followed by 35 mA until the bromophenol blue tracking dye reached the bottom of the gel. Samples were then transfered to PVDF membranes (Immobilon-P, Millipore) for 1 h at 70 Volts and stained with Coomassie R-250 (Bio-Rad), and peptide bands were cut out and stored at −20 °C prior to sequencing. Five or six PVDF bands of each internal peptide were used for one sequence determination. The bands were placed on a Polybrene preconditioned disk, stained side up, in order to maximize initial and repetitive yield (Simpson et al., 1989). Sequence determinations were performed as described above.

Complex Formation between Oprin and C. atrox α-Proteinase. Oprin (0.33 mg in 0.02 M Tris-HCl, pH 7.6) was incubated for 5 min at 25 °C with *C. atrox* α-proteinase (0.083 mg in the same buffer) in a total volume of 0.7 mL. This gave a 2:1 molar excess of oprin. The mixture was then charged onto a 0.5 × 5 cm column of Mono Q HR 5/5 and eluted using the Pharmacia FPLC system. Control samples of oprin and proteinase were also run under identical conditions. The protein peaks from the columns were pooled, concentrated, analyzed electrophoretically, and assayed for proteolytic activity on hide powder azure and for oprin inhibitory activity.

cDNA Cloning. An opossum liver cDNA library in λgt11 was custom synthesized by Clontech Laboratories. Monospecific rabbit antiserum to oprin was treated with *Escherichia coli* extract (Promega) to reduce background color development. The library was plated at a density of approximately 15 000 plaque-forming units per 150-mm LB plate and incubated at 42 °C for 3.5 h. Plates were then overlaid with dry nitrocellulose filters (BA 85, Schleicher & Schuell) previously saturated with 10 mM isopropyl thio-β-D-galactoside and incubated at 37 °C for 3.5 h. Filters were then screened using the Protoblot Immunoscreening System (Promega). Immunopositive plaques were picked, replated, and rescreened until pure. Positive clones were used to prepare 100-mL liquid lysates in LB broth (Quertermous, 1989). Recombinant λgt11 DNA was purified using Lambda-sorb phage absorbent (Promega), followed by phenol–$CHCl_3$ extraction and ethanol precipitation (Treco, 1989).

PCR. Recombinant inserts from the purified λgt11 DNA were amplified by the polymerase chain reaction in a Perkin-Elmer Cetus DNA Thermal Cycler using the Geneamp kit (Cetus) and λgt11 forward and reverse primers (24-mers) from New England Biolabs. dNTP's [200 μM (final)] and 0.2 μM (final) each primer were used in the amplification reaction. After an initial denaturation of 1.5 min at 94 °C, 40 cycles of the following cycler program were run: 1 min at 94 °C, 1 min at 60 °C, and 2 min at 72 °C. A final elongation was performed at 72 °C for 7 min. Double-stranded PCR product was electrophoresed on a 1.2% Seakem GTG agarose (FMC) minigel in TBE buffer, excised, and purified using the Geneclean II kit (Bio 101).

DNA Sequencing. Double-stranded DNA sequencing was performed on the amplified cDNA inserts by the dideoxy chain

Table I: Summary of Oprin Purification[a]

	total protein (A_{280})	total inhibitor (units)	specific activity (units/A_{280})	yield (%)	purification factor
opossum serum (30 mL)	2058	52 300	25	100	1
45–80% $(NH_4)_2SO_4$ ppt	729	42 300	58	81	2.3
DEAE-Sepharose	156	19 500	125	37	4.9
phenyl-Sepharose	74	14 900	201	28	7.9
Mono Q FPLC	30[b]	8 730	291	17	11.5

[a] Specific activity of opossum serum was not corrected for other metalloproteinase inhibitor(s) subsequently removed on DEAE- and phenyl-Sepharose [b] This corresponds to 27.3 mg of protein using the $E(1\%/1\text{ cm}) = 11.0$ determined for pure oprin.

termination method (Sanger et al., 1977) using sequenase (USB) according to Casanova et al. (1990) with 24-mer λgt11 forward and reverse sequencing primers (New England Biolabs) and [α-^{32}P]ATP (Du Pont). 7-Deaza-dGTP nucleotide mixtures (USB) were used to resolve sequencing compressions on 6% polyacrylamide gels (Mizusawa et al., 1986). Additional primers based on oprin cDNA sequencing results were synthesized using an Applied Biosystems 380A DNA synthesizer and purified on a Poly-Pak (Glen Research) cartridge followed by chromatography on Mono Q HR 5/5 and ProRPC HR 5/10 using the (Pharmacia) FPLC system. Double-stranded DNA sequencing was also performed using end-labeled ([γ-^{32}P]ATP) sequencing primers (Sambrook et al., 1989) and 7-deaza-dGTP nucleotide termination mixtures. A 5-day exposure of the dried gel to X-OMAT-AR5 film (Kodak) at −80 °C was required to achieve adequate band intensity with the end-labeling procedure.

Sequence Homology. Oprin cDNA and amino acid sequences were used to search the Genbank nucleotide (Release 67.0) and SWISS-PROT (Release 17.0) databases using the FASTA program (Pearson & Lipman, 1988) for sequence homology.

Results

Purification of Oprin. A summary of the purification procedures is presented in Table I. Saturated $(NH_4)_2SO_4$ solution (25 mL) was added to opossum serum (30 mL) to achieve 45% saturation. The solution was stirred for 1 h at 4 °C, left overnight, and then centrifuged 20 min at 23000*g* and the precipitate discarded. Solid $(NH_4)_2SO_4$ (198 g/L) was added to the supernatant to achieve 80% saturation, and the solution was stirred 1 h, kept at 4 °C for 4 h, centrifuged 20 min at 23000*g* and the supernatant discarded. The precipitate was dissolved in 10.0 mL of 0.02 M sodium phosphate, pH 6.5, and dialyzed 48 h against two changes (1 L each) of buffer. The dialyzed solution (729 A_{280} units) was charged onto a 2.5 × 37 cm column of DEAE-Sepharose equilibrated with buffer and eluted using a linear gradient from 0 to 0.3 M NaCl (750 mL per bottle); 6-mL fractions were collected at a flow rate of 90 mL/h. The void volume occurred at tube 60, and several peaks were eluted between tubes 60 and 155. Oprin was eluted in a single peak between tubes 160 and 180.

The DEAE-Sepharose column fractions were also assayed for several other inhibitory activities. Material analogous to human α_1-proteinase inhibitor (inhibition of trypsin and chymotrypsin) and to human α_1-antichymotrypsin (inhibition of chymotrypsin, but not of trypsin) was detected. The oprin peak lacked inhibitory activity on trypsin, but inhibition of chymotrypsin and of *C. atrox* HT-a hemorrhagic activity was detected. No inhibition of venom serine proteinases was noted.

The oprin peak was pooled, and solid $(NH_4)_2SO_4$ was added to 1.0 M. The pool was dialyzed 24 h against 2.0 L of 0.1 M sodium phosphate–1.0 M $(NH_4)_2SO_4$, pH 7.0, charged on a 1.5 × 17 cm column of phenyl-Sepharose, and eluted with a decreasing linear gradient (150 mL per bottle) from 1.0 to 0 M $(NH_4)_2SO_4$ in buffer. At fraction 150 elution was continued with buffer only, and a slight amount of inactive material was removed. Fractions (3.0 mL) were collected at a flow rate of 50 mL/h. The void volume occurred at tube 90, and two minor peaks eluted between tubes 90 and 120. Oprin activity was detected in a single major peak which eluted between tubes 125 and 150. The pool from this peak inhibited *C. atrox* metalloproteinases, but no inhibition of chymotrypsin or of HT-a hemorrhagic activity was detected. Electrophoretic analysis (not shown) indicated that two minor contaminants were still present.

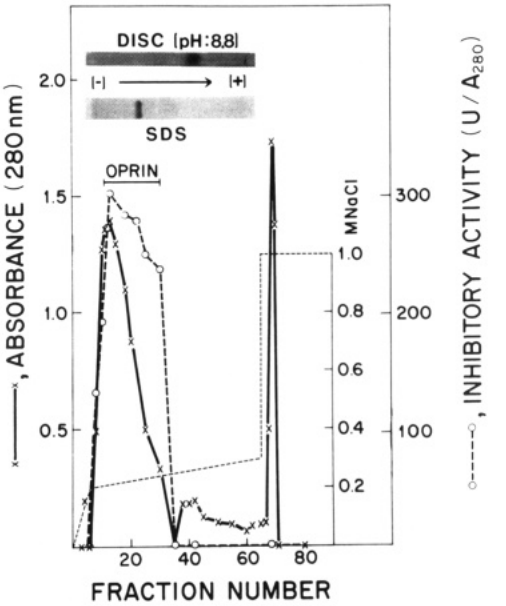

FIGURE 1: Chromatography on Mono Q HR 5/5. 65 A_{280} units of the oprin pool from the phenyl-Sepharose column were dialyzed against 0.02 M CHES, pH 9.5. 10 A_{280} units of the dialyzed oprin material was charged on a Mono Q HR 5/5 column. Elution was effected with a linear gradient from 0.2 to 0.3 M NaCl in CHES buffer. A stepwise increase to 1.0 M NaCl was employed to strip the column, and the column was then reequilibrated with starting buffer. This procedure was employed for six successive runs, and the oprin material from each run was pooled as shown. (—) Oprin pool; (---) M NaCl. Insert: Electrophoretic analyses of the combined oprin pools from six identical Mono Q column elutions.

The oprin pool was adjusted to pH 9.5 by careful addition of 0.5 M NaOH, dialyzed for 24 h against two changes (1 L each) of 0.02 M CHES, pH 9.5, concentrated to approximately 3 mg/mL using an Amicon YM-30 membrane, and either stored at −20 °C or chromatographed on Mono Q HR 5/5.

The concentrated material (65 A_{280} units) was divided into six aliquots containing approximately 10 A_{280} units of protein. Six identical chromatographic runs on a column of Mono Q HR 5/5 were then made using the Pharmacia FPLC system as shown in Figure 1. The oprin material was pooled as shown, and the combined pools from six identical runs were concentrated to approximately 3 mg/mL protein using an Amicon YM-30 membrane and stored at −20 °C. Under these

Table II: Amino Acid and Carbohydrate Composition of Oprin[a]

residue	mol/mol	nearest integer
lysine	13.7	14
histidine	6.1	6
arginine	19.1	19
aspartic acid	24.9	25
threonine	28.4	28
serine[b]	28.6	29
glutamic acid	37.7	38
proline	35.1	35
glycine	26.2	26
alanine	20.3	20
half-cystine[c]	7.9	8
valine	17.6	18
methionine	4.9	5
isoleucine	12.2	12
leucine	29.1	29
tyrosine	11.0	11
phenylalanine	15.3	15
tryptophan[d]	7.4	7
N-acetylneuraminic acid[e]	16.7	17
glucosamine	21.8	22
galactosamine	2.3	2
hexose[f]	28.7	29
molecular weight, protein:	38 243 (345 residues)	
molecular weight, carbohydrate:	13 537 (70 residues)	
total:	51 780	

[a] Average of duplicate analyses from 24-, 48-, and 72-h hydrolyses, assuming Tyr = 11.0. [b] Corrected by extrapolation to zero time. [c] Determined as cysteic acid; no free sulfhydryls were detected using Ellmann reagent. [d] Determined spectrophotometrically. [e] Thiobarbituric acid method. [f] Phenol–sulfuric acid method.

conditions, activity remained stable for at least 12 months, and no losses were noted with six freeze/thaw cycles. The specific activity of oprin pools from five preparations ranged from 285 to 310. The final product is fully active as judged by the stoichiometry of its interaction with α-protease.

Properties and Composition of Oprin. Purified oprin migrated as a single sharp band during electrophoresis in the presence and absence of SDS (Figure 1, insert). Only one band was detected in SDS gels run in the presence and absence of mercaptoethanol, indicating that oprin is composed of a single polypeptide chain. An estimated molecular weight of 52 000 was calculated from the electrophoretic mobility of oprin relative to standards of known molecular weight. There was color development on gels stained with the periodic acid–Schiff reagent, indicating that oprin contained carbohydrate residues. Three distinct protein bands present in approximately equal amounts and corresponding to pI = 3.4–3.6 were detected in isoelectric focusing experiments. Since the SDS/PAGE analyses had all indicated a single band, the results with isoelectric focusing were attributed to microheterogeneity of the oprin preparation. The bands were not assayed for activity. However, all are presumed to be active since complex formation between oprin and α-protease was stoichiometric (see below). Oprin is one of the most acidic proteins in the 45–80% $(NH_4)_2SO_4$ fraction of opossum serum, as judged by its elution position from DEAE-Sepharose. The inhibitor is also quite stable, with full activity being retained over the pH range 2.5–11.5 and at temperatures up to 70 °C.

The amino acid and carbohydrate analyses of oprin are shown in Table II. No free sulfhydryl groups were detected either in native oprin or in inhibitor denatured with guanidine hydrochloride. Therefore, oprin contains four disulfide bonds on the basis of the half-cystine content of the molecule. Oprin is rather highly glycosylated (26%), and the relatively high *N*-acetylneuraminic acid content is consistent with the low pI exhibited by this protein and may account for the observed microheterogeneity.

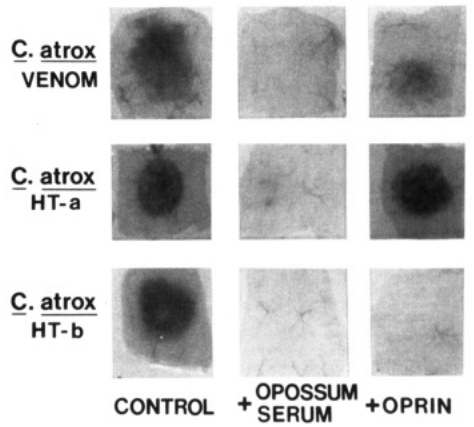

FIGURE 2: Effect of opossum serum and oprin on venom hemorrhagic activity. Hemorrhagic activity and its inhibition were determined as described under Experimental Procedures for aliquots of the following incubation mixtures, with the amounts in A_{280} units of each component given in parentheses: *C. atrox* venom (0.01) plus opossum serum (0.24) or oprin (0.50); *C. atrox* HT-a (0.003) plus opossum serum (0.12) or oprin (0.08); *C. atrox* HT-b (0.01) plus opossum serum (0.40) or oprin (0.02). The controls shown are the responses for the crude venoms or hemorrhagic toxins alone. Oprin, opossum serum, or saline alone (not shown) elicited no hemorrhagic response.

Inhibition Spectrum of Oprin. Oprin completely inhibited the metalloproteinase activity of crude venoms from *C. atrox*, *C. basiliscus*, and *B. arietans*. Purified *C. atrox* α-, β-, and γ-proteinases and *C. atrox* HT-b were also totally inhibited by stoichiometric amounts of oprin. However, *C. adamanteus* metalloproteinase activity was only partially inhibited (60%) as was proteinase II, a major metalloproteinase in this venom. A similar result (67% inhibition) was obtained when oprin was incubated with a partially purified metalloproteinase from *Dendroaspis angusticeps* venom. Incomplete inhibition of the proteinases was observed even when oprin was present at a 20-fold molar excess. It is not known whether the observed results are due to formation of partially active complexes or whether the complex which is formed dissociates under the assay conditions.

In contrast, metalloproteinases from several bacterial sources (clostridiopeptidase A, thermolysin, *P. aeruginosa* protease and elastase) were not inhibited by oprin. Serine proteinases from the above venoms were not inhibited, nor were bovine trypsin and chymotrypsin or porcine elastase. The cysteinyl proteinase, papain, the aspartyl proteinase, pepsin, and the exopeptidases, carboxypeptidases A and B, were also not inhibited.

Effect of Oprin on Venom Hemorrhagic Toxins. As shown in Figure 2, opossum serum inhibited the hemorrhagic activity of crude *C. atrox* venom, and this result was consistent with previous literature reports (Huang & Perez, 1980). In addition, opossum serum totally inhibited the hemorrhagic activity of *C. atrox* HT-a and HT-b (Figure 2). Incubation of equimolar amounts of oprin and *C. atrox* HT-b resulted in the complete inhibition of this hemorrhagic toxin (Figure 2), and a similar inhibition of hemorrhagic activity was obtained when oprin was incubated with *C. atrox* β-proteinase (data not shown). In contrast, *C. atrox* HT-a (the most active hemorrhagic toxin in this venom) still elicited a hemorrhagic response after exposure to a 30-fold excess of oprin, and a 50-fold excess of oprin only partially inhibited the hemorrhagic activity of *C. atrox* crude venom (Figure 2). This was somewhat unexpected, since total inhibition of the hemorrhagic activity of *C. atrox* crude venom had been observed with the partially purified oprin material from the DEAE-Sepharose column. Assays using pure hemorrhagic toxins rather than

Table III: Partial Nucleotide and Deduced Amino Acid Sequence of cDNA Coding for Oprin[a]

	CAT	GGC	TGG	AGC	TCC	CTG	AGC	GCC	CCA	GTG	GAA	GTG	ACG	GGA	AAA	GAG	CCT	51
	His	Gly	Trp	Ser	Ser	Leu	Ser	Ala	Pro	Val	Glu	Val	Thr	Gly	Lys	Glu	Pro	
	CTC	CCT	GCC	CCC	TCG	CTG	CGG	GCT	GAG	CCT	GGA	CCT	TGG	ATC	CTC	CGT	GGT	102
18	Leu	Pro	Ala	Pro	Ser	Leu	Arg	Ala	Glu	Pro	Gly	Pro	Trp	Ile	Leu	Arg	Gly	
	GTG	GAG	ACC	AAG	CTG	CAC	TGC	CGA	GGG	GTG	CTG	CTG	GGC	ATG	ATC	TTT	GAC	153
35	Val	Glu	Thr	Lys	Leu	His	Cys	Arg	Gly	Val	Leu	Leu	Gly	Met	Ile	Phe	Asp	
	CTG	TAC	CAG	GAA	GGA	GAG	CAA	GAG	CCG	GTG	AAG	AGT	TCC	CAC	ACA	CCA	GGA	204
52	Leu	Tyr	Gln	Glu	Gly	Glu	Gln	Glu	Pro	Val	Lys	Ser	Ser	His	Thr	Pro	Gly	
	ACA	GAA	GCC	ACC	TTC	ATC	GTC	AAC	AGT	ACT	GGG	AAC	TAC	AGC	TGC	CTC	TAC	255
69	Thr	Glu	***Ala***	***Thr***	***Phe***	***Ile***	***Val***	***Asn***	***Ser***	***Thr***	***Gly***	***Asn***	***Tyr***	***Ser***	***Cys***	***Leu***	***Tyr***	
	CGA	GCA	CCT	GCA	CCA	GCT	CCC	AGT	GTG	AAT	TCA	ACG	CCC	AGT	GAG	ACC	ATA	306
86	***Arg***	***Ala***	***Pro***	***Ala***	***Pro***	Ala	Pro	Ser	Val	Asn	Ser	Thr	Pro	Ser	Glu	Thr	Ile	
	CAC	GTT	GTG	ATA	CCA	GAC	TTC	CTG	CCA	AAG	GCC	AAC	TTC	TAT	ATT	TTG	AAC	357
103	His	Val	Val	Ile	Pro	Asp	Phe	Leu	Pro	Lys	Ala	Asn	Phe	Tyr	Ile	Leu	Asn	
	AAC	CGA	GTC	TTC	AGG	CCT	GGA	GAC	ATT	GTG	ACG	GTT	AGC	TGC	CAG	GCG	AGA	408
120	Asn	Arg	***Val***	***Phe***	***Arg***	***Pro***	***Gly***	***Asp***	***Ile***	***Val***	***Thr***	***Val***	***Ser***	***Cys***	***Gln***	***Ala***	***Arg***	
	TTC	TCT	GAG	CGG	GAA	TAC	GAT	CTG	GAA	TTC	AAG	CTG	TTC	AAA	GAT	GGA	CAG	459
137	***Phe***	***Ser***	***Glu***	Arg	Glu	Tyr	Asp	Leu	Glu	Phe	Lys	Leu	Phe	Lys	Asp	Gly	Gln	
	GAG	ACA	CTT	GTG	GAA	GTG	GTG	CTT	ACA	TCG	GAC	CAA	ATG	AAA	GTA	TTC	TTT	510
154	Glu	Thr	Leu	Val	Glu	Val	Val	Leu	Thr	Ser	Asp	Gln	Met	Lys	Val	Phe	Phe	
	GAT	CTG	ACA	GCT	GTT	GGC	CCT	GAG	GAT	GGG	GGC	AAG	TAC	AGC	TGC	AGG	TAC	561
171	Asp	Leu	Thr	Ala	Val	Gly	Pro	Glu	Asp	Gly	Gly	Lys	Tyr	Ser	Cys	Arg	Tyr	
	CGC	TTT	AGG	AAT	GGG	CCG	CCG	ATC	TGG	TCA	GAA	GAC	AGT	AAC	ATC	CTG	GAG	612
188	Arg	Phe	Arg	Asn	Gly	Pro	Pro	Ile	Trp	Ser	Glu	Asp	Ser	Asn	Ile	Leu	Glu	
	CTG	GTC	GTC	ACT	ACA	GGC	CAG	633										
205	Leu	Val	Val	Thr	Thr	Gly	Gln	211										

[a] The nucleotide sequence was determined for both strands. The deduced amino acid sequences which correspond to internal peptides generated by proteolytic digestion of oprin are shown in bold type. Polymorphism (shaded residues) was noted at positions 128 and 131 which had Ser and Phe, respectively, in the peptide analysis.

crude venoms showed that the hemorrhagic and proteolytic activities of HT-a and HT-b were readily inhibited by fractions 140–160 from the DEAE-Sepharose column, indicating that opossum serum contains at least two inhibitors which react with venom hemorrhagic toxins. The HT-a inhibitor peak just precedes the oprin peak on DEAE-Sepharose. At this stage in the purification, trace contamination of the oprin pool with the HT-a inhibitor accounts for the total inhibition of *C. atrox* venom hemorrhagic activity mentioned above. The HT-a inhibitor was totally removed from oprin during the phenyl-Sepharose purification step. The inhibitor of HT-a is present in much smaller amounts in opossum serum, has properties similar to oprin, and has thus been previously overlooked. Data concerning this second inhibitor will be presented separately.

Complex Formation between Oprin and C. atrox α-Proteinase. The mechanism of inhibition of α-proteinase by oprin is shown in Figure 3. A 2-fold molar excess of oprin was incubated with α-proteinase to assure that all enzymatic activity was blocked, and the reaction mixture was chromatographed on Mono Q HR 5/5 as shown in Figure 3C. Similar amounts of α-proteinase and oprin were chromatographed separately as shown in Figure 3 panels A and B. The reaction mixture (Figure 3C) contained a peak which did not correspond to either α-proteinase or oprin, and this was designated complex. Furthermore, no α-proteinase peak was detected, and the oprin peak (Figure 3C) was decreased by an amount expected for stoichiometric complex formation. In other experiments (data not shown) in which the oprin/α-proteinase ratio was 1/1 or 0.5/1, no excess oprin was eluted, indicating that the homogeneous oprin preparation is fully active. The protein peaks were pooled and analyzed electrophoretically (Figure 3, lower). Only the complex pool showed two bands in the presence of SDS, corresponding to α-proteinase and oprin. The material in this pool also migrated differently in nondenaturing electrophoresis. The electrophoretic data indicate that the complex is unstable in SDS/PAGE, and similar results indicating inhibitor/enzyme complex formation have also been noted when oprin was incubated with HT-b (not shown).

The complex pool showed no proteolytic activity on hide powder azure and had no inhibitory activity against the standard *C. atrox* proteinase mixture. The α-proteinase and oprin controls from the Mono Q columns showed full activity. It was concluded that oprin had formed an inactive inhibitor/enzyme complex with α-proteinase.

Oprin cDNA. Immunochemical screening resulted in the identification and isolation of three positive plaques. The PCR-amplified recombinant inserts were between 0.3 and 0.9 kb as determined by agarose gel electrophoresis. Preliminary sequencing indicated that the inserts all represented the same or very similar mRNA. The sequence of 633 nucleotides from one of the inserts and its deduced amino acid sequence are shown in Table III. Two internal peptide sequences of oprin were also determined, and they corresponded to nucleotide residues 211–270 and 364–420 as shown in Table III. Differences between the amino acid sequence deduced from cDNA and those determined by enzymatic digestion of the core molecule occurred as indicated in Table III. The differences could be due to genetic polymorphism similar to that found in human α1B-glycoprotein (Gahne et al., 1987), since the protein sequence determinations were done on oprin prepared from pooled opossum serum gathered in Texas, while the mRNA was from a single opossum captured in Pennsylvania.

The SWISS-PROT database was searched with the amino-terminal sequence (26 residues) of oprin and with the deduced

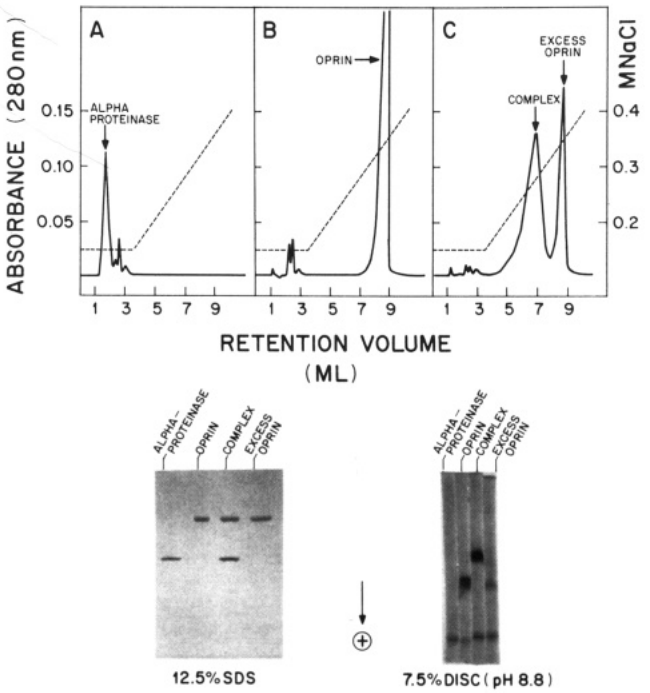

FIGURE 3: Complex formation between oprin and *C. atrox* α-proteinase. Upper panels: Oprin (0.33 A_{280} units) was incubated with *C. atrox* α-proteinase (0.083 A_{280} unit) for 5 min at 25 °C in 0.02M Tris-HCl, pH 7.6, to give a 2:1 excess of oprin. The mixture was charged on a Mono Q HR 5/5 column and initially eluted with 0.02 M Tris-HCl–0.15 M NaCl, pH 7.6. At fraction 4 a gradient from 0.15 to 0.46 M NaCl was employed, as shown in panel C. Fractions (0.3 mL) were collected at a flow rate of 1.0 mL/min. (A) α-Proteinase control; (B) oprin control. Lower panels: Electrophoretic analyses of above Mono Q column pools.

Table IV: Comparison of the Amino Acid Sequences of Oprin and Human α1B-Glycoprotein[a]

```
OPRIN  LKAMDTTPRLWIETESPSTPXTNVTL
          .: :.X: :.::  .:..::::
HUMAN  AIFYETQPSLWAESESLLKPLANVTLTCQARLETPDFQLFKNGVAQEPVHLDSPAIKHQF
α1B-G          10        20        30        40        50        60

OPRIN                  HGWSSLSAPVEVTGKEPLPAPSLRAEPGPWILRGVETKLHCRG
                       ::. ::  .:.:: ..:::: :. .: .:: .:..:.  :::
HUMAN  LLTGDTQGRYRCRSGLSTGWTQLSKLLELTGPKSLPAPWLSMAPVSWITPGLKTTAVCRG
α1B-G          70        80        90        100       110       120

OPRIN  VLLGMIFDLYQEGEQEPVKSSHTP-GTEATFIVNSTGNYSCLYRAPAPAPSVNSTPSETI
       :: :..: : .:::..X .. .... ..:::: :. .::::: ::. . ..  ..... ::
HUMAN  VLRGVTFLLRREGDHEFLEVPEAQEDVEATFPVHQPGNYSCSYRTDGEGALSEPSATVTI
α1B-G          130       140       150       160       170       180

OPRIN  HVVIPDFLPKANFYILNNRVFRPGDIVTVSCQARFSEREYDLEFKLFKDGQETLVEVVLT
       . . .  :   .  ...:..::. ::..: :..:    ...:.: ....: ::    :
HUMAN  EELAAPPPPVLMHHGESSQVLHPGNKVTLTCVAPLS----GVDFQLRRGEKELLVPRSST
α1B-G          190       200       210           220       230

OPRIN  SDQMKVFFDLTAVGPEDGGKYSCRYRFRNGPPIWSEDSNILELVVTTGQ
       : . ..::.:.::. .:::.:.::::.....  ::.::. .:::...
HUMAN  SPD-RIFFHLNAVALGDGGHYTCRYRLHDNQNGWSGDSAPVELILSDETLPAPEFSPEPE
α1B-G   240       250       260       270       280       290
```

[a] Residue numbering is that for human α1B-G (Ishioka et al., 1986). Oprin residues 1–26 were determined by amino-terminal sequence analysis, and residues 78–285 were deduced from cDNA (Table III). Two dots signify identical residues; one dot signifies a conservative mutation; X indicates the boundaries of the initial homology identification; gaps introduced during optimization by the computer program FASTA are shown as dashes.

amino acid sequence shown in Table III, and the results are presented in Table IV. The 211 amino acid residue overlap showed 36.5% identity with human α1B-glycoprotein, and the amino-terminal sequence of oprin showed 46.2% identity with human α1B-G.

Discussion

The data presented for oprin is the initial demonstration that the neutralization of venom metalloproteinases and some hemorrhagic toxins by opossum serum occurs via enzyme/inhibitor complex formation. Enzyme/inhibitor complex formation has previously been reported as the mechanism responsible for the neutralization of *Trimeresurus flavoviridis* H_2-proteinase by an inhibitor isolated from the snake's serum (Omori-Satoh, 1977). The complex dissociated during SDS/PAGE, and the extent of inhibition was approximately 75% at a 1:1 molar ratio. This is similar to the electrophoretic results shown in Figure 3 and the partial inhibition by oprin noted with some venom metalloproteinases. Recently, it was reported that a metalloproteinase inhibitor isolated from *Bothrops jararaca* plasma totally inhibited two metalloproteinases from *B. jararaca* venom, but only 70% of the proteolytic activity of the crude venom (Tanizaki et al., 1991). The inhibitor also neutralized the hemorrhagic activity of the crude venom. Inhibition was shown to occur via formation of a noncovalent enzyme/inhibitor complex. Similarly, three antihemorrhagic factors isolated from *C. atrox* serum neutralized the proteolytic activity of *C. atrox* venom and formed complexes with *C. atrox* hemorrhagic toxin *e* (Weissenberg et al., 1991). The properties of these snake plasma metalloproteinase inhibitors are similar to those observed for oprin, and it will be of interest to determine whether they possess any homology to α1B-G. The hedgehog (*Erinaceus europaeus*) is resistant to the hemorrhagic effects of viper (*Vipera berus*) venom, and a total of 14 proteinase inhibitors have been identified in hedgehog plasma on the basis of molecular weight and inhibition spectra (De Wit & Weström, 1987a). Neutralization of the viper venom hemorrhagic toxin was due to complex formation with hedgehog β_2-macroglobulin (M_r = 700 000) (De Wit & Weström, 1987b). The other metalloproteinase tested (clostridiopeptidase A) was inhibited only by the hedgehog macroglobulins. No metalloproteinase inhibitor similar to oprin was detected, but this may be due to the fact that the authors did not assay for inhibition of venom metalloproteinases other than the viper hemorrhagic toxin.

Oprin differs, in its properties and activity toward venom proteinases, from known mammalian plasma proteinase inhibitors. For example, human inhibitors (except for α_2-macroglobulin) are readily inactivated by catalytic amounts of venom metalloproteinases and venom proteolytic activity is retained (Kress, 1986). In contrast, oprin forms inhibitor/enzyme complexes with the venom metalloproteinases and proteolytic activity is neutralized (Figure 3). In addition, electrophoretic data indicate that oprin is not subjected to limited proteolytic cleavage by venom proteinases. Oprin binds venom metalloproteinases reversibly, does not inhibit bacterial metalloproteinases, and is quite stable to heating and low pH. In these properties it resembles tissue inhibitor of metalloproteinases (TIMP) (Cawston, 1986). However, oprin does not inhibit matrix metalloproteinases I–III (unpublished results), all of which are inhibited by TIMP (Woessner, 1991). The elution characteristics of oprin on DEAE-Sepharose resemble those of the previously reported opossum antihemorrhagic factor (Menchaca & Perez, 1981). However, oprin readily inhibited *C. atrox* HT-b, but had no effect on *C. atrox* HT-a, the most active hemorrhagic toxin in *C. atrox* venom.

Table V: Alignment of Amino-Terminal Sequences of α1B-Glycoprotein from Mammalian Sera[a]

	1									10										20						26
Opossum α1B-G	L	K	A	M	D	T	T	P	R	L	W	I	E	T	E	S	P	S	T	P	X	T	N	V	T	L
Human α1B-G	A	I	F	Y	E	T	Q	P	S	L	W	A	E	S	E	S	L	L	K	P	L	A	N	V	T	L
Pig Po2 F	A	L	F	L	D	P	P	P	N	L	W	A	E	A	Q	S	L	L	E	P	W	A	N	V	T	L
Pig Po2 S	A	L	F	L	D	P	P	P	N	L	W	A	E	A	Q	T/P	S	L	E	P	P	A	N	X	T	X
Horse α1B K	A	V	V	F	D	P	P	P	A	L	L/W	A	E	A	D	X	Q	Q	E	P	L	(R)	N	L	T	V/Q	
Donkey α1B	A	V	V	F	D	P	Q	P	A	L	(W)	A	E	A	D	T	Q	L	E	P							

[a] Sequence and naming are taken from the following: human (Ishioka et al., 1986); pig and horse (Van de Weghe et al., 1988); donkey (Patterson et al., 1991). The alignment and numbering follow that used for human α1B-G, starting at the amino terminus. Residues which occur at identical positions in all α1B-G's are shaded; X = residue unknown.

HT-a was, however, effectively inhibited by material present in fractions 140–160 of the DEAE-Sepharose column. This indicated that oprin differed from the previously reported antihemorrhagic factor and that opossum serum contains at least two inhibitors of venom metalloproteinases.

The amino acid and carbohydrate residue values for oprin shown in Table II are based on a best fit analysis for an M_r = 52000 as estimated from SDS/PAGE. Errors ranging from −22% to +10% have been observed for glycoprotein molecular weight estimations in SDS/PAGE (Leach et al., 1980). However, the oprin molecular weight appears to be 52000 ± 6%, since the best fit analyses beyond this range resulted in an odd number of cysteine residues and no free sulfhydryl groups were detected. The 26 amino-terminal residues determined by protein sequencing plus the 211 residues deduced from the cDNA insert account for about 69% of the total amino acids expected. No sequence homology was found between oprin and any previously described proteinase inhibitors. However, these partial sequences of oprin show 46.2% and 36.5% identity, respectively, with human α1B-glycoprotein. Human α1B-G is a well-characterized human plasma glycoprotein of unknown function (Schwick & Haupt, 1981). The amino acid sequence of human α1B-G has been determined, and computer analysis of the sequence indicated five repeating structural domains, each containing about 95 amino acids and one disulfide bond. The domains showed homology to the variable regions of immunoglobulin light and heavy chains and sequence similarities to other members of the immunoglobulin supergene family (Ishioka et al., 1986). The oprin structure thus far determined shows homology to domains I–III of human α1B-G. The disulfide bond residues of domains II and III are in identical positions, as are the consensus carbohydrate attachment sequences starting at residues Asn 23 and Asn 158 (Table IV). Oprin also contains two additional carbohydrate attachment points at residues 154 and 173, consistent with the higher carbohydrate content of oprin versus that of human α1B-G. It is very likely that carbohydrate chains in oprin do occur at positions 23, 154, and 158 (Table IV), since no PTH derivatives were detected at the corresponding cycles during the protein sequenator analyses. Human α1B-G has a molecular weight of 63000 (of which 17% is carbohydrate) and contains five disulfide bonds (Ishioka et al., 1986). Oprin has a molecular weight of 52000 (of which 26% is carbohydrate) and contains four disulfide bonds (Table II). These data suggest that oprin contains four of the five domains present in human α1B-G.

A comparison of the amino-terminal sequences of α1B-G from various mammalian sera is presented in Table V. Opossum α1B-G shows 30–46% identity with the other mammalian α1B-G's. Six residues are at identical positions in all forms, including the carbohydrate attachment point at Asn 23. Opossum α1B-G is a major constituent of opossum serum as judged by the yield of 27 mg from 30 mL of serum (Table I). This suggests a significant role for opossum α1B-G in controlling the rapid influx of metalloproteinases which accompanies rattlesnake envenomation and may also indicate that opossum α1B-G regulates endogenous metalloproteinase activity in the opossum. The α1B-G present in sera from other mammals resistant to the effects of rattlesnake venom may have a similar function.

Acknowledgments

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Registry No. Proteinase inhibitor, 9075-11-0; metalloproteinase, 81669-70-7; oprin (*Didelphis virginiana* reduced), 137540-00-2.

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