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# Inhibition of Platelet Aggregation of a Mutant Proinsulin Molecule Engineered by Introduction of a Native Arg-Gly-Asp Sequence

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## Abstract

A 13 amino acid sequence, CRVARGDWNDNYC, originated from disintegrin eristostatin, was introduced into an inactive human proinsulin molecule between the B29 and A2 sites to replace proinsulin C-peptide by molecular cloning techniques. The constructed Arg-Gly-Asp (RGD)-proinsulin gene was cloned into a temperature-inducible vector pBV220 and expressed in *Escherichia coli*. The expressed RGD-proinsulin was refolded and purified by Sephadex G50 and DEAE-Sephadex A25 separations. The chemical identity was confirmed by both amino acid composition and mass spectrometry analyses. This RGD-proinsulin showed an inhibitory activity of adenosine 5'-diphosphate–induced human platelet aggregation with an $IC_{50}$ value of 200 n*M*. Its insulin receptor binding activity remained as low as 0.03% with native insulin as a control, and its insulin immune activity retained 27.6% compared with proinsulin.

**Index Entries:** RGD motif; mutant proinsulin; RGD-proinsulin; recombinant; inhibition of platelet aggregation.

## Introduction

The Arg-Gly-Asp (RGD) sequence plays an important role as a cell recognition site to interact with integrin receptors on the cell surface *(1)*. This sequence exists in many adhesive proteins such as fibrinogen, fibronectin, and von Willebrand factor. Platelet aggregation is an important step in thrombotic events and requires the binding of fibrinogen to

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GPIIb/IIIa receptor on activated platelets. If RGD-containing proteins or peptides can simulate the functional conformation of the RGD motif, they may have potency to inhibit platelet aggregation, and may be used in the treatment of thrombotic diseases *(2)*. Many proteins or peptides from snake and leech venom contain RGD motifs and are potential antagonists of platelet aggregation *(3–7)*. Nuclear magnetic resonance (NMR) spectroscopic and X-ray crystallographic analyses of some purified RGD-containing proteins have revealed that the functional RGD motifs are usually located at solvent-exposing, highly mobile protein loops and always tend to assume a turn conformation *(8)*. The RGD motifs have been successfully introduced into many proteins. However, only short RGD-containing sequences, such as RGDS, RGDV, and CRGDSC were employed in these insertions *(8–11)*. Also, the protein scaffolds were so large that their clinical application was not promising. Because amino acids flanking the RGD sequence are important for maintaining the active conformation of the RGD motif, insertion of a longer RGD sequence into a smaller appropriate displaying protein frame may produce a molecule with high inhibitory activity of platelet aggregation.

To obtain a novel platelet aggregation inhibitor, a 13 amino acid peptide, CRVARGDWNDNYC, originated from functional motif of disintegrin eristostatin *(3)*, was selected to replace C-peptide of an inactive human proinsulin with no intra-A-chain disulfide bond *(12)*. Recombinant DNA techniques were used. The chimeric protein was purified and its platelet aggregation inhibitory activity, insulin immune activity, and insulin receptor binding activity were determined.

## Materials and Methods

### *Simulation of RGD-Proinsulin Structure*

The structure of the RGD-proinsulin was simulated with SGI workstation using Insight II software. The structural simulation was based on the NMR structure of native human insulin in a protein databank (Brookheaven, CA). The Biopolymer module in Insight II was employed to add the CRVARGDWNDNYC sequence to the B29 and A2 sites of insulin structure. At the same time, A6Cys and A11Cys of insulin were replaced by Ser to delete the intra–A chain disulfide bond. The Builder module was employed to carry out structural optimization (iterations: 1000; derivative: 0.01).

### *Construction of Mutant Gene, and Expression and Purification of RGD-Proinsulin*

The standard polymerase chain reaction method was employed to construct the mutant RGD-containing proinsulin gene. Plasmid pJG401, containing A6 and A11 Cys to Ser mutations of proinsulin gene, was used as template *(12)*. The sequences of the four primers were as follows: AAGGAG GAATTACGG (primer 1, 5' primer); GGACTGCTCCACAAT

ACAATAATTATCATT CCAATCCCCACGAGCCACTCTGCAGGGTGT GTAGAAGAA (primer 2, mutant primer); ATTGTGGAGCAGTCC (primer 3, complementary to the mutant primer); GTCGACGGCTCCTCA (primer 4, 3' primer). Primers 1 and 2 were used to obtain the upstream DNA fragment, and primers 3 and 4 were used to obtain the downstream DNA fragment. Then, the two DNA fragments were mixed as template, and primers 1 and 4 were used to obtain the full-length RGD-proinsulin gene. The gene was inserted into *Eco*RI and *Bam*HI sites of expression vector pBV220 under the control of a $P_RP_L$ promoter *(12)*. The constructed plasmid pJG225 was used to transform *Escherichia coli* strain DH5α. The mutation was confirmed by DNA sequencing.

The procedures for expression and refolding of the RGD-proinsulin were as described previously with some modifications *(13)*. After Sephadex G50 separation, the pH of the RGD-proinsulin fraction was adjusted to 3.0 with 2 *M* HCl, and solid NaCl was added to a final concentration of 12% (w/v) to salt out the RGD-proinsulin. The pellet was collected by centrifugation and dissolved in 0.05 *M* Tris-HCl and 40% 2-isopropanol, pH 7.0, and was further purified by DEAE Sephadex A25 chromatography.

### *Characterization and Determination of RGD-Proinsulin Activity*

The molecular weight of the RGD-proinsulin was determined by mass spectrometry. Amino acid composition and insulin receptor binding activity assay were done as described previously *(14)*. The standard method was used to determine the isoelectric point (p*I*) of the RGD-proinsulin. Insulin radioimmune assay and circular dichroism (CD) analysis were done as described *(15)*. Inhibition of platelet aggregation was determined as follows. Fresh human blood was anticoagulated with 0.01 *M* sodium citrate (10% [v/v]), pH 7.4, and centrifuged at 150*g* at 25°C for 10 min. The supernatant was collected as platelet-rich plasma. The protein samples were dissolved in 0.9% NaCl. Three hundred microliters of platelet-rich plasma were incubated with 25 µL of various concentrations of protein samples at 37°C for 2 min before the addition of 25 µL of 100 µ*M* adenosine 5'-diphosphate (ADP). The inhibition of platelet aggregation was determined by light transmission measurement.

## Results and Discussion

### *Design of RGD-Proinsulin*

Proinsulin is the precursor of insulin hormone and is converted to insulin by cleavage of the C-peptide and two pairs of basic amino acid residues flanking the C-peptide. The C-peptide is a loose loop, exposed on the surface of insulin. Our previous work has demonstrated that the intra-A-chain disulfide bond–deleted insulin showed almost no insulin receptor binding activity, but the whole immune activity was retained *(12,15)*. The structure of the mutant insulin is quite similar to that of native insulin

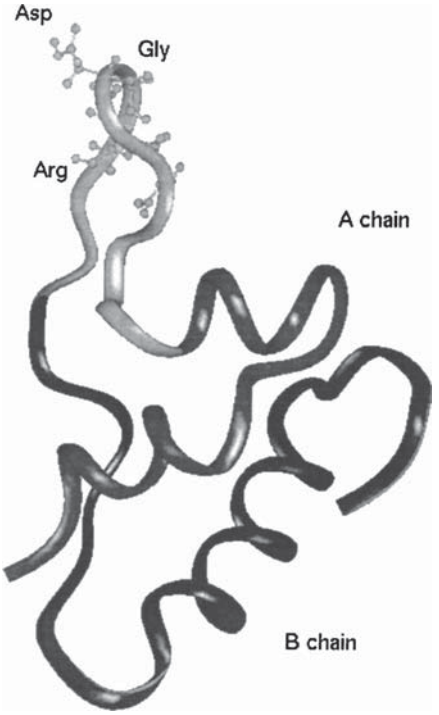


Fig. 1. Simulation of the three-dimensional structure of RGD-proinsulin. The structure was simulated with an SGI workstation using Insight II software. The peptide backbone is shown as a long slender coil shaded in black, with the RGD sequence (including the side chains) highlighted. The A- and B-chains are represented as deep black.

*(12,15,16)*. It seems that this mutant insulin molecule is a promising scaffold for displaying foreign peptides. It is of human origin and inactive in vivo and could be prepared easily as recombinant protein. The spatial distance between the B30 site of the B-chain and the A1 site of the A chain is about 5–10 Å, which is suitable for inserting a small peptide. Also, proinsulin and native RGD-containing platelet antagonists are all small proteins rich in disulfide bonds. The protein frame regions of native platelet antagonists are usually quite compact, whereas the RGD motifs are solvent accessible and are located at a surface loop region of the molecule.

With the similarity of structural characteristics between the C-peptide and the functional RGD motif, it is possible to display an RGD-peptide on the surface of the mutant proinsulin molecule. To reduce further the possibility of degradation of proinsulin to insulin, B29Lys, a possible protease digestion site was deleted. A1Gly and B30Thr were replaced by the two Cys on the two ends of the 13 amino acid RGD-containing peptide. These two Cys may form a disulfide bond to improve the platelet aggregation inhibitory activity of the RGD-containing protein. The structural simulation of RGD-proinsulin (Fig. 1) showed that the 13 amino acid peptide lies on the

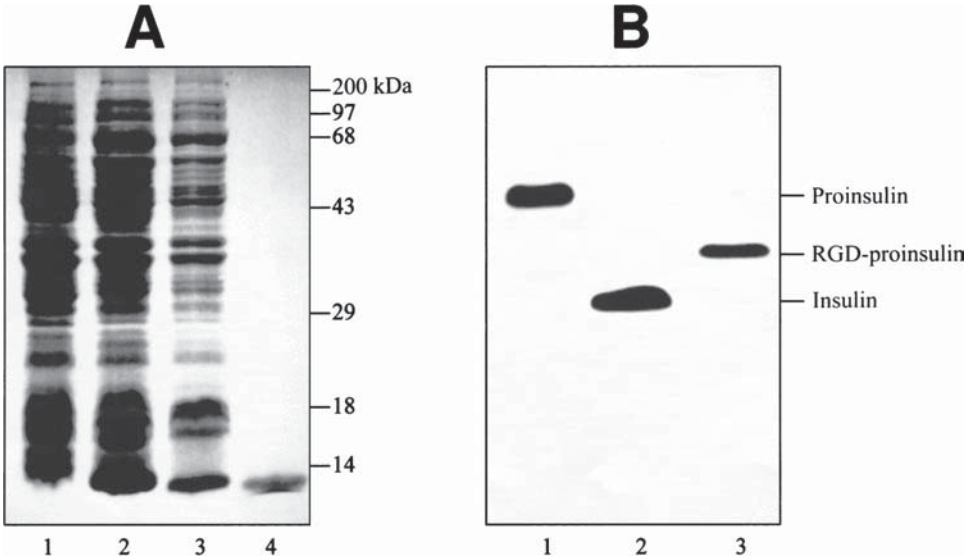


Fig. 2. Electrophoretic analysis of RGD-proinsulin. **(A)** Analysis of expression of recombinant clones by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Lane 1, total cellular proteins of cells transformed by pBV220 vector; lanes 2 and 3, total cellular proteins of cells transformed by recombinant expression plasmid pJG225; lane 4, purified RGD-proinsulin. **(B)** Electrophoretic analysis of the purified RGD-proinsulin on 12% polyacrylamide gel. Lane 1, proinsulin; lane 2, crystalline porcine insulin; lane 3, purified RGD-proinsulin. The gels were stained with Coomassie brilliant blue R-250.

surface of the insulin molecule and the side chains of RGD sequence are extended to the outside of the loop structure. This structural characteristic provides the necessary requirements of recognition and interaction with integrin receptor on the platelet cell surface. The insertion of the RGD-containing peptide to replace the C-peptide has little influence on the insulin structure. The RGD motif forms a native-like conformation and is far away from the core of insulin.

## *Expression, Purification, and Characterization of RGD-Proinsulin*

Routine molecular cloning techniques were used to construct a chimeric gene, and the gene was expressed in *E. coli*. The expression and purification of the RGD-proinsulin was mainly processed according to the method described previously *(13)*. The expressed protein was analyzed, as shown in Fig. 2A. The expression level was 15–20% of the total cellular proteins (lanes 2 and 3). After isolation of inclusion bodies, unfolding, and refolding, a Sephadex G50 chromatography was used to separate the refolded RGD-proinsulin, as shown in Fig. 3A. Peak 2 stands for RGD-proinsulin. The RGD-proinsulin was further purified by a DEAE-Sephadex A25 chromatography (Fig. 3B). A second DEAE-Sephadex A25 column was used to remove 2-isopropanol and NaCl in the first DEAE-Sephadex A25-

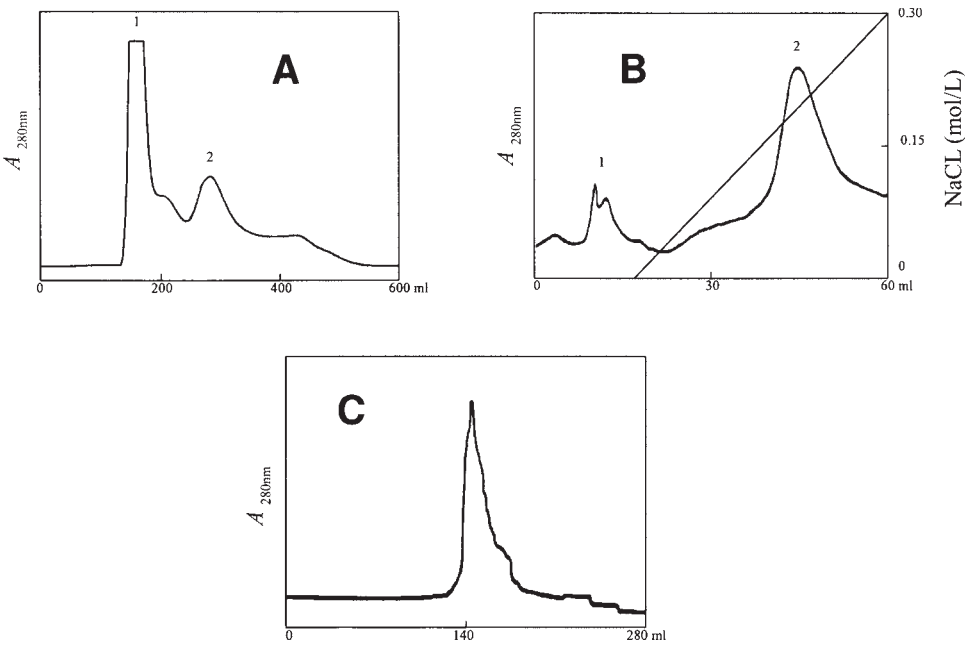


Fig. 3. Purification of RGD-proinsulin. **(A)** Sephadex G50 chromatography separation. The column (1 × 100 cm) was eluted with 0.05 *M* Gly-NaOH buffer (pH 10.8). Peak 2 represents RGD-proinsulin. **(B)** DEAE-Sephadex A25 chromatography to purify further the RGD-proinsulin. The column (0.5 × 1 cm) was equilibrated with 0.05 *M* Tris-HCl and 40% 2-isopropanol at pH 7.0. The RGD-proinsulin was eluted out with a linear NaCl gradient from 0 to 0.30 *M* in a total volume of 60 mL. Peak 2 represents RGD-proinsulin. **(C)** DEAE-Sephadex A25 chromatography to remove 2-isopropanol and NaCl in the RGD-proinsulin fraction from (B).

Table 1
Amino Acid Composition Analysis of RGD-Proinsulin

| | RGD-proinsulin[a] | Met-lys-proinsulin |
|---|---|---|
| Asx | 7.4 (7) | 4.0 (4) |
| Thr | 2.8 (2) | 2.6 (3) |
| Ser | 5.1 (6) | 5.8 (7) |
| Glx | 7.4 (7) | 15.3 (15) |
| Pro | 2.1 (1) | 3.4 (3) |
| Gly | 5.9 (4) | 9.9 (11) |
| Ala | 3.0 (2) | 5.1 (4) |
| Val | 5.0 (5) | 5.0 (5) |
| Met | 1.5 (1) | 1.0 (1) |
| Ile | 2.6 (2) | 1.5 (2) |
| Leu | 6.3 (6) | 12.5 (13) |
| Tyr | 4.6 (5) | 3.7 (4) |
| Phe | 2.9 (3) | 2.5 (3) |
| Lys | 0.6 (0) | 2.4 (2) |
| His | 1.8 (2) | 2.1 (2) |
| Arg | 3.3 (3) | 4.2 (4) |

[a]Data in parentheses are expected values.

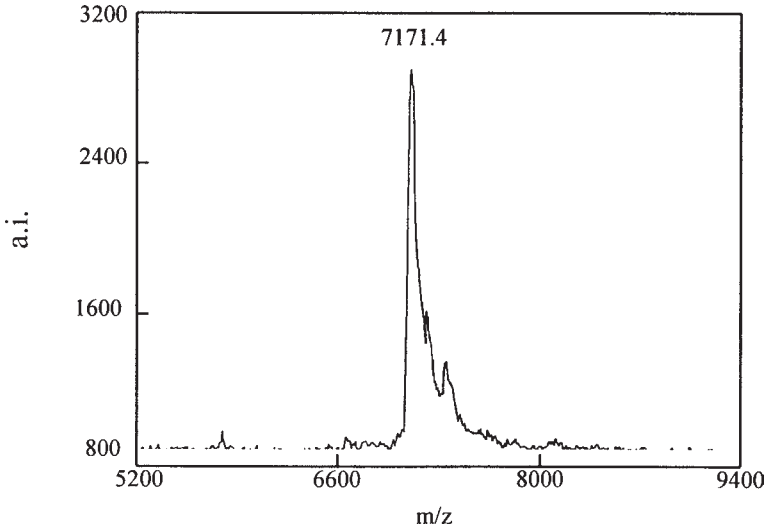


Fig. 4. Mass spectrometry analysis of purified RGD-proinsulin.

Table 2
CD Spectra Analysis of RGD-Proinsulin

| Secondary structure content (%) | RGD-proinsulin | Met-lys-proinsulin |
|---|---|---|
| α-Helix | 10.5 | 9.2 |
| β-Sheet | 23.1 | 19.3 |
| Random structure | 66.4 | 71.5 |

separated RGD-proinsulin fraction (Fig. 3C). High homogeneity of purified RGD-proinsulin can be seen in Fig. 2 by both SDS and native PAGE analyses. The p*I* of the RGD-proinsulin was determined to be 5.1, quite close to that of insulin. This is why our previous procedure for purification of recombinant human insulin could be used.

Table 1 showed the amino acid composition analysis of RGD-proinsulin. A proinsulin protein, met-lys-proinsulin *(13)*, was used as a control. The data agree with our expectation. Figure 4 shows the result of mass spectrometric analysis of RGD-proinsulin. Its molecular weight (7171.4) is almost identical to the expected value (7174) as calculated according to the amino acid composition of RGD-proinsulin. The CD spectra analysis of RGD-proinsulin indicated that it contains very close secondary structure contents compared with proinsulin (Table 2). The inhibitory activity of ADP-induced human platelet aggregation was tested, as shown in Fig. 5. The light transmission of inhibition of ADP-induced platelet aggregation is illustrated in Fig. 5A. The RGD-proinsulin showed quite high platelet aggregation inhibitory activity. The calculation of $IC_{50}$ is shown in Fig. 5B.

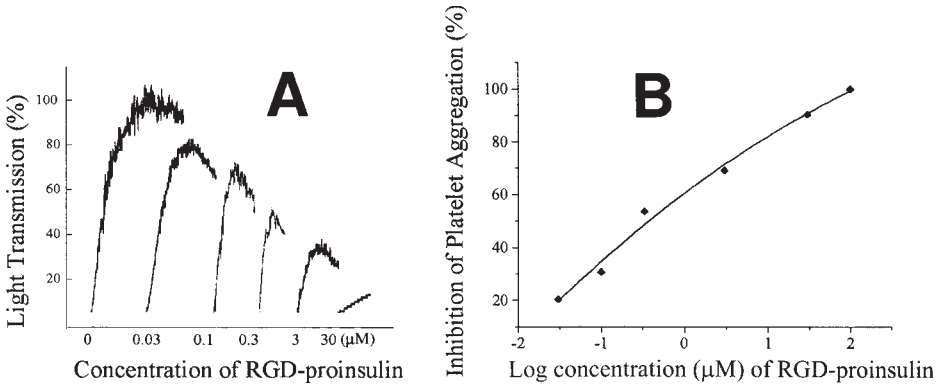


Fig. 5. Inhibitory activity analysis of ADP-induced platelet aggregation by RGD-proinsulin. **(A)** Light transmission measurement of platelet aggregation. Three hundred microliters of platelet-rich plasma were incubated with 25 µL of various concentrations of RGD-proinsulin at 37°C for 2 min before the addition of 25 µL of 100 µ*M* ADP. Samples 1–6 represent control buffer, 0.03, 0.1, 0.3, 3, and 30 µ*M* RGD-proinsulin, respectively. **(B)** Inhibition curve of ADP-induced platelet aggregation of RGD-proinsulin.

Table 3
$IC_{50}$ of Inhibition of Platelet Aggregation
of Some Native Disintegrins

| Native integrin | $IC_{50}$ (n*M*)[a] |
|---|---|
| Eristostatin | 59 ± 22 (3) |
| Barbourin | 309 ± 128 (4) |
| Tergeminin | 192 ± 73 (4) |
| Eristicophin | 104 ± 17 (4) |
| Echistatin | 160 ± 52 (4), 139 ± 17 (3) |
| Kistrin | 180 ± 10 (5) |
| Dendroaspin | 198 ± 28 (5) |
| Elegantin | 330 ± 30 (5) |
| Albolabrin | 392 ± 25 (6), 220 (7) |
| Flavoridin | 40 (7) |

[a]Numbers in parentheses are reference numbers.

The $IC_{50}$ value was 200 n*M*, which is within the potency of native disintegrins *(3–7)*. Table 3 gives the $IC_{50}$ of inhibition of platelet aggregation of some native disintegrins. Under the same conditions, both native insulin and proinsulin showed no inhibitory activity (data not shown). The RGD-proinsulin showed an insulin immune activity of 27.6% compared with proinsulin (Fig. 6A). This is as expected because the presence of a disulfide bond–fixed cyclic RGD-peptide may limit the movement of insulin A-chain N-terminus and B-chain C-terminus and result in a decrease in immune activity. The insulin receptor binding activity of the RGD-proinsulin was

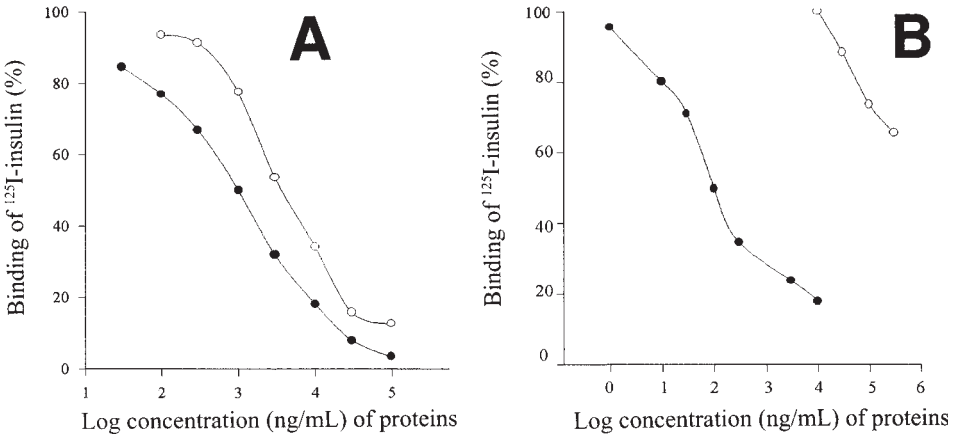


Fig. 6. Insulin radioimmune and receptor binding assays of RGD-proinsulin. **(A)** Result of radioimmune assay: (●) proinsulin as a control, (○) RGD-proinsulin. Insulin polyclonal antibodies were used. **(B)** Result of insulin receptor binding assay: (●) native insulin, (○) RGD-proinsulin.

only 0.03% native insulin, as shown in Fig. 6B. This agrees with our expectation that RGD-proinsulin would not exhibit insulin hormone activity.

These data indicate that the constructed RGD-proinsulin molecule does show advantages over some RGD motif–containing proteins or peptide, including larger chimeras, naturally occurring disintegrins, and synthetic peptides. Lee et al. *(10)* reported that a replacement of RGD sequence on the immunoglobulin $V_L$ domain REI yielded a chimera with an $IC_{50}$ of 4100 n*M* in inhibition of platelet aggregation assay. We made a trypsin-based RGD chimera with an $IC_{50}$ >7000 n*M (11)*. Some RGD-lysozymes were shown to possess only about 10% of cell adhesion activity compared with vitronectin *(9)*. The naturally occurring disintegrins usually gave an $IC_{50}$ of 40–400 n*M* in inhibition of platelet aggregation (Table 3), but they may show a high degree of immunogenicity as foreign proteins in clinical application. Very few synthetic peptides gave an $IC_{50}$ <400 n*M* in the inhibition of platelet aggregation assay *(6,17)*. We conclude from these results that the replacement of the C-peptide with the CRVARG DWNDNYC peptide in mutant proinsulin yielded a novel GPIIb/IIIa receptor antagonist with potent antithrombotic activity, but no insulin hormone activity.

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