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ISOLATION AND CHARACTERIZATION OF A PLATELET SURFACE COLLAGEN BINDING COMPLEX RELATED TO VLA-2

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SUMMARY: A heterodimeric, Mg^{++}-dependent, collagen binding protein has been isolated from platelet membranes. Electrophoretic properties and monoclonal antibody reactivity indicate that the heavy chain of the complex is platelet membrane glycoprotein Ia and that the light chain is glycoprotein IIa. Furthermore, the receptor appears to be identical with the recently defined VLA-2 complex found on activated T-lymphocytes, platelets and other cells. When incorporated into liposomes, the purified complex mediates the Mg^{++}-dependent adhesion of the liposomes to collagen substrates. These observations suggest that the VLA-2 complex mediates cellular adhesion to collagen in platelets and possibly in other cells.

Fibrillar collagen is the most thrombogenic vessel wall macromolecule (1). Upon vascular injury and exposure to collagen, blood platelets rapidly adhere, become activated, synthesize and/or secrete proaggregatory substances such as thromboxane A_2, ADP and adhesive proteins and express upon their surface activation-dependent receptors for adhesive proteins. These events result in the formation of a hemostatic platelet plug at the site of injury (2). Although many platelet surface molecules have been proposed as mediators of platelet-collagen adhesion, none has yet gained general acceptance as a physiologically relevant mediator of platelet-collagen adhesion.

We recently described a Mg^{++}-dependent process of platelet adhesion to collagen which exhibited many of the properties expected of a physiologically relevant platelet-collagen adhesive mechanism (3). The adhesive process was

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specific for native collagen as gelatin was not an effective substrate. Although platelets could adhere to either monomeric or fibrillar collagen substrates via this mechanism, only adhesion to the fibrillar substrates resulted in platelet activation and secretion. The vessel wall interstitial collagens types I and III, as well as type IV collagen, which is the major collagenous component of basement membranes, supported adhesion via the Mg^{++}-dependent mechanism. We have now exploited the Mg^{++}-dependence of the adhesive process to isolate and characterize a Mg^{++}-dependent collagen binding protein which appears to mediate platelet adhesion to collagen and possibly adhesion of other cell types to collagen as well.

MATERIALS AND METHODS

Purification of Collagen Binding Complex. Platelets were washed and freeze-thawed twice (4). Particulate material was collected by centrifugation and solubilized in buffer [0.05 M Tris·HCl (pH 7.4), 0.15 M NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1% Lubrol-PX]. Protease inhibitors [PMSF (2 mM) Trasylol (2 mM) and leupeptin (2 mM)] were included in the extraction buffer. After centrifugation (20,000 x g, 45 min, 4°C), the soluble supernatant was applied to a Concanavalin A-Sepharose column equilibrated with the above buffer in which the Lubrol concentration was reduced to 0.1%. After extensive washing, glycoproteins bound to the column were eluted by the addition of α-methyl mannoside (0.5 M) to the column buffer. Eluted proteins were dialized extensively against 0.05 M Tris·HCl (pH 7.4), 0.15 M NaCl, 2 mM $MgCl_2$ and 0.1% Lubrol to lower the Ca^{++} concentration and then subjected to affinity chromatography on a column of native triple helical collagen-Sepharose prepared by coupling collagen at 1 mg/ml in 0.194 M Na_2HPO_4 (pH 8.5) to cyanogen bromide activated Sepharose 4B for 24 hrs at 4°C. Unreacted sites were blocked with 0.5 M Tris (pH 8.8). After extensive washing of the collagen column, the column was eluted by substitution of 2 mM EDTA for the $MgCl_2$ in the column buffer.

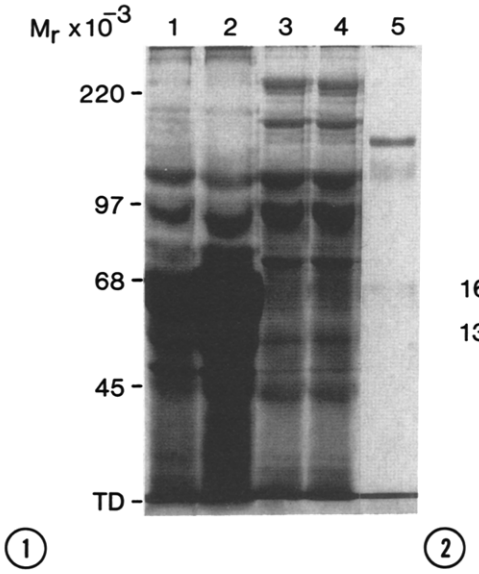
Radiolabeling. The purified collagen binding complex was radiolabeled with ^{125}I using 500 μCi Na ^{125}I (Amersham, Arlington Heights, IL) and two Iodobeads (Pierce Chemical Co., Rockford, IL) for 15 min according to the manufacturers instructions.

Analytical Procedures. Immunoprecipitations were performed as described by Pischel et al (5) using protein A-bearing Staphylococci to capture the immune complexes. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed according to the method of Laemmli (6). Gels were stained for protein with silver nitrate (7). For autoradiographic analysis, gels were dried under vacuum and overlaid with Kodak X-AR5 film at -70°C.

Liposomes. Phosphatidyl choline liposomes containing the purified radiolabeled collagen binding complex were prepared and then purified by floatation on discontinuous sucrose gradients as described by Pytela et al. (8). The liposomes were used in adhesion assays performed as described by Pytela et al (8) using collagen substrates prepared in 35 mm polystyrene dishes as previously described by us (4).

RESULTS AND DISCUSSION

The solubilized platelet membranes were subjected sequentially to affinity chromatography on Concanavalin A-Sepharose and collagen-Sepharose.



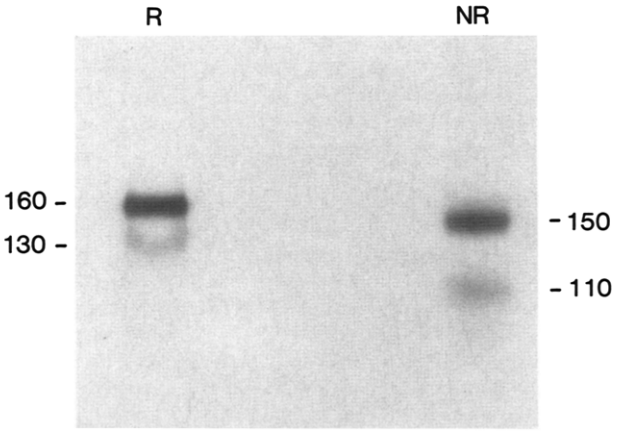


Figure 1. Purification of the 160/130 kDa collagen binding protein from platelet membranes. Platelet membranes were solubilized in buffer containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% Lubrol-PX and a cocktail of protease inhibitors. The soluble material was subjected to sequential affinity chromatography on Concanavalin A-Sepharose and collagen-Sepharose. Material bound to collagen in a Mg^{++}-dependent manner was eluted by the addition of EDTA to the column buffer. Fractions were subjected to analysis by SDS-PAGE under reducing conditions on 7 1/2% acrylamide gels. Protein was detected by silver staining. Lane 1: detergent solubilized membranes. Lane 2: material not bound to Concanavalin A-Sepharose. Lane 3: material bound and eluted from Concanavalin A-Sepharose. Lane 4: material not bound to collagen-Sepharose. Lane 5: material bound to collagen-Sepharose and eluted with EDTA. The lightly staining material at ~55 kDa represents a silver staining artefact present in all lanes including those which contained no sample.

Figure 2. The purified 160/130 kDa collagen binding complex was radiolabeled with ^{125}I and subjected to analysis by SDS-PAGE under both reducing (R) and nonreducing conditions (NR) on a 6% acrylamide gel. The proteins were detected by autoradiography.

The latter chromatographic step was carried out in the presence of 2 mM Mg^{++}. After extensive washing, elution was accomplished by substitution of EDTA for the Mg^{++} in the column buffer. Fractions were subjected to analysis by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) under reducing conditions. The analysis revealed that the material eluted from the collagen column upon the addition of EDTA was composed of two major polypeptides of 160 and 130 kDa (Figure 1).

The purified collagen binding complex was radiolabeled with ^{125}I and examined under both reducing and nonreducing conditions by SDS-PAGE. The radiolabeled complex was electrophoretically homogeneous under both sets of conditions (Figure 2), a further indication of its purity. The poor labeling of the 130 kDa subunit relative to the 160 kDa subunit probably accounts for our failure to detect the lower molecular weight subunit in our earlier study (3). In contrast to the 160 and 130 kDa molecular weights for the two

subunits observed under reducing conditions, the observed molecular weights under nonreducing conditions were 150 and 110 kDa respectively (Figure 2). The significantly reduced molecular weight of the lower molecular weight beta subunit observed under nonreducing conditions is suggestive of substantial intrachain disulfide bonding within the polypeptide and is a property shared in common with several other recently described cell surface extracellular matrix receptors and related proteins (9). This subunit composition resembles the heterodimeric glycoprotein IIb/IIIa complex which serves as a receptor for fibrinogen, fibronectin and von Willebrand factor on the platelet surface and the integrins, a related family of cell surface extracellular matrix receptor proteins (9).

The purified, radiolabeled collagen binding complex was incorporated into liposomes composed of phosphatidyl choline. As shown in Figure 3, the liposomes containing the complex bound to collagen substrates but not to the control substrate in a Mg^{++}-dependent manner. This observation indicates that the purified, radiolabeled complex retains the ability to bind collagen in a Mg^{++}-dependent manner, and that elution from collagen with EDTA during purification did not result in irreversible loss of collagen binding activity. This observation also serves to further increase the likelihood that the 160/130 kDa complex is, in fact, a mediator of Mg^{++}-dependent cell adhesion to collagen. The experiment shown in Figure 3 is representative of six such independent experiments.

The electrophoretic and lectin binding properties of the 160 kDa collagen binding protein are those of platelet membrane glycoprotein Ia (3,10). This tentative relationship is especially interesting in light of the recent report of Nieuwenhuis et al (10) who described a patient with a bleeding disorder with normal values for all assayed plasma coagulation factors but

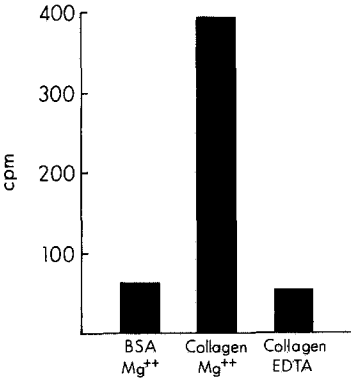


Figure 3. Adhesion of liposomes containing the 160/130 kDa complex to collagen. The purified complex was radiolabeled with ^{125}I and incorporated into phosphatidyl choline liposomes. Adhesion of the liposomes to BSA and collagen substrates was determined in the presence of 2 mM Mg^{++} or 2 mM EDTA as indicated.

with a prolonged template bleeding time indicative of a defect in platelet function. Detailed platelet function studies revealed normal platelet responsiveness to all agonists except collagen. Electrophoretic analysis of the patient's platelets revealed a deficiency of glycoprotein Ia.

In order to unequivocably establish the relationship of the 160/130 kDa complex to glycoprotein Ia, immunoprecipitation experiments with monoclonal antibody 12F1 were performed. This antibody is specific for an epitope on the alpha chain of the VLA-2 complex present on lymphocytes, fibroblasts, neural cells and platelets (5,11). Furthermore, the VLA-2 alpha chain has been shown to be immunochemically identical with platelet glycoprotein Ia which exists in a heterodimeric association with glycoprotein IIa (12,13). As shown in Figure 4, the radiolabeled preparation of collagen binding protein was specifically immunoprecipitated by monoclonal antibody 12F1 but was not precipitated in the absence of antibody or in the presence of control monoclonal antibody A2.5 which is specific for thrombospondin. Thus, glycoprotein Ia and IIa, as identified by both electrophoretic and immunochemical properties, are components of the platelet surface collagen binding complex.

Functional studies indicate that monoclonal antibody 12F1 is directed at an epitope on glycoprotein Ia which is not critical for the platelet-collagen interaction as judged by its inability to inhibit collagen-induced platelet aggregation or the collagen-induced display of fibrinogen receptors on

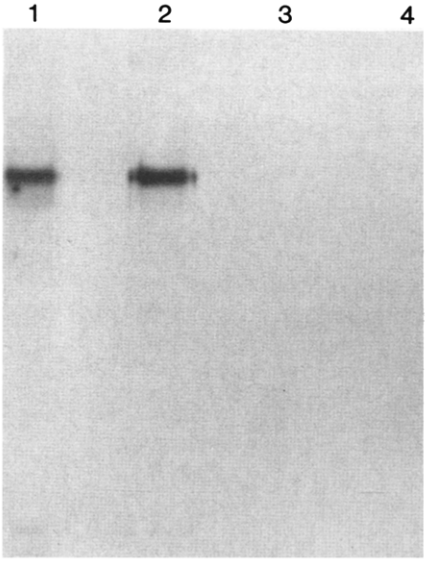


Figure 4. Immunoprecipitation of the 160/130 kDa collagen binding complex by monoclonal antibody 12F1. Purified ^{125}I-labeled 160/130 kDa complex (lane 1) was subjected to immunoprecipitation with monoclonal antibody 12F1 (lane 2) or monoclonal antibody A2.5 (lane 3) or in the absence of antibody (lane 4). The immunoprecipitates were subjected to analysis by SDS-PAGE on a 11% acrylamide gel. Precipitated complex was then detected by autoradiography.

platelets. Furthermore, 12F1 does not inhibit platelet aggregation induced by ADP or arachidonic acid and does not inhibit the induction of platelet receptors for fibrinogen, fibronectin or von Willebrand factor induced by thrombin or ADP. Finally, 12F1 itself does not induce platelet aggregation or induce platelet receptors for fibrinogen, fibronectin, or von Willebrand factor.

The significance of the immunoprecipitation by monoclonal antibody 12F1 goes well beyond establishing an immunochemical relationship between the 160/130 kDa collagen binding complex and glycoproteins Ia and IIa. It also establishes the identity or at least immunochemical relatedness of the collagen binding complex with the VLA-2 complex. The VLA (very late activation) antigens are so named because of their very late appearance (9-10 days) on the surface of T-cells following activation (14,15). The family consists of five heterodimeric membrane proteins which share a common ß subunit but which have unique α subunits (14,15). VLA-2 is a heterodimeric complex also composed of 160 and 130 kDa subunits (14,15). The present observations suggest not only a function for the VLA-2 complex as a platelet surface collagen binding complex but also suggest that the adhesive mechanism we have elucidated (3) is not unique to platelets but is shared by other cell types. Data have appeared which suggest that VLA-3 and VLA-5 serve respectively as cell surface laminin and fibronectin receptors (11,16). Thus, at this point in time three (VLA 2,3 and 5) of the five VLA family members appear to serve as cell surface receptors for extracellular matrix components.

Two distinct mechanisms of platelet-collagen adhesion can be distinguished, a divalent cation-independent mechanism (17) and the Mg^{++}-dependent mechanism recently described by us (3). The latter binding appears to be mediated by the 160/130 kDa complex described in this report. It remains to be established whether one of the many previous candidates proposed as mediators of platelet adhesion to collagen represents a physiologically relevant mediator of the divalent cation-independent mechanism. The relationship at the molecular level between the two operationally distinct mechanisms also awaits resolution.

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