Vol. 168, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 16, 1990 Pages 107-113

DIVALENT CATION-DEPENDENT STRUCTURE IN THE PLATELET MEMBRANE GLYCOPROTEIN Ia-IIa (VLA-2) COMPLEX

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Received February 21, 1990

SUMMARY: Recent studies have shown that the platelet membrane glycoprotein Ia-IIa (VLA-2) complex mediates the Mg^{++}-dependent adhesion of platelets to collagen and that this adhesion is inhibited by Ca^{++} in a simple, linear, noncompetitive manner. These findings suggested that separate binding sites for Mg^{++} and Ca^{++} stabilize different divalent cation-dependent structures within the receptor complex. To provide evidence for the existence of such structures purified platelet Ia-IIa complex was subjected to limited proteolytic digestion in the presence of Mg^{++}, Ca^{++}, Mg^{++} and Ca^{++}, or EDTA and the resulting peptides mapped by SDS-PAGE using both one- and two-dimensional techniques. Unique patterns of tryptic peptides were produced under each of the conditions. The results indicate that Mg^{++} and Ca^{++} stabilize different structures within the Ia-IIa (VLA-2) complex and that these structures influence both the collagen binding activity and proteolytic susceptibility of the complex.

Adhesion of platelets to collagen exposed by injury to the vascular endothelium is believed to be a major early step in formation of a primary hemostatic plug. We and others have recently identified a Mg^{++}-dependent mechanism of platelet adhesion to collagen (1,2) which is mediated by the platelet membrane glycoprotein Ia-IIa complex (3-6). This complex appears to be identical to the very late activation antigen-2 (VLA-2) complex present on fibroblasts, lymphocytes and other cells (3,7) and the extracellular matrix receptor II (ECMR II) collagen receptor on fibroblasts (4,8,9). The complex is a member of the integrin family of adhesive protein receptors which mediate the adhesive properties of many diverse cell types (10-13).

We have previously shown that Ca^{++} does not support the binding of the Ia-IIa complex to collagen and that the Mg^{++}-dependent adhesion to collagen of both intact platelets and liposomes containing the purified Ia-IIa complex is inhibited by Ca^{++} by a simple linear noncompetitive mechanism (1,5). The simplest interpretation of these data is that Mg^{++} and Ca^{++} bind to

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0006-291X/90 $1.50

different sites on the receptor complex and stabilize different divalent cation-dependent structures within the receptor. To provide direct evidence for the existence of such distinct divalent cation-dependent structures within the complex, we have digested purified platelet membrane Ia-IIa (VLA-2) complex with trypsin in the presence of Ca^{++} and Mg^{++} and mapped the resultant peptides by SDS-PAGE.

MATERIALS AND METHODS

Purification of the glycoprotein Ia-IIa complex. The glycoprotein Ia-IIa complex was purified from the particulate fraction of platelets produced by two cycles of freezing and thawing as recently described (3,5). Particulate material was collected by centrifugation and solubilized in extraction 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 at 20,000 x g for 45 min (4°C), the soluble supernatant was applied to a Concanavalin A-Sepharose column equilibrated in the above buffer in which the Lubrol concentration had been reduced to 0.1%. After extensive washing, glycoproteins bound to the column were eluted with column buffer containing 0.5 M α-methyl mannoside. 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 remove the sugar and the Ca^{++} and then subjected to affinity chromatography on a column of native triple helical collagen-Sepharose. This column was eluted by substitution of 2 mM EDTA for the $MgCl_2$ in the column buffer and the eluted fractions were collected into tubes containing sufficient 200 mM $MgCl_2$ to provide a final concentration of 5 mM free Mg^{++} in each fraction.

Radiolabeling. The purified Ia-IIa complex was radiolabeled with ^{125}I using 500 μCi of $Na^{125}I$ (Amersham, Arlington Heights, IL) and two Iodobeads (Pierce Chemical Co., Rockford, IL) for 15 min according to the manufacturer's instructions. The labeled protein was separated from free $Na^{125}I$ by gel filtration on a column of Sephadex G-10 equilibrated with the above column buffer. Radiolabeled Ia-IIa complex was then separated into material which retained collagen binding activity and material which lost collagen binding activity after iodination by a second cycle of affinity chromatography on collagen-Sepharose performed as described above.

Peptide mapping. Radiolabeled glycoprotein Ia-IIa complex was dissolved in 10 mM Tris·HCl (pH 7.4), 0.15 M NaCl, 0.1% Lubrol-PX and either 10 mM $MgCl_2$, 10 mM $CaCl_2$, 10 mM $MgCl_2$ and 10 mM $CaCl_2$ together, or 2 mM EDTA and subjected to digestion with 50 μg/ml of trypsin. Unless otherwise specified, digestion was for 60 min at 37°C.

Digests were then diluted with an equal volume of electrophoresis buffer containing 4% SDS and 2% 2-mercaptoethanol, boiled for 10 min, and subjected to one dimensional SDS-PAGE analysis performed according to Laemmli (14). For two dimensional SDS-PAGE mapping, digests were diluted with sample buffer from which the 2-mercaptoethanol was omitted. Samples were separated in the first dimension by SDS-PAGE performed under nonreducing conditions, equilibrated with 4% 2-mercaptoethanol for 1 hr and separated in the second dimension by SDS-PAGE under reducing conditions (15). For autoradiographic analysis, the gels were dried under vacuum and exposed to Kodak X-AR5 film at -70°C.

RESULTS AND DISCUSSION

The purified Ia-IIa complex was radiolabeled with ^{125}I and subjected to repurification by affinity chromatography on collagen-Sepharose after radiolabeling to insure that active material was studied. When

^{125}I-labeled glycoprotein Ia-IIa complex material was subjected to tryptic digestion and peptide mapping by SDS-PAGE, distinctly different patterns of peptides were produced depending upon whether the digestion was carried out in the presence of Mg^{++}, Ca^{++}, or EDTA (Figure 1). For example, major peptides of 146, 77, 73, 39, 35, and 29 kDa were produced in the presence of Mg^{++}. In the presence of Ca^{++}, however, the peptide doublet of 77 and 73 ka, which was present in the Mg^{++} digests, was not detected and the peptides at 39 and 35 kDa were replaced by a single peptide of 37 kDa. When the digestion took place in EDTA-containing buffer, a unique polypeptide of 50 kDa was produced, as was a relatively broad band of 37 kDa differing slightly in mobility from the somewhat similar polypeptides produced in the presence of Ca^{++} and Mg^{++}. A polypeptide of 30 kDa was present in both the Mg^{++} and EDTA tryptic digestions but not in the Ca^{++} digest.

One dimensional SDS-PAGE analysis of the peptides produced in the presence Mg^{++} and Ca^{++} together revealed a pattern of polypeptides not readily distinguishable from those produced in Ca^{++} alone. However, a two dimensional analysis (see below, Figure 3) revealed significant differences and the presence of polypeptides unique to the digestion containing both Mg^{++} and Ca^{++}. This suggests that the conformation and hence proteolytic susceptibility of the Ia-IIa complex in the presence of both Ca^{++} and Mg^{++} is different from that in the presence of either Mg^{++} or Ca^{++} alone. All of these observations are consistent with our recent conclusion that Ca^{++} and Mg^{++} bind to distinct sites on the Ia-IIa complex (5).

The experiments described above were carried out with material which retained collagen-binding activity following radiolabeling. We felt it might be instructive to perform similar experiments with material which although initially capable of binding collagen, no longer retained this activity following radiolabeling as judged by the ability to bind to collagen-Sepharose in a Mg^{++}-dependent manner. A comparison of the peptide maps of active and inactive Ia-IIa derived from tryptic digests performed in the presence of Mg^{++}, Ca^{++}, Ca^{++} and Mg^{++}, or EDTA is shown in Figure 2. The maps clearly indicate that different divalent cation-dependent structures are stabilized by Mg^{++} and Ca^{++} in both the active and inactive fractions of the complex based upon differences in the maps produced under the various conditions of tryptic digestion. Two major differences between the set of peptides derived from active material and those derived from inactive material are evident. A 50 kDa polypeptide (figure 2, upper arrow) was present in all of the various digests of Ia-IIa complex which had lost the ability to bind collagen but was only present in tryptic digests of active material carried out in EDTA. It seems likely that this polypeptide emanated from material in which the EDTA-induced, Mg^{++}-depleted conformation persisted even after readdition of Mg^{++} and that the persistence of this

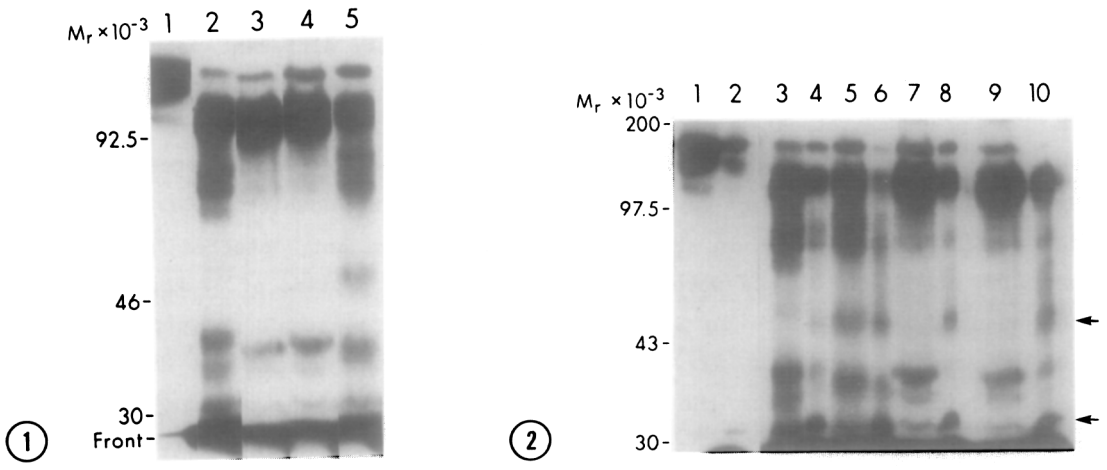

Figure 1. Peptides produced by tryptic digestion of platelet Ia-IIa complex in the presence of Mg^{++} and/or Ca^{++}. Purified ^{125}I-labeled Ia-IIa (lane 1) was digested for 60 min with 50 µg/ml trypsin in buffer containing either 10 mM $MgCl_2$ (lane 2), 10 mM $CaCl_2$ (lane 2), 10 mM each of $MgCl_2$ and $CaCl_2$ (lane 3), or 2 mM EDTA (lane 5). Aliquots were removed and examined by electrophoresis on 7.5% acrylamide gels.

Figure 2. Comparison of tryptic peptides derived from active and inactive platelet Ia-IIa complex. Purified platelet Ia-IIa was radiolabeled with ^{125}I. Samples of Ia-IIa which retained collagen binding activity (lane 1) and Ia-IIa which lost the activity during purification and iodination (lane 2) were digested with 50 µg/ml trypsin for 60 min. Both active (lanes 1,3,5,7,9) and inactive (lanes 2,4,6,8,10) Ia-IIa were digested in buffer containing either 10 mM $MgCl_2$ (lanes 3,4), 2 mM EDTA (lanes 5,6), 10 mM each of $MgCl_2$ and of $CaCl_2$ (lanes 7,8), or 10 mM $CaCl_2$ (lanes 9,10) and the samples were separated on a 7.5% acrylamide gel. Arrows indicate bands at 50 kDa and 35 kDa.

conformation was associated with the loss of collagen binding activity. A second 35 kDa polypeptide (Figure 2, lower arrow) was present in all digests derived from inactive material but in none of the digests derived from active material. This observation points to the existence of one or more iodination sensitive amino acid residues in the Ia-IIa complex, the iodination of which results in loss of Mg^{++}-dependent collagen binding activity. These two findings indicate that a significant amount of collagen binding activity may be lost during purification and subsequent iodination of the Ia-IIa complex. Furthermore, the differences observed between the affinity of Mg^{++} for the Ia-IIa complex of intact platelets and that for purified Ia-IIa inserted into liposomes (1,5) may also be due, at least in part, to these isolation and radiolabeling-induced changes.

The differences in peptides observed by one-dimensional SDS-PAGE mapping were examined in greater detail and confirmed by two-dimensional SDS-PAGE (nonreducing x reducing) mapping. An example of such an analysis is shown in Figure 3. Photographs of the autoradiograms are shown in panel A and schematic representations are shown in panel B. Major peptides which were uniquely present in digests performed in the presence of Mg^{++}, Ca^{++}, or

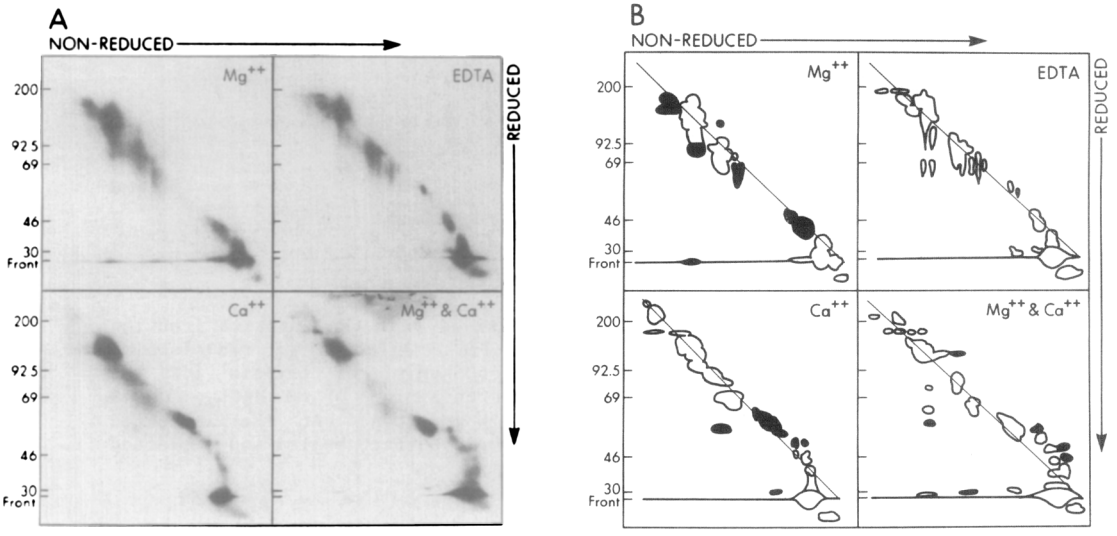

Figure 3. Two-dimensional electrophoretic analysis of peptides produced by tryptic digestion of platelet Ia-IIa complex. Purified ^{125}I-labeled Ia-IIa was digested with 50 μg/ml trypsin for 60 min in buffer containing either 10 mM $MgCl_2$, 10 mM $CaCl_2$, 10 mM each $MgCl_2$ and $CaCl_2$, or 2 mM EDTA. Digests were separated by SDS-PAGE under nonreducing conditions (first dimension) and then under reducing conditions (second dimension). A) Photographs of original autoradiograms; B) tracings of spots from the same autoradiograms. Peptides containing intrachain disulfide bonds appear above the diagonal while peptides containing interchain disulfide bonds appear below it and peptides containing no disulfide bonds are clustered along the diagonal. In panel B) major peptides produced by digestion in the presence of Mg^{++} but not in Ca^{++}, peptides produced by digestion in the presence of Ca^{++} but not in Mg^{++}, and peptides unique to the combination of Mg^{++} and Ca^{++} are indicated by shading.

Mg^{++} and Ca^{++} are indicated in the schematic diagram. It is evident from these figures that peptides uniquely produced in the presence of Ca^{++} and Mg^{++} together are apparently derived from disulfide bonded regions of the Ia-IIa complex, while those produced in the presence of Mg^{++} or Ca^{++} separately are derived from both disulfide-crosslinked and from non-crosslinked portions of the complex.

It was possible that the observed differences in peptide maps produced under the various conditions were due to differences in the rates of digestion rather than to the production of distinctly different sets of peptides. However, a detailed analysis of the time course of digestion over the period of 0 - 16 hr (Figure 4) clearly shows that the differences observed at 1 hr (Figures 1,2, and 3) are due to the presence of distinct sets of metastable fragments produced under the different conditions of digestion. These differences became apparent as early as 10 min and were maintained for up to 4-8 hrs of digestion.

Unique patterns of peptides were also produced when the Ia-IIa complex was digested for 60 min with chymotrypsin in the presence of either Mg^{++} or

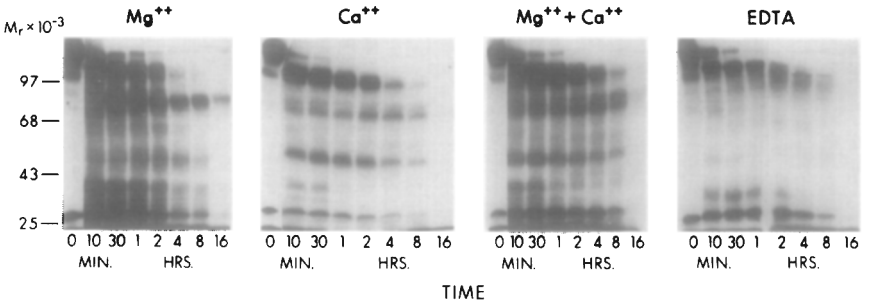

Figure 4. Comparison of tryptic peptides derived from the inactive fraction of ^{125}I-labeled Ia-IIa as a function of time. Aliquots of radiolabeled Ia-IIa were subjected to digestion with 50 μg/ml of trypsin for the indicated times in the presence of buffer containing 10 mM $MgCl_2$, 10 mM $CaCl_2$, 10 mM each $CaCl_2$ and $MgCl_2$, or 2 mM EDTA. At the indicated times, aliquots were diluted with SDS-PAGE sample buffer, boiled and subjected to analysis by SDS-PAGE on a 7.5% acrylamide gel.

Ca^{++} alone or Mg^{++} and Ca^{++} together (data not shown). Although the peptides differed somewhat in molecular weight from those produced with trypsin, the distinct cleavage patterns confirmed the overall conclusion from the tryptic digestion experiments that Mg^{++} and Ca^{++} stabilize distinct structures within the Ia-IIa complex. The results of the chymotryptic digestion also excluded the remote possibility that the production of the different peptides by trypsin were due to ion-specific effects on the protease.

Our results indicate divalent cation-dependent structures can form within the Ia-IIa (VLA-2) complex and that these structures alter the susceptibility of the complex to proteolysis. The simplest interpretation of these data is that these different structures reflect different, divalent cation-dependent conformations of the two polypeptides which constitute the Ia-IIa complex. Our previous studies have indicated that Ca^{++} and Mg^{++} bind to separate sites on the Ia-IIa complex and suggested that distinct Mg^{++}- and Ca^{++}-stabilized conformations of the complex exist. The present study has provided direct evidence for the existence of these structures which could only be inferred from our earlier adhesion studies. As the alpha subunit of the Ia-IIa complex is significantly more heavily labeled than the beta subunit, it is likely that the changes we have observed are primarily due to alterations occurring within the alpha subunit.

It seems likely that one or more of the divalent cation binding sites within VLA-2 has evolved to accommodate Mg^{++}, Mn^{++}, or other divalent cations which support activity and that these binding sites exclude Ca^{++}. When these sites are occupied by divalent cations which support activity, the complex assumes a conformation which facilitates binding to collagen. A distinct site on VLA-2 appears to be of relatively lower affinity and to be specific for Ca^{++}. Occupation of this latter site by Ca^{++} results in a

conformation of the complex which is unable to bind collagen (5). The results of the present study provide direct evidence for the existence of these different divalent cation-dependent conformations in the Ia-IIa (VLA-2) complex.

ACKNOWLEDGMENTS

This investigation was supported by grants from the National Institutes of Health (HL 40506) and the American Heart Association with funds contributed in part by the Missouri Affiliate. SAS is an Established Investigator of the American Heart Association.

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