Cloning, Characterization, and Functional Studies of a Nonintegrin Platelet Receptor for Type I Collagen

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Abstract

A cDNA (1.6 kb) encoding a platelet protein receptor that binds type I collagen has been isolated from a human bone marrow cDNA library by using a degenerate oligonucleotide probe derived from the amino acid sequence of a CNBr fragment of the purified receptor. Computer search revealed that this cDNA represents the coding sequence of a unique protein. Using the prokaryotic expression system pKK 223-3-65 cDNA, a 54-kD recombinant protein was obtained and purified to apparent homogeneity. In an eukaryotic expression vector (pcDNA3-65 cDNA), a 65-kD protein was identified that was recognized by monoclonal anti-65 kD antibody (anti-65m). The recombinant protein binds to type I, but not to type III collagen by affinity column chromatography. The binding of the recombinant protein to type I collagen–coated Petri dishes is inhibited by anti-65m in a dose-dependent manner. The pcDNA3-65 cDNA-transfected nonadherent T cells express the protein, allowing them to attach to a type I collagen matrix, and are inhibited by anti-65m in a dose-dependent manner. Like the receptor protein purified from platelet membranes, the recombinant protein inhibits type I collagen–induced platelet aggregation and the adhesion of [^{14}C]serotonin-labeled platelets to type I collagen in a dose-dependent manner. The recombinant protein neither binds to type III collagen–coated Petri dishes nor inhibits type III collagen and ADP-induced platelet aggregation, indicating specificity for type I collagen. (*J. Clin. Invest.* 1997. 100:514–521.) Key words: platelet aggregation • platelet aggregation inhibitor • collagen • receptor

Introduction

After injury to the blood vessel wall, circulating platelets adhere to the underlying connective tissue, spread, become activated, and aggregate, forming a hemostatic plug. Although several components of the connective tissue (such as fibronectin, laminin, thrombospondin, and von Willebrand factor) have been shown to be active in platelet adhesion, collagen is thought to be the most thrombogenic matrix molecule in that in addition to adhesion, it also causes activation and aggregation of platelets. Platelet adhesion and activation are also cru-

cial for pathologic thrombosis where atherosclerotic processes lead to exposure of subendothelial connective tissue to the flowing blood. Thus, an understanding of the mechanisms by which platelets adhere to collagen may be useful in devising therapeutic strategies to intervene in thrombosis.

Adhesion of platelets to collagen is clearly complex. Many platelet membrane proteins have been proposed as mediators of platelet adhesion to collagen. Several of these belong to the integrin family; perhaps the most extensively studied and characterized is the α2β1 integrin, which is probably identical to the platelet membrane glycoprotein Ia–IIa complex and the very late activation antigen-2 complex (1, 2). Other integrins reported as platelet surface receptors for collagen include GPIIb/IIIa and Ia/IIa (3, 4), and GPIb-IX (5, 6). Other nonintegrin proteins have also been proposed as collagen receptors, including GPIV (7), a 61-kD protein (8), a 62-kD protein called p62 (9), and a 65-kD protein (10), among others (11–19).

Several years ago we isolated, purified, and characterized a nonintegrin platelet membrane glycoprotein (with a 65 kD M_r) that functioned as a receptor for type I collagen (10). As a further extension of these investigations, in this study we isolated a cDNA clone encoding the 65-kD receptor protein, and have expressed a recombinant protein in both a prokaryotic and eukaryotic expression system. We show that the recombinant protein binds specifically to type I collagen in a dose-dependent manner. When expressed in naturally nonadherent T cells, the protein renders them adherent to a type I collagen–coated matrix that can be inhibited by a specific monoclonal antibody raised against the 65-kD protein (anti-65m).[1] Finally, the purified recombinant protein can inhibit type I collagen–induced platelet aggregation and the release of ATP in a manner identical to the native 65-kD protein isolated from platelets as previously described by us.

Methods

Cloning of the platelet receptor for type I collagen. The platelet receptor protein (65 kD M_r) was purified from isolated human platelet membranes as previously described (10), and was digested with CNBr as follows: 10 mg of purified protein was dissolved in 4 ml of 75% formic acid, and was saturated with N_2 for 10 min before 0.5 g cyanogen bromide was added. The test tube was sealed and incubated at 40°C for 4 h. At the end of incubation, the sample was diluted to 50 ml with cold distilled water, and was frozen and lyophilized. The lyophilized material was dissolved in SDS-PAGE sample buffer, and was subjected to 15% SDS-PAGE (20). The protein was then electrophoretically transferred onto a polyvinylidene difluoride membrane (6 h at 4°C) (21) followed by Amido black staining before destaining with

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1. *Abbreviations used in this paper:* anti-65m, monoclonal anti-65 kD antibody; anti-47p, anti-type III collagen receptor antibody; CEM, nonadherent T lymphoma cell line; CHO, Chinese hamster ovary; LB, liquid broth; PRP, platelet-rich plasma; TBS, 20 mM Tris, 500 mM NaCl, Tween 20 (0.25 ml/liter), pH 7.4.

distilled water. The distinct bands were cut individually and frozen at $-20°C$, and were sequenced with a vapor phase protein sequenator (Protein Core Laboratory, Veterans Administration Medical Center, Memphis, TN).

The amino-terminal sequence (14 amino acids residues) of a large CNBr fragment (25 kD M_r) was determined and used to design degenerate oligonucleotide probes according to the method developed by Lathe (22). Probe 1 (21 mers) is as follows: 5′- GA(T/C) AT(C/A/T) CC(G/A/T/C) (T/C)T(G/A/T/C) GA(A/G) GG(G/A/T/C) TA(T/C)-3′ derived from amino acid sequence D-I-P-L-E-G-Y. Probe 2 (21 mers) is as follows: 5′-CC(G/A/T/C) GT(G/A/T/C) GG(G/A/T/C) AC(G/A/T/C) GA(A/G) (T/C)T(G/A/T/C) AC(G/A/T/C)-3′ derived from amino acid sequence P-V-G-T-E-L-T.

The degenerate oligonucleotides were end-labeled with T4 polynucleotide kinase using $[^{32}PO_4]$-γ-ATP (Amersham Corp., Arlington Heights, IL) and purified through a G-25 column (Stratagene Inc., La Jolla, CA). These end-labeled probes were used to screen the human bone marrow cDNA library (CLONTECH, Palo Alto, CA). Two positive clones were isolated from the screening of 10^6 plaques.

Initial screening of a human bone marrow λgt 11 cDNA library (10^6 plaques) was carried out by using a mixture of both kinased probes. The preliminary positive clones obtained from the initial screening were further screened with each individual probe separately. At the final screening, each oligonucleotide was used separately to assess the positive clones in duplicate. The positive plaque was expanded, and phage DNA was prepared and cut with EcoR1. A 1.6-kb cDNA insert was obtained, gel-purified, and subcloned into pCR II vector (TA Cloning Kit; Invitrogen Corp., San Diego, CA). Plasmid DNA preparation was subjected to restriction enzyme digestion, and was analyzed on agarose gel electrophoresis (data not shown). An intact 1.6-kb full length cDNA insert was recovered by EcoRI digestion, and two fragments were identified by PstI (4.5 and 1 kb), whereas the pCR II-cDNA construct was only linearized with ScaI (5.5 kb).

The cDNA was ligated into a pKK 223-3 prokaryotic expression vector (Pharmacia Biotechnology, Piscataway, NJ), heat-shocked into *Escherichia coli* (JM 105), and grown on liquid broth (LB) agar. Single colonies were picked and regrown in LB with streptomycin and ampicillin in the presence of 1 mM isopropylthio-β-D-galactoside (Gibco Laboratories, Grand Island, NY) overnight. Protein from cell extracts was analyzed by Western analysis using anti-65m (23).

Western blot. Samples were separated by 7.5 or 10% SDS-PAGE (20), and were electrophoretically transferred onto a nitrocellulose sheet (23–25), followed by treatment with 3% dried milk in 20 mM Tris, 500 mM NaCl, Tween 20 (0.25 ml/liter vol/vol), pH 7.4 (TBS) to eliminate nonspecific binding. The nitrocellulose sheet was then washed three times with TBS and incubated with anti-65m (2 μg/ml) overnight at 4°C. After washing three times in TBS, the nitrocellulose sheet was incubated with a second antibody (peroxidase-conjugated goat anti–mouse IgG, 1/2,000) for 3 h at room temperature. After washing three times with TBS and once with 20 mM Tris-HCl, pH 7.4, the nitrocellulose sheet was developed with enhanced chemiluminescence solution as described by the manufacturer (Amersham Corp.).

Purification of recombinant protein. Transformed bacteria (*Escherichia coli*, JM 105) containing the pKK 223-3-65 cDNA for the platelet receptor for type I collagen were grown overnight in LB containing ampicillin (50 μg/ml) and streptomycin (25 μg/ml). Cells were harvested and washed with 20 mM phosphate buffer containing 130 mM NaCl, pH 7.4, followed by resuspension in 50 mM Tris-HCl, pH 7.4, were sonicated for 2 min, and were centrifuged for 20 min at 15,000 *g*. The supernatant was subjected to an affinity column using specific anti-65m coupled to Sepharose 4B (23). The column was equilibrated and washed with 20 mM phosphate, 130 mM NaCl, pH 7.4 (PBS). The bound protein was eluted with 0.2 M glycine, pH 3.5, neutralized with 1 M Tris base, and dialyzed against distilled water (three changes) at 4°C. The dialyzed pool fraction was lyophilized, reconstituted in PBS, and redialyzed against the same buffer. The protein concentration was determined by the method of Lowry et al. (26).

Preparation of platelet-rich plasma (PRP), platelet aggregometry, and washed platelets. PRP was prepared from healthy volunteer donors as described earlier (27). Platelet aggregometry was performed by the turbidimetric method of Born (28) ChronoLog Lumi aggregometer; Chrono-Log Corp., Havertown, PA). Washed platelets were prepared by mixing equal volumes of PRP and 20 mM Tris, 130 mM NaCl, 1 mM EDTA, pH 7.4 (Tris-NaCl-EDTA) and were centrifuged at 1,000 *g* for 5 min. The pellet was washed once with Tris-NaCl-EDTA, and was then suspended in the same buffer or autologous platelet-poor plasma at a concentration of 400,000–600,000 platelets/mm³.

Preparation of type I collagen. Neutral salt-soluble collagen (type I) was extracted from the skins of 3-wk-old white Leghorn chicks according to the method described by Kang et al. (29). Human type I collagen was prepared from placenta in our laboratory, or was purchased from Chrono-Log Corp.

Collagen-coating of microtiter wells. Aliquots of 100 μl containing various amounts of type I collagen in phosphate buffer, pH 7.6, were added into microtiter wells and incubated at 4°C overnight. The amount of collagen bound to the wells was determined in parallel experiments using ¹⁴C-labeled type I collagen. Wells were washed with PBS and incubated with 0.1% bovine serum albumin (150 μl/well) for 30 min at room temperature. The wells were washed with PBS again before the addition of ⁵¹Cr-labeled transfected cells. At the end of incubation, the wells were washed five times with PBS. The radioactivity recovered from each well after solubilization with 5% SDS was detected by scintillation counting in the presence of 10 ml ScintiVerse BD (Fisher Scientific Co., Pittsburgh, PA).

Microtiter cell adhesion assay. A nonadherent T lymphoma cell line (CEM cells) (5×10^9) was electroporated in the presence of pcDNA3-65 cDNA (200 μg), and was grown on type I collagen–coated Petri dishes at 37°C for 3 d in a 5% CO_2 incubator. Adherent cells were detached with a buffer solution containing trypsin-EDTA, and were transferred to a tube containing medium with 15% FCS and soybean trypsin inhibitor (1 mg/ml). This cell suspension was centrifuged, and the cell pellets were resuspended in growing medium for 4 h in non–collagen-coated Petri dishes. At the end of incubation, cells were harvested and washed with growing medium without FCS and antibiotics. Cell suspensions (adjusted to 2×10^7/ml) in growing medium containing 0.1% (wt/vol) bovine serum albumin and 200 μCi/ml of sodium [⁵¹Cr]chromate (Amersham Corp.) were incubated at 37°C for 30 min in a shaking water bath. After incubation, the cells were repeatedly washed until free [⁵¹Cr] in the supernatant was at a minimum (30, 31). The washed and labeled cells were suspended in PBS (10^5/ml), applied to collagen-coated microtiter wells, and incubated for another 30 min. Then the wells were washed with PBS (150 μl) seven times. The attached cells were solubilized with 2% SDS, and the radioactivity recovered was assessed by gamma counting. The specificity of adhesion of cDNA-transfected cells to type I collagen–coated wells was tested using specific antibodies to the receptor protein as specified (see Table III).

Results

Amino acid sequence of isolated CNBr fragments of the 65-kD protein. The purified 65-kD protein was digested with CNBr, and two large fragments (25 and 6 kD M_r) were isolated by SDS-PAGE. The amino-terminal sequence of the 25-kD CNBr peptide was determined by Edman degradation in an automated peptide sequencer. The sequence of D-I-P-L-E-G-Y-P-V-G-T-E-L-T was obtained. A search of computer databases (gene and protein) (National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD) failed to yield a match to any known protein.

Establishment of the primary structure of the 65-kD protein. The pKK 223-3-65 cDNA was sequenced by using cus-

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CTAAGTCATTCACTTTATCTTATCTTATAAGCGTTTTATCATCTCACTTCAACACAAGAA          60
GGGTGAGCACGGTACTATGAGATATTTTGAGAATGAGCCGAGACCATGTTCACATAACTTT         120
TGTATGGTATATTGTTCCGTTTTCTTATTGGTTATTGCTGTTCATTTGTTACTGTGCCAG         180
          M  V  Y  C  S  V  F  L  L  V  I  A  V  H  L  L  L  C  Q       19

ATTTATAAATTTAACTTGATTATAGGTATATACCTATACGAAAAAACTGTGTGTGTGTGT         240
I  Y  K  F  N  L  I  I  G  I  Y  L  Y  E  K  T  V  C  V  C        39

TTGTGTGTGTGTTTATTTATTTACTGTTCAGTACTCTGTATGGTTTCAGGCATCCCCTGG         300
L  C  V  C  L  F  I  Y  C  S  V  L  C  M  V  S  G  I  P  W       59

GAATCCTGCAGTGGGATCTCCCGAGGATCAAGGGGACTACTGTATTTCCTTCCTTCTTGC         360
E  S  C  S  G  I  S  R  G  S  R  G  L  L  Y  F  L  P  S  C       79

TTTCTTCATTGCAGAAGAGCTAATGCAGCTTTAAAAGCTGGGGAGCTTTATAAGTGTATA         420
F  L  H  C  R  R  A  N  A  A  L  K  A  G  E  L  Y  K  C  I       99

TTGTATCATCACTTGCACTGTTATCAGAATTGTAAACCACATGCATTATTTCTAGCAAAA         480
L  Y  H  H  L  H  C  Y  Q  N  C  K  P  H  A  L  F  L  A  K      119

ACACCTATAGCTACAAGACTGAGGTTGGCCGGACACAATGCTCACATGTATAATCCCAGC         540
T  P  I  A  T  R  L  R  L  A  G  H  N  A  H  M  Y  N  P  S      139

ACTTTATGGGAGGCCAAGGTGGGAGAATTGCTTCAGGCCAGGAGCTCAAGACCAGCCTGG         600
T  L  W  E  A  K  V  G  E  L  L  Q  A  R  S  S  R  P  A  W      159

CAACATAGCCCATCTCTATATAAAAGTAAAAATTTGCCAGTATGGTGGTACATGGATATA         660
Q  H  S  P  S  L  Y  K  S  K  N  L  P  V  W  W  Y  M  D  I      179

CCCCTGGAGGGCTATCCTGTGGGCACTGAGCTGACTACACCCAGGACGGCTTTGAATAGG         720
P  L  E  G  Y  P  V  G  T  E  L  T  T  P  R  T  A  L  N  R      199

GCCAACACAAATTCATATACTTTCTTAAAACATTACGAGCCTTTTTTTTCTGTGATTTCA         780
A  N  T  N  S  Y  T  F  L  K  H  Y  E  P  F  F  S  V  I  S      219

TTGTTGTTGTTCGTATTTTTGCTCATCAGCTGTTGTTACTGTTGGTGTGTTTTATGTGTG         840
L  L  L  F  V  F  L  L  I  S  C  C  Y  C  W  C  V  L  C  V      239

GCTCAAGACAATTCTTTCGACGTGGCCCAGGAAAGCCAAGATTGGACACCCCTGCTGTAC         900
A  Q  D  N  S  F  D  V  A  Q  E  S  Q  D  W  T  P  L  L  Y      259

TCCAAGCTCCTCAGGAGGCTGAAGGGAGAGGATCACTTGAGCCCAGGAGTTCAAGACTGC         960
S  K  L  L  R  R  L  K  G  E  D  H  L  S  P  G  V  Q  D  C      279

AGTGAGCCGATGTTACCACTGCACTCAAGCCTTGGAGACTGCAGGAGACCCTATCTCAAAC        1020
S  E  R  C  Y  H  C  T  Q  A  L  E  T  A  G  D  P  I  S  N      299

AAAGTCAGCACAATATACTTCGGTCACGTTGTGTCAGATGCTTTGACCAACAATTGACA         1080
K  V  S  T  I  Y  F  G  H  V  V  S  D  A  L  T  K  N*  L  T     319

GACACGCAAGACAGCATCGGCTTTATCAAGGCCTTCCAAAAGTTGGTAGATCAAGGCCTT        1140
D  T  Q  D  S  I  G  F  I  K  A  F  Q  K  L  V  D  Q  G  L      339

CCAAAAGTTGGTGGTTGCAGATCAAGCTTACACCATTCCAACAACTGTCGGCATTGCTGC        1200
P  K  V  G  G  C  R  S  S  L  H  H  S  N  N  C  R  H  C  C      359

ACAATTTGTGAATGGTCGTGTGACGGGTTGGACAACAGCACCAACGAATGCTTTGTATTG        1260
T  I  C  E  W  S  C  D  G  L  D  N*  S  T  N  E  C  F  V  L     379

CACAACTGGGTGTTTCTCAAGATAAGCCAGTGCCATGTAGACCATATCCAAGAAAGTACG        1320
H  N  W  V  F  L  K  I  S  Q  C  H  V  D  H  I  Q  E  S  T      399

TTCGTGCTCGTCGTGCAACGGAAGTCTGAGTACCAGTATCCTCCCAATACCATGTCTCAA        1380
F  V  L  V  V  Q  R  K  S  E  Y  Q  Y  P  P  N  T  M  S  Q      419

TACTTCGTTGCACTATATAACCCGATGTGCTCAGTTGACCACTGCTCACATCGGTTTTGC        1440
Y  F  V  A  L  Y  N  P  M  C  S  V  D  H  C  S  H  R  F  C      439

TTTGCTTACTGCAAAAGTGCACGATGCTTTGAGGTGAGGAGTGCCGATTTGGCAATGATG        1500
F  G  Y  C  K  S  A  R  C  F  E  V  R  S  A  D  L  A  M  M      459

TTAATAAAAATGTGATATTGATGTTATCATGACTATATTTATAAAAAACGAGTGCTTGGAG        1560
L  I  K  C  D  I  D  V  I  M  T  I  F  I  K  N  E  C  L  E      479

CGGTACTGGTCTAAGAACCTTAGAGCTTGGAGAAGTTTGCACTGTTCAAGTTTGTCATAT        1620
R  Y  W  S  K  N  L  R  A  W  R  S  L  H  C  S  S  L  S  Y      499

TCAGTAAATTTCAGAACTCTTTTACTTTTATTGCTCTACCATTGCTCTAGGAATCCCAAC        1680
S  V  N  F  R  T  L  L  L  L  L  L  Y  H  C  S  R  N  P  N      519

CTGGGGGAGATTGATATTATTAAGATGTGAAAATACAGCATAA                        1722
L  G  R  L  I  L  L  R  C  E  N  T  A  #                          532
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Figure 1. Nucleotide sequence and the deduced amino acid sequence of the cloned cDNA. Custom-designed primers of pKK 223-3 were used to determine the initial nucleotide sequence of the cloned cDNA by chain termination reaction. Other primers (underlined with arrow to indicate the direction of the sequence) were made according to the sequenced portion of the cDNA. The deduced amino acid sequence was determined with a computer program (DNA Strider; Pasteur Institute, Paris, France). The sequence that is identical to the degenerate probes is boxed. The hypothetical signal peptide (===) and the glycosylation sites (*) are indicated in the figure. 123 bp of the untranslated region were also shown upstream from the ATG start codon (position 124). The stop codon (TAA) was located at position 1719.

tom-designed primers (Fig. 1). The open reading frame represented by 1596 bases of this cDNA starts with the ATG start codon at position 124, and stops with the TAA stop codon at position 1720 of the sequenced nucleotide. The deduced amino acid sequence derived from this open reading frame indicated that the mapping of the 25-kD fragment was between amino acid residues 177 and 417. A computer search of the gene bank, however, did not find any significant homology to our cDNA. A segment of 123 bp from the untranslated region of the 5'-end was also identified. The amino acid sequence corresponding to the degenerate oligonucleotide probes of the isolated CNBr peptide is shown in boxes. The recombinant protein is 60 kD M_r. In addition, two N-glycosylation sites were also observed from the deduced amino acid sequence of this receptor. They are located at the amino acid residues 317–319 (N-L-T) and 371–373 (N-S-T) as shown in Fig. 1.

Purification of the recombinant protein. The recombinant protein made in *E. coli* generated from the pKK 223-3-65 cDNA expression vector was isolated and assessed by using anti-65m in Western blot analysis (Fig. 2 *A*). In this assessment, two colonies expressed a protein that is recognized by the anti-65m (lanes *2* and *4*). These two colonies have the same sequence. Protein extracts in lanes *1* and *3* did not react because the cDNA was inserted in the reverse direction as proven in the subsequent sequencing studies (inverted sequence of Fig. 1).

The crude protein from the cell extract was further purified by affinity column chromatography using anti-65m coupled to Sepharose 2B. The degree of purification of the recombinant protein was examined by 7.5% SDS-PAGE. A single band with a 54 kD M_r was obtained (Fig. 2 *B*).

The 54-kD M_r purified recombinant protein is somewhat lower than that of the 65-kD protein isolated from platelet membranes. We considered the possibility that the difference in M_r might be due to glycosylation. We therefore treated the purified receptor protein isolated from platelets with endoglycosidase H. The result shown in Fig. 2 *C* indicates that the apparent 65-kD M_r platelet protein (Fig. 2 *C*, lane *1*) was indeed reduced to 54 kD after treatment with endoglycosidase H (Fig. 2 *C*, lane *2*). Staining with periodic acid Schiff stain of the control showed a reactive band of 65 kD M_r, but the sample treated with endoglycosidase H did not show a stainable band at 54 kD M_r (Fig. 2 *D*). These data strongly support the likelihood that the difference in M_r between these two proteins is due to glycosylation.

In a eukaryotic expression vector (pcDNA3-65 cDNA), a 65-kD protein was indeed expressed in the transfected Chinese hamster ovary (CHO) cells, and this protein product is recognized by the anti-65m (Fig. 3 *A*). The expression product was observed after 2 d (lane *9*), significantly increased after day 3 (lane *10*) and day 4 (lane *11*), but decreased at day 5 (lane *12*). Each band was screened with a densitometer. The area density (OD × mm^2) is 1, 1.7, 2.1, 3.9, and 2.7 for 2–5 d culture (lanes *9–12*), respectively. Isolated platelet membranes were used for control (density = 4.9, lane *13*). The same time course experiment was performed with inverted cDNA insert transfected CHO cells (lanes *1–6* are 0–5 d, respectively). There is no reac-
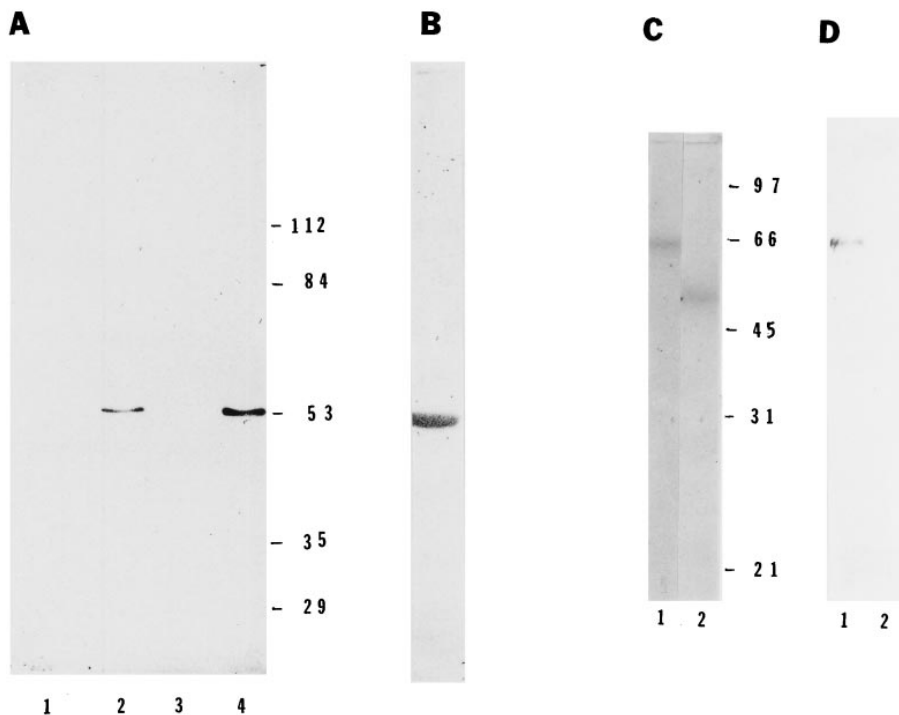
Figure 2. Characterization of protein expressed by pKK 223-3-65 cDNA. *A* shows Western blot (anti-65m) of four colonies (lanes *2* and *4* with correct inserted cDNA; lanes *1* and *3* with inverted insert; confirmed by cDNA sequence). The prestained M_r markers (10^3) are phosphorylase B (112), BSA (84), ovalbumin (53), carbonic anhydrase (35), and soybean trypsin inhibitor (29). *B* shows the purified recombinant protein (10 μg) analyzed by 7.5% SDS-PAGE and stained with Coomassie brilliant blue. *C* and *D* show the purified platelet 65-kD protein before (15 μg, lane *1*) and after incubation with endoglycosidase H (5 U, lane *2*). One set of samples was stained with Coomassie brilliant blue (*C*) and the other set was stained with periodic acid Schiff stain (*D*). Markers (10^3) are phosphorylase B (97), BSA (66), ovalbumin (45), bovine carbonic anhydrase (31), and soybean trypsin inhibitor (21).
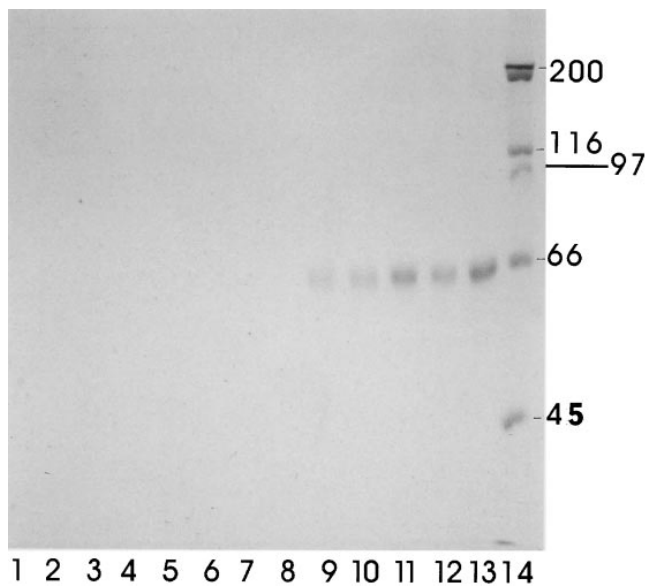

Figure 3. Expression of the recombinant protein in CHO cells. The 65-kD cDNA was ligated into a eukaryotic expression vector (pcDNA3-65 cDNA). The control (inverted sequence, lanes *1–6*) and experimental (correct sequence, lanes *7–12*) plasmids were electroporated (10 μg) into CHO cells. These cells were grown for 0 d (lanes *1* and *7*), 1 d (lanes *2* and *8*), 2 d (lanes *3* and *9*), 3 d (lanes *4* and *10*), 4 d (lanes *5* and *11*), or 5 d (lanes *6* and *12*) for controls and experiments, respectively. At the end of the experiment, the cells were sonicated in a protease inhibitor buffer, and the protein concentration was determined. An equal amount of protein (150 μg) at each time point, and isolated platelet membranes (lane *13*), were analyzed by 10% SDS-PAGE, and were electroblotted onto nitrocellulose membrane. The nitrocellulose sheet was Western blotted with the anti-65m, and was developed with ECL solution. Bio-Rad (Hercules, CA) high M_r markers (10^3) are myosin (200), β-galactosidase (116), phosphorylase B (97), BSA (66), and ovalbumin (45) (lane *14*).

tive band corresponding to 65-kD platelet protein in the inverted cDNA controls.

Binding of the recombinant protein to type I collagen. Several experiments were carried out next to investigate whether the isolated recombinant protein binds to type I collagen specifically. In the first set of experiments, the [^{32}PO$_4$]-recombinant protein was applied to an affinity column of type I collagen–Sepharose or type III collagen–Sepharose 2B. As shown in Fig. 4, the radiolabeled recombinant protein bound to type I collagen–Sepharose 2B (Fig. 4, □-□) but not to type III collagen–Sepharose-2B (Fig. 4, ◆-◆). The first peak eluting unretarded from the type I-Sepharose 2B column represents labeled protein not bound to the affinity column due to overloading.

In additional experiments, varying amounts of the radiolabeled recombinant protein were incubated on type I collagen–coated Petri dishes, and the amount of radioactivity bound was measured. As shown in Table I, binding was dependent on the amount of radiolabeled recombinant protein added. Binding was inhibited by a 10-fold excess of unlabeled recombinant protein, indicating that the binding was specific. Moreover, binding could be inhibited in a dose-dependent manner by anti-65m, but not by anti–type III collagen receptor antibody (anti-47p) (32) (Table II). There was no demonstrable binding of the labeled recombinant protein to type III collagen– or fibronectin-coated Petri dishes (data not shown). These data strongly indicate that the recombinant protein binds to type I collagen specifically.

Effect of expression of the recombinant protein in a eukaryotic cell line. To gain an insight into the biologic significance of the 65-kD protein, the effect of expression of the recombinant protein in a nonadherent eukaryotic cell line was studied. CEM cells were transfected with the cDNA construct (pcDNA3-65 cDNA). Cells were then grown on type I collagen–coated Petri dishes (100 cm^2 in RPMI medium contain-
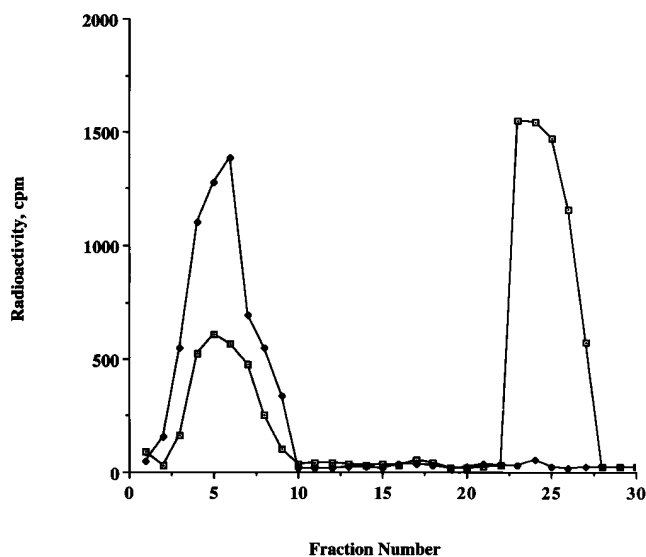
Figure 4. Binding of the isolated recombinant protein to type I collagen–Sepharose 2B column. The purified recombinant protein (3 mg) was incubated with cAMP-dependent protein kinase (25 U) and 10 μM [^{32}PO$_4$]-γ-ATP for 30 min and purified on a Sephadex G-25 column. The [^{32}PO$_4$]-recombinant protein was collected, freeze-dried, redissolved in 20 mM Tris-HCl, pH 7.4, and dialyzed against the same buffer. One aliquot was applied to type I (□-□), and another aliquot to type III (◆-◆) collagen affinity column (20-ml gel beads). The columns were washed with 40 ml Tris-HCl, pH 7.4, followed by 40 ml Tris-HCl, pH 7.4, containing 4 M urea (starting with fraction 20). Fractions were collected, and the radioactivity in each was detected with a counter in the presence of 10 ml ScintiVerse BD (Fisher Scientific Co.). Y axis, radioactivity/fraction; x axis, fraction numbers.

ing pen-strep and 15% FCS) for 3 d. Two cell populations were obtained; one population of cells attached to type I collagen–coated plates, and the second population of cells did not attach but floated in the medium. Both the adherent and nonadher-

Table I. Binding of Recombinant Protein to Type I Collagen

[^{32}PO$_4$]-recombinant protein-added	[^{32}PO$_4$]-recombinant protein-bound
	cpm±SD
2.5 μg	305±56
5 μg	1202±871
10 μg	1651±328
20 μg	3336±687
20 μg + nonlabeled recombinant protein (200 μg)	451±72

Biocoat Petri dishes (60 mm; Becton Dickinson, Bedford, MA) were coated with 1 ml type I collagen (300 μg) in 120 mM phosphate buffer, pH 7.6, at 4°C overnight. The wells were washed with PBS three times. Various amounts of [^{32}PO$_4$]-recombinant protein (1 ml) were added to the dishes, followed by incubation at room temperature for 30 min. At the end of incubation, each dish was washed with PBS (2 ml) five times. The bound recombinant protein was detached from the dish by two washes with a solution of 2% SDS-PBS (1 ml each). The washes were combined in a vial, and the radioactivity in each vial was assessed with a scintillation counter in the presence of 10 ml ScintiVerse BD (Fisher Scientific Co.). Data are expressed as mean±SD of triplicate experiment.

Table II. Effect of Anti-65m and Anti-47p on Binding of Labeled Recombinant Protein to Type I Collagen-coated Petri Dishes

| IgG	Bound [^{32}PO$_4$]-recombinant protein (cpm±SD)	
μg	*Anti-65m**	*Anti-47p‡*
0	1630±126	1630±126
1	1043±113	1545±174
5	485±184	1651±45
10	293±97	1687±85
20	137±78	1648±117

Various amounts of anti-65m and anti-47p were added to the test tubes containing 10 μg of [^{32}PO$_4$]-recombinant protein in a final volume of 1 ml, and were incubated at room temperature for 30 min. The incubated materials were then transferred to PBS-washed Biocoat Petri dishes (60 mm) (Becton Dickinson Labware, Bedford, MA) that were coated with type I collagen and incubated for another 30 min. At the end of incubation, the Petri dishes were washed with PBS, and the radioactivity in each dish was assessed as described in Table I. Data are expressed as mean±SD of triplicate experiments. *Anti-65m is monoclonal IgG raised against type I collagen receptor (65 kD) (23). ‡Anti-47p is polyclonal IgG raised against type III collagen receptor (47 kD) (32).

ent cells were collected separately, lysed by sonication, and analyzed by Western blot as shown in Fig. 5. Both the adherent cells (lane *3*) and platelet membranes (used as positive control, lane *4*) showed a band (65 kD) that is recognized by the anti-65m. By contrast, neither the vector-transfected cells (used as
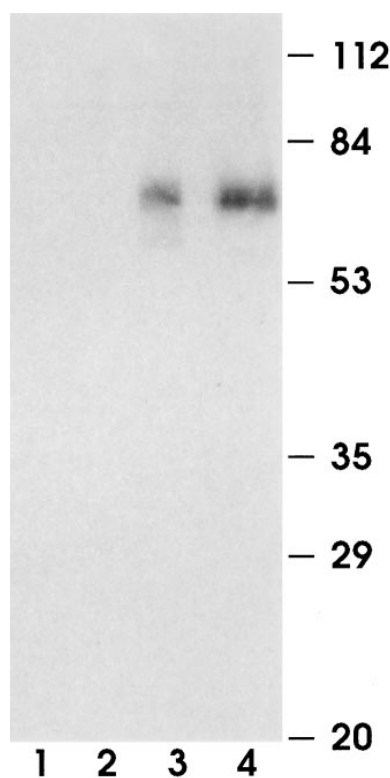
Figure 5. Expression of the recombinant protein in nonadherent eukaryotic cells. The pcDNA3-65 cDNA was electroporated (20 μg) into CEM cells. The transfected cells were grown on type I collagen–coated Petri dishes (100 cm^2 in RPMI medium containing pen-strep and 15% FCS) for 3 d. The vector-transfected cells (lane *1*) and transfected, nonadherent cells (lane *2*) were collected from the suspension. The Petri dishes were then washed three times with PBS (10 ml each), and the adherent cells (lane *3*) were detached from the Petri dishes with a rubber policeman. Western blot was performed with anti-65m and developed with ECL solution. The isolated platelet membranes (lane *4*) served as a positive control. The prestained M_r markers are the same as Fig. 2 *A*.
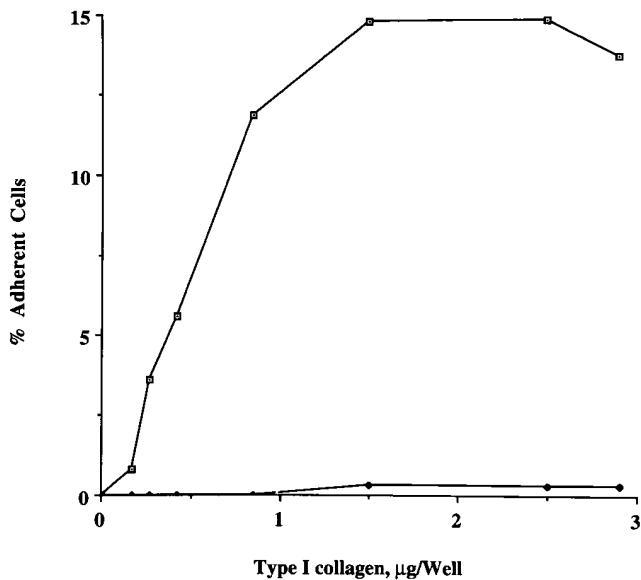
Figure 6. Binding of pcDNA3-65 cDNA–transfected cells to microtiter wells coated with varying concentrations of type I collagen. Microtiter wells were coated with various amounts of type I collagen (0, 0.17, 0.27, 0.42, 0.85, 1.5, 2.5, and 2.9 µg/well) in phosphate buffer at 4°C overnight and blocked with 100 µg/ml BSA in PBS for 1 h. The wells were then washed with PBS and 100 µl of ^{51}Cr-labeled, transfected cells (1,400 cpm) (□-□) or control (vector-transfected) cells (1,370 cpm) (◆-◆) were added and then incubated at 37°C for 30 min. At the end of incubation, the wells were washed five times with PBS, and the adherent cells were detached with two aliquots of 100 µl of 5% SDS. The radioactivity in each sample was assessed in a scintillation counter in the presence of 10 ml ScintiVerse BD. Data are shown as mean of duplicate experiments.

negative control, lane *1*) nor the nonadherent cells (lane *2*) expressed the anti-65m–reactive protein. These data suggest that the adherence of the positively transfected cell population is the result of interaction between the receptor for type I collagen on the cell membrane and the type I collagen immobilized on the plate.

The effect of the coating concentration of type I collagen on the binding of transfected cells was investigated next. Microtiter wells were coated with varying concentrations of type I collagen, and ^{51}Cr-labeled pcDNA3-65 cDNA–transfected cells (□-□) or control (vector-transfected) cells (◆-◆) were incubated for 30 min at 37°C. As shown in Fig. 6, the binding of pcDNA3-65 cDNA-transfected cells was collagen concentration–dependent. Control (vector-transfected) cells did not bind to type I collagen–coated microtiter wells. Furthermore, the binding of the labeled transfected cells to type I collagen–coated microtiter wells was inhibited by the addition of anti-65m in a dose-dependent manner (Table III). By contrast, anti-47p had no effect on binding of the transfected cells to type I–coated microtiter wells. Finally, there was no detectable binding of the transfected cells on type III collagen–coated microtiter wells, or on fibronectin-coated microtiter wells (data not shown). These data indicate that the expression of the 65-kD recombinant protein on nonadherent eukaryotic cells renders them adherent to type I collagen specifically, supporting the importance of the 65-kD protein in interaction with type I collagen.

Table III. Binding of pcDNA3-65 cDNA–transfected CEM Cells to Type I Collagen–coated Petri Dishes and the Effect of anti-65m and anti-47p

IgG	Bound [^{51}Cr]-CEM cells (cpm±SD)	
µg	*Anti-65m*	*Anti-47p*
0	1085±113	1085±113
1	670±38	1032±8
5	370±43	1062±21
10	224±24	1014±35
20	254±13	1024±24

Aliquots of the [^{51}Cr]-CEM cells (12,391 cpm) were incubated with various amounts of IgG for 30 min at room temperature. At the end of incubation, cells were transferred to microtiter wells that were coated with type I collagen and incubated for another 30 min. The free [^{51}Cr]-cells were aspirated, and the microtiter wells were washed six times with PBS (150 µl each). The attached [^{51}Cr]-cells were solubilized with 50 µl of 2% SDS three times. The radioactivity in the solubilized material was assessed by gamma counting. Data are expressed as mean±SD of triplicate experiments without subtracting background count (135 cpm).

Effects of the purified recombinant protein on platelet function. Previous studies showed that the 65-kD protein isolated and purified from platelets could competitively inhibit type I collagen–mediated platelet aggregation, and the release of ATP (13). We therefore tested the effect of the purified recombinant protein on type I collagen–induced platelet aggregation, and the release of ATP. As shown in Fig. 7 (*top*), both platelet aggregation and the release of ATP were inhibited after preincubation of the recombinant protein with type I collagen in a dose-dependent manner. The degree of inhibition by 5 µg of the recombinant protein in the assay (curves *C* and *c* for aggregation and the release of ATP, respectively) was 70%, whereas 100% inhibition was attained by adding 10 µg of the recombinant protein (curves *D* and *d* for aggregation and the release of ATP, respectively) compared to collagen alone (curves *B* and *b* for aggregation and the release of ATP). Buffer control is shown in curves *A* and *a* where no aggregation (curve *A*) or release of ATP (curve *a*) was obtained.

The inhibitory effect of the recombinant protein could be overcome by increasing the dose of type I collagen used to stimulate platelets. As shown in Fig. 7 (*bottom*), 10 µg of the recombinant protein (curve *B* and *b*, first *arrow*) clearly inhibits both platelet aggregation and the release of ATP induced by 0.5 µg type I collagen. At the second *arrow*, an additional dose of 0.5 µg type I collagen was added, and platelet aggregation was restored (Fig. 7, *bottom*, curve *B*) but the release of ATP (curve *b*) was only partially restored.

Increasing the amount of recombinant protein to 20 µg, as may be expected, inhibited the platelet aggregation and the release of ATP mediated by 0.5 µg type I collagen (Fig. 7, *bottom*, curves *C* and *c* for aggregation and the release of ATP, respectively; first *arrow*). The addition of another 0.5 µg type I collagen could not reverse the inhibition (Fig. 7, *bottom*, curves *C* and *c* for aggregation and the release of ATP, respectively; second *arrow*). At the end of the experiment (curve *C*, third *arrow*) an aliquot of 4 µM ADP was added to the same cuvette. Both platelet aggregation and the release of ATP
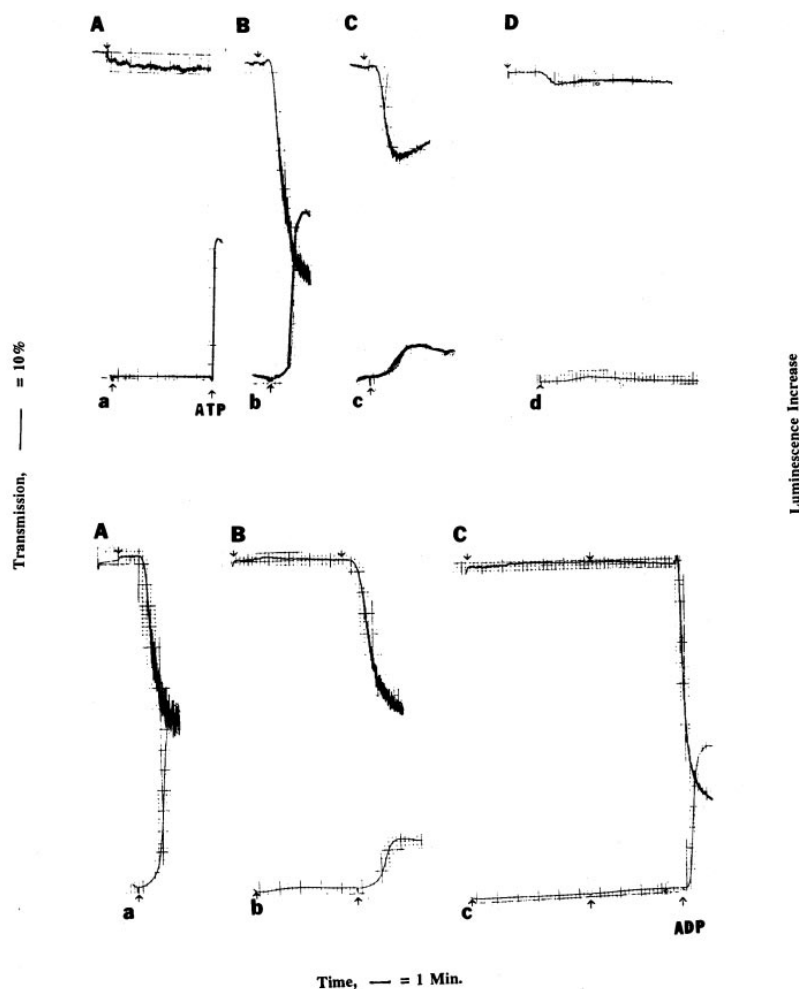
Figure 7. Effect of recombinant protein on type I collagen–induced platelet aggregation and the release of ATP. (*Top*) The effect of recombinant protein on type I collagen–induced platelet aggregation and the release of ATP. Type I collagen (0.4 μg) was preincubated with various amounts of the recombinant protein for 10 min in aggregation cuvettes. Each incubated sample was added to PRP (0.45 ml) and luciferin luciferase (25 μl). A reduction in transmission (*A–D*) and an increase in luminescence due to the release of ATP (*a–d*) were recorded as shown in the figure. *A* and *a* are the aggregation and the release of ATP by the vehicle control, respectively. At the end of the experiment, ATP (1 μM) was added to the cuvette to calibrate the luminescence increase. *B* and *b* are 0.4 μg type I collagen–induced aggregation and release of ATP, respectively. *C* and *c* are tracing for 0.4 μg collagen + 5 μg recombinant protein. *D* and *d* are 0.4 μg collagen + 10 μg recombinant protein. (*Bottom*) The inhibitory effect of recombinant protein can be overcome by increased concentrations of type I collagen. *A* and *a* represent 0.5 μg type I collagen–induced platelet aggregation and the release of ATP, respectively. *B* and *b* represent platelet aggregation and the release of ATP by the addition of the recombinant protein (10 μg) and type I collagen (0.5 μg at the first *arrow*). At the second *arrow*, an additional dose of 0.5 μg type I collagen was added. *C* and *c* are platelet aggregation, and the release of ATP by the addition of 20 μg of the recombinant protein and type I collagen (0.5 μg at the first *arrow*). At the second *arrow*, another 0.5 μg of type I collagen was added. At the end of the experiment, 4 μM ADP was added to test the response of platelets.

were observed, suggesting that the platelet was not damaged by the addition of the recombinant protein.

Discussion

We have cloned a 1.6-kb cDNA with a functional open reading frame from the human bone marrow cDNA library using two degenerate oligonucleotide probes designed from the amino acid sequence of a CNBr fragment (25 kD M_r) of a platelet nonintegrin type I collagen receptor (65 kD M_r). This cDNA was subcloned into a prokaryotic expression vector (pKK 223-3), and a recombinant protein was expressed and purified to apparent homogeneity as shown on 7.5% SDS-PAGE. This recombinant protein is 54 kD M_r, and it is recognized by anti-65m specific for the platelet type I collagen receptor by Western blot analysis. The observed M_r of the prokaryotic recombinant protein is smaller than the platelet receptor protein that has a 65 kD M_r. To investigate whether the difference could be attributed to glycosylation, two sets of experiments were carried out. When the 65-kD protein isolated from platelet membranes is treated with endoglycosidase H, the protein was reduced to 54 kD M_r. The 54-kD protein is not stained by periodic acid Schiff stain. In a eukaryotic expression vector, a recombinant protein was expressed in CHO cells with a 65 kD M_r. These data suggest that the receptor has a 54 kD M_r and is

posttranslationally modified by glycosylation. The glycosylation of this protein does not seem to affect the function of the protein, since both the recombinant protein and the purified platelet protein inhibit type I collagen–induced platelet aggregation.

Type I collagen–induced platelet aggregation and the release of ATP were both inhibited by preincubation of the recombinant protein and collagen in a dose-dependent manner. Preincubation of platelets with the recombinant protein did not inhibit type I collagen–induced platelet aggregation or the release of ATP. This effect was observed when the recombinant protein was preincubated with type I collagen, suggesting that the recombinant protein binds to collagen.

Binding of pcDNA3-65 cDNA–transfected T cells to type I collagen–coated microtiter wells is type I collagen–dose-dependent. Importantly, the transfected cells did not bind to type III collagen–coated microtiter wells, suggesting that the 65-kD protein plays a role in cell adhesion, specifically for type I collagen.

Although chick type I collagen was used in our initial work on the isolation of the 65-kD receptor protein, we have subsequently found that the same results are obtained by using type I collagen from other mammalian species, including humans. For example, the binding of the recombinant protein to the type I collagen–Sepharose 2B column (Fig. 4), the adhesion of

cDNA-transfected CEM cells to collagen-coated wells (Fig. 5), and the platelet aggregation studies (Fig. 7) were performed using human collagens prepared by us. Comparable results are obtained by using Chrono-Log Corp. collagen, or rat type I collagen. It would appear, therefore, that the specific structural elements of collagen involved in interaction with 65-kD platelet receptor protein are common to type I collagen from various species.

Taken together, our current data suggest that the recombinant protein encoded by the 1.6-kb cloned cDNA is a nonintegrin platelet receptor for type I collagen. This postulation is based on five supporting data. First, the recombinant protein is recognized by the monoclonal antibody raised against the platelet-purified 65-kD protein. Second, the purified recombinant protein inhibits type I collagen–induced platelet aggregation and the release of ATP in a dose-dependent manner. Third, the inhibitory effect of the recombinant protein on platelet aggregation and the release of ATP requires the preincubation of the recombinant protein with collagen. Fourth, the binding of the recombinant protein to type I collagen–coated Petri dishes is inhibited by anti-65m but not anti-47p in a dose-dependent manner. And finally, the binding of the transfected [^{51}Cr]-cells to type I collagen–coated microtiter wells is inhibited in a dose-dependent manner by the addition of anti-65m.

Although our data presented in this paper clearly indicate that the 65-kD protein serves as platelet receptor for type I collagen, it does not exclude the possibility that other platelet surface proteins (1–19) may also play a role in collagen–platelet interaction. Moreover, the relationship between the cloned receptor described here and other nonintegrin receptors described by others is not known. A search of gene and protein databases did not find a matching sequence, suggesting that the 65-kD protein is a novel protein. More studies will be needed to clarify the complexities involved in collagen–platelet interaction.

Acknowledgments

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