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Interaction of Specific Platelet Membrane Proteins with Collagen: Evidence from Chemical Cross-Linking†

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ABSTRACT: Two recently developed membrane-impermeant cross-linkers, 3,3′-dithiobis(sulfosuccinimidyl propionate) (DTSSP) and bis(sulfosuccinimidyl) suberate (BS^3), have been used to examine the interaction of human platelets with collagen. Reaction of human platelets with either of the two cross-linking reagents at micromolar concentrations completely inhibited platelet aggregation in response to collagen but not in response to thrombin. Platelet adhesion to collagen was, however, not affected by these reagents. Inhibition of collagen-induced platelet aggregation by DTSSP or BS^3 appears to be due to cross-linking and not simply to the chemical modification of membrane proteins, since the homologous monofunctional reagent sulfosuccinimidyl propionate had no effect on platelet aggregation. Inhibition of platelet aggregation by BS^3 was accompanied by a decrease in the intensity of glycoprotein bands IIb, IIIa, and IV when analyzed on sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gels. In order to determine if collagen is directly interacting with a specific platelet membrane glycoprotein, ^3H-labeled platelets were allowed to adhere to collagen and then cross-linked with various concentrations of DTSSP. Proteins which remain associated with collagen after lysis and washing were analyzed on $NaDodSO_4$ gels. At concentrations of 16–50 μM DTSSP, glycoproteins IIb and IIIa appeared to be specifically cross-linked to collagen. These results suggest that the glycoprotein IIb–IIIa complex, which has previously been implicated as the fibrinogen receptor in activated platelets, may also be directly involved in collagen-induced platelet aggregation.

The sequence of events initiated when injury to a blood vessel exposes the subendothelial layer to circulating platelets presumably first involves specific interactions of platelet membrane components, primarily glycoproteins, with the matrix. In addition, through the intervention of soluble proteins including von Willebrand factor (Weiss et al., 1978; Kao et al., 1979; Fujimoto et al., 1982), and fibrinogen (Marguerie et al., 1979; Bennett & Vilaire, 1979), platelets adhere to one another to form the platelet "plug". Despite intensive study, the identities of the platelet membrane glycoproteins involved in these events have been determined only partially. Thus, for example, the role of a glycoprotein IIb–IIIa complex as a fibrinogen receptor, after platelet activation, seems relatively well established (Nachman & Leung, 1982; Di Minno et al., 1983; Bennett et al., 1983). Both glycoprotein I and glycoprotein V have been implicated as sites of interaction of thrombin (Tollefsen et al., 1974; Workman et al., 1977; Larsen & Simons, 1981; Berndt & Phillips, 1981), but the identification of the membrane component responsible for specific interaction with collagen, or with von Willebrand factor, is less clear (Jamieson & Okumura, 1978; Jenkins et al., 1983; Chiang & Kang, 1982).

Chemical cross-linking reagents have been applied in a limited way to the analysis of topography and function of the platelet membrane (Larsen & Simons, 1981; Davies & Palek, 1982; Lahav et al., 1982). The availability of a new family of such reagents, 3,3′-dithiobis(sulfosuccinimidyl propionate) (DTSSP)[1] and bis(sulfosuccinimidyl) suberate (BS^3), which exhibit considerable stability toward hydrolysis and which are membrane impermeant (Staros, 1982) has led us to examine their effect upon cross-linking of membrane glycoproteins to one another and to collagen fibrils. In the course of these investigations, it was found that the exposure of platelets to micromolar levels of these cross-linking reagents led to the specific inhibition of collagen-induced aggregation but did not affect either thrombin-induced aggregation or adhesion to collagen.

We report here also the effects of these cross-linking reagents on platelet membrane glycoproteins as evidenced by changes in the electrophoretic pattern and the selective cross-linking of certain membrane glycoproteins of adhering platelets to fibrillar collagen.

Experimental Procedures

Materials. Tritiated sodium borohydride (22 Ci/mM) was obtained from Research Products International, Mount Pro-

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[1] Abbreviations: DTSSP, 3,3′-dithiobis(sulfosuccinimidyl propionate); BS^3, bis(sulfosuccinimidyl) suberate; SSP, sulfosuccinimidyl propionate; $NaDodSO_4$, sodium dodecyl sulfate; MbA, methylenebis(acrylamide); DATD, *N,N′*-diallyltartardiamide; DTT, dithiothreitol; TEMED, *N,N,N′,N′*-tetramethylethylenediamine; ACD, acid–citrate–dextrose; EDTA, disodium ethylenediaminetetraacetate; HPLC, high-performance liquid chromatography; $HOSu(SO_3)$, *N*-hydroxysulfosuccinimide; Tris, tris(hydroxymethyl)aminomethane; NEM, *N*-ethylmaleimide; BSA, bovine serum albumin; M_r, relative molecular weight.

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spect, IL. ^{14}C-Labeled protein molecular weight standards (5–22 μCi/mg of each protein) were purchased from Bethesda Research Laboratories, Inc., Bethesda, MD. Sodium metaperiodate and disodium ethylenediaminetetraacetate (EDTA) were from Fisher Scientific, Fair Lawn, NJ, and human fibrinogen (fraction 1) was from Calbiochem, La Jolla, CA. Human skin insoluble collagen was prepared as described by Cunningham & Ford (1968). DTSSP and BS^3 were prepared as previously described (Staros, 1982), and sulfosuccinimidyl propionate (SSP) was prepared from propionic acid and *N*-hydroxysulfosuccinimide by the general method described for the preparation of sulfosuccinimidyl active esters (Staros, 1982). Bovine serum albumin, *N*-ethylmaleimide, tris(hydroxymethyl)aminomethane, and glycine were obtained from Sigma, St. Louis, MO. Sodium dodecyl sulfate, acrylamide, *N*,*N*′-methylenebis(acrylamide)(MbA), *N*,*N*′-diallyltartardiamide (DATD), dithiothreitol (DTT), ammonium persulfate, and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) were from Bio-Rad, Richmond, CA. All other chemicals were high-grade commercial products. Human thrombin was a generous gift of Dr. John W. Fenton, New York State Department of Health, Albany, NY.

Isolation of Human Platelets. Human blood was obtained from healthy donors who had not ingested, within the preceding 2 weeks, any drug known to affect platelets. Blood was collected into 0.1 volume of the anticoagulant ACD (38 mM citric acid, 75 mM sodium citrate, and 136 mM glucose). Platelets were then isolated by the multiple centrifugation method previously described (Santoro & Cunningham, 1979). Blood was centrifuged at 180*g* for 10 min at room temperature, and platelet-rich plasma was slowly removed into another tube containing 0.1 blood volume of ACD. Platelets were separated from plasma by centrifugation at 1200*g* for 10 min, suspended to the original blood volume in buffer A (137 mM NaCl, 2.7 mM KCl, 4.25 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 5 mM glucose, and 2 mM EDTA, pH 7.4) containing 0.1 volume of ACD, and again separated by centrifugation. This last step was repeated once. Platelets were then suspended in the appropriate buffer at a concentration of 1×10^9/mL.

Surface Labeling of Human Platelets. Platelets were radiolabeled by the periodate–[^{3}H]borohydride method with some modification of the previously described procedures (Gahmberg & Andersson, 1977; Sixma & Schiphorst, 1980). Platelets were suspended in 1 mL of buffer B (buffer A without glucose) at a concentration of 10^9/mL and placed on ice for 3 min. Ten microliters of 100 mM $NaIO_4$ was added, and the mixture was kept on ice in complete darkness for another 5 min. Five milliliters of buffer B was added, and the suspension was centrifuged at 1200*g* for 10 min at room temperature. The platelet pellet was washed with 5 mL of buffer B, centrifuged, and then resuspended in 1 mL of the same buffer. NaB^3H_4 (0.5 mCi) was added to the platelet suspension which was then incubated for 15 min at room temperature. Five milliliters of buffer B was added, the suspension was centrifuged, and the resulting pellet was then washed once with buffer A. Finally, platelets were suspended in buffer A at a concentration of 1×10^9/mL. Tritiated platelets prepared by this procedure were fully active in both the aggregation and adhesion assays employed in this study (see below).

Characterization of SSP and Its Reaction with Amino Groups. Analyses of SSP and its hydrolysis products and its products of reaction with model compounds were carried out by high-performance liquid chromatography on a system consisting of a Spectra-Physics SP8700 pump and ternary gradient controller, an Alltech 70061 column (C_{18} reversed phase, 5-μm spherical silica packing), an ISCO V^4 monitor (set at 257 nm), and a Hewlett-Packard HP3390A recording integrator. The mobile phase consisted of 60:40 (v/v) aqueous 10 mM tetrabutylammonium formate:methanol for 5 min, followed by a 10-min linear gradient to a final 20:80 aqueous buffer:methanol mixture, delivered at a constant flow rate (1.00 mL/min).

A sample of SSP was dissolved in 50 mM sodium phosphate buffer, pH 7.3, to a concentration of 20 mM. HPLC analysis of the solution yielded a major peak corresponding to SSP and a minor peak corresponding to free *N*-hydroxysulfosuccinimide [HOSu(SO_3)]. Incubation in this buffer at room temperature led to a progressive decrease in the SSP peak and a corresponding increase in the HOSu(SO_3) peak (and no others), with a half-time of hydrolysis of approximately 1.5 h. When, under the same conditions, a fresh solution of SSP was added to a solution of phenylalanine, to concentrations of 10 and 15 mM, respectively, a new peak, corresponding to *N*-propionylphenylalanine, was formed in >90% yield within 50 min.

Treatment of Human Platelets with DTSSP, BS^3, or SSP. In a typical experiment, radiolabeled or unlabeled platelets, at a concentration of 5×10^8/mL, were incubated with various concentrations of the cross-linker (DTSSP or BS^3) or the monofunctional reagent (SSP) in buffer A for 20 min at room temperature. The reaction was quenched by adding Tris-HCl (pH 7.4) to a final concentration of 0.2 M and incubating for 5 min. Buffer A (6 volumes) was added to the samples which were then centrifuged at 1200*g* for 10 min. For aggregation studies, reacted unlabeled platelets were washed once by resuspension in 6 volumes of aggregation buffer (buffer A supplemented with 0.35% bovine serum albumin and with EDTA omitted) and then suspended in aggregation buffer at a platelet count of 2.5×10^8/mL. For gel electrophoresis studies, reacted labeled platelets were washed 2 times by resuspension in 6 volumes of buffer A containing 20 mM NEM, followed by centrifugation, and 1 time with buffer A without NEM. Platelet pellets were stored frozen at −70 °C until further use.

Cross-Linking of the Platelet–Collagen Complex. Human skin insoluble collagen was extensively washed by cycles of suspension in buffer A containing 1% $NaDodSO_4$ followed by centrifugation. The extracted collagen was then washed in buffer to free it of $NaDodSO_4$. This extracted insoluble collagen was fully effective in initiating platelet adhesion and aggregation. Radiolabeled platelets (850 μL, containing 2.5×10^8 cells) in buffer A were incubated with 150μL of human skin insoluble collagen homogenate (2 mg/mL) with continuous gentle horizontal mixing at 37 °C for 20 min to allow adhesion (Santoro & Cunningham, 1979). Samples were centrifuged at 1200*g* for 10 min. The pellets were suspended in buffer A and cross-linked with various concentrations of DTSSP in an incubation volume of 0.5 mL at room temperature for 20 min. The reactions were quenched by the addition of Tris-HCl (to 0.2 M), pH 7.4, and further incubated for 5 min. Samples were centrifuged, and the pellets were washed once with 6 volumes of buffer A containing 20 mM NEM. Platelets were lysed in 6 volumes of buffer A containing 20 mM NEM and 1% $NaDodSO_4$. The lysates were centrifuged, and the protein–collagen pellets were washed by centrifugation 2 more times with the lysing buffer until no radioactivity was detected in the supernatant. Finally, samples were washed with buffer A, and the pellets were incubated in 200 μL of $NaDodSO_4$–sample buffer containing 50 mM DTT (to cleave the cross-linker) for 45 min at 37 °C and then centrifuged at

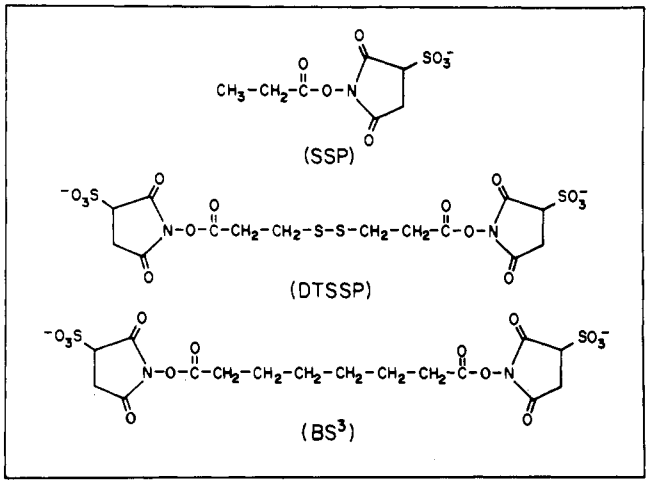

FIGURE 1: Structures of the cross-linking reagents and the homologous monofunctional compound: 3,3′-dithiobis(sulfosuccinimidyl propionate) (DTSSP); bis(sulfosuccinimidyl) suberate (BS^3); and sulfosuccinimidyl propionate (SSP).

20000*g* for 10 min. The supernatants were counted for radioactivity and stored frozen at −70 °C.

NaDodSO₄–Polyacrylamide Gel Electrophoresis. Electrophoresis was performed on 1.5-mm slab gels by utilizing a procedure modified from Laemmli (1970). Percent T is the percent of total monomers, and percent C is the percent of cross-linking monomer in the total monomers; resolving gels contained 6% T and 2.6% C (MbA), and stacking gels contained 5% T and 15% C (DATD). Samples were incubated in sample buffer containing 50 mM DTT for 5 min at 90 °C. As molecular weight standards, the following mixture of ^{14}C-proteins was used: cytochrome *c*, M_r 12 300; β-lactoglobulin, M_r 18 400; α-chymotrypsinogen, M_r 25 700; ovalbumin, M_r 43 000; bovine serum albumin, M_r 68 000; phosphorylase *b*, M_r 92 500; and myosin, M_r 200 000. After electrophoresis, gels were processed for fluorography (Bonner & Laskey, 1974).

Platelet Aggregation. Platelet aggregation was measured in a Chronolog aggregometer (Santoro & Cunningham, 1977). Platelets were suspended in aggregation buffer at a platelet count of 2.5×10^8/mL. 0.4 mL of platelet suspension was aliquoted into the aggregometer cell, and the suspension was adjusted to 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.05% fibrinogen by adding 8 μL of a concentrated stock. Aggregation was induced by the addition of 10 μL of human skin insoluble collagen suspension containing 10–20 μg of collagen.

Platelet–Collagen Adhesion. Platelets were radiolabeled by the periodate–[^3H]borohydride method and were then cross-linked with various concentrations of DTSSP in buffer A for 20 min at room temperature. The reaction was quenched by adding Tris-HCl (to 0.2 M), pH 7.4, and then 6 volumes of buffer A. Samples were centrifuged at 1200*g* for 10 min and then washed once with the same buffer. Pellets were suspended in buffer A ± 20 mM NEM for 10 min, centrifuged, and finally resuspended at a concentration of 2.5×10^8/mL in buffer A containing 0.35% BSA. Finally, platelet–collagen adhesion assays were performed by the method of Santoro & Cunningham (1981).

Results

Effects of Cross-Linking Reagents and a Related Monofunctional Reagent on Platelet Aggregation and Adhesion to Collagen. The reaction of human platelets with the two membrane-impermeant cross-linking reagents (Figure 1) at concentrations as low as 10 μM DTSSP and 50 μM BS^3 completely inhibited platelet aggregation in response to collagen (Figure 2). In order to determine if such inhibition is due to cross-linking of platelet membrane proteins or simply to the chemical modification of these proteins, platelets were treated with various concentrations of the monofunctional reagent (SSP), and the experiment was carried out exactly as before. The monofunctional reagent did not inhibit platelet aggregation over the concentration range used for both DTSSP and BS^3 (Figure 2). Inhibition of platelet aggregation by various concentrations of DTSSP was not reduced when higher doses of collagen were used to induce aggregation (Figure 3).

The bifunctional reagent DTSSP did not, however, inhibit platelet aggregation induced by 0.2 unit/mL thrombin (Figure 3), suggesting that the cross-linker is affecting proteins which are involved in collagen but not thrombin activation of platelets. Furthermore, although strongly inhibitory to collagen-induced aggregation, DTSSP had no effect on adhesion of platelets to collagen (Table I). As a control, and under

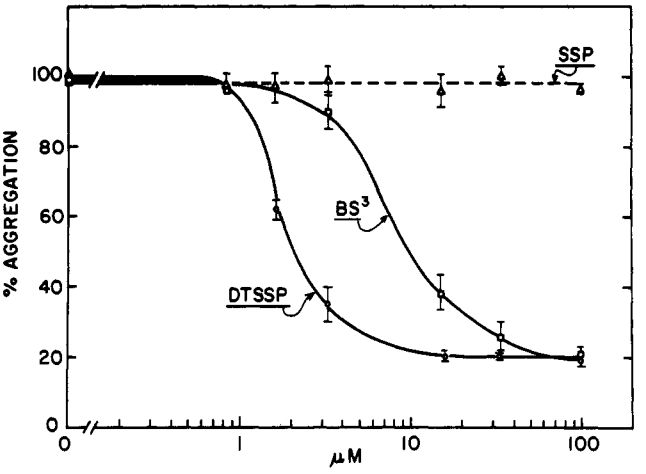

FIGURE 2: Effects of DTSSP, BS^3, and SSP on collagen-induced platelet aggregation. The concentration of the monfunctional reagent (SSP) used is twice that indicated on the abscissa so that the concentration of reactive groups is the same as in the experiments with the cross-linkers. Percent aggregation was calculated from the slope of the aggregation profiles, and the values shown represent the average of three determinations ±SD.

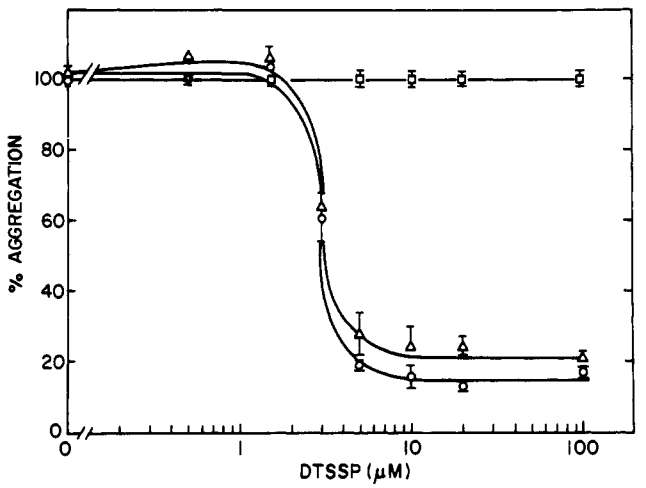

FIGURE 3: Effects of DTSSP on platelet aggregation induced by two collagen concentrations and by thrombin. Platelets were cross-linked with various concentrations of DTSSP and then tested for aggregation as described under Experimental Procedures. Platelet aggregation was induced by thrombin (0.2 unit/mL) (□) and by collagen (50 μg/mL, 200 μg/mL) (○, △). Percent aggregation was calculated from the slope of the aggregation profiles, and the values shown represent the average of two determinations ±SD.

Table I: Effect of DTSSP on Platelet–Collagen Adhesion

[DTSSP] (μM)	% adhesion[b] –NEM	% adhesion[b] +NEM[a]	% trapping[b]
0	40 ± 2[a]	4 ± 2	2 ● 0.1
10	39 ± 4	6 ± 0.3	3 ± 1
100	42 ± 6	10 ± 2.4	5 ± 2
1000	41 ± 5	9 ± 1	8 ± 1

[a] Values represent the average of two determinations ±SD. [b] Determined by the method of Santoro & Cunningham (1981).

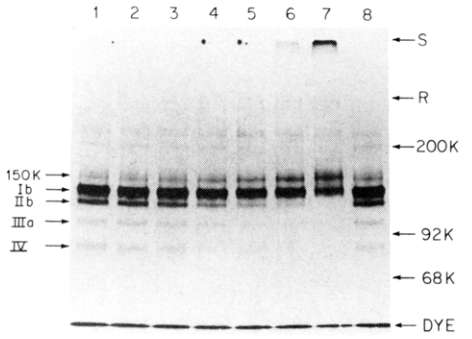

FIGURE 4: Fluorograph of $NaDodSO_4$–polyacrylamide gel electrophoresis of ^{3}H-platelet proteins cross-linked with various concentrations of BS^3. Lanes 1 and 8, no cross-linker; lane 2, 1 μM; lane 3, 10 μM; lane 4, 50 μM; lane 5, 100 μM; lane 6, 200 μM; lane 7, 2000 μM. S and R indicate the top of the stacking and resolving gels, respectively. Molecular weight standards are labeled in the right hand margin.

these same conditions, 20 mM NEM does inhibit adhesion as previously reported (Spaet & Lejnieks, 1969).

Cross-Linking of Platelets. In order to determine which platelet membrane glycoproteins had been intermolecularly cross-linked by the bifunctional reagent BS^3, platelets were radiolabeled and then reacted with various concentrations of BS^3 as described under Experimental Procedures. Samples were analyzed on $NaDodSO_4$ gels, and a fluorograph of such a gel is shown in Figure 4. There was a marked change in the membrane protein profile of cross-linked platelets (lanes 4–6) compared to the control sample (lane 1). With increasing amounts of BS^3, the intensity of the bands corresponding to glycoproteins IIb, IIIa, and IV gradually decreased, almost disappearing at very high cross-linker concentrations. In addition, an increase in the intensity of a protein band of M_r 150000 was associated with treatment with increasing amounts of BS^3. To determine if the change in the protein profile after cross-linking occurred at the same BS^3 concentration which inhibits platelet aggregation, a fluorograph of the same gel was scanned with an integrating densitometer. The result, shown in Figure 5, indicated that when 50 μM BS^3 was used [a concentration of BS^3 which inhibits >90% of platelet aggregation (see Figure 2)] approximately 50% of the bands corresponding to glycoproteins IIb and IIIa had disappeared while there was no change in the amount of glycoprotein Ib. The approximate nature of both the aggregation assay and the densitometric procedure limits the precision of this comparison.

Platelet–Collagen Cross-Linking. In order to determine if collagen is directly interacting with specific platelet membrane glycoproteins, experiments were conducted according to the protocol described under Experimental Procedures, in which ^{3}H-platelets were allowed to adhere to collagen and were then cross-linked with the cleavable cross-linker DTSSP. In preliminary experiments, the platelet–collagen complex was cross-linked with 2 mM DTSSP for various periods of time; samples were processed and then counted for radioactivity.

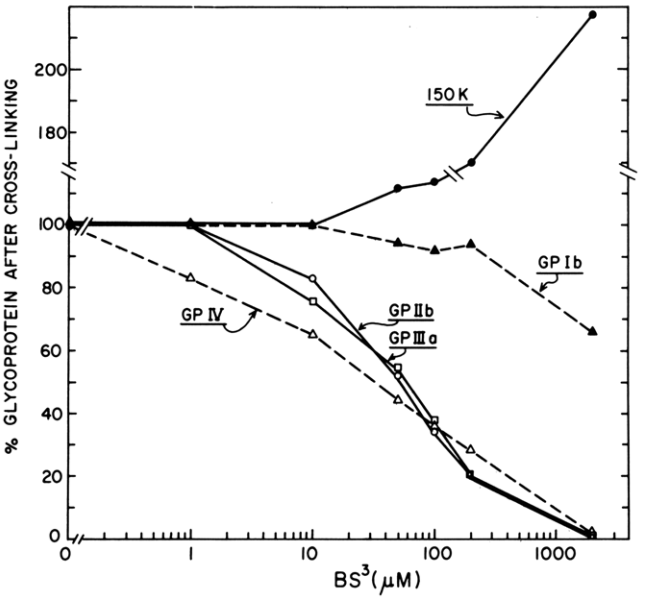

FIGURE 5: Densitometric scan of a gel fluorograph of glycoproteins after cross-linking with BS^3. The gel of Figure 4 was scanned with an integrating Kontes fiber optic scanner (Model 800). The percent of each glycoprotein at various BS^3 concentrations was calculated with reference to an un-cross-linked sample.

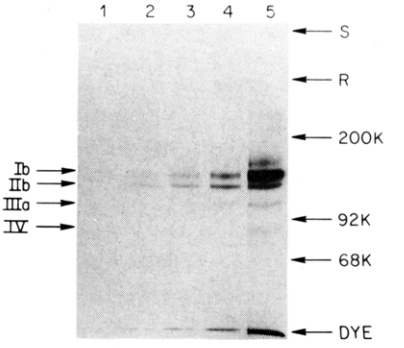

FIGURE 6: Fluorograph of $NaDodSO_4$–polyacrylamide gel electrophoresis of radiolabeled glycoproteins which were cross-linked to collagen by DTSSP. ^{3}H-Platelets were allowed to adhere to collagen and were then cross-linked with various concentrations of DTSSP for 20 min (see Experimental Procedures). Lane 1, no cross-linker; lane 2, 16 μM; lane 3, 49 μM; lane 4, 164 μM; lane 5, normal profile of ^{3}H-platelets. Molecular weight standards are labeled in the right hand margin. The same quantity of insoluble collagen was present in each sample, and an equal aliquot of reaction product was applied to lanes 1–4.

Maximum cross-linking of glycoproteins to collagen occurred after 20 min at room temperature. Samples of the platelet–collagen adhesion complex were therefore cross-linked with various concentrations of DTSSP for 20 min. Samples were analyzed on $NaDodSO_4$ gels, and a fluorograph is shown in Figure 6. These results suggest that glycoproteins Ib, IIb, and IIIa (lanes 2 and 3) were cross-linked to the collagen fibrils since they remained associated with the insoluble pellet after platelet lysis and washing and were released by DTT. The predominant glycoproteins cross-linked to collagen, relative to the control profile of radiolabeled whole ^{3}H-platelets (lane 5), were glycoprotein IIb and glycoprotein IIIa. Glycoprotein Ib was present but at a markedly lower relative concentration. The control sample which was not cross-linked showed no radioactive bands (lane 1).

The ratio of collagen-bound radiolabel in glycoprotein IIb to that in glycoprotein IIIa was the same as that found for the radiolabel in these glycoproteins in whole platelets (lane 5),

where, when the lower initial incorporation of ^3H into glycoprotein IIIa is taken into account, it has been shown to reflect a molar ratio of approximately 1:1 (Nachman & Leung, 1982).

Discussion

Normal circulating human platelets respond extremely rapidly to a number of stimuli by shape change, secretion of granule contents, and aggregation. Under most conditions, it would appear that the most typical stimulus is adhesion of platelets to collagen in the exposed subendothelial matrix, although exposure to thrombin may also be a frequent initiator of platelet aggregation.

Both of these initiating events appear to involve platelet membrane glycoproteins, although the case for glycoprotein V, in the Phillips & Agin (1977) nomenclature, as a thrombin substrate appears to be considerably better than any case which has yet been made for a specific glycoprotein "receptor" for collagen. Indeed, it is not clear whether the platelet membrane interacts directly with collagen in the matrix or through a bridging molecule such as von Willebrand factor (Weiss et al., 1978; Kao et al., 1979; Fujimoto et al., 1982). However, the failure of normal circulating platelets to bind von Willebrand factor before they have been activated by thrombin (Fujimoto et al., 1982) strongly suggests an initial direct interaction of platelet membrane components with the collagenous matrix. Following activation of the platelet, other membrane changes occur which result in the generation of receptor activity for fibrinogen (Marguerie et al., 1979), fibronectin (Ginsberg et al., 1980), and von Willebrand factor (Fujimoto et al., 1982). The complexity of these changes is suggested by the observation, for example, that although glycoproteins IIb and IIIa are at least partly exposed on the outer surface in unactivated platelets, they are inactive as fibrinogen receptor until platelet activation occurs (Nachman & Leung, 1982).

It would clearly be of great interest to understand the topography of the exposed surface of the platelet membrane, and of its response to activation. The application of membrane-impermeant cross-linking reagents has given insight into the extracytoplasmic surface of the red cell membrane (Staros et al., 1981; Staros, 1982; Staros & Kakkad, 1983), and we have now applied two of these recently developed cross-linking reagents to the study of the platelet. These reagents, based on sulfosuccinimidyl active esters, are relatively stable to spontaneous hydrolysis and, by virtue of the sulfonate groups, essentially impermeant to the plasma membrane. They are effective at relatively low concentrations, and their effects can be ascribed solely to reaction on the outer surface of the plasma membrane in intact cells. Complications arising from the cross-linking of membrane glycoproteins to cytoskeletal elements can thereby be eliminated.

In order to characterize the effects of these cross-linking reagents, we examined a broad range of concentrations, ranging from 1 μM to 2 mM, looking both at the effects on platelet function, as evidenced by platelet adhesion to collagen and platelet aggregation induced by collagen and thrombin, and at the direct effects on membrane glycoproteins by Na-DodSO_4–polyacrylamide gel electrophoresis.

Both cross-linkers were found to be extremely effective inhibitors of collagen-induced platelet aggregation (Figure 2), with half-maximal inhibition occurring near 2 μM with DTSSP and near 8 μM with BS^3. The lower limit of 20% aggregation found in these experiments (Figures 2 and 3) appears to be an artifact due to the effect of residual cross-linker upon the turbidity of the collagen fibrils added. The monofunctional reagent SSP, which approximates, structurally, half of the cross-linker molecules, caused no inhibition of aggregation (Figure 2). These results suggest that it is the generation of a covalent bridge between two membrane glycoproteins, or within a single membrane glycoprotein, and not simply the chemical modification of a particular amino group which leads to loss of susceptibility to collagen.

The inhibition of collagen-induced aggregation by the cross-linker cannot be reversed by a high collagen concentration (Figure 3). In contrast, thrombin-induced platelet aggregation proceeded normally, at least up to a concentration of 100 μM DTSSP, well beyond the point where collagen-induced aggregation had failed (Figure 3). The basis for this unexpected and potentially informative difference is not yet understood, but it clearly suggests that one very sensitive action of the cross-linking reagents is to block the *effect* of collagen "recognition", but not "recognition" (adhesion) itself, without interfering with the action of thrombin or the generation of the second wave of receptors created by the action of thrombin. A problem with this analysis, however, is that the autocatalytic nature of the platelet response may require or permit activation of the platelet by very different levels of collagen and thrombin. Further study of these systems may provide a resolution to this interesting question.

A direct look at the effect of the noncleavagle cross-linker BS^3 upon platelet membrane glycoproteins does not provide a ready explanation for these effects (Figure 4). At concentrations up to 100 μM, where the maximal inhibition of aggregation by this reagent is observed, the major effects seen are a decrease in glycoproteins IIb, IIIa, and IV while glycoprotein Ib remains unchanged. There is an easily seen increase in very high molecular weight material which enters the stacking gel only poorly or which collects near the top of the separating gel. The loss of glycoproteins IIb, IIIa, and IV cannot be accounted for simply by increases in specific higher molecular weight bands. The postulated IIb–IIIa complex which exists in activated and (it would appear from these results) in unactivated platelets even in the presence of EDTA (Fujimura & Phillips, 1983) should, when covalently linked, give a band of M_r 230 000. There is a slight increase in this region at high cross-linker concentrations, but it is hardly sufficient to account for all the glycoproteins IIb and IIIa which have disappeared. These results support the existence of a complex in the unactivated platelet membrane of, at least, glycoproteins IIb and IIIa. Glycoprotein IV does not appear to be directly associated with glycoproteins IIb and IIIa despite its similar rate of reaction with BS^3 since it is not found with these glycoproteins in association with collagen (see below). Further clustering of these complexes may occur normally or may be induced by the conditions of the cross-linking experiment as evidenced by the increasing quantity of very high molecular weight material (Polley et al., 1981). Of particular interest, however, is glycoprotein Ib, which remains free of these putative complexes. It is affected only at extremely high, above 200 μM, concentrations of BS^3.

Under conditions similar to those of Figure 3 where collagen-induced aggregation is completely inhibited by DTSSP, the adhesion of platelets to collagen fibrils is not affected (Table I). This was surprising in view of the differing effects of the cross-linkers upon thrombin and collagen-induced platelet aggregation, but it offered an opportunity for a direct chemical exploration of the existence and nature of a platelet membrane "receptor", i.e., adhesion site, for collagen. The cleavable cross-linker DTSSP was allowed to react with platelets adhering to fibrillar collagen for the same time and at

similar concentrations to those used in the membrane cross-linking and inhibition experiments, except that EDTA was present to prevent aggregation. The entire system was then lysed and washed with $NaDodSO_4$, so that only a residual pellet of insoluble collagen, and any covalently cross-linked platelet membrane components, was left and recoverable by centrifugation. The radiolabeled platelet components were freed from collagen by treatment with DTT and examined by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 6). At low concentrations of DTSSP, 16 μM or even 50 μM, where the aggregation-inhibitory activity of this reagent is complete, glycoproteins IIb and IIIa or glycoprotein bands with the same molecular weights as glycoproteins IIb and IIIa, are preferentially linked to collagen, suggesting the presence of the IIb–IIIa complex. Glycoprotein Ib is cross-linked in greatly reduced amounts from its ratio to glycoprotein IIb in intact platelets, and it is not clear whether this reflects simply a small contamination with this major ^3H-labeled glycoprotein of platelet membranes or whether it is actually linked to collagen, or to glycoprotein IIb–IIIa, by the cross-linker. The very extensive washing of the collagen pellet which precedes the analysis and the total absence of glycoprotein Ib in the control suggest that these small amounts of glycoprotein Ib are covalently incorporated. The complete absence of glycoprotein IV is especially interesting in view of its similar rate of disappearance to glycoproteins IIb and IIIa in the presence of cross-linking reagents noted previously (Figure 4). Under our conditions, the membrane component of M_r 65 000 reported by Chiang & Kang (1982) to exhibit affinity for collagen could not be detected. However, this component may not be labeled by ^3H under our conditions. On the whole, it is not possible to draw final conclusions from these studies, but taken at face value, they suggest that the IIb–IIIa complex may be the collagen adhesion site, or form a part of that site, in unactivated platelets, in addition to its role as the fibrinogen receptor following activation. Furthermore the inhibition of collagen-induced aggregation by DTSSP, under conditions shown to affect neither adhesion to collagen nor thrombin-induced aggregation, indicates a sensitive step in the mechanism of collagen-induced aggregation, subsequent to adhesion but not common to the thrombin pathway. The inhibition of this step by low concentrations of these membrane-impermeant cross-linkers further suggests that the external portion of a membrane component is involved and offers, with further study, the possible identification of that component.

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