Cloning of the cDNA and Expression of Moubatin, an Inhibitor of Platelet Aggregation*

(Received for publication, September 29, 1992)

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Moubatin, a new type of specific inhibitor of collagen-induced platelet aggregation, has been isolated from the soft tick Ornithodoros moubata (Waxman, L., and Connolly, T. M. (1993) J. Biol. Chem. 268, 5445–5449). A polymerase chain reaction-generated hybridization probe, produced using primers based on moubatin protein sequence, identified phage containing the entire cDNA sequence of moubatin. Analysis of the predicted amino acid sequence yielded a mature protein of 156 amino acids with a putative prepeptide of 15 amino acids. Comparison of the sequence of moubatin to that of other proteins in the Swiss PROT data base revealed no significant homology.

The cDNA sequence was cloned into the yeast expression vector pKH422, producing a biologically active protein which inhibited collagen-stimulated aggregation of washed human platelets with an IC50 of about 100 nM, which is similar to the potency of native tick moubatin. A concentration of recombinant moubatin that fully inhibited collagen-stimulated aggregation did not inhibit aggregation induced by a variety of other platelet agonists, again demonstrating comparable properties of the recombinant and native proteins. Moubatin did not inhibit platelet adhesion to collagen even at a concentration up to 16 times its IC50 for the inhibition of aggregation. This specificity for inhibiting collagen-stimulated aggregation and not adhesion to collagen indicates that moubatin is unique among the natural product inhibitors of collagen stimulation of platelets. Further examination of the mechanism of moubatin-mediated inhibition of collagen-stimulated aggregation revealed that 1–6 μM moubatin diminished the second phase of aggregation induced by ADP, inhibited aggregation in response to submaximal concentrations of the thromboxane A2 mimetic U46619, and competed for the binding of a thromboxane A2 receptor antagonist to platelet membranes. Therefore, at higher concentrations, moubatin may affect more than one aspect of platelet signal transduction including the thromboxane A2 receptor. The availability of recombinant moubatin will allow further investigation of its unique activities in vitro and in vivo.

*The abbreviations used are: PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography.

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Experimental Procedures

Materials—Restriction endonucleases were purchased from New England Biolabs (Beverly, MA) and Boehringer Mannheim; PCR reagents were from Perkin-Elmer Cetus; sequencing reagents were from United States Biochemical Corp.; the cDNA library kit was from Stratagene (La Jolla, CA); SQ29548 was from Du Pont-New England Nuclear; U46619 was kindly provided by Dr. Ronald Shebuski, Merck Research Laboratories; L-670,596 was synthesized at Merck Research Laboratories; all other materials were as reported previously (4).

cDNA Library—A Bluescript SK (Stratagene) tick cDNA library

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was conducted following the manufacturer’s instructions using total tick poly(A) RNA.

**PCR Analysis**—Total phage DNA from the tick cDNA library (6) was isolated using DNA isolation procedures described in Ref. 7. Using standard PCR protocols (8), the DNA was screened for the presence of moubatn sequences by PCR amplification. PCR analysis was performed on these plaques by using the computer program SIGSEQB, which can be used to reveal a major hydrophobic region at the amino terminus between amino acids 15 and 16 (Ser and Ile). This cleavage sequence was located 15 nucleotides before the poly(A) tract. Only 12 nucleotides of 5' untranslated region were found in any of the sequenced clones. This may be due to premature translation (3, 4). The purity of the inhibitory activity was assessed by analysis of the samples on SDS-PAGE. The peak fractions were pooled, concentrated in a Speed Vac concentrator, and applied to a reverse phase HPLC column. Purified recombinant moubatin was eluted with a linear gradient of isopropanol alcohol (17-50%), containing 0.08% trifluoroacetic acid over 45 min. The fractions were dried in a Speed Vac concentrator and dissolved in 20 mM sodium acetate, pH 7.2, and assayed for aggregation inhibitory activity and purity by SDS-PAGE. The final isolated recombinant moubatin was quantified by amino acid composition analysis and its identity and purity confirmed by amino-terminal amino acid sequence analysis.

**RESULTS**

A PCR analysis of the tick cDNA library was performed using degenerate oligonucleotides based on two Lys-C peptide sequences, L1 and L7, and the 3' arm primers. Since the library was not unidirectional (the cDNA can be inserted in both directions within the Bluescript SK vector), product was obtained from a given arm primer and both the coding and complementary Lys-C primer in all cases. The size of these products was used to order the Lys-C fragments relative to one another within the cDNA, with peptide Lys-C fragment L7 located amino-terminal to peptide L1.

The PCR product of primer pair L1,4, which was approximately 500 base pairs, was digested with NotI and SpeI, subcloned into NotI and SpeI-cut and gel-purified Bluescript SK (Stratagene) plasmid. Inserts were then sequenced using primers which anneal to the Bluescript SK plasmid upstream and downstream of the subcloned insert.

**Nucleotide Sequencing**—Candidate clones were excised with helper phage and subclones generated in the Bluescript SK phagemid vector according to the manufacturer’s instructions. Inserts were then sequenced using a United States Biochemical Corp. sequencing kit.

**Computer-aided Signal Peptide Selection**—Computer-aided signal peptide selection analysis was performed using the computer program SIGSEQB (9, 10).

**Preparation of the Moubatin Expression Vector**—The predicted mature protein coding sequence was expressed in yeast using the expression vector pKH42 which contains the galactose-regulated GAL10 promoter and α-mating factor prepro secreter leader. The coding sequence for moubatin was PCR-generated as described previously (4) with minor modifications. The coding sequence, with appropriately modified ends for subcloning into pKH42, was primer pair L1 (5'-GGGATCCGATTAGCTCCG-3') or primer L2 (5'-CTCAAGTTGCGTGCATGGC-3') and degenerate oligonucleotide primers whose sequences were based on two Lys-C peptide fragments.

The peptide sequence and the corresponding coding and complementatory oligonucleotides were the following: YQWQING, which is found within Lys-C fragment L7 (5), and Primer 1 (5'-ATTGCAGGCCTTCACT-3') and Primer 2 (5'-ATGGGCCTGCACCAATCTGGG-3'). The second Lys-C fragment peptide DQVCDDEK, L1 (5), and the corresponding coding and complementary oligonucleotides were primer 3 (5'-ATGGGCCTGCACCAATCTGGG-3') and Primer 3 (5'-ATGGGCCTGCACCAATCTGGG-3') and Primer 4 (5'-ATTTGGGCGCCTTCTGGC-3') and Primer 4 (5'-ATTTGGGCGCCTTCTGGC-3').

Oligonucleotide primers coding for these peptides were chosen from the right available oligo sequences because of their low degeneracy. Initial PCR analysis was performed using 10 ng of total library DNA as a substrate. Each arm primer was used with each of the four degenerate primers to generate product. As a control for nonspecific product formation each primer was also run alone with the total library DNA as a substrate. Sizes of the PCR products were determined by comparing their migration through a 1.5% agarose gel relative to a X174 RF DNA/HaeIII marker (Bethesda Research Laboratories).

The initial PCR generated product produced using the oligonucleotide primer pair, L1,4, was cut with the restriction endonucleases NotI and SpeI and subcloned into NotI, SpeI-cut and gel-purified Bluescript SK (Stratagene) plasmid. Inserts were then sequenced using primers which anneal to the Bluescript SK plasmid upstream and downstream of the subcloned insert.

**Screening of O. moubata Tick cDNA Library**—The tick cDNA library was screened with a PCR-generated probe corresponding to an 62-base pair pair product produced by oligonucleotide primer pair, L1 and 4, using the subcloned fragment described above produced by primers L1,4 as a substrate. Generation of radiolabeled probe and hybridization protocols were performed as described (4).

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**Transformation of S. cerevisiae BJ1995**—Transformation of the yeast with the pKH42-moubatin expression vector was performed as described previously (4). Transformant clones were grown at 30°C in a defined synthetic medium and induced with galactose. After 24-96 h of induction at 30°C, the cells were removed by centrifugation, and the cell-free culture medium was analyzed by assaying for the inhibition of human platelet aggregation (3, 4) and by SDS-PAGE of these samples. Quantitation of the expression level of recombinant moubatin from yeast transformed yeast compared to moubatin standards whose concentration had been determined by amino acid composition analysis.

**Expression of Recombinant Moubatin**—Undiluted yeast culture medium and culture medium concentrated on a Centriicon-10 10 kDa molecular mass cutoff microcentrifuge (Amicon) from yeast transformed by the expression vector or the pKH42-moubatin expression vector were evaluated for the expression of recombinant moubatin. The concentrated media were analyzed by assaying for the inhibition of human platelet aggregation (3, 4) and by SDS-PAGE of these samples. Quantitation of the expression level of recombinant moubatin from yeast transformed yeast compared to moubatin standards whose concentration had been determined by amino acid composition analysis.

**Purification of Recombinant Moubatin**—Yeast cells were sedimented by centrifugation, and the cell-free culture medium was diluted 5-fold with 20 mM sodium acetate buffer, pH 4.5, and applied to a Fast S Sepharose column that had been equilibrated previously with the same buffer. The column was washed with 5 column volumes of this buffer, and the recombinant moubatin was eluted with a 0-1.0 M NaCl gradient in the same buffer. The fractions were screened for recombinant moubatin by assay for platelet aggregation inhibitory activity (3, 4). The purity of the inhibitory activity was assessed by analysis of the samples on SDS-PAGE. The peak fractions were pooled, concentrated in a Speed Vac concentrator, and applied to a reverse phase HPLC column. Purified recombinant moubatin was eluted with a linear gradient of isopropanol alcohol (17-50%), containing 0.08% trifluoroacetic acid over 45 min. The fractions were dried in a Speed Vac concentrator and dissolved in 20 mM sodium acetate, pH 7.2, and assayed for aggregation inhibitory activity and purity by SDS-PAGE. The final isolated recombinant moubatin was quantified by amino acid composition analysis and its identity and purity confirmed by amino-terminal amino acid sequence analysis.

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The PCR product of primer pair L1,4, which was approximately 500 base pairs, was digested with NotI and SpeI, subcloned into NotI and SpeI-cut Bluescript SK vector, and sequenced. The sequencing confirmed that this PCR product contained within it both of the Lys-C fragments used to construct the primers as well as the Lys-C peptide sequences of fragments L4, L5, L6, and L7 (5).

The PCR product of primer pair L1,4 was then used as a hybridization probe to screen the cDNA library directly. This was done to eliminate any possible PCR generated sequence errors, rather than generating the entire cDNA directly using PCR. Screening initially identified numerous positive phage plaques. Twenty plaques were isolated, and PCR analysis was performed on these plaques using primer pair L1,4, thus amplifying the 5' end of the cDNA. Five of the 20 plaques gave a product of ~500 base pairs; the remainder gave a smaller product or none at all. Presumably the absence of a product was due to inserts which were oriented in the opposite direction. Two of the phagemids containing the longest inserts were grown and sequenced. Sequencing revealed an uninterpretable open reading frame of 513 nucleotides (Fig. 1) which contained within it sequences corresponding to all of the Lys-C fragments (Fig. 1 and Ref. 5). The open reading frame was followed by a 60-nucleotide 3' untranslated region and a poly(A) tract. A hexanucleotide AAATAA consensus sequence was located 15 nucleotides before the poly(A) tract. Only 12 nucleotides of 5' untranslated region were found in any of the sequenced clones. This may be due to premature termination of the reverse transcriptase during the construction of the tick library.

A hydrophathy plot (11) of the deduced amino acid sequence revealed a major hydrophobic region at the amino terminus of the translation product (the putative prepeptide), whereas the remainder of the protein was hydrophilic (Fig. 2). By using the computer program SIGSEQQ2, which can be used to predict which region of the protein contains the signal peptide, the predicted signal peptidase cleavage site probably occurs between amino acids 15 and 16 (Ser and Ile). This cleavage
Recombinant Tick Platelet Aggregation Inhibitor

results in a mature protein of 156 amino acids with a predicted molecular weight of 17,256. When the predicted moubatin protein sequence was compared with other protein sequences in the Swiss PROT data base using the FASTA program of the GCG package, no significant homology was found. The best score was a 31% identity over only a 42-amino acid stretch.

Expression of Recombinant Moubatin in Yeast—The coding region for the mature protein was PCR-generated using the primer pair 5,6 (which anneal to the amino and carboxyl termini, respectively) and the cDNA clone as a substrate. The PCR-generated product was then cloned into the yeast expression vector pKH4a2 to form the pKH4a2.moubatin vector (Fig. 3). The pKH4a2 expression vector has been used successfully to express and secrete a number of other proteins of similar size (4, 6, 12).

The media supernatants from yeast cultures transformed with the pKH4a2-moubatin vector or the control vector pC1/1 were initially analyzed for expression of moubatin by SDS-PAGE. As shown in the inset of Fig. 4, the yeast transformed with the pKH4a2-moubatin expression vector produced a protein of approximately 17.5 kDa, the same size as native moubatin (see below). The yeast transformed with the pC1/1 control vector did not produce a protein of this size. The yeast media supernatants were next examined for their ability to inhibit collagen-stimulated platelet aggregation. As shown in Fig. 4, the medium from pKH4a2.moubatin transformed yeast inhibited aggregation by 100%, whereas the medium from control yeast cultures had no effect on aggregation. This result suggests that the transformed yeast can produce correctly folded active moubatin. The media supernatant was applied directly to a C18 reverse phase HPLC column, and the elution of moubatin was compared against moubatin standards which had been quantitated by amino acid analysis. The yeast expression levels of moubatin were from 2.5 to 12 μg/ml culture medium depending on the time of harvest and growth conditions.
Purification and Characterization of Recombinant Moubatin—Preliminary characterization of the media from yeast secreting moubatin suggested that the optimal expression levels were obtained at 48–72 h after galactose induction. A longer growth period provided no significant increase in expression. Information obtained purifying native tick moubatin (5) and a different yeast secreted protein, rLAPP (4), was utilized to purify the recombinant inhibitor protein from the medium. The culture medium was first diluted with buffer, to lower both the sample conductivity and the pH to 4.3 in order to promote its interaction with a cation exchange resin. The sample was applied to the resin followed by washing and then elution with a NaCl gradient. The recombinant moubatin was then further purified by reverse phase HPLC on a C₁₈ column. An isopropanol gradient eluted the majority of the material in a symmetrical peak at 32–35% isopropanol, 0.08% trifluoroacetic acid as shown in Fig. 5A. After removal of the organic solvent under vacuum, assay of the fractions for aggregation inhibitory activity revealed that all the activity was in the major protein peak eluting at 21 min. The concentration and identity of this protein was determined by quantitative amino acid analysis. The recombinant protein migrated on SDS-PAGE at ~17.5 kDa, the same as tick moubatin, as shown in Fig. 5B. The purity of the recombinant moubatin was further assessed by amino-terminal sequence analysis. Thirty to forty percent of the sample had the correct amino-terminal sequence, whereas the remainder had an additional Arg residue at its amino terminus. Why the yeast secreted moubatin without cleaving this Arg, which is the last amino acid of the α-mating factor leader sequence and should be cleaved by the KEX2 protease, is not known. A protease in yeast has been described which cleaves between paired basic residues (13), and the activity of this enzyme may explain this processing of moubatin that was observed.

Evaluation of the biological activity of the yeast-expressed moubatin was carried out by examining its ability to inhibit collagen-induced platelet aggregation. The recombinant moubatin isolated from two different growths inhibited aggregation with an IC₅₀ of ~100 nM as shown in Fig. 6A. This activity is comparable with that of the moubatin isolated from ticks. The similarity of the IC₅₀ values is remarkable when one considers that the NH₂-terminal amino acid of the mature tick moubatin is unknown and that at least 10 of 156 amino acids of the recombinant moubatin are different from those of the tick moubatin (see underlined amino acids of the moubatin sequence in Fig. 1 and the discussion below). Recombinant moubatin also inhibited collagen-induced platelet Ca²⁺ mobilization in a concentration-dependent manner, with an IC₅₀ of 200 nM as shown in Fig. 6B. In some experiments the moubatin-mediated inhibition of Ca²⁺ mobilization was demonstrated as only a delay in Ca²⁺ mobilization. In these cases the inhibited elevation of intracellular Ca²⁺ levels observed after collagen stimulation eventually approached that
of the control after several minutes. However, taken together, moubatin is not only an inhibitor of platelet aggregation, but it is also an inhibitor of other events that follow collagen stimulation of platelets.

In order to further assess the effect of moubatin on other platelet-collagen interactions, its effect on platelet adhesion to collagen was evaluated. As shown in Fig. 7A, culture medium from yeast transformed with either the control vector or the pKH4a2-moubatin vector did not inhibit platelet adhesion to collagen. Purified recombinant moubatin, at a concentration 20-fold higher than its IC50 to inhibit platelet aggregation, also did not inhibit platelet adhesion to collagen. This assay used an antibody against the VLA-2 integrin complex (22) and the leech inhibitor of platelet adhesion to collagen, LAPP (4), both inhibited platelet adhesion to collagen by 100% as shown in Fig. 7A and as reported previously. In contrast, media from yeast transformed with the pKH4a2-moubatin vector and purified recombinant moubatin inhibited collagen-induced platelet aggregation as shown in Fig. 7B. Thus, as moubatin only inhibits aggregation and not adhesion of platelets to collagen, it is not like the other previously described natural product inhibitors of collagen stimulation of platelets (14, 15).

In further studies to analyze the inhibitory mechanism of moubatin, its effect on aggregation induced by collagen in plasma was examined. Moubatin also inhibited collagen-provoked aggregation in the presence of plasma, but only at a higher concentration (IC50 = 3 μM) than that required to inhibit washed platelets (IC50 = 0.1 μM). The specificity of moubatin for inhibiting collagen-stimulated aggregation of washed platelets or platelets in plasma was next examined. At a concentration of recombinant moubatin that maximally

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**Fig. 5.** Purification of recombinant moubatin. Culture medium from yeast transformed with the pKH4a2-moubatin vector was subjected to Mono S cation exchange chromatography and ~4.5 mg of partially purified protein was applied to a 0.46 × 25 cm Vydac C-18 reverse phase column equilibrated and eluted as described under "Experimental Procedures" (A). B: lane 1, SDS-PAGE on a 20% acrylamide Phast Gel followed by silver staining of 110 ng of tick moubatin; lane 2, 110 ng of recombinant moubatin.

**Fig. 6.** Effect of tick and recombinant moubatin on platelet activation. Washed human platelets and the indicated amount of either tick or recombinant moubatin were incubated together followed by the addition of collagen and the aggregation monitored as described in Fig 5 and "Experimental Procedures" (A). The extent of aggregation was determined by the Chronolog Aggrolink system: A, n = 3, tick moubatin; ○, n = 7, recombinant moubatin. B, washed human platelets loaded with Fura 2 were challenged with 10 μg/ml collagen and Fura 2 fluorescence continuously determined for 2 min in a Photon Technology International fluorometer. Intracellular Ca2+ concentrations at 80 s (time of the maximum control response) were determined as described (29). The results are the mean ± S.E. for four separate determinations. The basal platelet Ca2+ concentration was 40 nM and was subtracted from all values.

**Fig. 7.** Effect of recombinant moubatin on collagen-stimulated platelet activation. A, washed platelets and the indicated amount of culture medium or purified moubatin were added to collagen-coated 96-well plates and platelet adhesion monitored as described previously (4). The control adhesion is an optical density of 0.50 at 562 nm. Column 1, 1:10 dilution pC1/1 control medium; column 2, 1:10 dilution pKH4a2-moubatin medium; column 3, 1.6 μM recombinant moubatin; column 4, 20 μg/ml Gi9 antibody; column 5, 1 μM LAPP. B, aggregation of washed platelets as described in Fig. 5. The control extent of aggregation is 70 arbitrary units. Column 1, 1:10 dilution pC1/1 control medium; column 2, 1:10 dilution pKH4a2-moubatin medium; column 3, 1.6 μM recombinant moubatin. Results are from a representative experiment of 3–10 separate experiments.
inhibited collagen-stimulated aggregation, little effect on aggregation induced by a series of standard platelet agonists, including 0.4 nM thrombin, 1.25 mg/ml ristocetin, 1 μM A23187, 0.5 mM arachidonic acid, or 20 μM ADP, was observed in agreement with the results obtained with native tick moubatin (5). With the availability of recombinant protein, these studies were extended to include the effect of a ~20-fold greater concentration of moubatin and lower agonist concentrations for those stimuli whose effects could be measured at concentrations below those listed above. As shown in Table I, under these conditions, inhibition of the final extent of aggregation in response to ADP could be observed. This result suggested that moubatin may inhibit the cyclooxygenase-dependent pathway of platelet activation. We therefore re-examined the effect of moubatin on arachidonic acid and the arachidonic acid metabolite thromboxane A₂ (using the mimetic U46619)-stimulated aggregation. U46619-provoked aggregation was inhibited by moubatin in a manner dependent on both the concentration of inhibitor and agonist. In further studies, moubatin at >1 μM competed for the binding of the thromboxane A₂ receptor antagonist SQ29548 to platelet membranes shown in Fig. 8. It was a less potent competitor than the thromboxane A₂ receptor antagonist L-670,596 (16) or the thromboxane A₂ receptor agonist U46619. The leech-derived inhibitor of collagen stimulation of platelets, LAPP, inhibited collagen-stimulated aggregation, little effect on aggregation induced by a series of standard platelet agonists, including 0.4 nM thrombin, 1.25 mg/ml ristocetin, 1 μM A23187, 0.5 mM arachidonic acid, or 20 μM ADP, was observed in agreement with the results obtained with native tick moubatin (5). With the availability of recombinant protein, these studies were extended to include the effect of a ~20-fold greater concentration of moubatin and lower agonist concentrations for those stimuli whose effects could be measured at concentrations below those listed above. As shown in Table I, under these conditions, inhibition of the final extent of aggregation in response to ADP could be observed. This result suggested that moubatin may inhibit the cyclooxygenase-dependent pathway of platelet activation. We therefore re-examined the effect of moubatin on arachidonic acid and the arachidonic acid metabolite thromboxane A₂ (using the mimetic U46619)-stimulated aggregation. U46619-provoked aggregation was inhibited by moubatin in a manner dependent on both the concentration of inhibitor and agonist. In further studies, moubatin at >1 μM competed for the binding of the thromboxane A₂ receptor antagonist SQ29548 to platelet membranes shown in Fig. 8. It was a less potent competitor than the thromboxane A₂ receptor antagonist L-670,596 (16) or the thromboxane A₂ receptor agonist U46619. The leech-derived inhibitor of collagen stimulation of platelets, LAPP, did not compete for this binding at up to 30 μM. Finally, in further studies of the mechanism of inhibition of aggregation by moubatin, it did not compete for the binding to platelets of the monoclonal antibody G69, which inhibits platelet adhesion to collagen and interacts with the proposed platelet collagen receptor, the α5β3 integrin complex (data not shown).

**DISCUSSION**

The current results describe the identification and characterization of the cDNA for moubatin, the *O. moubata* tick-derived inhibitor of collagen-stimulated platelet aggregation (5). Based on the amino acid sequence of peptides derived from tick moubatin available when these studies were initiated, the full-length cDNA was cloned and sequenced from a tick cDNA library. Analysis with the program SIGSEQ2 of the amino acid sequence deduced from the cDNA predicted a peptide cleavage site after amino acid Ser-15 to yield a mature protein of 156 amino acids with a molecular weight of 17,256. This hypothetical amino terminus of moubatin cannot be compared with that of the tick protein as its terminus appears to be blocked (5). Interestingly, there are 10 amino acid differences (6.4%) between the sequence deduced from the cDNA compared with that of the protein peptide sequences derived from the isolated tick protein. Although the ticks used for the isolation of moubatin and those used to prepare the cDNA library are of the same species, they are from different sources (see "Experimental Procedures"). Since the ticks are inbred, it may be that these differences are due to intraspecies variability that has developed after many years of breeding. A similar number of differences in amino acid sequence (4 of 60, 6.7%) of the tick anticoagulant protein (TAP) derived from the two tick colonies has also been observed (17). We are currently planning to obtain more ticks from the various colonies to address this issue.

Consistent with moubatin's unique functional activity is its lack of sequence homology to LAPP, which is the only protein inhibitor of collagen stimulation of platelets whose sequence has been reported. Moubatin also does not share sequence homology with any other platelet inhibitor, collagen, or to any other protein and does not contain the Arg-Gly-Asp sequence important in cell adhesive function (18). As it has not been possible to deduce functional information from the primary amino acid sequence, future studies using mutagenesis and chemical modification will hopefully provide some clues as to the mechanism of moubatin's inhibitory properties. The pKH4α2 expression vector was used to express secreted moubatin, since the expression of a secreted protein simplifies its subsequent isolation. We have successfully expressed other proteins using this vector, including echistatin (19), TAP (6), LAPP (4), and antistasin (20). These proteins contain 8, 6, 6, and 20 cysteines, respectively, all of which are probably disulfide-bonded. Since the isolated recombinant moubatin is of comparable specific activity to tick moubatin, yeast must be able to correctly fold this protein.

Moubatin displays unique functional activity, at a concentration that fully inhibits collagen-induced aggregation it selectively inhibits collagen stimulation of platelet aggregation. Under these conditions the aggregation of platelets initiated by other agonists is not inhibited by moubatin; however, at significantly higher concentrations of moubatin and at submaximal concentrations of ADP, a diminished final extent of aggregation was observed. This result suggested the possibility that the cyclooxygenase-dependent pathway of platelet aggregation was impaired by moubatin. Although 0.5 mM arachidonic acid-induced aggregation was not significantly inhibited, the cyclooxygenase inhibitor indomethacin inhib-

**TABLE I**

*Effect of recombinant moubatin on platelet aggregation*

Platelet-rich plasma or washed platelets in the presence of 0.2 mg/ml fibrinogen, and the indicated concentration of moubatin were incubated at 37 °C for 2 min followed by the addition of a stimulus and the extent of aggregation was monitored as described under "Experimental Procedures" and Refs. 3 and 4. The results are from a representative experiment of two to five separate experiments.

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<tr>
<th>Stimulus</th>
<th>[Moubatin] (μM)</th>
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<tr>
<td>Collagen</td>
<td>0.3</td>
<td>70</td>
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<tr>
<td>ADP</td>
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<td>U46619</td>
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*In platelet-rich plasma.

**FIG. 8. The effect of moubatin on the binding of SQ29548 to human platelet membranes.** Human platelet membranes (125 μg of protein) were incubated with 3.4 nM [3H]SQ29548 alone or in the presence of L-670,596 (V), U46619 (II), moubatin (O), or APP (A) as described (30). Binding to filters alone represented <1% of total binding. The total bound was approximately 3000-5000 cpm depending on the experiment. The results are the average of two separate experiments.
cloned collagen-stimulated aggregation and the final extent of aggregation in response to lower concentrations of ADP in a manner comparable with that observed with higher concentrations of moubatin (data not shown). Therefore, the effect of moubatin on another component of the cyclooxygenase-dependent pathway of platelet aggregation, thromboxane A2-induced aggregation was examined. Moubatin in a concentration-dependent manner inhibited aggregation induced by the thromboxane A2 mimetic U46619. At the highest concentration of inhibitor and lowest U46619 concentration tested, the stimulus provoked platelet shape change and aggregation were inhibited. These results are comparable with those observed with a nonpeptide thromboxane receptor antagonist (16) and suggested a possible mechanism for moubatin’s mode of inhibition. Also, dihydropyridines, which interfere with voltage-sensitive Ca2+ channels, at >1 μM have been shown to also block U46619-stimulated aggregation and the second phase of ADP-induced aggregation (21). It was proposed that these effects were mediated through an effect on the thromboxane receptor. To address this issue we found that moubatin at >1 μM competed for the binding of a thromboxane A2 receptor antagonist to platelet membranes. Thus, at least at these higher concentrations of moubatin, part of its inhibitory effect is likely mediated by blockade of the thromboxane A2 receptor. Whether all the effects of moubatin are mediated via this mechanism is the subject of current investigations.

The adhesion of platelets to collagen is not markedly affected by moubatin. The other described inhibitors specific for the activation of platelets by collagen either inhibit both platelet aggregation and platelet adhesion to collagen or only adhesion. The inhibitor protein we recently isolated from leeches, LAPP (3, 4), and the 50-kDa protein from medicinalis leech saliva (15) all inhibit both collagen-induced aggregation and platelet adhesion to collagen. Other types of inhibitors of collagen interaction with platelets prevent only platelet adhesion to collagen. These include the peptide DGEA (22), and the monoclonal antibodies directed against the αβ1 integrin, P1H5 (23, 24) and G19 (25). G19 does not inhibit collagen-stimulated platelet aggregation.2 The monoclonal antibody 6F1, raised against the platelet αβ1 integrin, glycoprotein IIa/IIIa, inhibits platelet adhesion to collagen and collagen-stimulated platelet aggregation in a purified system, whereas it is a less effective inhibitor of these processes in plasma (26). A polyclonal antibody against platelet glycoprotein IV has been shown to partially inhibit platelet adhesion to collagen and to inhibit collagen-stimulated aggregation (27). This antibody is not completely specific for collagen stimulation of platelets, however, as ADP and epinephrine-induced aggregation are also inhibited (28). Only a nonapeptide derived from Type III collagen has been reported to inhibit platelet aggregation induced by collagen; however, its effect on platelet adhesion to collagen is unknown (29).

In summary, the current results describe the successful cloning and expression of a new type of platelet inhibitor, moubatin. As discussed above, the other inhibitors of collagen stimulation of platelets assert their effects through their interaction with either a known platelet surface protein or with collagen itself. The mechanism by which moubatin inhibits collagen-stimulated platelet aggregation remains unclear. In preliminary studies we could not demonstrate a specific interaction of moubatin with either platelets or Type I collagen. However, moubatin does compete for binding to the thromboxane A2 receptor via a low affinity interaction. The significance of this finding remains to be explored. The availability of sufficient quantities of recombinant moubatin should facilitate unraveling this problem and lead to new information on the importance of inhibiting collagen-stimulated platelet aggregation.

Acknowledgments—We thank Jerzy Karczewski for assistance with the adhesion assays, Mohinder Sardana and John Rodney for assistance with the protein sequence determinations, Carl Bennett and Mary Jo Zaborowski for analysis of the protein sequences, Richard Endris for supplying ticks used to generate the cDNA library and helpful discussions on tick physiology, and Mike Neeper for constructing the tick cDNA library.

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