Expression of a Fibrinogen Fusion Peptide in Escherichia coli: A Model Thrombin Substrate for Structure/Function Analysis

By Susan T. Lord and Dana M. Fowkes

The initial event in fibrin clot formation is the thrombin-catalyzed cleavage of the Aα chain of human fibrinogen. Most of the information required for thrombin recognition and cleavage of the Aα chain lies in the amino terminal 51 residue CNBr fragment. By selective modification of residues in this region, we probed the features that participate in thrombin interactions. We constructed a vector which expressed a tripartite protein (tribrid) consisting of amino acids 1 to 50 of the Aα chain followed by 60 amino acids of chicken collagen and the β-galactosidase protein from Escherichia coli. Cell lysates run on NaDodSO4-polyacrylamide gels contained the predicted band of molecular weight (mol wt) 125,000. The tribrid reacted with a monoclonal antibody, Mab-Y18, which recognizes the amino terminus of the Aα chain. When cell lysates were incubated with thrombin, FPA was released. By including one heterogeneous oligonucleotide in the construction, we generated plasmids that encoded three specific amino acid substitutions. Surprisingly, changing Gly14 to Val did not alter thrombin cleavage, although recognition by Mab-Y18 was lost. Substitution of Ile for Arg23 did not alter either thrombin cleavage or monoclonal recognition. Substitution of Leu for Arg16 altered thrombin cleavage; unexpectedly, recognition by Mab-Y18 was not changed. © 1989 by Grune & Stratton, Inc.

The CENTRAL EVENT in blood clot formation is conversion of the soluble plasma protein fibrinogen into an insoluble fibrin clot. This conversion is catalyzed by thrombin, which cleaves the Aα chain, releasing fibrinopeptide A (FPA), and the Bβ chain, releasing fibrinopeptide B (FPB). In fibrinogen, thrombin cleaves the Arg16/Gly17 bond in Aα; in Aα chain fragments, thrombin also cleaves the Arg19/Val20 bond, although at a much reduced rate. Previous investigators studied the interaction of thrombin with Aα chain fragments isolated from fibrinogen, short synthetic peptides, and fibrinogen variants containing substitutions in the Aα chain. These studies demonstrated that virtually all residues required for thrombin recognition and cleavage are in the amino terminal 50 residues of the Aα chain.

Peptides could be synthesized based on this fragment and a variety of amino acid substitutions incorporated to probe the chemical nature of the thrombin–fibrinogen interaction. We decided, however, that this goal could be more efficiently obtained with genetic engineering techniques. Our approach permitted simultaneous synthesis of a number of fibrinogen variants. We fused the Aα fragment to an enzyme, permitting rapid quantitation of the Aα product independent of the mutated structure and providing a convenient handle for purification. Specifically, we constructed a plasmid vector which expresses a recombinant protein containing the first 50 residues of the Aα chain linked to E. coli β-galactosidase by a segment of collagen. The Aα chain segment was assembled from synthetic oligonucleotides. One oligonucleotide was synthesized as a heterogeneous mixture so that specific amino acid substitutions were encoded. After cloning these sequences, we isolated independent colonies that express recombinant peptides with specific mutations in the Aα segment. These changes in primary structure affect the interaction of the recombinant proteins with thrombin and with Aα-specific antibodies. The data demonstrate the feasibility of using this tribrid peptide as a model substrate for thrombin.

MATERIALS AND METHODS

Oligonucleotide assembly. Oligonucleotides were synthesized with CED phosphoramidites and tetrazole from American Bionetics. Oligonucleotide 3 (Fig 1) was a mixture of 16 products obtained by incorporating equimolar amounts of G and T at the four indicated positions that are G in the natural cDNA sequence. Oligonucleotides, except 1 and 6, were kinased with T4 polynucleotide kinase (New England Biolabs, Beverly, MA). The kinase was inactivated by heating at 65°C, oligonucleotides 1 and 6 were added (to give 0.15 μmol/L of each oligonucleotide), and the mixture was annealed by being heated at 85°C for 15 minutes and cooled slowly to room temperature. The annealed oligonucleotides were ligated with 10 U T4 ligase (provided by J. Griffith, University of North Carolina, Chapel Hill), the ligated products were separated on a 6% polyacrylamide gel, and the 158-base pair (bp) fragment was recovered by electroelution.

Plasmid constructions. Plasmid DNA was prepared by the alkaline lysis method. Reactions with restriction enzymes and T4 ligase were carried out as recommended by the manufacturer (New England Biolabs). E. coli (strain JM101, P-L: Pharmacia, Piscataway, NJ) were transformed as described. Plasmid pJG200, provided by Dr D. Bastia (Duke University, Durham, NC), was modified by inserting a synthetic linker between the EcoRI and BamHI sites to add a BglII site and an NcoI site, both of which are unique in the modified plasmid. Subsequently, the trc promoter was excised as an EcoRI-NcoI fragment from pKK233-2 (P-L Pharmacia) and inserted between the EcoRI and NcoI sites to make the new expression vector p277. Plasmids encoding the natural Aα and the mutated peptides were constructed by inserting the assembled oligonucleotide fragment between the NcoI and BamHI sites of p277.

Because oligonucleotide 3 was a mixture of 16 sequences, many of the plasmid products were heteroduplexes; thus, any cell transformed with this DNA could contain a mixture of two plasmids. To

From the University of North Carolina at Chapel Hill. Submitted January 5, 1988; accepted August 18, 1988. Supported by a research grants HL-31048 and HL-31012 from the National Heart, Lung and Blood Institute of the National Institutes of Health, Bethesda, MD.

Address reprint requests to Susan T. Lord, MD, Department of Pathology, University of North Carolina, UNC Medical School, CB #7525, 601 Brinkhous-Bullitt Building, Chapel Hill, NC 27599-7525.

© 1989 by Grune & Stratton, Inc. 0006-4971/89/7301-0020$3.00/0
which are designated with a star were synthesized as an equimolar mixture of G and T. Residues in oligonucleotide 3 were synthesized based on the cDNA sequence for the Act segment of the recombinant protein. The Act segment of the recombinant protein was measured by tricolor detection using the RIA kit and the latter were affinity-purified, goat anti-mouse IgG antisera (1:7,500 dilution, Promega Biotech) using bromo-chloro-indolyl phosphate and nitroblue tetrazolium, as recommended by Promega Biotech.

**Cell lysis and trbrid assay.** Except for the buffer, the cell lysis procedure is as described by Germino et al. E. coli were harvested by centrifugation, and the cell pellets were suspended in one-fifth volume 0.05 mol/L Tris-HCl, pH 8, 0.05 mol/L EDTA. 15% sucrose with freshly dissolved lysozyme at 1 mg/mL. After being incubated in room temperature for 15 minutes, the lysates were frozen at −70°C, thawed rapidly in a 37°C water bath, and sonicated briefly to shear DNA. Tribrid synthesis was quantitated by colorimetric assay for β-galactosidase activity using o-nitrophenyl-β-D-galactopyranoside as substrate. Activities are reported as micromoles of o-nitrophenol per minute per milliliter of cell lysate.

The Aα segment of the recombinant protein was measured by radioimmunoassay (RIA) with a kit from Mallinckrodt Diagnostics (Maryland Heights, MO) using a rabbit antiserum raised against human FPA. FPA is measured in a competitive assay by comparison to a standard curve from 1 to 40 ng/mL. We diluted our lysates in 50 mmol/L Tris-HCl, pH 8, 0.15 mol/L NaCl to concentrations within the range of the standard curve.

**Thrombin cleavage.** The thrombin used in these studies was National Institutes of Health (NIH) standard H-1. The activity of this preparation was measured using the synthetic substrate Spectrozyme TH as recommended by the supplier (American Diagnostica, New York). To compare the release of FPA from natural and mutant proteins, we first assayed β-galactosidase activity in the lysates and then diluted the samples with 0.05 mol/L Tris-HCl, pH 8, 0.05 mol/L EDTA, and 15% glucose such that all lysates contained the same concentration of β-galactosidase. The lysates were then incubated either with or without thrombin for one hour at 37°C, and the reaction was terminated either by diluting an aliquot 1:10 in 10−3 mol/L (D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (P-PACK)) (Calbiochem, San Diego) in 50 mmol/L Tris-HCl, pH 8, 0.15 mol/L NaCl, or by adding NaDodSO4-polyacrylamide gel electrophoresis (PAGE) loading buffer. The former were assayed with the RIA kit and the latter were assayed by protein blotting, as described above.

**RESULTS**

**Synthesis of the fragment encoding amino acids 1 to 50 of Aα fibrinogen.** The seven oligonucleotides shown in Fig 1 were synthesized based on the cDNA sequence for the Aα chain of human fibrinogen with the following modifications: (a) An initiating methionine was included as part of the overhang ending of the 5′ NcoI site; (b) a BamHI overhanging end was added to the 3′ end of the fragment; and (c) oligonucleotide 3 was a heterogeneous mixture synthesized by substituting an equimolar mixture of G and T at four positions that are G in the fibrinogen gene.

**Construction of the plasmid expression vector.** The final expression vector, pAA, is shown in Fig 1. We sequenced 24 isolates and identified four of the possible 16 sequences. One of these, pAA, had the natural Aα sequence and the other three encoded the following changes: (a) pG14V: Gly14 is replaced by Val; (b) pR23I: Arg23 is replaced by Ile; and (c) pR16L-R23I, Arg16 is replaced by Leu and Arg 23 is replaced by Ile.

**Expression of the tribrid proteins in E. coli.** The Coomassie-stained NaDodSO4-polyacrylamide gel in Fig 2A shows the synthesis of an IPTG-dependent band only in the lysates harboring the tribrid plasmids. The intensity of this band was quantitated by densitometric analysis with a computer system (model Densidyne Scientific).
product of thrombin cleavage. The data (Fig 2B) clearly showed that the trbrid which expressed the normal $\Lambda\alpha$ fragment reacted strongly with Mab-Y18, as did the trbrid expressed by pR23I and pR16L-R23I. However, there was no reactive band in the pG14V lysate. A second filter from this same gel was developed with a Moab to $\beta$-galactosidase. This blot (Fig 2C) clearly showed that pG14V lysates contained an IPTG-induced trbrid with the same mobility as that in pAa, pR23I, and pR16L-R23I. Thus, the single substitution of Val for Gly14 altered the $\alpha$A epitope so that it was no longer readily recognized by Mab-Y18. Figure 2C also shows the presence of a second band at mol wt 110 Kd. This band appeared in all induced samples and is probably the truncated (lacZAM15 encoded) $\beta$-galactosidase expressed in the parental cell line (JM101).

To quantitate the amount of trbrid synthesized by these clones, we did colorimetric assays for $\beta$-galactosidase activity. The results from these assays are presented in Table 1. As expected, p277 lysates had low levels of $\beta$-galactosidase activity. All the trbrid clones had approximately equal levels of $\beta$-galactosidase activity, which increased four to fivefold after IPTG induction.

To measure the fibrinogen $\alpha$A segment of the trbrid and to demonstrate cleavage by thrombin, we used a competitive radiomunoassay (RIA) for FPA. The antiserum reacted not only with FPA but also with the trbrid. We therefore treated E coli extracts with bentonite to remove any trbrid not cleaved by thrombin. As shown in Table 1, the thrombin-dependent release of FPA from the natural $\alpha$A trbrid was demonstrated by comparing samples treated with thrombin (25 U/mL) and bentonite to untreated samples or samples treated with bentonite alone. With no treatment, the lysates contained an average of 6 ng/mL FPA. On treatment with bentonite, this crossreactivity was lost. When lysates were incubated with an excess of thrombin and then treated with bentonite, an average of 31 ng/mL FPA was found, consistent with the release of FPA from the bentonite precipitable protein. Using this concentration of released FPA (31 ng/mL in a diluted cell lysate), we calculated that the induced cells contain $\sim$80 $\mu$g trbrid/mL bacterial culture.

Assay of pR23I lysates produced similar results. Samples treated with thrombin and bentonite showed a fivefold increase in the level of FPA as compared with untreated samples, indicating that the substitution of Ile for Arg23 did not substantially alter thrombin cleavage of the trbrid or recognition by the FPA antisera. In contrast, results with pG14V and pR16L-R23I were significantly different. The levels of FPA in untreated pR16L-R23I lysates were higher than in untreated pAa lysates. After treatment with thrombin and then with bentonite, FPA levels in the pR16L-R23I lysates decreased, indicating incomplete thrombin cleavage. The levels of FPA in untreated pG14V lysates were similar to the natural trbrid lysate, but after treatment with thrombin the levels of FPA did not change. This result is consistent with either a reduction in thrombin cleavage or a reduced affinity of the antisera for the altered FPA.

To discriminate between these two possibilities, cell lysates were incubated with lower concentrations of thrombin and the reactions stopped with $10^{-5}$ mol/L P-PACK, an
irreversible thrombin inhibitor. Samples were assayed for FPA with or without bentonite treatment. Data from one experiment is presented in Fig 3. With the pG14V lysates, incubation with the lowest concentration of thrombin released a low level of FPA. The same level was found with the highest thrombin concentration. Since increased thrombin did not increase FPA, these data indicate that the pG14V tribrid was completely cleaved at the lowest thrombin concentration. This implies that the Val substitution at position 14 reduces the peptide reactivity in the radioimmunoassay, but does not alter thrombin cleavage.

The data in Fig 3 confirmed the slow FPA release from pR16L-R23I. The pR16L-R23I lysates incubated with the lowest levels of thrombin contained little or no FPA in the bentonite-treated samples. At higher thrombin concentrations, FPA increased. Thus, the amount of FPA released increased with increasing thrombin concentration, as expected if the mutant tribrid is cleaved less efficiently. The data in Fig 3 also confirmed the similarity of pAα and pR23I. At the lowest levels of added thrombin there was a significant release of FPA that was not increased by further thrombin addition.

To substantiate the extent of tribrid cleavage with these concentrations of thrombin, we took advantage of the specificity of Mab-Y18 for the uncleaved substrate. Aliquots of the cell lysates prepared as for the experiment shown in Fig 3 were analyzed by protein blotting. Because the tribrid product from pG14V did not react with Mab-Y18, analysis with this MoAb was limited to pAα, pR23I, and pR16L-R23I. The results (data not shown) demonstrated that the samples incubated without thrombin show a strong crossreactive band for all three tribrid products. At the lowest thrombin concentration, the intensity of the crossreactive band decreased for both pAα and pR23I but remained virtually unchanged for pR16L-R23I. At higher thrombin concentrations, the Mab-Y18 reactive band disappeared with pAα and pR23I, indicating that these tribrids were readily cleaved by thrombin. In contrast, even at the highest thrombin concentration (100 U/mL), a crossreactive band remained with pR16L-R23I. At ten times this thrombin concentration, the band with pR16L-R23I was lost, demonstrating that this tribrid can be completely cleaved by thrombin.

**DISCUSSION**

We expressed the amino terminal 50 residue fragment as part of a tripartite protein including the E coli β-galactosidase enzyme because (a) the N-terminal 50 residues include the amino acids required to study the mechanism of thrombin recognition and cleavage of the Aα chain; (b) a simple method to make multiple simultaneous mutations by oligonucleotide assembly was feasible for a peptide of this size; (c) fusion of eukaryotic proteins or peptides to β-galactosidase is reported to stabilize them in E coli; (d) the β-galactosidase activity provides a rapid, quantitative measure of recombinant protein synthesis; and (e) the β-galactosidase marker offers the possibility for rapid purification of the tribrid based on affinity chromatography (for the β-galactosidase activity). Our data indicate that a single recombinant protein of expected size is synthesized and that this protein is recognized and cleaved by thrombin.

The expression of the natural Aα recombinant protein was readily demonstrated by protein blotting using Mab-Y18. This antibody has been extensively characterized. It cross-reacted with Aα, and this immunoreactivity was lost upon treatment with thrombin. Mab-Y18 did not crossreact with fibrinogen fragment Y (which includes Aα 1-51) from pig, rat, cat, or cow, and crossreacted only slightly with fragment Y from abnormal human fibrinogen Metz, where Arg16 is replaced by Cys. Thus, Koppert et al conclude that Arg16 is essential for the Mab-Y18 epitope. However, the data shown in Fig 2 contradict this. The mutant protein from

---

**Table 1. Tribrid Induction and Thrombin Cleavage**

<table>
<thead>
<tr>
<th>Assay</th>
<th>IPTG Addition</th>
<th>p277</th>
<th>pAα</th>
<th>pG14V</th>
<th>pR23I</th>
<th>pR16L-R23I</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-galactosidase activity</td>
<td></td>
<td>0.2 ± 0.1</td>
<td>1.2 ± 0.2</td>
<td>1.5 ± 0.4</td>
<td>1.7 ± 0.1</td>
<td>1.8 ± 0.5</td>
</tr>
<tr>
<td>(μmol/min/mL)*</td>
<td>+</td>
<td>0.2 ± 0.1</td>
<td>5.5 ± 0.9</td>
<td>6.8 ± 1.5</td>
<td>6.9 ± 0.4</td>
<td>7.2 ± 1.6</td>
</tr>
<tr>
<td>β-galactosidase activity</td>
<td></td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>plus bentonite</td>
<td></td>
<td>&lt;1</td>
<td>1.7 ± 0.5</td>
<td>2.1 ± 0.1</td>
<td>1.2 ± 0.8</td>
<td>3.8 ± 0.3</td>
</tr>
<tr>
<td>FPA (ng/mL)†</td>
<td></td>
<td>&lt;1</td>
<td>5.7 ± 1.4</td>
<td>6.5 ± 0.7</td>
<td>5.3 ± 2.6</td>
<td>11.2 ± 2.8</td>
</tr>
<tr>
<td>No thrombin</td>
<td></td>
<td>&lt;1</td>
<td>3.6 ± 0.6</td>
<td>9.9 ± 2.6</td>
<td>3.9 ± 2.7</td>
<td></td>
</tr>
<tr>
<td>No bentonite</td>
<td></td>
<td>&lt;1</td>
<td>5.4 ± 1.8</td>
<td>11.2 ± 2.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plus thrombin</td>
<td></td>
<td>&lt;1</td>
<td>30.6 ± 5.7</td>
<td>7.8 ± 3.3</td>
<td>24.0 ± 9.9</td>
<td></td>
</tr>
<tr>
<td>Plus bentonite</td>
<td></td>
<td>&lt;1</td>
<td>1.5 ± 0.9</td>
<td>6.5 ± 0.7</td>
<td>1.2 ± 0.3</td>
<td></td>
</tr>
</tbody>
</table>

*Tribrid synthesis was quantified by colorimetric assay for β-galactosidase using o-nitrophenyl-β-D-galactopyranoside. Activities are reported as micromoles of o-nitrophenol/min/ml of cell lysate.
†FPA was measured using a competitive radioimmunoassay (Mallinckrodt Diagnostics) with antisera raised against human FPA. FPA is quantitated by o-nitrophenyl-β-D-galactopyranoside activity plus bentonite/β-galactosidase activity.
pR16L-R23I (Arg16 changed to Lue) crossreacted readily with Mab-Y18. In contrast, the product from pG14V did not crossreact with Mab-Y18. We conclude that Mab-Y18 recognizes not only primary structure but also secondary conformation in this region.

When assaying the recombinant proteins in crude lysates, we found that a substitution of Ile for Arg23 or a substitution of Val for Gly14 did not substantially alter the extent of thrombin cleavage as compared to the natural Aα segment. The results with the Arg23 substitution were consistent with the previous data which showed that two synthetic peptides differing only by the presence of this Arg residue have similar kinetic constants (i.e., \( K_m \) and \( k_{cat} \)) for hydrolysis by thrombin.21

In contrast, the results with the Gly14 substitution differed from those we expected. A comparison of the N-terminal amino acid sequences in mammalian fibrinogens shows that this residue is virtually invariant in known FPA sequences.20 In addition, the results from experiments with synthetic peptides have led Scheraga and his colleagues23 to propose that thrombin recognizes a hairpinlike structure in this Aα segment and that Gly14 may be part of a tight turn. A substitution of Val for Gly appeared likely to disrupt this structure. Indeed, a change in structure is substantiated by the lack of crossreactivity between the trivrid from pG14V and Mab-Y18. As a corollary, one might expect this single amino acid substitution to alter the interaction of the pG14V trivrid with thrombin. However, within the limits of the assays we present, the pG14V Trivrid was cleaved as efficiently as the natural recombinant substrate. Preliminary amino acid analysis of the peptide released from pure pG14V demonstrated that thrombin cleaved this recombinant between Arg16 and Gly17 (P.A. Byrd and S.T. Lord, unpublished observations, November 1987). The results from more quantitative experiments on purified substrate will be required to interpret this result.

Thrombin cleavage of the pR16L-R23I protein was reduced, as expected. Whenever Arg16 has been changed to any other amino acid, whether in abnormal human fibrinogens or in synthetic peptide substrates, cleavage by thrombin has been reduced. The thrombin-dependent peptide released from the pR16L-R23I Tribrid probably came from cleavage between Arg19 and Val20 because thrombin can cleave this bond in the CNBr 1-51 fragment, although at a substantially slower rate. (Preliminary results support this hypothesis; P.A. Byrd and S.T. Lord, unpublished observations, November 1987). This release of an FPA-like peptide from the pR16L-R23I trivrid demonstrates that the Arg16 to Leu and Arg23 to Ile substitutions do not prevent binding to thrombin. The crossreactivity of this mutant with Mab-Y18 shows that Arg at position 16 is not required for antibody recognition.

These results clearly indicate that the Aα segment 1-50 synthesized in E coli as part of a tripartite protein retained the characteristics relevant to its role as a model substrate for thrombin. Peptide sequences readily cleaved by thrombin were also cleaved in the tribrids pAα and pR23I, whereas a peptide that is not expected to be cleaved by thrombin was not cleaved in the trivrid pR16L-R23I. Thus, we conclude that the trivrid serves as a good model substrate for thrombin and have begun a quantitative analysis of thrombin recognition of several peptides with mutations in this segment.

ACKNOWLEDGMENT

We thank Dr D. Bastia for pJG200, Dr W. Nieuwenhuizen for Mab-Y18, John Stebbins and Cheng Wei for excellent technical assistance, and Drs B. Errede and G. White for constructive comments on the manuscript.

REFERENCES


5. Mosesson M, Siebenlist KR, Diorio JP, Hainfeld JF, Wall JS, Soria J, Soria C, Samama M: Evidence that proximal NH₂-terminal portions of fibrinogen Metz (Aα 16Arg → Cys) Aα chains are oriented in the same direction, in Muller-Berghaus G, Scheefers- 


Expression of a fibrinogen fusion peptide in Escherichia coli: a model thrombin substrate for structure/function analysis

ST Lord and DM Fowlkes