Thrombin-induced Platelet Aggregation Is Inhibited by the
Heptapeptide Leu²⁷¹-Ala²⁷⁷ of Domain 3 in the Heavy Chain of High
Molecular Weight Kininogen*

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The ability of kininogens to modulate thrombin-induced aggregation of human platelets has been assigned to domain 3 (D3) in the common heavy chain coded for by exons 7, 8, and 9 of kininogen gene. We expressed each of the exons 7, 8, and 9, and various combinations as glutathione S-transferase fusion proteins in Escherichia coli. Each of the exon products 7 (Lys²³⁶–Gln²⁹²), 8 (Val²⁹⁳–Gly³⁰⁸), and 9 (Gln³²⁹–Met³⁵⁷), and their combinations were evaluated for the ability to inhibit thrombin-induced platelet aggregation. Only products containing exon 7 inhibited platelet aggregation induced by thrombin with an IC₅₀ of >20 μM. A deletion mutant of exon 7 product, polypeptide 7A product (Lys²³⁶–Lys²⁷⁰) did not block thrombin-induced platelet aggregation, while 7B product (Thr²⁵⁵–Gln²⁹²) and 7C product (Val²⁹³–Gly³⁰⁸) did not block thrombin, and Asn²⁷⁵–Phe²⁷⁹ had only minimal inhibitory activity. A heptapeptide Leu²⁷¹-Ala²⁷⁷ inhibited thrombin-induced aggregation of platelets with an IC₅₀ of 65 μM. The effect is specific for the activation of platelets by thrombin but not ADP or collagen. No evidence for a thrombin-kininogen complex was found, and neither HK nor its derivatives directly inhibited thrombin activity. Knowledge of the critical sequence of kininogen should allow design of compounds that can modulate thrombin activation of platelets.

Platelets are critical cells in physiological hemostasis and pathological thrombosis. Platelet activation can be defined by distinct cellular events initiated by thrombin, as well as other agonists such as collagen, ADP, and thromboxane A₂. Stimulation of platelets results in an orderly process consisting of shape change, followed by aggregation and/or secretion, finally resulting in the formation of a platelet plug (Holmsen, 1994). Thrombin has been shown to bind to two receptors on the platelet surface: platelet glycoprotein Ib-IX complex (Takamatsu et al., 1986) and a seven-membered transmembrane receptor coupled to G proteins. The latter polypeptide is cleaved to expose a new NH₂-terminal sequence SFLLRN (Vu et al., 1991), which can bind to distal sequences in the receptor to initiate signal transduction. Glycoprotein Ib-IX complex modulates thrombin action since, in its absence in Bernard-Soulier disease, 10 times as much thrombin is required for aggregation of platelets (J amieson and Okumura, 1978).

The multifunctional protein high molecular weight kininogen (HK) serves as the cofactor of the kallikrein-kinin system (also known as the contact system), a surface-mediated defense system (Colman, 1984). HK binds to platelets (Gustafson et al., 1986), neutrophils (Gustafson et al., 1989), and endothelial cells (Schmaier et al., 1988) and serves as a source of bradykinin after cleavage by a number of plasma proteases (Scott et al., 1984, 1985). HK and low molecular weight kinogen (LK) are derived from the same gene by alternate splicing of the primary transcript and have identical heavy chains, which consist of three segments (domains 1, 2, and 3), each highly homologous to cystatin (Salvesen et al., 1986; Ishiguro et al., 1987). The sequence HK diverges from LK at a position on their light chains 12 residues COOH-terminal of the bradykinin moiety. Each molecule then exhibits a unique sequence (Takagaki et al., 1985). Kinogens can inhibit papain and cathepsins B, H, and L, and are the most potent plasma inhibitors of the calcium-activated cellular protease, calpain (Schmaier et al., 1986). The concentration of the kinogens in plasma is 3.95 μM (Kerbiriou-Nabias et al., 1984) comprising HK (0.67 μM) and LK (3.28 μM).

HK (Puri et al., 1991) and LK (Meloni and Schmaier, 1991) both inhibit the aggregation of human gel-filtered and washed platelets by thrombin. The primary determinants for thrombin-induced platelet aggregation are present on the heavy chain, which is shared in both HK and LK. Unlike calpain, which exclusively requires domain 2 of kininogens (Bradford et al., 1990), and papain, which can be inhibited by either domain 2 or 3, the inhibition of thrombin-induced platelet aggregation is uniquely found in domain 3 of kininogens (Jiang et al., 1992).

In the present study, we have expressed kininogen domain 3 and several fragments of D3, in E. coli as glutathione S-transferase (GST) fusion proteins, and then tested each polypeptide

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primers modified the EcoRI recognition site. The nucleotide sequences of the sense and antisense primers used for these constructions are given in Table I. Products of exons 7, 8, and 9 (Gly235-Gln292, Gin292-Gly288, and Gin285-Met287, respectively) were constructed using SPK-82A/B, SPK-82C/D, and SPK-82E/F primers, respectively. Gly235-Gly288 (exon products 7 and 8) and Gin292-Met287 (exon products 8 and 9) were constructed using SPK-82A/D and SPK-82C/F, respectively. Thr295-Gln292 (exon 7B product) was constructed using SPK-89 and SPK-828 primers.

Construction of Δ311-346 D3 Expression Plasmid

Deletion of residues 293–328 in D3 was accomplished by construction of D3 expression plasmid lacking the coding region for Val293–520 HK LC (Kunapuli et al., 1993). The first PCR was carried out using SPK-82A as a sense primer and SPK-3B (complementary to nucleotides 912–930 and 1039–1050) as an antisense primer. The second PCR was carried out using SPK-83A (corresponding to nucleotides 916–930 and 1039–1053) as a sense primer and SPK-82B as an antisense primer. The products of PCR 1 and 2 were used as a template in a third PCR with SPK-82A and SPK-82B as primes. The product of the third PCR was cleaved with BamHI and EcoRI and inserted into BamHI- and EcoRI-cut pGEKT as described earlier (Kunapuli et al., 1993). This plasmid is capable of expressing D3 lacking residues 293–328 (exon 8 product) and represents a fusion of exons 7 and 9 products.

Construction of Gly235–Lys270 Fragment Expression Plasmid

The plasmid DNA capable of expressing Gly235-Gln292 (exon 7A product) was digested with HindIII and EcoRI, filled in with Klenow fragment of DNA polymerase in the presence of four dNTPs, and religated. The unique HindIII site at nucleotide 862 thus eliminates the coding segment of Asn272–Gln292.

Constructions of Lys270–Gln292 Fragment Expression Plasmid

The plasmid DNA capable of expressing Lys270–Gln292 (exon 7C product) was constructed by phosphorylated synthetic oligonucleotides SPK-84 (A–F). These oligonucleotides were annealed to form a fragment with BamHI- and EcoRI-compatible sites, which was ligated into BamHI- and EcoRI-digested pGEKT. All of these plasmids were checked for the authenticity of coding sequences and junction sequences by DNA sequence analysis (Sanger et al., 1977). Fig. 2 summarizes the protein sequences coded by the recombinant fragments of D3.

Purification of Recombinant Proteins

The recombinant proteins were purified by the procedure of Smith and Johnson (1988) as described earlier (Kunapuli et al., 1993). Briefly, E. coli harboring the expression plasmid of interest were grown overnight in 100 ml of LB broth containing 100 µg ampicillin at 37°C. This culture was then added to 1 liter of fresh LB broth containing 100 µg/ml ampicillin (1:10) and the culture grown for 2–5 h to an A600 of 0.6. Then, 1 mM isopropyl-1-thio-β-D-galactopyranoside was added and grown for an additional 2 h. Cells were harvested by centrifugation at 3000 × g for 20–30 min at 4°C. The cells were resuspended in 20 ml of PBS (0.1 mM sodium phosphate, pH 7.4, 0.15 w NaCl) containing 1 mM p-mercuribenzenesulfonfyl fluoride, 10 mM EDTA, and 0.5 µM pepstatin. The cells were disrupted by sonication, and 0.1 volume of 10% Triton X-100 was added. The cell debris was removed by centrifugation at 12,000 × g for 15 min at 4°C. The clear supernatant was then passed through a glutathione-Sepharose column (Pharmacia), pre-equilibrated in PBS containing 1% Triton X-100 and protease inhibitors. The breakthrough was passed through the column once more, and the column was washed thoroughly with PBS containing Triton X-100, followed by wash with PBS alone. The recombinant fusion protein was eluted with 5 mM reduced glutathione in 20 mM Tris-HCl, pH 8.0, and fractions (1 ml) were collected. The fractions containing the protein were identified by A280 and by SDS-polyacrylamide gel, followed by Coomassie staining.

Cleavage and Purification of Fusion Products

The proteins were concentrated, exchanged into Tyrode's buffer, and incubated with thrombin at 1/100 of the molar concentration of the recombinant polypeptide exon product for 1 h at 37°C, a-Phe-Pro-Arg chloromethyl ketone was then added at a 10-fold molar excess to thrombin to inactivate any remaining active thrombin. After verification by SDS-PAGE that the fusion peptide had been cleaved, reverse phase HPLC using a linear gradient from 0–80% acetonitrile in a trifluoroacetic acid buffer on a C-18 column was utilized to purify the expressed polypeptide from GST and thrombin. The recovered polypeptides were

MATERIALS AND METHODS

Purified Proteins

Human fibrinogen (20.7 mg/ml) and HK (1 mg/ml) was purchased from Enzyme Research Laboratories, South Bend, IN. HK was supplied as a single chain and migrates as a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of 120,000 daltons. Human α-thrombin was a generous gift of Dr. J. John W. Fenton II (Division of Laboratories and Research, New York State Department of Health, Albany, NY) and had an activity of 2393 NIH units/mg containing 99.24% α-thrombin. Hirudin (9230 units/mg) was purchased from Enzyme Research Laboratories, South Bend, IN. HK was supplied with 99.24% a-thrombin. Hirudin (9230 units/mg) was purchased from Enzyme Research Laboratories, South Bend, IN. HK was supplied with 99.24% a-thrombin. Hirudin (9230 units/mg) was purchased from Enzyme Research Laboratories, South Bend, IN. HK was supplied with 99.24% a-thrombin. Hirudin (9230 units/mg) was purchased from Enzyme Research Laboratories, South Bend, IN. HK was supplied with 99.24% a-thrombin. Hirudin (9230 units/mg) was purchased from Enzyme Research Laboratories, South Bend, IN. HK was supplied with 99.24% a-thrombin.

Construction of Various D3 Deletion Mutant Expression Plasmids

The primary structure of D3 is illustrated (Fig. 1). The details of bacterial expression and purification of D3 (Gly235–Met287) are described earlier (Wachtufog et al., 1994). We have utilized similar methodology using LK cDNA (pHKG36) (Kitamura et al., 1994), kindly provided by Dr. S. Nakashishi, to template to express various D3 fragments in bacteria. The DNA coding for various fragments was separately amplified by polymerase chain reaction (PCR) using specific sets of primers and inserted in-frame into pGEKT vector (Pharmacia Biotech Inc.) by the procedures described earlier (Wachtufog et al., 1994; Kunapuli et al., 1993). This construct expresses the protein products as fusion proteins with GST as the N-terminal region (Fig. 2). All the sense primers contained a BamHI recognition site, and the antisense

![Fig. 1. Primary structure of kininogen domain 3. Domain 3 (amino acids 235–357) is coded for by exons 7 (Gly235–Gln292), 8 (Val293–Gly288), and 9 (Gln292–Met287). Boxed N is N-linked carbohydrate. The shaded area indicates the site for cysteine protease binding. Figure is modified from Dela Cadena et al. (1994a).](http://www.jbc.org/Downloaded from)

for its ability to inhibit thrombin-induced platelet aggregation. Recombinant fragments that demonstrated inhibition were further dissected for critical regions by deletion mutagenesis. Smaller peptides were synthesized and tested for functional inhibition of platelet aggregation. Identification of the inhibitory sequences of kininogen should yield new insights into the structural requirements for the modulation of thrombin-induced platelet aggregation by kininogen.

Materials and Methods

Primary structure of kininogen domain 3. Domain 3 (amino acids 235–357) is coded for by exons 7 (Gly235–Gln292), 8 (Val293–Gly288), and 9 (Gln292–Met287). Boxed N is N-linked carbohydrate. The shaded area indicates the site for cysteine protease binding. Figure is modified from Dela Cadena et al. (1994a).
FIG. 2. Recombinant fragments of kininogen domain 3. The schematic representation of the 10 recombinant HK D3 GST-linked constructs. The corresponding amino acid sequence location for each construct is appropriately indicated. The resulting recombinant protein product identification is listed for each exon product construct. The position of the thrombin cleavage site located between GST and the NH₂ terminus of each construct is indicated with an arrow.

Table I
Oligonucleotides used for the construction of various D3 expression plasmids

| Oligonucleotide | Strand | Corresponding Sequence in pHKG36 | Sequence |
|-----------------|--------|----------------------------------|----------|
| SPK-82A         | +      | nt 757–772                       | 5'-ATTGGGATCCCCGGAAGGATTTTGACAC-3' |
| SPK-82B         | -      | nt 910–930                       | 5'-GCGCAATTCCGTACCTGCTTTTCCAC-3' |
| SPK-82C         | +      | nt 925–942                       | 5'-ATTAGGATCCGTACAGGTGGTGGCTGGC-3' |
| SPK-82D         | -      | nt 1021–1038                     | 5'-GCGGAAATTCGCCAAGTTTTTTGGCTCT-3' |
| SPK-82E         | +      | nt 1039–1056                     | 5'-AGTTGGGATCAAAGCCTAGATTGCAAC-3' |
| SPK-82F         | -      | nt 1108–1125                     | 5'-GCTAGAAATTCGACAG9AAAGGCTTAGTTG-3' |
| SPK-83A         | +      | nt 916–930 and nt 1039–1053      | 5'-ATCTAGGCGCTTTTCTTGTCTC-3' |
| SPK-83B         | -      | nt 990–912 and nt 1039–1050      | 5'-GATCAAAGCTTATGCAAGAAGAAGCCACT-3' |
| SPK-83C         | +      | nt 862–888                       | 5'-TTCTATTTCAAGATTGCAAACTTG-3' |
| SPK-83D         | -      | nt 898–912                       | 5'-AAAAAGGCAAGTACAGAGG-3' |
| SPK-83E         | +      | nt 913–930                       | 5'-AACCTGTACTGTCTGTTTCTTACATTGTC-3' |
| SPK-83F         | -      | nt 904–930                       | 5'-AAATTTTTCAATTGCAAGAAGCCACT-3' |
| SPK-83G         | +      | nt 880–903                       | 5'-ATCTTTGAAGAAGTTCGTTGTTG-3' |
| SPK-83H         | -      | nt 826–879                       | 5'-ATTGAGATGCACCAACACGGCAGCTG-3' |

Electrophoresis
SDS-PAGE (10%) was used to monitor protein purification and cleavage was performed by the method of Laemmli (1970).

Peptide Synthesis
The peptides were synthesized using a modified standard t-butoxycarbonyl solid phase chemistry on an Applied Biosystems model 430 fully automated synthesizer and purified on HPLC as described previously (Bradford et al., 1993). The average yield of peptide was 40 mg, and the purity by HPLC analysis was 90–95%.

Platelet Preparation
Fresh human blood was collected into acid citrate dextrose and centrifuged for 18 min at 990 x g to separate the red cells from the platelet rich plasma component used in the following step. Five ml of platelet rich plasma is applied onto a 50-ml packed Sepharose 2-B complex with HK (Page et al., 1994).

Clotting time was monitored by a BBL Fibrometer (BBL, Cockeysville, MD) equipped with a 0.3-ml probe. The reciprocal of the clotting time is directly proportional to the thrombin concentration. We also tested whether HK could prevent the access of hirudin (25 units/ml, 330 nM) to the active site and/or exosite, both of which are occupied by the inhibitor. The concentration of hirudin was used at a value that resulted in a 50% inhibition of the reciprocal of the thrombin-fibrinogen clotting time. Kininogen or its derivatives were added to thrombin, followed by hirudin and fibrinogen.

Binding of Fluorescein Isothiocyanate (FITC)-Thrombin to HK—
Thrombin was adjusted to pH 9.5 with 0.5 M sodium carbonate, after which 0.2 mg of FITC was added to 1 mg of thrombin and then incubated on ice for 2 h in total darkness. The unbound FITC was removed by gel filtration on a G-25 Sephadex column, equilibrated in PBS. The labeled thrombin present in the first colored band was collected from the column. The thrombin binding assay was performed by capturing HK on latex beads (Seradyn, Inc. (Division of Mitsubishi Chemical Corp.) Indianapolis, IN) with a monoclonal antibody, C1C11 (Schmaier et al., 1987), directed against the light chain of HK (domain 5), which was chosen to avoid interference with the D3 domain. Increasing amounts of FITC labeled thrombin (50-100 µg/ml) were then added and incubated for 60 min. The beads were then collected on a porous membrane microplate and washed with PBS. The fluorescence was measured in a fluorescent concentration analyzer (Idexx Laboratories, Westbrook, ME).

Cross-linking of HK with Thrombin—HK (3.8 µM) and thrombin were incubated in PBS for 10 min at 25 °C. Bis(sulfosuccinimidyl) suberate (Pierce), in Me₆SO was added to yield a concentration of 250 µM and incubated for 30 min, then quenched for 15 min with Tris- OH (5 mM). Samples were withdrawn and analyzed by SDS-PAGE under reduced conditions. HK and thrombin were each treated separately for comparison with the test samples.

Complex Formation of HK with Radiolabeled Thrombin
Thrombin was labeled by the IODOGEN method with [125I] (Fraker and Speck, 1978). [125I]-thrombin (at 20 µg/ml, 13,000 cpm/µl) was incubated with HK at molar ratios of 0.5, 1.0, and 2.0. The mixture was electrophoresed on 10% PAGE (without SDS), and the gels were dried and autoradiographed on Cronex 4 film, DuPont Medical Products, Wilmington, DE.

RESULTS
Generation and Purification of Recombinant Kinogen Domain 3 Fragments—Using procedures described under “Methods and Materials,” we have generated various recombinant fragments of kinogen domain 3, and the structures of these fusion proteins are shown in Fig. 2. All the recombinant fragments contain GST at their amino terminus, unless otherwise stated. These recombinant fragments were purified using glutathione-Sepharose as affinity matrix in a single step. The purified recombinant fragments were electrophoresed on SDS-polyacrylamide gels and stained with Coomassie Brilliant Blue R250. As can be seen in Fig. 3, the GST fusion proteins show mainly single components at the predicted molecular size. Some heterogeneity was observed in the recombinant fragments for exons 7A and 8, both of which contained a second band (27 kDa) consistent with GST that apparently had leached from the glutathione-Sepharose affinity column along with each of the resulting fusion proteins. The resulting purified fragment for exon 7 showed three bands comprising exon 7, GST, and an undetermined component migrating between GST and exon 7.

Inhibition of Thrombin-induced Platelet Aggregation by Recombinant Kinogen Domain 3 Fragments—Various kinogen domain 3 recombinant fragments were evaluated for their ability to block the aggregation of gel-filtered platelets by (2 nM) thrombin. Initial screening of the seven initial recombinant fusion proteins showed that D3 (Gly235–Met357), coded for by exons 7, 8, and 9, and exon 7 product (Gly235–Gln292); and products of exons 7 and 8 and exons 7 and 9, were capable of blocking thrombin-induced platelet aggregation with an IC₅₀ less than or equal to 20 µM (Table II). These results suggest that only those polypeptides containing the sequence coded by exon 7 had inhibitory activity. The IC₅₀ of the GST-free exon 7 product is 13.4 µM, only 3 times more than D3. These results indicate that the primary determinants for inhibition of thrombin-induced aggregation reside in the polypeptide coded by exon 7. In order to further define the sequence responsible for inhibition, three additional recombinant fragments were made from exon 7, namely 7A (Gly235–Leu270), 7B (Thr255–Gln292), and 7C (Lys270–Gln292). When these recombinant polypeptides were tested for their ability to inhibit thrombin-induced aggregation, 7B, containing the COOH-terminal half of exon 7 product, was the most effective inhibitor (25 µM). Since GST (27 kDa) could potentially modify the inhibitory activity of the much smaller kinogen polypeptides, the recombinant polypeptides were deaffed from fusion proteins, purified to homogeneity, and retested in the aggregation assay (Table II). Several attempts were made to deaffve and remove GST from exon 7A product, but this construct proved resistant to thrombin cleavage even at 10-fold higher thrombin concentrations and incubation times of up to 18 h; therefore, only the IC₅₀ value for the GST-linked product is reported. However, the IC₅₀ of the fusion protein exon product 7A was the least potent of the exon 7 products. When the deaffed polypeptides were repurified on HPLC (see “Materials and Methods” and Table II), D3 was the most potent (IC₅₀ = 4 µM, similar to native HK (2 µM). Exon 7, 7B, and 7C products were highly active, only 3–5 times less potent than D3 (IC₅₀ 13.4–19 µM) (Table II, Fig. 4). The shortest inhibitory sequence 7C was the 23-amino acid Lys²⁷⁰–Gln²⁹² (exon 7C).

Inhibition of Thrombin-induced Platelet Aggregation by Synthetic Peptides—In order to further delineate the minimal structure responsible in Lys²⁷⁰–Gln²⁹², we synthesized 4 overlapping peptides. Neither Ile²⁸¹–Gln²⁹² nor Phe²⁷⁹–Ile²⁸¹ had any inhibitory activity at 500 µM and Asn²⁷⁵–Phe²⁷⁹ inhibited...
at 275 μM (Table III). In contrast, Leu271-Ala277 showed an IC50 of 65 μM (about 15 times that of recombinant D3) (Fig. 4).

Molecular Model of Leu271-Ala277—Within D3—We retested a homology model of D3 constructed as described previously (Bradford et al., 1993), based on the crystalline structure of egg white cystatin (Bode et al., 1988). The polypeptide L271NAENNA277 appears as a subdomain on the surface (Fig. 5). An α carbon trace (data not shown) indicates that it is in a helix-turn conformation.

Specificity of Leu271-Ala277—Previous studies from this laboratory (Puri et al., 1991) showed that the action of HK or thrombin-induced platelet aggregation was specific for thrombin since ADP, collagen, and the thromboxane A2 analog U46619 were not inhibited. Leu271-Ala277 (LNAENNA) completely inhibited platelet aggregation by thrombin (1 nm) and partially inhibited aggregation by thrombin (2 nm) (Fig. 6). In contrast, platelet aggregation induced by ADP (10 μM), collagen (2 μg/ml) (Fig. 6), or SFLLRN (10 μM, the thrombin receptor tethered peptide) (data not shown) were unaffected.

Thrombin Binding to Kininogen—HK was evaluated to determine if the observed inhibition of platelet aggregation by HK could be due to direct complexing of thrombin to HK by four different methods. The ability of HK to inhibit the thrombin conversion of fibrinogen to fibrin was first evaluated and compared with hirudin. Hirudin could completely block thrombin clotting of fibrinogen, while HK showed no effect up to a 50-fold molar excess to thrombin. Second, when HK (2 μg/ml) or a control BSA (2 μg/ml) was coated onto an Immobilon plate overnight, washed, reacted with increasing amounts of thrombin, washed, and probed for bound thrombin with S-2238, modest amounts of thrombin activity could be detected bound to both HK and BSA, but no specific binding of thrombin to HK was observed when the amount of thrombin bound to the BSA control was subtracted. Similar results were observed when HK bound to a C1C11 monoclonal antibody (directed to HK domain 5) linked to latex beads (see “Materials and Methods”) were used and reacted with increasing concentrations of FITC-labeled thrombin. Fluorescence concentration analyzer detection of FITC-labeled thrombin bound to HK immobilized to latex beads yielded negative results.

125I-Thrombin was reacted with kininogen at 1/0.5, 1/1, and 1/2 molar ratios and run on 10% native gels (no SDS), then autoradiographed to determine if a mobility shift could be observed or the presence of a new band could be seen. No difference could be found between the thrombin control and the lanes containing thrombin and kininogen mixtures (data not shown). These experiments provide further evidence against the formation of a thrombin-HK complex to explain the ability of HK to inhibit thrombin-induced platelet aggregation.

Kininogen Peptide Effects on the Amidolytic Activity of Thrombin—Selected synthetic peptide was evaluated for the ability to block the thrombin cleavage of the chromogenic substrate S-2238 (H-D-Phe-Pip-Arg-p-nitroanilide). The synthetic peptides Leu271-Ala277 or the intact HK molecule did not inhibit the active site of thrombin at concentrations below 500 μM, while d-Phe-Pro-Arg-chloromethyl ketone completely abolished thrombin activity at concentration of 50 μM or above. The addition of 1 unit of hirudin could inhibit 80% of thrombin amidolytic activity, and the addition of 2 μM HK could not compete with hirudin for thrombin regardless of whether HK was added before or after hirudin addition (data not shown). Taken together, kininogens or their derivatives do not block the active site of thrombin.

DISCUSSION

The central role of thrombin in the pathogenesis of venous thrombosis has emphasized the actions of thrombin not only to clot fibrinogen and form the fibrin plug, but also to convert procofactors factor V and VIII to active cofactors factor Va (Colman et al., 1970) and VIIIa (Eaton et al., 1986), and thereby enhance the formation of thrombin. In contrast, on the arterial

**TABLE III**
The inhibition of thrombin-induced platelet aggregation by HK exon 7 derivatives

| Amino acid sequence of recombinant HK exon 7 products | IC50 (μM) |
|------------------------------------------------------|-----------|
| 235GKDFVQPPTK1CVGCPRIDNSPELETTLHTITKLNAENNAFYYKIDNVKKARVQ292 | <sup>12</sup>13.4 |
| 235GKDFVQPPTK1CVGCPRIDNSPELETTLHTITKLNAENNAFYYKIDNVKKARVQ292 | <sup>14</sup>86 |
| 235GKDFVQPPTK1CVGCPRIDNSPELETTLHTITKLNAENNAFYYKIDNVKKARVQ292 | 30 |
| 235GKDFVQPPTK1CVGCPRIDNSPELETTLHTITKLNAENNAFYYKIDNVKKARVQ292 | <sup>15</sup>275 |
| 235GKDFVQPPTK1CVGCPRIDNSPELETTLHTITKLNAENNAFYYKIDNVKKARVQ292 | <sup>16</sup>65 |

*GST-linked.

**FIG. 4.** Inhibition of the thrombin-induced platelet aggregation by D3-derived peptide products. Thrombin-induced platelet aggregation was evaluated in the presence of three polypeptides derived from exon 7 of HK. Gel-filtered platelets were incubated for 2 min with increasing amounts of each polypeptide and then activated with 2 nM thrombin to determine the relative IC50 inhibitory potencies. The recombinant polypeptide 7C (Gly235–Gln292) resulted in an IC50 of 13.4 μM (○). The COOH-terminal half of the polypeptide coded for by exon 7, 7B (Lys270–Gln292), has an IC50 of 30 μM (▲), and the synthetic peptide LNAENNA (Leu271–Ala277) inhibited thrombin-induced platelet aggregation with an IC50 of 65 μM (●).
side, coronary or cerebral vessels are occluded by thrombi composed of both platelets and fibrin. The importance of thrombin in re-occlusion (Puri and Colman, 1993) after angioplasty (Heras et al., 1989) or thrombolysis (Gash et al., 1986) has emphasized the role of this serine protease in activating platelets. Various strategies have been suggested to prevent this process, including preventing platelet aggregation by all agonists and by blocking the binding of fibrinogen to its platelet receptor glycoproteins IIb/IIIa (Bennett and Vilaire, 1979). Unfortunately, monoclonal antibodies to this platelet integrin, while effective in inhibiting platelet aggregation by thrombin, cause excessive bleeding (Hanson et al., 1988). This result is not unexpected, since the hereditary absence or functional impairment of glycoprotein IIb/IIIa results in a hemorrhagic disease, Glanzmann’s thrombasthenia (Nurden and Caen, 1974; Phillips et al., 1975). A second strategy uses direct thrombin inhibitory polypeptides such as hirudin (Markwardt, 1991) or small organic inhibitors such as argatroban (Kikumoto et al., 1984). Since these compounds inhibit the active site of thrombin, they can prevent thrombin activation of platelets, which requires proteolytic activity. By inhibiting the actions of thrombin on the coagulation process, they, like heparin, also increase the possibility of hemorrhage.

Spurred by the cloning of a seven-transmembrane thrombin receptor (Coughlin et al., 1992) and discovery of a tethered peptide revealed after cleavage that initiated changes in intracellular calcium, investigators have attempted, thus far without remarkable success, to develop peptides that inhibit the binding of thrombin to this receptor. We found that the presence of physiological concentrations of HK, not only in purified systems but also in plasma, modulates thrombin-induced platelet aggregation by shifting the concentration dependence so that 10 times more thrombin is required to stimulate platelets than in washed platelets (Puri et al., 1991). Thus, we hypothesized that HK contained critical sequences that could modulate thrombin-induced platelet aggregation.

Jiang et al. (1992) demonstrated that D3 of kininogen was able to prevent binding of thrombin to platelets. Using monoclonal antibodies, they found that they could distinguish two activities in D3, one of which blocked HK binding to platelets and the other thrombin binding. However, the location of their binding sites was unknown. Recently, Herwald et al. (1995) reported that a peptide Leu$^{331}$–Met$^{357}$ present in the polypeptide encoded for by exon 9 inhibited HK binding with an IC$_{50}$ = 60 µM. We have studied the similar oxidized peptide Cys$^{333}$–Cys$^{352}$ for its ability to inhibit platelet aggregation induced by thrombin. At 84 µM, no inhibition was observed (data not shown). It should be noted that the exon 7 peptides blocked thrombin-induced aggregation, while the exon 9 peptides blocked HK binding, and therefore the biological activities of the peptides were distinct.

Domain 3 is coded for by 3 exons designated exons 7, 8, and 9 (Kitamura et al., 1985). We reasoned that each exon might express distinct functions. Therefore, we expressed each exon product as a GST fusion protein and tested its ability to inhibit thrombin-induced aggregation of platelets from normal human donors, and compared the potency to HK and D3. Recombinant D3 was similar in potency to HK, with only a 2-fold difference in the concentration needed to inhibit the maximum aggrega-
tation by 50%. Only the recombinant polypeptide coded by exon 7, Gly235–Gln292, exhibited potency equivalent to HK or D3. In fact, exon 7 product when used as GST fusion protein showed an IC50 = 0.2 μM, 10 times more potent than D3. This increase in potency is probably due to more favorable folding, since, after cleavage of the thrombin-sensitive linker and purification of free exon product 7, the potency decreased 67-fold and was one-third as potent as D3 (Table I). Cleavage of the fusion protein to yield the free recombinant fragment of kininogen did not always result in a less potent polypeptide. In fact, in the other cases of exon product 7 fragment, potency was increased (see below) by a factor of 2–3. Since two activities were distinguished within D3, we also made all three combinations of the exon product, 7+8, 8+9, and 7+9, to test whether the difference between exon 7 product and D3 could be accounted for by another site on a different exon product. No increase in potency was found in products of exons 7+8 or exons 7+9, and the product of exons 8+9 showed no activity.

Since exon 7 product comprises 56 amino acids, we sought to localize the responsible sequence more precisely. We generated three overlapping recombinant polypeptides. Exon product 7A, Gly235–Leu271, comprised the NH2-terminal half of the exon product 7, while 7C represented the COOH-terminal portion, Lys270–Gln292. In order to avoid splitting the active sequence, we also expressed 7B, Thr255–Gln292. Since 7B and 7C both inhibited thrombin-induced activation, but 7A did not, the functional region lay in the COOH-terminal 23-mer, Lys270–Gln292. Examination of the molecular model of D3 revealed that the proximal portion of the sequence of 7C was on the surface, but much of the rest was associated with the protein core. To test the hypothesis that the NH2-terminal portion of 7C contained all or part of the site responsible for inhibition of thrombin-induced platelet aggregation, we synthesized four peptides that subsisted the sequence Lys270–Gln292. Only the peptide Leu271–Ala277, which exists in a surface loop at the junction of a helix-turn configuration in the molecular model, inhibited platelet aggregation by thrombin. A search of the protein data base indicates no significant homologies except to domain 3 of rat and bovine kininogen; the latter shows complete identity for LNAENNA. The rat HK and LK contains an identical heptapeptide except for substitution of histidine for Ala277. Rat T-kininogen, which is a potent inhibitor of thrombin and factor XI using portions of domain 3 (Tait and Fujiwara, 1987), with plasmogen via the light chain (Humphries et al., 1994), and with thrombospondin (DeLa Cadena et al., 1994b), involving both HK light and heavy chains. A peptide in D3 within exon 7 product is involved in HK binding to thrombospondin on the activated platelet surface (DeLa Cadena et al., 1993). However, it is contained within exon product 7A, which did not inhibit thrombin-induced aggregation. We found that HK did not inhibit thrombin cleavage of fibrinogen or a peptide substrate (amidolytic activity). Moreover, thrombin failed to bind to immobilized HK or to HK in the fluid phase or to HK on native gel electrophoresis. While this study was in progress, Hasan and colleagues (Hasan et al., 1995) also were unable to demonstrate a thrombin-HK complex using several techniques different from those used in this report. The second possibility is that the sequence LNAENNA in D3 directly blocks the binding of thrombin to the seven transmembrane receptor. Three pieces of evidence make this unlikely. First, HK does not inhibit the tethered peptide SFLLRN from aggregating platelets (Jiang et al., 1992) nor did LNAENNA in our study. Second, higher concentrations of thrombin can reverse the inhibition by HK (Puri et al., 1991). Finally, exon 7C product and LNAENNA fail to block shape change induced by thrombin.2 Shape change is the earliest morphological change following ligand binding and is associated with an increase in intracellular Ca2+ and with phosphorylation of myosin light chain (Daniel et al., 1984). The calcium increase has been shown to be a consequence of the interaction of SFLLRN or thrombin with the seven-transmembrane receptor. We cannot rule out an effect on this receptor at a site distant from the tethered peptide, but consider this unlikely. We are currently testing the hypothesis that HK may inhibit the binding of thrombin to glycoprotein Ib.

Finally, our studies show that the HK D3 and its constituents inhibit neither ADP and collagen-induced aggregation nor thrombin protease activity. This specificity would allow selective inhibition of thrombin-induced platelet aggregation, thus modulating thrombin-platelet interaction without either compromise of platelet function or thrombin effects on blood coagulation. These properties make it an attractive template for construction of peptidomimetic drugs to inhibit reocclusion and thrombosis.

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Platelet Aggregation Inhibited by Heptapeptide of Kinogen
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