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-transformed 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 [14C]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 crucial 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 αβ1 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 Mr) 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). 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 Mr) 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 N2. After 10 min before 0.5 g cyanogen bromide was added. The test tube was scaled and incubated at 4°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 elecrophoretically transferred onto a polyvinylidene difluoride membrane (6 h at 4°C) (21) followed by Amido black staining before destaining with 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.

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Received for publication 26 February 1997 and accepted in revised form 2 May 1997.

constituted in PBS, and redialyzed against the same buffer. The protein was generated using D-I-P-L-E-G-Y probes according to the method developed (Kang et al. (29)). The bound protein was eluted with 0.2 M glycine, pH 3.5, and was recovered from each well after solubilization with 5% SDS. The amino-terminal sequence (14 amino acids residues) of a large CNBr fragment (25 kD M,) 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’- GAG(T/C) AT(T/A/ C) CC(G/A/T/C) (T/C)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) TG(T/A/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 [γ-32P]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 106 plaques.

Initial screening of a human bone marrow cDNA library (105 plaques) was carried out by using a mixture of both kinase-labeled 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 plaques were expanded, and plasmid DNA was prepared and cut with EcoRI. 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 (5.4 kb and 1.1 kb), whereas the pCR II-cDNA construct was only linearized with Scal (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 each extract was analyzed by Western analysis using anti-65m (23).

**Western blot.** Samples were separated by 7% or 10% SDS-PAGE (20), and were electrophoretically transferred onto a nitrocellulose sheet (20-25), followed by treatment with 3% dried milk in 20 mM Tris, 300 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 (250 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 pooled fraction was lyophilized, reconstituted in PBS, and reanalyzed with the same buffer. The protein concentration was determined by the method of Lowry et al. (26).

**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 µg/ml) for 30 min at room temperature. The wells were washed with PBS again before the addition of 32P-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 × 106) 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% CO2 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 × 107/ml) in growing medium containing 0.1% (wt/vol) bovine serum albumin and 200 µCi/ml of sodium [35Cr]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 of [35Cr] in the supernatant was at a minimum (30, 31). The washed and labeled cells were suspended in PBS (107/ml), applied to collagen-coated microtiter wells, and incubated for another 5 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 assayed 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,) 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-
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'-H11032 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 Mr. 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. 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 Mr was obtained (Fig. 2B). The 54-kD Mr 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 Mr might be due to glycosylation. We therefore treated the purified receptor protein isolated from platelets with endoglycosidase H. The result shown in Fig. 2C indicates that the apparent 65-kD Mr platelet protein (Fig. 2C, lane 1) was indeed reduced to 54 kD after treatment with endoglycosidase H (Fig. 2C, lane 2). Staining with periodic acid Schiff stain of the control showed a reactive band of 65 kD Mr, but the sample treated with endoglycosidase H did not show a stainable band at 54 kD Mr (Fig. 2D). These data strongly support the likelihood that the difference in Mr 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. 3A). 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/H11003 mm²) 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/H11005 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 reaction 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.
A Nonintegrin Platelet Receptor for Type I Collagen

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, \(\text{H11623}\)) but not to type III collagen–Sepharose 2B (Fig. 4, \(\text{H17004}\)). The first peak eluting unretracted 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–Sephadex 2B column. The purified recombinant protein (3 µg) was incubated with cAMP-dependent protein kinase (25 U) and 10 µM [32PO4]γ-ATP for 30 min and purified on a Sephadex G-25 column. The [32PO4]-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.

Table I. Binding of Recombinant Protein to Type I Collagen

<table>
<thead>
<tr>
<th>[32PO4]-recombinant protein-added</th>
<th>[32PO4]-recombinant protein-bound</th>
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<tbody>
<tr>
<td></td>
<td>cpm±SD</td>
</tr>
<tr>
<td>2.5 µg</td>
<td>305±56</td>
</tr>
<tr>
<td>5 µg</td>
<td>1202±871</td>
</tr>
<tr>
<td>10 µg</td>
<td>1651±328</td>
</tr>
<tr>
<td>20 µg</td>
<td>3336±687</td>
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<tr>
<td>20 µg + nonlabeled recombinant protein (200 µg)</td>
<td>451±72</td>
</tr>
</tbody>
</table>

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 [32PO4]-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 experiments.

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 cm²) 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 prestained M markers are the same as Fig. 2A.

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

<table>
<thead>
<tr>
<th>IgG</th>
<th>Bound [32PO4]-recombinant protein (cpm±SD)</th>
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<tr>
<td></td>
<td>Anti-65m*</td>
</tr>
<tr>
<td>0</td>
<td>1630±126</td>
</tr>
<tr>
<td>1</td>
<td>1043±113</td>
</tr>
<tr>
<td>5</td>
<td>485±184</td>
</tr>
<tr>
<td>10</td>
<td>293±97</td>
</tr>
<tr>
<td>20</td>
<td>137±78</td>
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</tbody>
</table>

Various amounts of anti-65m and anti-47p were added to the test tubes containing 10 µg of [32PO4]-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
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 

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\text{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 
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\text{collagen–mediated platelet aggregation, and the release of } \text{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

<table>
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<tr>
<th>IgG</th>
<th>Bound [%Cr]-CEM cells (cpm±SD)</th>
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<tbody>
<tr>
<td>μg</td>
<td>Anti-65m</td>
</tr>
<tr>
<td>0</td>
<td>1085±113</td>
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<tr>
<td>1</td>
<td>670±38</td>
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<tr>
<td>5</td>
<td>370±43</td>
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<tr>
<td>10</td>
<td>224±24</td>
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<tr>
<td>20</td>
<td>254±13</td>
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Aliquots of the [35S]-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 [35S]-cells were aspirated, and the microtiter wells were washed six times with PBS (150 μl each). The attached [35S]-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).
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 $M_t$) of a platelet nonintegrin type I collagen receptor (65 kD $M_t$). 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 $M_t$, and it is recognized by anti-65m specific for the platelet type I collagen receptor by Western blot analysis. The observed $M_t$ of the prokaryotic recombinant protein is smaller than the platelet receptor protein that has a 65 kD $M_t$. 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 $M_t$. 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 $M_t$. These data suggest that the receptor has a 54 kD $M_t$ 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

**References**


**Acknowledgments**

We wish to thank Ms. V. Rasberry, Mr. J. West, and Mr. K. Alderick for their expert technical assistance.

This research was supported by the Department of Veterans Affairs.

We thank the following colleagues for their expert technical assistance.

This research was supported by the Department of Veterans Affairs.