Identification of a Third Component of Complement-binding Glycoprotein of Human Platelets

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Abstract

Utilizing affinity chromatography, a C3-specific binding protein was isolated from ^{125}I surface-labeled human platelets. Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis demonstrated two bands with mean M_r of 64,000 and 53,000, characteristic variability in the relative density of the two bands in a given individual, and the presence of N-linked complex oligosaccharides as well as sialic acid residues not associated with N-linked sugars. These characteristics are similar to those of a human leukocyte iC3- and C3b-binding glycoprotein, termed gp45-70. Further analysis showed that leukocyte gp45-70 and the platelet C3-binding glycoprotein have identical M_r and other similar structural features. Functional characterization of solubilized platelet preparations indicated that gp45-70 has cofactor activity. This membrane glycoprotein is structurally and antigenically distinct from decay accelerating factor (DAF), a complement regulatory protein previously identified on human platelet membranes. DAF and gp45-70 have complementary activity profiles inasmuch as DAF can prevent assembly of and dissociate the C3 convertases but has no cofactor activity, whereas gp45-70 has cofactor activity but no decay accelerating activity. We suggest that these two proteins function conjointly to prevent autologous complement activation.

Introduction

A number of membrane proteins capable of binding the third component of complement (C3) have been identified (1–3). On human peripheral blood cells, the C3b/C4b receptor (CR1) was the first to be described biochemically (4, 5) and is a single-chain integral membrane glycoprotein with an M_r of ~200,000. This molecule has been identified on erythrocytes, granulocytes, monocytes, macrophages, B lymphocytes, and a subpopulation of T cells and glomerular podocytes (6). It exhibits a number of interesting cell-specific structural features and a polymorphism in which four autosomal codominantly inherited alleles have M_r between 160,000 and 250,000 (7–11). CR2, the C3d or C3dg receptor, is a single-chain integral membrane glycoprotein with a M_r of 140,000 (12–14). This molecule is found on B lymphocytes (1–3) and has also recently been shown to be the "receptor" for the Epstein-Barr virus (15, 16). CR3, the receptor for C3bi, is a glycoprotein composed of two polypeptide chains of M_r 165,000 and 95,000 and is found on neutrophils and monocytes (17–22).

An additional C3b/C4b-binding membrane protein, termed gp45-70, has been identified (23, 24). It binds iC3 and C3b but not C3d and is widely distributed on human peripheral blood mononuclear cells. On most cells this glycoprotein is a doublet with a predominant upper band and has an M_r between 45,000 and 70,000. Gp45-70 has a similar M_r to C3b-binding proteins previously described on rabbit macrophages (25, 26) and, more recently, on murine cells (27). The human and murine molecules do not appear to mediate rosette formation and the physiologic function of gp45-70 is unknown.

Human platelets do not possess CR1, CR2, or CR3 but have been shown to possess decay accelerating factor (DAF)[1] (28, 29). This ~70,000-M_r regulatory membrane protein is also found on erythrocytes and leukocytes and facilitates the decay of both the classical and alternative pathway C3 convertases (28–30). However, DAF does not bind to C3b or C4b affinity columns and, in contrast to other human peripheral blood cells, no C3b-binding molecules have been identified on platelets (1–3). In this report, we have identified a C3-binding membrane protein of human platelets and provided evidence that it has regulatory activity for the complement system.

Methods

Purification of platelets and leukocytes. Platelets and leukocytes were purified from 150 ml of blood collected in 3.5 ml of 0.25 M Na_2EDTA from normal individuals on the day of use. Platelets were purified as described (31) with minor modifications. All procedures were performed at room temperature using plastic ware only. After centrifugation of whole blood at 1,000 *g* for 3 min at 25°C, platelet-rich plasma was removed. Platelets were then sedimented by centrifugation at 2,000 *g* for 10 min at 25°C. The pellet was then resuspended with wash buffer (0.113 M NaCl, 4.3 mM K_2HPO_4, 4.3 mM Na_2HPO_4, 24.5 mM NaH_2PO_4, 5.5 mM dextrose, pH 6.5) and centrifuged at 120 *g* for 10 min to sediment contaminating erythrocytes and leukocytes. This centrifugation step was then repeated twice more. Platelets were then again sedimented and washed twice with wash buffer. Approximately 4×10^9 platelets were obtained from 150 ml of whole blood. Microscopic examination of the purified platelet preparations revealed less than one leukocyte per 10^4 platelets.

Mononuclear leukocytes were purified from whole blood by dextran sedimentation followed by bouyant density centrifugation employing Ficoll-Hypaque (24, 32). U937, a human monocytelike cell line, was obtained from the Center of Basic Cancer Research, Washington University School of Medicine, and grown under conditions as reported (24).

Surface-labeling and solubilization of cells. Platelets were prepared for surface radioiodination by resuspension in labeling buffer (0.14 M Tris-HCl, 0.13 M NaCl, 52 mM glucose, pH 7.4) (31) at a concentration

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1. *Abbreviations used in this paper:* DAF, decay accelerating factor; Endo F, endoglycosidase F; PAGE, polyacrylamide gel electrophoresis.

of 1×10^9 cells/ml. Purified leukocytes were prepared for radiolabeling by suspension in phosphate-buffered saline (PBS) (0.15 M NaCl, 1.92 mM NaH_2PO_4, 8.7 mM K_2HPO_4, pH 7.4) at a concentration of 2×10^7 cells/ml. Surface iodination with ^{125}I (Amersham) was performed by a modified lactoperoxidase method (8, 33). Cells were then solubilized at 4°C in PBS, 1% Nonidet P-40 (NP-40, Sigma Chemical Co., St. Louis, MO), 2 mM phenylmethylsulfonyl fluoride, 3 mM EDTA, 1 μM pepstatin, and 20 mM iodoacetamide. Platelets were solubilized at 5×10^9/ml and leukocytes at 10^8 cells/ml.

Affinity chromatography. Affinity chromatography was peformed as previously described (8, 24, 34). Affinity columns were prepared by two methods: (*a*) iC3, BSA, or human IgG was coupled to cyanogen bromide-activated Sepharose (Sepharose 6B, Sigma Chemical Co.) as previously described (7, 8) or (*b*) C3b- and C3d-thiol-Sepharose were prepared by methods also described previously (13, 24).

Solubilized preparations were first preincubated with IgG-Sepharose for 1 h with constant mixing at room temperature (0.2 ml of packed beads per 1 ml of solubilized preparation). The supernatant was then removed, diluted threefold to 0.05 M NaCl with distilled water, and incubated with the protein-coupled Sepharose for 1 h at room temperature with constant mixing. The affinity column was then washed with 0.05 M PBS, 1% NP-40 and eluted with 400 mM NaCl, 1% NP-40. After dialysis against distilled H_2O, lyophilization and acetone precipitation, samples were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or enzyme treatments.

Evaluation of the efficiency of this methodology showed that >80% of the leukocyte or platelet-binding proteins specific for a given column were removed by the initial incubation. A second or third incubation removed >95% of specific binding proteins. The pattern on SDS-PAGE of the eluates from these additional incubations was identical to that of the first eluate. Also, C3b-affinity columns were eluted first with high salt and then with 6 M guanidine. The eluates were compared by SDS-PAGE. No new specific bands were eluted by 6 M guanidine. Further, >70% of the counts in the C3b-specific bands were eluted by high salt.

Immunoprecipitation. Immunoprecipitation was performed as previously described (8, 34). Antibodies utilized were monoclonal anti-CR1 antibody 57F (gift of V. Nussenzweig, Department of Pathology, New York University, New York City) and polyclonal antibodies raised in rabbits to H, C4-binding protein (C4bp), DAF, and CR1 (8, 35). For all immunoprecipitations, parallel conditions were run with MOPC-21 (as a nonspecific monoclonal antibody of the same isotype as 57F) and rabbit serum or rabbit IgG (as a control for the polyclonal antisera). The antibody to DAF was a generous gift of Dr. Michael Pangburn (Department of Biochemistry, University of Texas Health Science Center, Tyler) and was an IgG fraction.

Briefly, solubilized preparations were first precleared of nonspecific proteins with *Staphylococcus aureus* (Cowan I strain) and then incubated with either specific or nonspecific polyclonal or monoclonal antibody for 1 h at 4°C. For the monoclonal immunoprecipitation, a rabbit anti-mouse second antibody was then added and incubation continued for 1 h at 4°C. This preparation was then incubated in Staph Cowan for 15 min and, after several washings with PBS and 1% NP40, bound proteins were eluted.

Enzyme treatment. Enzyme treatments were performed as described (34, 36) with minor modifications. Radioiodinated material was resuspended in 0.10 ml of the appropriate buffer for treatment with neuraminidase (50 mM sodium acetate, 1 mM $CaCl_2$, 0.2% NP-40, pH 5.5) or endoglycosidase (Endo) F (100 mM NaH_2PO_4, 50 mM EDTA, 1% NP-40, 0.1% SDS, 1% β-mercaptoethanol, pH 6.1). The preparation was heated at 80°C for 10 min and then allowed to cool to room temperature. For most experiments Endo F or neuraminidase was then added in a 0.02-ml aliquot containing 60 mU and a second 60-mU was added at 3 h. After a total incubation period of 6 h at 37°C, treatment was terminated by precipitating the samples with 1 ml of acetone at −20°C for 5 h. Precipitated samples were then recovered by centrifugation and prepared for additional enzyme treatments or SDS-PAGE.

SDS-PAGE and autoradiography. SDS-PAGE was performed as previously described (7, 8, 37, 38), using a 7.5% or 6–18% polyacrylamide gel. All samples were loaded onto a 3% stacking gel. On reduced gels, samples were treated with 5% β-mercaptoethanol and heated at 80°C for 10 min. Autoradiographic methodology has been described (8). Quantification of the relative intensity of bands observed on autoradiographs was performed with a Zeineh Soft Laser Scanning Densitometer (Biomed Instruments, Chicago, IL) (8, 24).

Determination of cofactor activity in the solubilized platelet preparation. Plasma complement regulation proteins H and I and C3 were purified as reported (35). I was also purchased from Cordis and one C3 sample was a gift from Dr. R. P. Levine, Dept. of Genetics, Washington University School of Medicine. C3 was freeze-thawed to prepare hemolytically inactive C3 (iC3) and then radioiodinated by the chloramine-T method (35).

For these functional studies, purified platelets solubilized in the presence of protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 3 mM EDTA, 1 μM pepstatin, 20 mM iodoacetamide) were first precleared. Anti-human C4bp and anti-human CR1 rabbit polyclonal antibodies were prepared as described (35). Anti-human H rabbit antibody was purchased from Calbiochem-Behring Corp. (San Diego, CA). To remove C4bp, CR1, and H, sufficient antibody to remove >90% of C4bp or H from 0.1 ml of serum or of CR1 from 5×10^8 solubilized E was first bound to Staph Cowan at 4°C for 15 min. After several washings with PBS, 1% NP40, the pellet was incubated with 0.5 ml (5×10^9 cell equivalents) of solubilized platelet preparation for 1 h at 4°C and the supernatant was removed and tested for cofactor activity. These samples or H (5 μg) were incubated with ^{125}I-labeled iC3 (5 μg) and I [50 inhibitory units (Cordis)] at 37°C for 1 h. These experiments were also performed using purified I (35) and identical results were obtained. The buffer used throughout this assay was PBS, pH 7.4. At timed intervals, samples were removed for analysis by SDS-PAGE (reducing conditions) and autoradiography.

For assessment of cofactor activity of iC3- or BSA-Sepharose eluates, samples were first dialyzed against dH_2O and then against PBS, pH 7.4. The assay was then performed as described above. Alpha chain cleavage was determined by densitometric scanning of the α_2 and α'_2 fragments.

Results

Identification of a C3-binding platelet membrane protein. Incubation of labeled solubilized platelet preparation with IgG-, BSA-, or iC3-Sepharose beads, elution of these columns, and analysis of the eluates by SDS-PAGE followed by autoradiography revealed an iC3-binding protein with an M_r between 50,000 and 68,000 (Fig. 1). This molecule specifically binds to iC3 (track *3*) and is a doublet consisting of two relatively broad bands. The mean M_r of the "upper" and "lower" bands is 64,000 and 53,000, respectively. Molecules with M_r of 42,000 and 53,000 are prominent in the IgG lane; this M_r is in the range expected for the Fcγ receptor (39–41). Two prominent bands with an M_r of 132,000 and 90,000 bound to all three columns. These bands also appear in autoradiographs of immunoprecipitations of solubilized platelet preparations (see below) and are the most heavily labeled bands of solubilized material applied directly to gels. Furthermore, while the bands at 64,000 and 53,000 M_r are specifically inhibited by fluid phase iC3 (see below), the 132,000- and 90,000-M_r bands are unaltered.

Consistent with previously reported functional studies (1, 2), CR1 with an M_r of ~200,000 or CR2 with an M_r of 140,000 was not found in the iC3 eluate (also see Fig. 7). This lack of CR1 was further confirmed by an immunoprecipitation employing a polyclonal anti-CR1 antibody (not shown). Finally, no band with an M_r of 70,000–75,000 corresponding to that reported for DAF is present on the autoradiograph in Fig. 1. To further substantiate this finding, DAF was immunoprecipitated from solubilized platelet preparations and analyzed in parallel

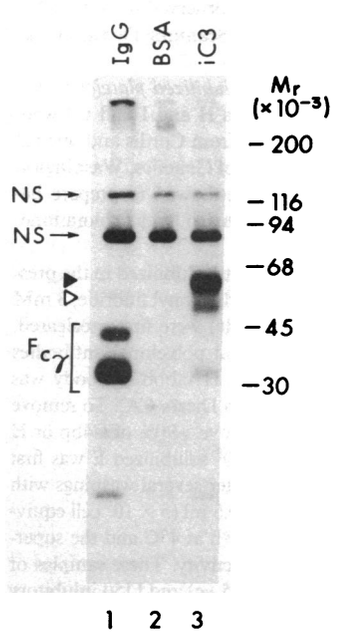

Figure 1. Autoradiograph of eluates of IgG-, BSA-, and iC3-Sepharose to which surface-labeled solubilized platelet preparations were applied. Two nonspecific contaminants at 132,000 and 90,000 M_r (marked by arrows) bind to all three columns (see Results). In addition, the putative two-band Fcγ receptor (mean M_r of 42,000 and 35,000 and marked by brackets) bind specifically to IgG-Sepharose (track *1*). Eluate of proteins binding iC3-Sepharose (track *3*) include the contaminants and an iC3-specific binding protein (plt-C3BP) consisting of two bands with mean M_r of 64,000 and 53,000. On this and subsequent autoradiographs, the higher M_r band is marked with a solid arrowhead and the lower M_r band is marked with an open arrowhead. 6–18% gradient gel, nonreducing.

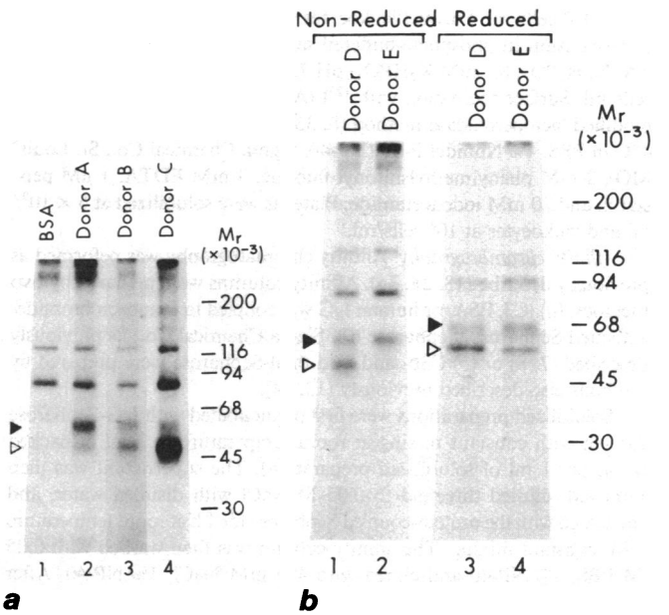

Figure 3. (*a*) An analysis of the platelet iC3-binding protein of three selected individuals. The three patterns observed for this molecule are shown here: predominant upper band (track *2*), approximately equal doublet (track *3*) and predominant lower band (track *4*). Autoradiograph of 6–18% gradient gel, non-reducing. (*b*) Effect of reduction on M_r of plt-C3BP. Reduction produces a 6,000 increase in M_r for both bands of the molecule (tracks *3* and *4*, also see text). 6–18% gradient gel.

with eluates of iC3 affinity chromatography of the same preparations (Fig. 2). The M_r of DAF was greater than that of plt-C3BP and plt-C3BP was not immunoprecipitated by anti-DAF (the variability in the density of the two bands of plt-C3BP is addressed below).

A screen of the platelet iC3-binding protein (plt-C3BP) of eight unrelated individuals demonstrated that both bands of the doublet migrate at identical M_r; however, the relative distribution in the "upper" and "lower" bands of the molecule varied among donors (Fig. 3 *a*). The most common pattern observed was one in which >70% of the radioactivity was located in the higher M_r band, as determined by direct counting of the gel slice or by densitometric scanning. This ratio (Fig. 1, track *3* and Fig. 3, track *2*) was observed in six of the eight donors studied. The other two patterns, observed in one donor each, are an approximately "equal doublet" in which at least 30% of counts are in each band (Fig. 3, track *3*), and one in which >70% of the counts are in the lower M_r band (Fig. 3, track *4*). Multiple (more than three) purifications of one individual of each type over a 12-mo period have shown that the pattern is stable.

Reduction of plt-C3BP with β-mercaptoethanol results in a 6,000-M_r increase for both bands, suggesting the presence of intrachain disulfide linkages. Upon reduction, the lower band is more sharply focused and thus appears to be more prominent (Fig. 3 *b*, two additional gels comparing reducing and nonreducing patterns are shown in Fig. 7). Also, with reduction a third band slightly below the lower band, although faint, is usually observed.

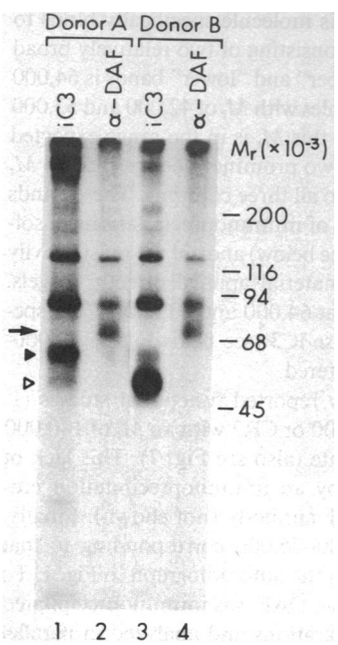

Figure 2. Comparison of proteins isolated from platelet solubilized preparations by iC3 affinity chromatography and by immunoprecipitation with anti-DAF. The arrow points to DAF; the open and closed arrowheads identify plt-C3BP. Nonspecific rabbit IgG did not lead to the precipitation of DAF (not shown). In this and most other gels, a variable quantity of labeled material whose identity is unknown is observed at the top of the gel. Autoradiograph of a 6–18% gradient gel, nonreducing.

Specificity of plt-C3BP for C3 and its fragments. To further demonstrate that plt-C3BP isolated by iC3-Sepharose affinity chromatography is a C3-specific binding protein, fluid-phase inhibition and binding specificity studies were performed.

In fluid-phase inhibition studies, proteins were preincubated or simultaneously incubated with the solubilized platelet preparation and iC3-Sepharose. Fluid-phase iC3 inhibited binding of plt-C3BP to iC3-Sepharose (Fig. 4) but had no effect on the two proteins of 90,000 and 132,000 M_r (not shown). Increasing amounts of fluid-phase iC3 led to increased inhibition and a threefold excess of fluid-phase iC3 over that coupled to the Sepharose resulted in >90% inhibition of binding of plt-C3BP. Incubation of the solubilized platelet preparation with fluid-phase human IgG resulted in <30% inhibition, even up to a 10-fold excess. In these studies, inhibition of binding by fluid phase iC3

FLUID-PHASE INHIBITION OF BINDING TO iC3-SEPHAROSE

iC3 (mg)	% Inhibition Exp.1	Exp. 2
0	-	-
0.07	-	64
0.14	68	83
0.28	-	90
0.42	90	-
0.70	-	>99
1.40	96	>99

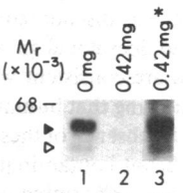

Figure 4. Effect of fluid-phase proteins on binding of plt-C3BP to iC3-Sepharose. The tabular data present results for two experiments, including the one (exp. 1) corresponding to the autoradiograph. In both experiments <30% inhibition was observed with the same concentrations of IgG. In exp. 1 the solubilized platelet preparation and iC3-Sepharose beads (0.1 ml of packed beads coupled at 1.40 mg/ml) were incubated together with the fluid-phase inhibitory proteins (iC3 or IgG), whereas in exp. 2 the solubilized platelet preparation was pre-incubated with a fluid-phase protein for 1 h before incubation with iC3-Sepharose. The autoradiograph is of labeled platelet material bound to iC3-Sepharose (under conditions of exp. 1) in the presence of 0 mg (track *1*) or 0.42 mg (track *2*) of fluid-phase iC3 (1.64 mg/ml). The presence of a reduced amount of plt-C3BP in track *2* is confirmed by an eightfold greater exposure (track *3*). Also, a condition with 0.42 mg of IgG was employed in this same experiment and the resulting gel track was identical to that of track *1* (not shown). Percent inhibition was calculated from data obtained by densitometric scanning of the autoradiograph. 6–18% gradient gel, nonreducing.

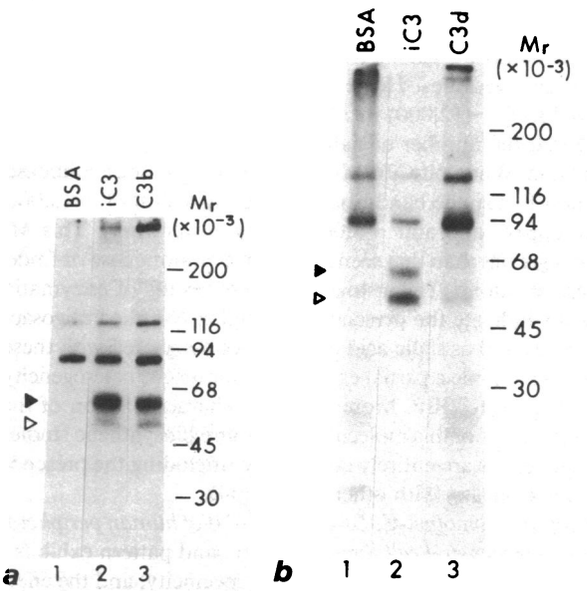

Figure 5. Analysis of the binding specificity of plt-C3BP (*a*) This autoradiograph shows those molecules on human platelet membranes that bind BSA-Sepharose (track *1*), iC3-Sepharose (track *2*), and C3b-thiol-Sepharose (track *3*). 6–18% gradient gel, nonreducing. (*b*) Autoradiograph of human platelet membrane proteins which bind BSA-Sepharose (track *1*), iC3-Sepharose (track *2*), and C3d-thiol-Sepharose (track *3*). Tracks *1* and *3* were exposed twice as long as track *2*. 6–18% gradient gel, nonreducing. Elution of the C3b and C3d by 2-mercaptoethanol from the thiol-Sepharose indicated that >90% of the α-chain of C3 was α' and only a single fragment of 32,000 M_r, respectively.

was greater than anticipated based on the quantity of iC3 bound to the Sepharose (assuming that there is equal affinity of the binding protein for fluid phase and Sepharose-bound iC3). This may be due to a number of factors, including the possibility that a fraction of the iC3 coupled to the Sepharose is oriented such that the binding site is not optimally exposed.

In a second type of study binding specificity of plt-C3BP for C3 and its derivatives was evaluated. Radiolabeled platelet membranes were evaluated by affinity chromatography with iC3-Sepharose and C3b- and C3d-thiol-Sepharose (Fig. 5). Multiple experiments indicated that plt-C3BP bound to iC3-Sepharose and to C3b-thiol-Sepharose (Fig. 5 *a*), but binds minimally to C3d-thiol-Sepharose (Fig. 5 *b*). Taken together, these results indicate that the binding region for plt-C3BP resides in the C3c portion of C3 (24).

Effect of glycosidase treatments on the M_r of plt-C3BP. One explanation for the M_r heterogeneity observed for plt-C3BP is a variation in oligosaccharide structure. To address this possibility, labeled plt-C3BP was purified by affinity chromatography and subjected to treatment with neuraminidase, an enzyme that cleaves sialic acid residues, and Endo F, an enzyme that removes N-linked oligosaccharides. The effects of these enzymatic treatments were assessed by SDS-PAGE and autoradiography using an individual with a predominant upper band pattern (Fig. 6).

As seen in this and other similar experiments, treatment with Endo F results in the upper and lower M_r bands of plt-C3BP each being resolved into two bands (track *2*). There is a 3,000–4,000 reduction in M_r of part of each band of the molecule, suggesting the presence of at least one and possibly two N-linked oligosaccharide unit(s). However, the M_r difference between the upper and lower bands is not reduced. Incomplete digestion (42, 43) or the presence of two separate classes of molecules could explain this result.

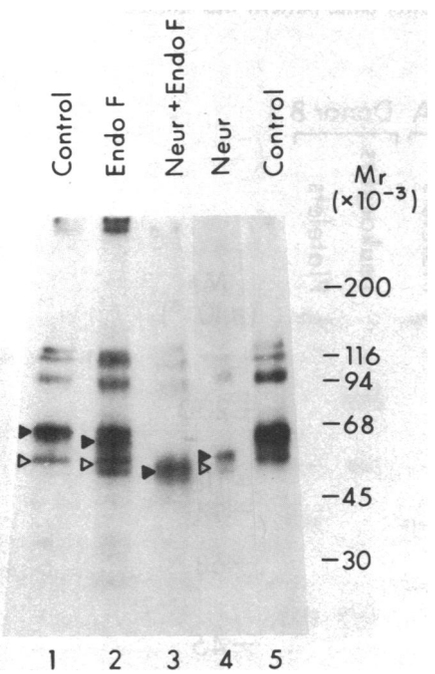

Figure 6. Effect of glycosidase digestions on M_r of plt-C3BP. This autoradiograph shows affinity purified plt-C3BP treated with Endo F (track *2*), neuraminidase (track *4*), or both neuraminidase and Endo F (track *3*). In track *3*, a definite distinction between the upper and lower band is not possible. 6–18% gradient gel, reducing.

Upon treatment with neuraminidase (track *4*), the upper and lower bands are again reduced in M_r, indicating the presence of sialic acid residues. However, the upper band is relatively more reduced (~12,000) in M_r than the lower (~5,000), suggesting that the number of sialic acid residues is greater on the higher band. When plt-C3BP is treated with both neuraminidase and Endo F, a single band is seen reflecting a shift of ~15,000 for the upper band and ~8,000 for the lower band. This M_r change is greater than that seen for either neuraminidase or Endo F treatment alone. Taken together, these results of enzymatic digestions indicate the presence of complex N-linked oligosaccharides, as well as sialic acid residues not associated with these sugars, and provide a partial explanation for the M_r heterogeneity observed for plt-C3BP. More complete characterization of the oligosaccharides of this molecule will require biosynthetic studies but these results are entirely compatible (including the presence of O-linked sugars) with other studies (44).

A comparison of plt-C3BP and gp45-70 of human peripheral blood leukocytes and cell lines. The two band pattern exhibited by plt-C3BP on SDS-PAGE, its ligand specificity, and the effect of glycosidase digestions on the M_r of this molecule suggested that this glycoprotein shares many characteristics with a recently described C3-binding glycoprotein of human peripheral blood leukocytes and monocyte-derived cell lines, termed gp45-70 (24). To further compare these proteins, human mononuclear leukocytes (Ficoll-Hypaque interface) and platelets were purified from the same donor and surface radioiodinated and solubilized. C3-binding proteins were then isolated by affinity chromatography and analyzed. Autoradiographs demonstrate that plt-C3BP and gp45-70 align by SDS-PAGE (Fig. 7). In addition, the relative band density of the two major bands of this protein is similar for both cell types for a given individual. This is most clearly demonstrated in tracks *3* and *4* (donor B) in which an individual with a predominant lower band pattern was studied.

No band that aligns with CR1 is seen but a faint band migrating at the M_r of approximately 140,000 is present (Fig. 7 *a*). The possibility that this band represents CR2 (marked on leukocyte lanes of Fig. 7) was discounted as it also binds to BSA-Sepharose columns and does not align with CR2 upon reduction (Fig. 7 *b*). These data are consistent with prior functional and structural studies indicating that human platelets do not possess CR1 or CR2 (1–3). The absence of these receptors also indicates that there is no significant contamination of the platelet preparations with leukocytes, confirming our microscopic studies.

Cofactor activity of plt-C3BP. In preliminary experiments, J. Turner (45) showed that eluates of an iC3-Sepharose column to which solubilized preparations of U937 had been applied possessed cofactor activity for the I-mediated cleavage of C3. Our laboratory has recently found that this cofactor activity is largely attributable to gp45-70 and that purified gp45-70 from U937 and HSB-2 has cofactor activity (46). We therefore determined if the platelet preparations contained such an activity (Fig. 8).

Purified human platelets solubilized in the presence of protease inhibitors were precleared of known cofactors, H, C4bp, and CR1, and then incubated with ^{125}I-labeled iC3 and I. As controls, ^{125}I-iC3 was incubated with H, H + I, or I alone. Analysis of iC3 cleavage fragments by SDS-PAGE and autoradiography (Fig. 8) shows that solubilized platelet preparations contain I-dependent cofactor activity (lanes *3–7*). The α-chain of iC3 was cleaved into α_1 (M_r 75,000) and α_2 (M_r 46,000), which was further cleaved to α'_2 (M_r 43,000). These cleavage products are of identical M_r to those generated by I + H (Fig. 8, lane *8*). In addition, this cleavage exhibited dose-dependency since increasing concentrations of platelet preparation resulted in greater α-chain cleavage.

To evaluate whether this activity binds to iC3, platelet preparations were incubated with IgG-Sepharose and then with either BSA- or iC3-Sepharose. Eluates of these columns were then

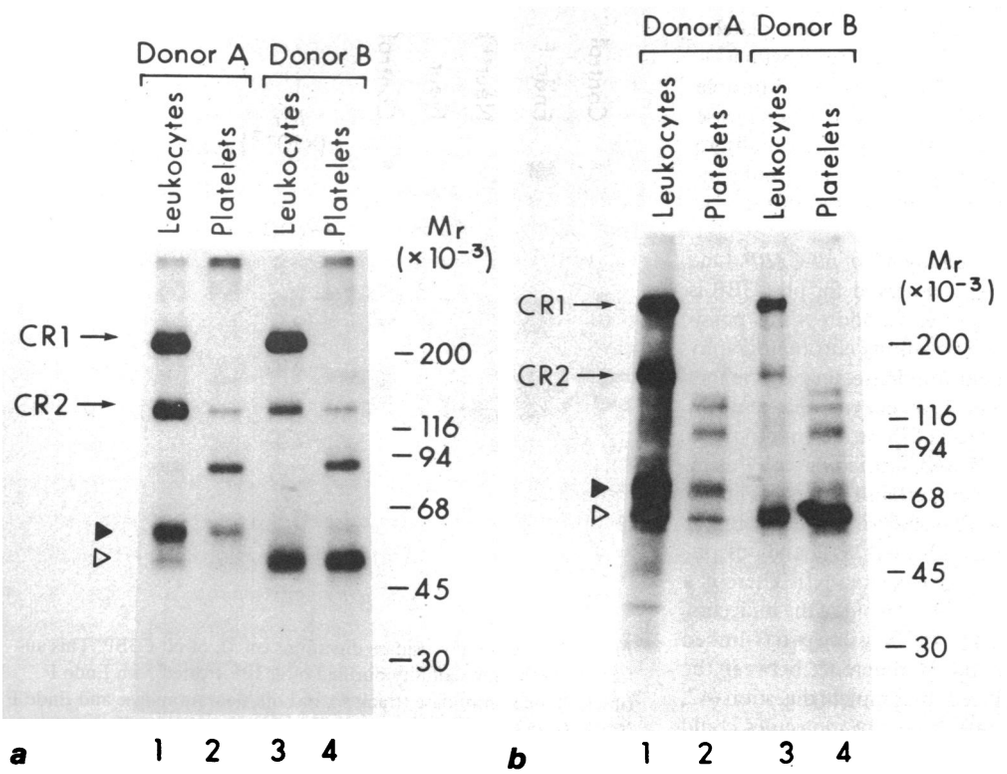

Figure 7. Comparison of C3-binding proteins isolated from mononuclear leukocytes (Ficoll-Hypaque interface) and from platelets. These autoradiographs (*a*, 6–18% gradient, nonreducing; *b*, 6–18% gradient, reducing) are derived from two individuals, one with a predominant upper band pattern (donor A) and one with a predominant lower band pattern (donor B).

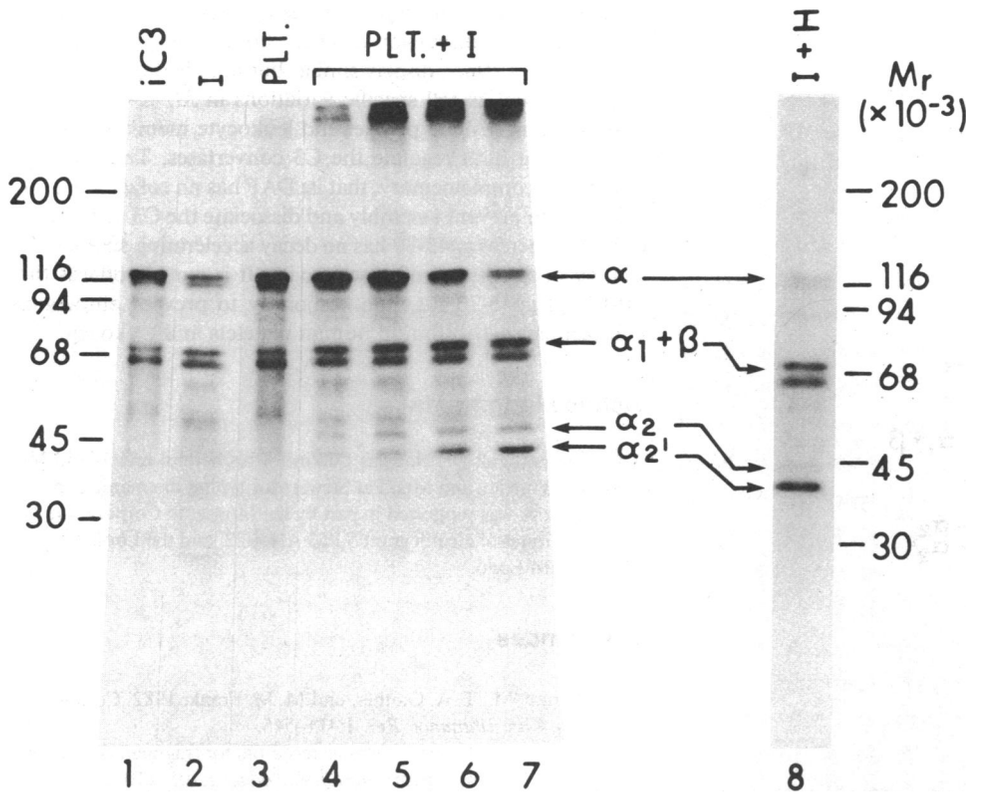

Figure 8. Cofactor activity of solubilized platelet preparations. Samples include ^{125}I-iC3 alone (track *1*), I alone (track *2*), and platelet solubilized preparation alone (track *3*). Tracks 4–7 show ^{125}I-iC3 incubated with I and increasing amounts of solubilized platelet preparation: 1.5×10^7 cell equivalents (track *5*); 6.1×10^7 cell equivalents (track *6*); 1.5×10^8 cell equivalents (track *7*); and 6.1×10^8 cell equivalents (track *8*). ^{125}I-iC3 was also incubated with H and I (track *8*). I-mediated cleavage of iC3 is detected by cleavage of the α-chain into α_1, α_2 and α'_2 fragments. Because of the large number of samples in this experiment, track *8* was run on a separate gel. In other similar experiments, I + H and plt + I samples were run on the same gel and the α_2 and α'_2 fragments aligned. A band of unknown identity is seen below the β-chain in this iC3 preparation. Autoradiograph of 6–18% gradient gel, reducing.

evaluated for cofactor activity for iC3 α-chain cleavage (Fig. 9). The eluate from iC3-Sepharose possessed cofactor activity, whereas that from BSA-Sepharose did not, suggesting that the cofactor present in the platelet preparation is an iC3-specific binding molecule. In other experiments, the supernatants from solubilized platelet preparations previously incubated with IgG-, BSA-, or iC3-Sepharose were assessed for cofactor activity. No depletion of activity was observed in the IgG or BSA supernatants but there was >50% depletion in cofactor activity in the iC3 supernatants (not shown), again suggesting an iC3-specific cofactor. The cofactor is likely to be plt-C3BP as previous autoradiographs demonstrate it to be the sole iC3-specific binding molecule of human platelet membranes.

Discussion

In this report we have identified a specific C3-binding membrane glycoprotein of human platelets. With the exception of the regulatory protein, DAF (28, 29), complement receptors, binding proteins, or regulatory molecules have not previously been described on human platelets. DAF does not bind to iC3 or iC4-Sepharose affinity columns and is structurally, antigenically, and functionally distinct from the molecule identified in this work. This glycoprotein, termed platelet C3-binding protein (plt-C3BP), is labeled by surface radioiodination and appears on autoradiographs as two bands, an upper band with a mean M_r of 64,000 and a lower band with a mean M_r of 53,000. Studies of the binding specificity of plt-C3BP established it as an iC3 or C3b binding protein with ligand specificity for the C3c portion of C3. Lastly, evidence was obtained that this molecule possesses cofactor activity.

In the course of these studies, we noted the similarities between plt-C3BP and gp45-70 of human leukocytes (24). The M_r of the two bands of the molecule of both platelets and leukocytes were identical on SDS-PAGE. Furthermore, individuals with a predominantly upper or lower band pattern for their leukocyte gp45-70 display the same pattern on their platelets. In addition, results of glycosidase digestions of gp45-70 of U937 and HSB-2 are identical to those obtained here for platelets (44–46). Analysis by two-dimensional gels also shows plt-C3BP (unpublished data) and gp45-70 (44) to both have very acidic isoelectric points (43, 44), compatible with the large amount of sialic acid on the molecule. Both molecules also bind to the C3c portion of C3 (24). These similarities of structure and binding specificity indicate that plt-C3BP and gp45-70 of human mononuclear leukocytes are identical.

For all donors evaluated the two bands that comprise this C3 binding protein have identical M_r. In contrast, the relative amount of the upper and lower band varied widely among donors but was reproducible in a given individual. A partial explanation for the M_r difference between the two bands was provided by glycosidase digestions. Platelet gp45-70 possesses complex N-linked oligosaccharides and evidence was obtained for sialic acid residues that were not associated with N-linked sugars (probably O-linked oligosaccharides). An increased number of these sialic acid residues was found on the higher M_r form of this molecule. In progress are studies of the inheritance pattern of this molecule and further comparative structural analyses of the two bands. Such experiments should permit us to determine if this unusual two band pattern represents allelic variants or two distinct glycoprotein species.

In addition to the initial identification of this glycoprotein on platelets and its structural and functional similarity to gp45-

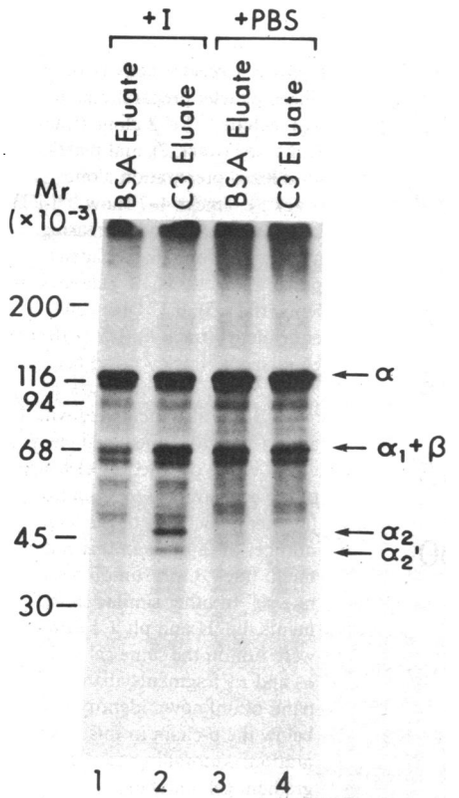

Figure 9. Cofactor activity for I-mediated cleavage by eluates of iC3- or BSA-Sepharose. Solubilized platelet preparation was precleared with IgG-Sepharose and then applied to BSA- or iC3-Sepharose columns. The eluate of these columns was then assessed for cofactor activity. Eluates were incubated with I (tracks *1* and *2*) or, as a control, with buffer (tracks *3* and *4*). Autoradiograph of a 6–18% gradient gel, reducing.

70, another important finding was that platelet gp45-70 has cofactor activity. The possibility that this cleavage was caused by other known cofactors such as H, C4bp and CR1 was assessed and excluded. In addition, the activity observed was not likely to be due to nonspecific proteases as appropriate inhibitors were present and the activity was I-dependent. This platelet-associated cofactor activity was assigned to a C3-specific binding molecule; that is, the activity in solubilized platelet preparations specifically bound to and eluted from an iC3-Sepharose column. Affinity chromatography of solubilized surface labeled platelets leads to the isolation of platelet gp45-70 as the only C3-specific binding protein on platelet membranes. It is likely, therefore, that platelet gp45-70 is responsible for the cofactor activity. Finally, gp45-70 has recently been purified from leukocytes and shown to have cofactor activity (46, 47).

Though human platelets do not possess complement receptors (CR1 or CR2), they have previously been shown to possess another protein which regulates complement activation, decay accelerating factor or DAF (28, 29). DAF dissociates (30) and prevents assembly of the C3 convertases (48); however, it has no cofactor activity (30). DAF on human erythrocytes has several other properties of note including the ability to insert into erythrocyte membranes; in addition, it possesses decay accelerating activity only for C3b or C4b inserted into the same membrane upon which the DAF itself is located (so called intrinsic decay accelerating activity) (48, 49).

The findings in this report on platelet gp45-70 are pertinent to the preceding discussion of DAF. Both molecules, although antigenically distinct, display similar but not identical molecular weights as well as cell specific variations in M_r (24, 50). Also, DAF and gp45-70 are platelet and leukocyte membrane glycoproteins that both regulate the C3 convertases. Their activity profiles are complementary; that is, DAF has no cofactor activity (30) but can prevent assembly and dissociate the C3 convertases (30, 48), whereas gp45-70 has no decay accelerating activity (46, 47) but does possess cofactor activity. It is our hypothesis that DAF and gp45-70 function conjointly to prevent autologous complement activation on human platelets and leukocytes.

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