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MODULATION OF AN RGDS BINDING SITE ON THE PLATELET MEMBRANE GLYCOPROTEIN IIb-IIIa COMPLEX

Samuel A. Santoro[1] and William J. Lawing, Jr.

Division of Laboratory Medicine - Box 8118
Departments of Pathology and Medicine
Washington University School of Medicine
St. Louis, Missouri 63110

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SUMMARY: Fibronectin, von Willebrand factor, and fibrinogen each bind to the glycoprotein IIb-IIIa complex on activated platelets via an arg-gly-asp-ser (RGDS) sequence present within the adhesive proteins. Both the IIb and IIIa polypeptides of the IIb-IIIa complex on thrombin activated platelets are specifically and extensively labeled by a radiolabeled, photoactivatable arylazide derivative of the RGDS sequence when the labeling is performed in the presence of concentrations of Ca^{++} or Mg^{++} approaching 0.5 mM. In contrast, labeling of unactivated platelets, ADP activated platelets, or thrombin activated platelets in the presence of low concentrations of divalent cations resulted in restriction of labeling to the IIb polypeptide of the complex.

The platelet membrane glycoprotein IIb-IIIa complex exists as a noncovalently associated heterodimeric complex within the platelet membrane (1). The complex serves as an activation-dependent receptor on the platelet surface for the adhesive proteins fibrinogen, fibronectin, and von Willebrand factor (2). The binding of these proteins to the IIb-IIIa complex appears to be mediated in large part by a sequence of 3-4 amino acids, RGD(S), present within each of the three adhesive proteins. Small peptides containing the RGDS sequence effectively inhibit the binding of all three adhesive proteins to the IIb-IIIa complex (3-5). We (6) have recently demonstrated that radiolabeled, photoactivatable aryl azide derivatives of RGDS-containing peptides covalently labeled both the IIb and IIIa polypeptides of the IIb-IIIa complex in a specific manner. In this report, we present evidence that this pattern of labeling is attributable to the existence of two conformations of the receptor complex, one of which favors labeling of the IIb polypeptide while the other results in preponderant labeling of the IIIa polypeptide.

[1]To whom all correspondence should be addressed.

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MATERIALS AND METHODS

Materials. The iodinatable, photoactivatable crosslinking reagent N-hydroxysuccinimydyl-4-azido-salicylic acid (NHS-ASA) and Iodobeads were obtained from Pierce Chemical Co. (Rockford, IL). Carrier-free Na ^{125}I was purchased from Amersham (Arlington Heights, IL). Highly purified human thrombin (3300 U/mg) was generously provided by Dr. Joseph P. Miletich, Washington University School of Medicine.

The synthetic peptides GRGDSC and GDGRSC were prepared using standard automated solid-phase technology with an Applied Biosystems model 430 peptide synthesizer. Peptides were purified by chromatography on TSK SP-5W columns eluted with 20 mM NaH_2PO_4 and 0 - 0.5 M NaCl gradient and by chromatography on a Vydac C-18 column eluted with 0.1% trifluoroacetic acid and a 0 - 100% gradient of acetonitrile. Structures of peptides were confirmed by analysis of amino acid composition and sequence.

Preparation of photoaffinity derivatives. Peptides were derivatized with NHS-ASA and radiolabeled with Na ^{125}I as recently described (6). Briefly, a 150 µl aliquot of NHS-ASA (400 mM) dissolved in a 1:1 mixture of dimethyl sulfoxide and acetone was mixed with a 150 µl aliquot of peptide (10 - 12 mM) dissolved in a 1:1 mixture of dimethyl sulfoxide and 0.05 M Na_2HPO_4, 0.15 M NaCl (pH 7.4). The mixture was incubated at room temperature for 1 hr. Derivatized peptide was then separated from NHS-ASA and its hydrolysis products by chromatography on a 0.9 x 50 cm column of Sephadex G-10 (Pharmacia, Inc., Piscataway, NJ) equilibrated with 0.05 M Na_2HPO_4 (pH 7.4), 0.15 M NaCl. Fractions containing the derivatized peptide were pooled and then radiolabeled by incubation with 350 µCi of Na^{125}I and four Iodobeads for 1 hr at room temperature. Radiolabeled peptide was then separated from free Na ^{125}I by gel filtration on a 0.9 x 50 cm column of Sephadex G-10 equilibrated with phosphate buffered saline as described above. All procedures were carried out in the dark.

Labeling of Platelet Receptors. Platelets were washed by a combination of centrifugation and gel filtration as described earlier (3). The washed platelets were resuspended in 0.05 M Na_2HPO_4 (pH 7.4), 0.15 M NaCl, 5 mM glucose, 0.5% (w/v) bovine serum albumin. EDTA, Ca^{++}, or Mg^{++} were added as indicated in specific experiments.

Photoaffinity labeling reactions were conducted in a volume of 800 µl. The reaction contained platelets (3 x 10^8/ml) and the photoaffinity reagent (85 µM). The platelets were then activated by the addition of thrombin (1 U/ml) or ADP (20 µM) and were incubated at 37°C for 15 min. All of the above procedures were carried out in the dark. The reaction mixtures were then irradiated on ice for 5 min from a distance of 10 cm with a Spectroline model E-16 longwave UV light source.

The mixture was then made 3 mM in EDTA, and the platelets were washed twice by centrifugation with EDTA-containing buffer. The platelet pellet was then dissolved in 150 µl of 0.0625 M Tris (pH 6.8), 2% SDS, 1% ß-mercapto-ethanol, 10% glycerol, 0.001% bromphenol blue and heated at 100°C for 2 min. The samples were then subjected to analysis by SDS-PAGE and autoradiography.

Electrophoretic procedures. SDS-PAGE was carried out using gels composed of 7.5% acrylamide according to the method of Laemmli (7). The gels were stained for protein with Coomassie blue. Autoradiography of dried gels was carried out at -70°C using Kodac XAR-5 film.

RESULTS AND DISCUSSION

The IIb-IIIa complex exists as a divalent cation-dependent heterodimer. However, only low concentrations of divalent cations, reportedly less than 1 μM, are required to maintain the integrity of the complex (6,8). To explore the effects of divalent cations upon the photoaffinity labeling of the IIb-IIIa complex with the RGDS-derived probes, labeling experiments were performed in the absence of divalent cations (2 mM EDTA), very low concentrations of divalent cations (no additions), and in the presence of 2 mM Mg^{++}.

When thrombin activated platelets were subjected to photoaffinity labeling with the RGDS probe in the presence of 2 mM Mg^{++}, both the IIb and IIIa polypeptides were labeled as expected (Fig.1, lane 3). As also expected, no detectable labeling of the IIb-IIIa complex was detected in the presence of EDTA (Fig.1, lane 1). However, when the labeling reaction was carried out in the absence of exogenously added divalent cations, labeling was almost exclusively confined to the IIb subunit of the IIb-IIIa complex (Fig.1, lane 2).

Either Ca^{++} or Mg^{++} is capable of supporting the enhanced labeling of the IIIa polypeptide by the RGDS probe. As shown in Fig. 2, in the absence of added divalent cations labeling is confined almost exclusively to the IIb polypeptide of the complex (lane 1). In the presence of either 2 mM Ca^{++} or 2 mM Mg^{++}, both polypeptides of the complex were significantly labeled and to nearly identical extents (lanes 2 and 3). The total extent of labeling of thrombin activated platelets in the presence of either Ca^{++} or Mg^{++} was significantly greater than in the absence of added divalent cations.

The influence of Mg^{++} on the pattern of labeling was examined in greater detail over the range of 0 - 2 mM Mg^{++}. Thrombin activated

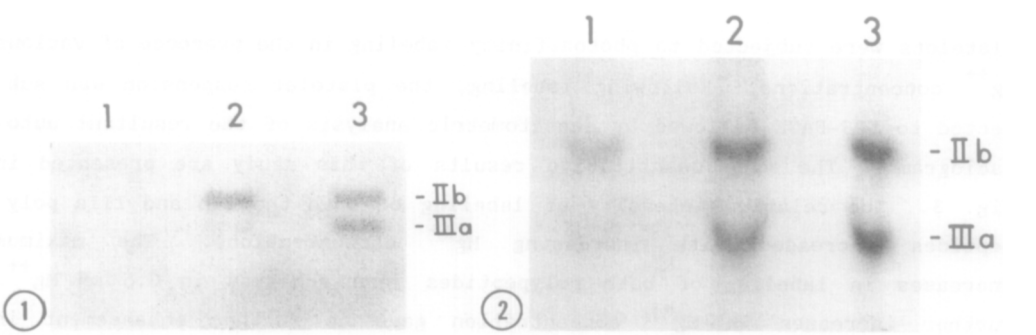

Figure 1: Divalent cation-dependent modulation of the RGDS labeling pattern of the IIb-IIIa complex. Platelets were labeled with the GRGDSC probe in the presence of 2 mM EDTA (lane 1), no additions (lane 2), or 2 mM Mg^{++} (lane 3).

Figure 2: Both Ca^{++} and Mg^{++} support modulation of the IIb-IIIa labeling pattern. Platelets were photoaffinity labeled with the GRGDSC derivative in the absence of exogenously added divalent cations (lane 1), in the presence of 2 mM Mg^{++} (lane 2), or in the presence of 2 mM Ca^{++} (lane 3).

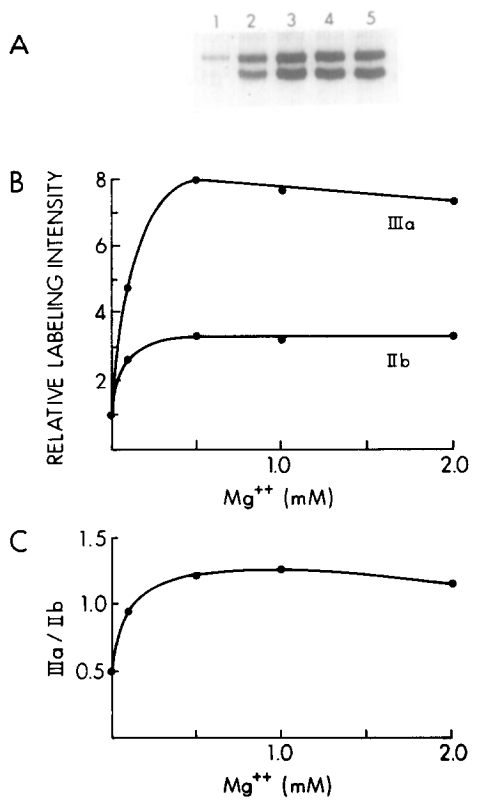

Figure 3: Effect of Mg^{++} concentration on the pattern of labeling of the IIb-IIIa complex by the GRGDSC probe. A. Autoradiogram of labeling patterns observed in the absence of exogenously added divalent cations (lane 1), 100 μM M Mg^{++} (lane 2), 500 μM Mg^{++} (lane 3), 1 mM Mg^{++} (lane 4), and 2 mM Mg^{++} (lane 5). B. Densitometric analysis of the gel in A demonstrating the effect of increasing Mg^{++} concentration on the intensity of labeling of both the IIb and IIIa polypeptides. The intensity of labeling of each polypeptide observed in the absence of exogenously added divalent cations was arbitrarily assigned a value of 1. C. Densitometric analysis of the effect of increasing Mg^{++} concentration on the IIIa:IIb labeling ratio.

platelets were subjected to photoaffinity labeling in the presence of various Mg^{++} concentrations. Following labeling, the platelet suspension was subjected to SDS-PAGE followed by densitometric analysis of the resultant autoradiograms. The more quantitative results of this study are presented in Fig. 3. The relative intensity of labeling of both the IIb and IIIa polypeptides increased with increasing Mg^{++} concentrations. The maximum increases in labeling of both polypeptides were achieved in 0.5 mM Mg^{++}. Further increases in Mg^{++} concentration gave no further enhancement in labeling of either polypeptide (Fig. 3B). Although the extent of labeling of both polypeptides increased, the relative intensity of labeling of IIIa increased much greater (approximately 8-fold) than did the intensity of labeling of the IIb polypeptide (approximately 3-fold).

The IIIa:IIb ratio of labeling also increased as the concentration of Mg^{++} was increased. In the absence of added Mg^{++} the IIIa:IIb ratio was

always found to be 0.5 or less, whereas in the presence of 2 mM Mg^{++} the ratio increased to 1.2 (Fig. 3C).

The increased labeling of the IIIa subunit of the IIb-IIIa complex with the RGDS-containing photoaffinity probe was not solely a consequence of either the presence of exogenously added Mg^{++} or of platelet activation with thrombin. As shown in Fig. 4, when unactivated platelets were subjected to reaction with the probe in the absence of exogenously added Mg^{++}, a limited extent of labeling confined almost exclusively to the IIb polypeptide was observed (lane 1). Upon activation of the platelets with thrombin in the absence of added divalent cations, the extent of labeling was significantly increased relative to that observed with unactivated platelets. The labeling, however, was still confined almost exclusively to the IIb polypeptide of the complex (Fig. 4, lane 2). When unactivated platelets were subjected to labeling with the RGDS probe in the presence of 2 mM Mg^{++}, again only limited labeling of the IIb polypeptide was observed (Fig. 4, lane 3). The labeling of thrombin activated platelets in the presence of 2 mM Mg^{++} resulted in the greatest extent of labeling of the IIb-IIIa complex. It was only under this latter condition of thrombin activation in the presence of exogenously added divalent cations that substantial labeling of the IIIa polypeptide was observed (Fig. 4, lane 4).

It is interesting to note that Ginsberg et al (9) have recently presented immunochemical evidence for a divalent cation-dependent conformational change of the IIb-IIIa complex which occurs over a similar range of divalent cation concentrations.

The experiments described thus far were all conducted with either unactivated platelets or with platelets activated with thrombin. ADP is also capable of inducing adhesive protein binding sites on the platelet surface IIb-IIIa complex (10-12). A major difference appears to exist, however, in the binding sites induced by ADP and thrombin. For example, it now seems clear that both von Willebrand factor and fibronectin bind to the IIb-IIIa complex in an activation-dependent manner via RGDS sequence present in each of the adhesive proteins (3,4,13,14). However, whereas the binding of von Willebrand factor can be induced by either thrombin or ADP activation, the binding of fibronectin is induced by thrombin but not by ADP (12,15-17). This discordance prompted us to probe the topography of the RGDS binding site(s) on the IIb-IIIa complex induced by ADP or thrombin activation.

As shown in Fig. 5, platelet activation with thrombin (1 U/ml) but not activation with 20 μM ADP, markedly enhanced the labeling of the IIIa polypeptide relative to that of the IIb polypeptide by the RGDS photoaffinity probe. Although ADP activation approximately doubled the extent of labeling of the IIb-IIIa complex when compared to unactivated platelets, the labeling was essentially confined to the IIb component of the complex. Activation of

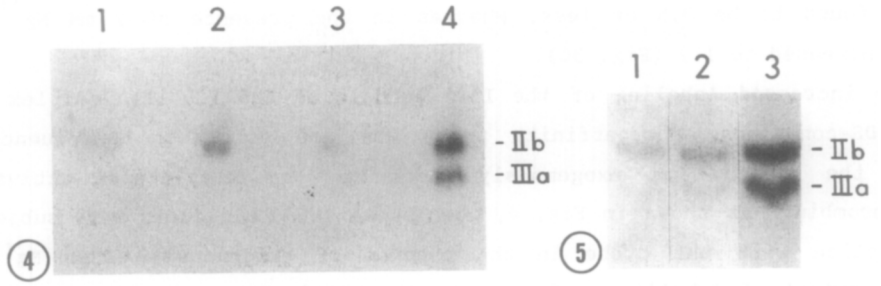

Figure 4: Modulation requires both thrombin activation and increased divalent cation concentrations. Photoaffinity labeling was carried out with the GRGDSC probe. Experiments were conducted in the absence of exogenously added divalent cations (lanes 1 and 2) and in the presence of 2 mM Mg^{++} (lanes 3 and 4). Thrombin was either omitted (lanes 1 and 3) or present at 1 U/ml (lanes 2 and 4).

Figure 5: Thrombin but not ADP produces a divalent cation-dependent modulation of the labeling pattern of the IIb-IIIa complex. Labeling was accomplished with the GRGDSC probe in the presence of 2 mM Mg^{++}. Platelets were unactivated (lane 1), activated with 20 μM ADP (lane 2), or with 1 U/ml of thrombin (lane 3).

the platelets by ADP was confirmed by microscopic examination which revealed the morphologic appearance characteristic of activated platelets. Examination of Coomassie blue stained gels of unactivated platelets, ADP-activated platelets, and thrombin activated platelets revealed no differences in regions corresponding to the IIb-IIIa complex which could have been due to proteolysis (data not shown).

D'Souza et al (18) have recently confirmed many of the findings described in our original report (6). They, however, did not observe a difference between thrombin and ADP-activated platelets such as we describe here in the patterns of labeling produced with RGDS probe. This was likely due to inherent differences in crosslinking chemistry employed. D'Souza et al (18) employed homobifunctional agents for crosslinking the probe to the receptor which required close proximity of amino groups on both the peptide probe and receptor for crosslinking to occur. The photoaffinity probes employed in this and our earlier study (6) impose no such structural constraints on the receptor site.

In summary, the data described in this report provide evidence for the existence of at least two distinct, active RGDS-binding conformations of the platelet membrane IIb-IIIa complex as reflected in the pattern of labeling produced with RGDS-derived photoaffinty probes. The conformational equilibrium appears to be controlled by the extracellular divalent cation concentration and by the agent giving rise to platelet activation.

Our observations suggest that whereas the RGDS sequence as displayed on von Willebrand factor is accessible to the binding site on IIb-IIIa induced by ADP or thrombin, the RGDS sequence as displayed on fibronectin is

accessible only to the conformation induced by thrombin. The nature of the conformations and their mechanism(s) of induction at this point remain unknown but are likely relevant to an understanding of the specificity of other RGD binding proteins.

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