KRDS—A Tetrapeptide Derived From Lactotransferrin—Inhibits Binding of Monoclonal Antibody Against Glycoprotein IIb-IIIa on ADP-Stimulated Platelets and Megakaryocytes

By Sanghamitra Raha, Christine Dosquet, Jean-François Abgrall, Pierre Jolles, Anne-Marie Fiat, and Jacques P. Caen

Short peptides isolated from fibrinogen and K-casein have been shown to inhibit platelet aggregation and fibrinogen binding to stimulated platelets. We studied the effects of synthetic peptides occurring in milk proteins (bovine K-casein, KNQDK, and human lactotransferrin, KRDS) and in fibrinogen (RGDS and L10) on subsequent binding of monoclonal antibodies (MoAb) against the glycoprotein (GP) IIb-IIIa complex (AP2 and P2) on adenosine diphosphate (ADP)-stimulated and unstimulated human platelets and megakaryocytes (MKs) by using an immunoperoxidase method to visualize antibody binding. Only KRDS (900 μmol/L) inhibited the binding of AP2 and P2 on ADP (5 μmol/L)-stimulated platelets, but not on unstimulated platelets. However, the binding of P2 was considerably more inhibited than that of AP2 as judged by immunoperoxidase intensity. Radiolabeled AP2 binding was inhibited by 30% with KRDS on ADP-stimulated platelets as compared with platelets incubated in the absence of ADP. KRDS did not inhibit the binding of MoAbs against GP IIa (SZ 21), GP IIb (SZ 22), and GP Ib (SZ 2) on ADP-stimulated human platelets. Inhibition of P2 binding by KRDS was also observed in a section of MKs isolated from human bone marrow and stimulated by 15 or 20 μM ADP. A lower concentration of ADP (5 or 10 μmol/L) failed to produce any inhibition of binding. This indicates that MKs may not be equally responsive to agonists as platelets. Moreover, P2 binding inhibition was observed in a larger (P < .001) percentage of mature MKs (29%) as compared with younger, maturing MKs (11%). The observations suggested that a functional ability possessed by platelets, namely, agonist-induced exposure of the site of interaction of KRDS, may occur at a late stage of MK development.

EVIDENCE accumulated in recent years indicates that the fibrinogen receptor is situated on a Ca2+-dependent, heterodimeric complex of two platelet glycoproteins (GP) on the platelet plasma membrane.1,2 Fibrinogen binding to the platelet receptor requires activation of platelets, presumably resulting in the exposure of fibrinogen binding sites.3,4 Peptide sequences present in fibrinogen and some other adhesive proteins are known to inhibit platelet aggregation and binding of fibrinogen to stimulated platelets.5,6 Many monoclonal antibodies (MoAbs) directed against the platelet–GP IIb-IIIa complex also inhibit fibrinogen binding to stimulated platelets.7,8 Homologies between the sequences of cow K-casein and human fibrinogen γ chain have been evidenced by Jolles and Henschen.9,10 It was shown that an undecapeptide isolated from cow K-casein inhibited platelet aggregation and fibrinogen binding; the behavior of this peptide was very similar to that of the C-terminal dodecapeptide of human fibrinogen γ-chain.11

Bone marrow megakaryocytes (MKs) are the precursor cells of blood platelets and possess many of the granule constituents initially observed in platelets.12,13 MoAbs against platelet membrane glycoproteins bind to a majority of MKs including the small lymphoid-like mononuclear cells.14,15 Surface properties and some functional abilities are also shared by platelets and some MKs. These common properties include lectin binding, agonist-induced aggregation and spreading; adenosine diphosphate (ADP), adenosine triphosphate (ATP), and serotonin release; and attachment to subendothelial and extracellular matrix.16-23 However, binding of fibrinogen or fibrinogen receptor expression by agonists has not yet been demonstrated in MKs.

It is apparent that for the proper evaluation and application of platelet fibrinogen binding inhibitors as antithrombotic agents a better knowledge of specific MK responses such as agonist-induced expression of surface receptors is necessary.

Our aim was to study, on human platelets and MKs, the interaction between peptide sequences that inhibit fibrinogen binding and MoAbs directed against GP IIb-IIIa and characterize MK responses to agonists.

MATERIALS AND METHODS

The synthetic peptides RGDS (residues 572 to 575 of human fibrinogen α chain) and L10 (LGGAKQAGDV residues 402 to 411 of human fibrinogen γ chain) were gifts from Dr. G. Marguerie (Grenoble, France). The synthetic peptides KRDS (residues 39 to 42 of human lactotransferrin) and KNQDK (residues 112 to 116 of cow K-casein) were prepared with an Applied Biosystems (Foster City, CA) 430-A peptide synthesizer. Preparation of washed platelets. Blood (5.5 vol) was drawn from healthy human donors into a solution of 318 mmol/L citric acid, 62 mmol/L trisodium citrate, and 133 mmol/L glucose (1 vol) and centrifuged (100 g, ten minutes, 20°C) to obtain platelet-rich plasma. The platelets were washed according to the method of Lee et al.3 The wash buffer contained 36 mmol/L citrate, 20 mmol/L prostaglandin E1 (PGE1), 25 μg/mL apyrase, and 3.5 mg/mL bovine serum albumin (BSA). The platelets were finally suspended in modified Tyrode's buffer that consisted of 137 mmol/L NaCl, 3 mmol/L KCl, 12 mmol/L NaHCO3, 0.3 mmol/L Na2HPO4, 2H2O,
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2 mmol/L CaCl₂, 1 mmol/L MgCl₂, 5.5 mmol/L glucose (pH 7.4), and 0.35% BSA. To incubate the platelets with peptides, different peptides (at a concentration range between 450 and 900 μmol/L) were added to the platelet suspension containing 5 × 10⁶ platelets/mL and subsequently stimulated by the addition of 5 μmol/L ADP. The control incubations received either 5 μmol/L ADP or just the peptide alone. After the additions, the platelet suspensions were mixed by gentle pipetting and left at room temperature for 20 minutes. After the incubation, platelet suspensions (1 vol) were fixed by the addition of 90% methanol (20 vol) and immediately centrifuged.

The platelet button was suspended and washed once with modified Hanks' basal salt solution Ca²⁺ and Mg²⁺ free (calcium magnesium free Hanks' buffer [CMFH]: 135 mmol/L NaCl, 5 mmol/L KCl, 3 mmol/L Na₂HPO₄, 4 mmol/L KH₂PO₄, 4 mmol/L NaHCO₃, 10 mmol/L HEPES, 12.5 mmol/L Na₃ citrate, and 5 mmol/L glucose, pH 7.0). Cytocentrifuge (Shandon Southern Products, Ltd, Cheshire, England) preparations of the platelet suspensions were made on glass slides and kept frozen for immunoperoxidase staining.

Megakaryocyte isolation. Bone marrow was obtained from patients without any hematologic abnormality who were undergoing hip replacement surgery. Bone marrow was suspended in CMFH containing 1 mmol/L adenosine, 2 mmol/L theophylline, and 3.5 mg/mL BSA and isolated on discontinuous Percoll gradients by modification of the method of Raha et al.²⁵ Isolated MK-containing cell fractions (5% MKs) were washed twice with wash buffer (2 mmol/L CaCl₂, 2 mmol/L MgCl₂, 20 mmol/L PGE₁, 25 μg/mL apyrase, 3.5 mg/mL BSA) and suspended in modified Tyrode's buffer, pH 7.4. A quantity of 2 × 10⁶ cells/mL were incubated and subsequently treated in a manner similar to that for platelets.

Immunoperoxidase staining of MKs and platelets. Immunoperoxidase technique was performed on cytocentrifuge preparations of washed human platelets treated as described in the text. Abbreviation: ND, not determined.

### RESULTS

Effects of peptides from fibrinogen and milk proteins (K-casein and lactotransferrin) on binding of MoAbs against platelet membrane glycoproteins on ADP-stimulated and unstimulated platelets. In brief, Table 1 depicts the effect of preincubation with KRDS on subsequent binding of MoAbs to the GP IIb-IIIa complex, GP IIb, GP IIIa, and GP Ib on both ADP-stimulated and unstimulated platelets as evaluated qualitatively by immunoperoxidase. Among the four peptides (KRDS, KNQDK, RODS, and L 10) studied, only KRDS (900 μmol/L) inhibited the binding of MoAbs against the GP IIb-IIIa complex (AP₂ and P₂), but this property of KRDS was only in evidence on the ADP-stimulated platelet and not on the unstimulated platelet.

Binding of the two MoAbs (AP₂ and P₂) was slightly enhanced by preincubation with 900 μmol/L RDS or 900 μmol/L KNQDK (results not shown). Although the binding of both MoAbs was inhibited by KRDS, P₂ binding is affected considerably more than is AP₂, as judged by the intensity of the immunoperoxidase staining. The binding of S22 (GP Ib), S22 (GP IIIa), and S22 (GP IIb) was not inhibited by KRDS.

Figure 1 illustrates the difference in P₂ binding between platelets incubated in the presence of KRDS alone (Fig 1A) and platelets stimulated by 5 μmol/L ADP in the presence of KRDS (Fig 1B). The peroxidase reaction (brown) was enhanced by further incubation for five minutes with 0.5% CuSO₄. After counterstaining for 1.5 minutes in Gill's hematoxylin 2, the slides were dehydrated and mounted.

Classification of MKs in cytocentrifuge preparations. About 100 MKs were observed from each slide and classified by using the following criteria (a) cell size; (b) shape, size, and location of the nucleus; and (c) nucleus:cytoplasm ratio.²⁹ In each class of MKs, immunoperoxidase positive-MKS and MKs showing varying degrees of negative reaction were counted and expressed as a percentage of MKs in the respective classes.

Binding of ¹²⁵I-AP₂ to platelets. Washed platelets were incubated with KRDS (900 μmol/L) with or without 5 μmol/L ADP as described earlier, and followed by fixation in 90% methanol. Fixed platelets were resuspended in PBS, pH 7.4, containing 2% horse serum (10³ platelets/mL). A quantity of 7.5 μg/mL of ¹²⁵I-AP₂ was added to the platelet suspension and incubated for 30 minutes. One hundred–microliter aliquots were taken after incubation and layered over 1 mL of 20% sucrose solution and centrifuged at 12,000 g for three minutes. Supernatants were carefully withdrawn, and the radioactivity of the pellets was counted in a Beckman 7000 gamma counter (Beckman Instruments, Gagny, France).

### Table 1. Effect of Preincubation With KRDS on Subsequent Binding of MoAbs to Platelet Glycoproteins to Unstimulated and ADP-Stimulated Platelets

<table>
<thead>
<tr>
<th>ADP (μmol/L)</th>
<th>Without KRDS</th>
<th>With KRDS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AP₂</td>
<td>P₂</td>
</tr>
<tr>
<td>0</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>5</td>
<td>++</td>
<td>++</td>
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Immunoperoxidase staining intensity is graded from 0 to ++ + + in cytocentrifuge preparations of washed human platelets treated as described in the text.
intense following incubation with KRDS alone where only faint staining (light yellow-gray) was observed when the preincubation mixture also included 5 μmol/L ADP. In some cases the peroxidase reaction is completely abolished (not shown).

Platelets incubated in the presence of ADP alone or in the absence of both KRDS and ADP also showed intense immunoperoxidase reaction with both AP2 and P2.

Binding of radioiodinated AP2 on ADP-stimulated platelets was reduced by 30% with pretreatment of KRDS compared with platelets that were incubated with KRDS in the absence of ADP stimulation (Table 2).

Role of KRDS on binding of MoAb against GP IIb-IIIa (P2) on human MKs. Table 3 demonstrates the effect of preincubation with KRDS on the binding of P2 on isolated human MKs. A positive reaction was demonstrated by a dark yellow-brown reaction product, and a negative reaction was seen as blue–light purple. P2 binding was observed all over the cytoplasm and on the plasma membrane in 98% of the recognizable MKs from MK-enriched samples incubated in the absence of both KRDS and ADP. Strong positivity was

Table 2. Effect of KRDS on Binding of 125I-AP2 to ADP-Stimulated Platelets

<table>
<thead>
<tr>
<th>ADP (μmol/L)</th>
<th>KRDS (900 μmol/L)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>14,385.00 ± 426.81</td>
</tr>
<tr>
<td>5</td>
<td>10,176.32 ± 2,194.55 (30)%</td>
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Values are expressed as molecules IγG per platelet and as means ± SD of three experiments. The figure in parentheses denotes percentage reduction in binding. *P < .05.

Table 3. Effect of Preincubation With KRDS and RGDS on Subsequent Binding of P2 on Isolated Human MKs

<table>
<thead>
<tr>
<th>ADP (μmol/L)</th>
<th>Without Peptides</th>
<th>With Peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AP2</td>
<td>P2</td>
</tr>
<tr>
<td>0</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>5</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>10</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>15</td>
<td>ND</td>
<td>+++</td>
</tr>
<tr>
<td>20</td>
<td>ND</td>
<td>+++</td>
</tr>
</tbody>
</table>

Immunoperoxidase staining is graded 0 to +++ by the expanse of the positive reaction. The value +++ is given to MKs demonstrating fully positive and 0 to MKs showing a very small area of positive reaction. An MK is designated ± when it is partially positive and shows both positive and negative reactions.

*In one experiment, AP2 binding on MKs was considerably less inhibited by KRDS than was P2 binding.

†Variations observed in P2 binding within the same MK-enriched sample. For further details, please see the text.
also noted in small immature MKs. Incubation of the MK-enriched cell suspension in the presence of ADP alone (5, 10, 15, or 20 μmol/L), KRDS (900 μmol/L) without ADP, or KRDS with 5 or 10 μmol/L ADP showed similar binding of P2 in MKs. KRDS inhibited the binding of P2 to a section of MKs after ADP (15 or 20 μmol/L) stimulation. Inhibition of P2 binding by KRDS in MKs required a higher concentration of ADP (15 or 20 μmol/L) than that required in platelets (5 μmol/L). Within the same MK-enriched suspension, completely positive MKs and also MKs with varying areas of negatively were observed. Only a positive reaction was seen in MKs incubated with 900 μmol/L RGDs and 15 or 20 μmol/L ADP.

Table 4 shows the effect of KRDS on the different morphological stages of human MKs. Only a small percentage of the total MK population (21.45% ± 2.76%) demonstrated a difference in P2 binding. The percentage of MKs showing change was also significantly higher among mature MKs (29%) as compared with maturing MKs (10.9%).

Figure 2 illustrates the various localization patterns of P2 binding in MKs treated with KRDS and 15 or 20 μmol/L ADP. The expanse of the negative reaction area (inhibition of P2 binding) varied widely between MKs. Negativity located only on plasma membrane was very rarely observed. A negative reaction on the plasma membrane was almost always accompanied by negativity on the adjoining cytoplasm. A completely negative MK was never observed. Maximum inhibition was observed as a slight positive reaction at one pole of the cell where the nucleus was located (Fig 2A).

This type of reaction was also not seen frequently. Figure 2B shows a mature MK with intense staining all over the cytoplasm and a much smaller MK, also intensely positive, from the same sample as Fig 2A. The most commonly observed pattern of inhibition was that clearly dividing the cells into positive and negative areas (Fig 2C, 2D, 2E). The perinuclear regions of MKs were most often found to be intensely positive even though the surrounding cytoplasm was negative (Fig 2F).

**DISCUSSION**

RGDS and the C-terminal decapetide of fibrinogen γ chain (residues 402 to 411) are potent inhibitors of fibrinogen binding to stimulated platelets.**4** GP IIb-IIIa has been shown to be a component of the RGDS binding site on platelets that serves as a common receptor for fibrinogen, fibronectin, and von Willebrand factor (vWF). Furthermore, the two peptide sets (RGDS and γ chain sequences) interact with the same receptor that contains GP IIb-IIIa.**40**

Compared with the RGDS peptides and the fibrinogen γ chain sequences, less information is presently available on platelet effects of milk protein sequences. Jollès et al**13** have reported that natural and synthetic undecapeptides from cow K-casein (residues 106 to 116) are good inhibitors of ADP-induced fibrinogen binding and aggregation to human platelets and are comparable in this respect to fibrinogen γ chain peptides. MoAbs to platelet GP IIb-IIIa (AP2 and P2) also inhibit platelet aggregation and thrombin and ADP-stimulated fibrinogen binding to platelets.**7,10** To the best of our knowledge, little information is presently available on the interaction between antibodies against GP IIb-IIIa and the platelet-inhibitory peptides.**31**

In this study, one peptide sequence from human lacto-transferrin (KRDS) and one from cow K-casein (KNQDK) were studied together with the better-known peptides RGDs and L10. Among the peptides studied, only KRDS affected the binding of antibodies to the GP IIb-IIIa complex, but not antibodies to GP Ib, IIb, or IIIa. Moreover, this inhibition was only observed in ADP-stimulated platelets and not on unstimulated platelets. KRDS has also been observed to inhibit fibrinogen binding and platelet aggregation (L. Her- mant, S. Levy-Toledano, P. Jollès, and A.M. Fiat, personal communication). It is likely that KRDS can only bind on stimulated platelets. Alternately, it cannot be excluded that possibly KRDS can interact with unstimulated platelets and that the extent of interaction may increase greatly by platelet stimulation. Recently, D'Souza et al**12** have presented evidence that RGD-containing peptides could be cross-linked to both subunits of GP IIb-IIIa on unstimulated platelets. Upon stimulation, there is a dramatic increase in cross-linking, but only to one of the subunits of GP IIb-IIIa. Parise et al**13** have also demonstrated a conformational change in purified GP IIb-IIIa when bound to peptides from fibrinogen and fibronectin. The site of interaction of KRDS on the stimulated platelet, therefore, is perhaps in close proximity to the determinant recognized by P2 on GP IIb-IIIa, and occupation of this site by KRDS may lead to changes detrimental to P2 binding.

Bone marrow MKs have many structural and functional similarities to their progeny, the blood platelets. Not only do MKs possess many platelet constituents such as fibrinogen, platelet factor V, platelet factor VIII–vWF, and platelet

<table>
<thead>
<tr>
<th>Table 4. Inhibition of P2 Binding on MKs by KRDS</th>
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<tr>
<td>MKs</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Immature</td>
</tr>
<tr>
<td>Maturing</td>
</tr>
<tr>
<td>Mature</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD of two to three experiments and as the percentage of MKs belonging to each morphological class. The numbers in parentheses denote means ± SD of the percentage of MKs in each class showing changes in P2 binding. Control samples are either with ADP stimulation in the absence of KRDS or without ADP stimulation in the presence of KRDS.

*Percentage of MKs showing changes in P2 binding that is significantly higher than the corresponding value in control maturing MKs. P < .001.
†Percentage of MKs showing changes in P2 that is binding significantly higher than the corresponding value in control mature MKs. P < .001.
‡Significantly higher than the corresponding value in maturing MKs treated with KRDS and 15 to 20 μmol/L ADP.
demonstrated that MKs can attach themselves to extracellular matrix, thus indicating possession of adhesive properties similar to platelets. ADP-induced spreading has also been documented in cultured guinea pig MKs. Moreover, only half of the MKs are observed to undergo spreading, which indicates that this ability may be restricted to a section of MKs. Our results established that KRDS, when added in the presence of 15 to 20 μmol/L ADP, could inhibit binding of P₃ on the plasma membrane and intracellular sites of some (20%) isolated human MKs; inhibition of P₃ binding is

Fig 2. Immunoperoxidase reaction using P₃ in isolated human MKs after incubation in the presence of 900 μmol/L KRDS and 15 or 20 μmol/L ADP and subsequent methanol fixation. (A) Mature MK showing positive peroxidase reaction at only one pole (arrow), with the rest of the cytoplasm being negative (original magnification ×400). (B) Mature MK showing intense positive reaction. One small MK is also strongly positive (arrow), original magnification ×400 (C, D, and E). Mature MK showing variations in the positive reaction area, original magnification ×1,000 (C and D) and ×400 (E); (D) an immature MK showing intense positivity is attached to the mature MK. (F) Intense positive reaction localized around the nucleus in a mature MK (original magnification ×400).
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very prominent part of the cytoplasm in mature MKs, its calcium on the stability of the platelet membrane glycoprotein studies, have shown that only mature MKs in which the DMS has reached continuity with the plasma membrane and on the demarcation membrane system (DMS) in the MK cytoplasm. While DMS forms a very prominent part of the cytoplasm in mature MKs, its presence has also been observed in younger members of the MK series. Although DMS is known to be continuous with the extracellular space, in electron microscpic studies, have shown that only mature MKs in which the DMS has reached continuity with the plasma membrane may be able to expose intracellular antigens to the extracelluar space. These MKs accounted for 50% of all fresh and cultured MKs from normal mouse bone marrow and 50% to 75% of all MKs in bone marrow of immune thrombocytepnia patients. We, however, noted that only a much smaller percentage (20%) of the total isolated MK population and only 29% of the mature MKs showed inhibition of P2 binding. It seems more likely that only a section of mature MKs with surface-connecting DMS can expose sites of interaction to KRDS on ADP stimulation. In accordance with this concept, the inhibition of P2 binding on the MK plasma membrane rarely occurred without simultaneous inhibition on intracytoplasmic sites. This underscores the acquisition of an important function toward the end stage of differentiation. Moreover, MKs can respond to ADP stimulation and expose sites of interaction to KRDS only when a larger concentration of ADP is present than that required for platelets. Thus, responsiveness to agonists, acquired earlier, is not fully developed until the terminal stage of the MK-platelet maturation sequence. Fedorko has also demonstrated that guinea pig MKs required a higher concentration of ADP for serotonin release than platelets.

In our study, P2 binding was always observed to be present in the perinuclear region of MKs. Beckstead et al have indicated the presence of GP IIb-IIIa on perinuclear cisterne (PC) of MKs. Since PC is not connected with the DMS, GP IIb-IIIa located here may not be accessible to externally applied KRDS and ADP in contrast to the GP IIb-IIIa located on the DMS. Our observation of an intense reaction around the nucleus of MKs even when the peripheral cytoplasm was negative seems to be compatible with this concept.

The data presented here demonstrated that MK-like platelets could be stimulated by ADP to expose sites of interaction for a peptide that inhibits fibrinogen binding; this ability is limited to a section of MKs that are predominantly mature as judged by morphological criteria.

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KRDS--a tetrapeptide derived from lactotransferrin--inhibits binding of monoclonal antibody against glycoprotein IIb-IIIa on ADP-stimulated platelets and megakaryocytes

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