Red Cell ICAM-4 Is a Novel Ligand for Platelet-activated αIIbβ3 Integrin*

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ICAM-4 (LW blood group glycoprotein) is an erythroid-specific membrane component that belongs to the family of intercellular adhesion molecules and interacts in vitro with different members of the integrin family, suggesting a potential role in adhesion or cell interaction events, including hemostasis and thrombosis. To evaluate the capacity of ICAM-4 to interact with platelets, we have immobilized red blood cells (RBCs), platelets, and ICAM-Fc fusion proteins to a plastic surface and analyzed their interaction in cell adhesion assays with platelets and platelets from normal individuals and patients, as well as with cell transfectants expressing the αIIbβ3 integrin. The platelet fibrinogen receptor αIIbβ3 (platelet GPIIb-IIIa) in a high affinity state following GRGDS peptide activation was identified for the first time as the receptor for RBC ICAM-4. The specificity of the interaction was demonstrated by showing that: (i) activated platelets adhered less efficiently to immobilized ICAM-4-negative than to ICAM-4-positive RBCs, (ii) monoclonal antibodies specific for the β3-chain alone and for a complex-specific epitope of the αIIbβ3 integrin, and specific for ICAM-4 to a lesser extent, inhibited platelet adhesion, whereas monoclonal antibodies to GPIb, CD36, and CD47 did not, (iii) activated platelets from two unrelated type-I glanzmann’s thrombasthenia patients did not bind to coated ICAM-4. Further support to RBC-platelet interaction was provided by showing that dithiothreitol-activated αIIbβ3-Chinese hamster ovary transfectants strongly adhere to coated ICAM-4-Fc protein but not to ICAM-1-Fc and was inhibitable by specific antibodies. Deletion of individual Ig domains of ICAM-4 and inhibition by synthetic peptides showed that the αIIbβ3 integrin binding site encompassed the first and second Ig domains and that the G65-V74 sequence of domain D1 might play a role in this interaction. Although normal RBCs are considered passively entrapped in fibrin polymers during thrombus, these studies identify ICAM-4 as the first RBC protein ligand of platelets that may have relevant physiological significance.

The main physiological function of red blood cells (RBCs), which encapsulate hemoglobin, is to ensure the respiratory gases transport throughout the human body. However, the recent demonstration that mature RBCs express a growing number of adhesion molecules, many of which exhibit blood group specificities (1–3), reinforces the necessity to revisit the functional interaction of RBCs with leukocytes, platelets, and vascular endothelium under normal and pathological conditions.

It is interesting that many RBC adhesion molecules contain protein domains characteristic of the immunoglobulin superfamily, suggesting some recognition function. These molecules might participate in the normal RBC physiology by playing a role during erythropoiesis (differentiation, maturation, enucleation, release), self-recognition mechanisms, red cell turnover, and cell aging through cellular interactions with counter receptors present on macrophages from bone marrow or reticuloendothelial system in spleen and liver (1, 4–9). Along this process, some adhesion molecules are rapidly down-regulated and others are expressed at different stages and remain on RBCs (Refs. 10 and 11, and references therein). Finally, mature RBCs still express adhesion molecules which are usually associated with leukocytes (CD44, CD47, CD58) and others that have potential adhesion properties such as LW/ICAM-4 (CD242), Lu (CD239), Okα (CD147), CD99/Xg, JMH (CD108), and DO (1–3). Nevertheless, normal RBCs do not adhere to circulating cells and vessel walls under normal circumstances, suggesting that the RBC adhesion molecules are inaccessible to their ligands. In contrast, the conversion of non-adherent RBCs to adherent state arises in several diseases. In such circumstances, adhesion molecules might be involved in the pathophysiology of malaria (12, 13), sickle cell disease (14–17), and diabetes (18, 19), mainly through an abnormal adhesion to the vascular endothelium (1, 20). Additionally, both phosphatidylserine exposure at the RBC surface and adhesion molecules on these cells might also play a role in hemostasis and thrombosis, for instance through interaction with cells expressing integrins, like activated leukocytes, monocytes, platelets, and endothelial cells (21, 22). Interestingly also, RBCs have the necessary signal transduction pathways to mediate these functions (23).

Among RBC adhesion molecules, ICAM-4 (LW blood group glycoprotein, CD242) emerges from the others by its structural similarities to the ICAM family and its interaction characterized in vitro with different members of the β integrin subfamilies (α4β1 (LFA-1), α5β1 (Mac-1) (24–26), α4β1 (VLA-4), α4 integrins (α4β1 and α4β2); Ref. 27). These two families of proteins are well known to play crucial role in cell-cell interactions binding inhibitor; TSP, thrombospondin; VWF, von Willebrand Factor; mAb, monoclonal antibody; PGE1, prostaglandin-E1; TNF-α, tumor necrosis factor-α; HUVEC, human umbilical vein endothelial cells; DTT, dithiothreitol; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline.

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§ The abbreviations used are: RBCs, red blood cells; FBI, fibrinogen.
and to be involved in a large range of biological functions (28–31). For instance, ICAM-4/integrin interaction might play a role during erythroid maturation in bone-marrow or in the red cell turn-over by spleen macrophages that express the α2β1 integrin (25, 27, 32). Additionally, ICAM-4 as well as the Lu blood group protein might be involved in adhesion of sickle RBCs to TNF-α-activated endothelial cells (HUVEC) (7) and to laminin (33, 34), respectively. It is suspected that abnormal adhesion of sickle RBCs to endothelial cells and extracellular matrix proteins might be responsible for the painful crisis of the disease that result from vaso-occlusive episodes (35).

The purpose of this report was to examine the potential role of ICAM-4 in RBC-platelet interaction and to demonstrate that this protein interacts in vitro with the high affinity state of activated platelet αIIbβ3 integrin.

MATERIALS AND METHODS

Blood Samples, Reagents, and Antibodies—RBC from donors with common and rare phenotypes (Doantil, Luantil, of the Lu(a–b–) type, LWantil, JMHantil) came from the frozen RBC collection of the Centre National de Reference pour les Groupes Sanguins (Paris, France). Fresh blood samples from two unrelated type-I glanzmann thrombasthenia patients were obtained after informed consent. Apyrase, prostaglandin-E1 (PGE1), thrombin from human origin and anti-glycophorin-A mAb (clone E4), and the peptide Arg-Gly-Glu (RGE) were purchased from Sigma. Peptides Gly-Ary-Gly-Asp-Ser-Pro (GRGDSP), Arg-Gly-Asp (RGD) and the fibrinogen binding inhibitor (FBI) peptide (residues 401–411 of the fibrinogen γ-chain, Fγ) were from Bachem (Budendorf, Switzerland). Other peptides Gly-Trp-Val-Ser-Tyr-Gln-Leu-Asp-Val (Gly–Val, residues 65–74 of ICAM-4), Cys-His-Ala-Arg-Leu-Asn-Leu-Asp-Gly-Leu-Val-Val (C–R, residues 180–192 of ICAM-4) and corresponding random (rd) peptides used were synthesized and purified by Neosystem (Strasbourg, France). Specific mAbs used in this study include clones P2 and SZ22 recognizing the αmβ1 (CD41) in the presence and the absence of the β3 (CD61, CD41a), respectively, clone SZ2 and SZ22 specific for the β3 (CD61 and GP IIb/IIIa) protein (CD42b), respectively, clone FA6.152 specific for CD56, and clone AICD58 specific for CD58, which were purchased from Coulter/Immunotech (Marseille, France). The mAb AP-2 specific for a complex-specific epitope of the αIIbβ3 integrin came from GTI (Brookfield, WI). PAC-1 and AK-4 mAbs specific for activated αIIbβ3 complex and P-selectin (CD62P), respectively, came from BD PharMingen (San Diego, CA). The mAb 3E12 to CD47 was from BioAtlanstique (Nantes, France). The murine mAb BS66 to ICAM-4/LWαβ was previously described (36). ImmunoPure mouse IgG from Pierce was used as negative control IgG. Chimeric ICAM-pIgI constructs derived from intact ICAM-4 (LW αβ) cell carrying the two IgG heavy and light chain, respectively, of the parental CHO-ICAM-4 (residues 1–101) or D2-ICAM-4/iges102–208 were used to produce soluble Fc-fusion proteins as described (26). ICAM-1- and ICAM-2-pIgI constructs (kindly provided by Dr. D. Simmons and E. Ferguson, Oxford, UK) were used to produce ICAM-Fc soluble fusion proteins as above.

GRGDSP-activated Platelets—Human platelets were obtained from fresh ACD-anticoagulated blood from volunteers not taking any medication and were washed three times in modified Tyrode’s albumin buffer (5 mM Hepes, 150 mM NaCl, 2.5 mM KCl, 12 mM NaHCO3, 5.5 mM glucose, 0.1% (w/v) bovine serum albumin (pH 6.5), 250 ng/ml PGE1, 25 μg/ml apyrase) by centrifugation at 1,200 × g for 10 min. Platelets were activated as previously described (37, 38). Briefly, 1 × 10^7 washed platelets resuspended in 0.1 ml of Tyrode’s-albumin buffer (pH 7.4) containing 2 mM CaCl2 and 1 mM MgCl2, were incubated at 22 °C for 5 min with 1 mM GRGDSP peptide. Then, an equal volume of phosphate-buffered saline (PBS, 10 mM phosphate buffer in 0.15 mM NaCl, pH 7.2) containing PGE1 (1.5 mg/ml), 250 ng/ml apyrase, 1 mM MgCl2, and 1% (w/v) paraformaldehyde, was added, and the mixture was incubated for 1 h at 22 °C. Then, 0.2 ml of 500 mM NH4Cl was added to stop the reaction in PBS. Fixed activated platelets were washed several times to remove the activating peptide prior to assays and reuspended in modified Tyrode’s buffer, pH 7.4 containing divalent cations. Fixed unactivated platelets used as control, were prepared by omitting divalent cations and the activating peptide in the different buffers.

Platelet Adhesion Assays to Immobilized RBCs—RBCs were immobilized on microtiter plates through binding to coated anti-glycophorin A. Briefly, mAb E4 at 20 μg/ml (50 μl/well) in 25 μl Tris, pH 8, 150 mM NaCl, was adsorbed overnight at 4 °C on flat-bottom 96-well microtiter plates (Nunc A/S, Roskilde, Denmark). After two washes of wells with the same buffer, RBCs (2.0 × 10^5/well in a final volume of 300 μl) resuspended in modified Tyrode’s buffer, pH 7.4 with or without cations (2 mM MgCl2 and 2 mM CaCl2) were added. After 1 h of incubation at 22 °C, fixed GRGDSP-activated or unactivated platelets (5.0 × 10^7/well in a final volume of 100 μl) in modified Tyrode’s buffer, pH 7.4 with or without divalent cations, respectively, were added to RBC-coated wells. After 90 min at 22 °C, non-adherent cells were removed by filling the wells with binding buffer, and the microplates were put to float upside down in a PBS solution. Cells that adhered to the plastic wells were recovered by vigorous shaking in 400 μl of PBS and were counted by flow cytometric analysis using a FACScalibur. Platelets and RBCs were distinguished by forward scatter and platelet staining with the fluorescein isothiocyanate (FITC)-anti-human CD61 mAb (clone VI- PL2, BD Biosciences).

RBCs Adhesion to Adherent Platelets—Following isolation, unactivated platelets (1 × 10^7/well in a final volume of 100 μl) resuspended in RPMI 1640, 10 mM Hepes containing PGE1, and apyrase were added to wells to adhere overnight at 37 °C. After washing, RBCs (3.3 × 10^7/well in a final volume of 300 μl) resuspended in HBSS with 2 mM CaCl2, 1 mM MgCl2, were added to each well. After 90 min at 22 °C, non-adherent RBCs were removed by filling the wells with binding buffer, and the microplates were put to float upside down in a PBS solution. Then RBCs enumeration was done using a Nikon Eclipse TE300 microscope (Nikon, Paris, France) (×10 objective) coupled to a Biocom informatic system of images integration (Biocom, les Ulis, France). For blocking experiments, RBCs and adherent platelets stimulated by thrombin were pretreated with specific mAbs (2.5 μg/ml) and ICAM-Fc protein (2.5 μg/ml), respectively, for 30 min at 22 °C. αmβ1, CHO Transfectants and DTT Activation—The Chinese hamster ovary cell line (CHO) was grown in Isco’s modified Dulbecco medium with Glutamax-1 (Invitrogen) supplemented with amphotericin-B-penicillin-streptomycin and 10% fetal calf serum. CD41 (αmβ1 complex) and CD61 (β3-chain) cDNAs subcloned into pcDNA3.1 vector (Invitrogen). Kindly provided by Dr. P. J. Newman (Blood Center of Southeastern Wisconsin, Milwaukee, WI), were cotransfected into CHO cells using the lipofectin reagent according to the manufacturer’s instructions (Invitrogen). Stable transformants resistant to G418 (0.6 mg/ml of genetin) were selected for CD41 and CD61 expression by immune-magnetic separation using mAb AP-2 and magnetic beads coated with anti-mouse IgG (Dynalbeads-M-450, DYNAL, Oslo, Norway). CD41 and CD61 expression of stable clones was analyzed and quantified by flow cytometric analysis with Qifikit calibration beads, used according to the manufacturer’s instructions (Dako, Denmark). One clone with the strongest expression of αmβ1 integrin was selected. For adhesion assays, αmβ1, CHO-transfected and wild-type (parental) CHO cells were treated with or without 10 mM DTT in RPMI 1640, 10 mM Hepes, at 22 °C for 20 min to activate the αmβ1 complex receptor (39).

Cell Adhesion Assays to Immobilized Proteins—Purified ICAM-Fc proteins diluted in 25 μl Tris, pH 8.0, 150 mM NaCl, 2 mM MgCl2, and 2 mM CaCl2, were absorbed to flat-bottom 96-well microtiter plates overnight at 4 °C, at 2.5–20 μg/ml (50 μl/well in triplicate). The wells were then blocked for 2 h at 22 °C with 1% nonfat milk in the same buffer. For adhesion assays, either fixed GRGDSP-activated or unactivated platelets (5.0 × 10^7/well in a final volume of 100 μl) in modified Tyrode’s buffer, pH 7.4, with or without divalent cations, respectively, were added to CHO cells. DTT-activated or unactivated transfectants (1.0 × 10^7/well in a final volume of 100 μl) resuspended in RPMI 1640, 10 mM Hepes containing 2 mM MgCl2 and 2 mM CaCl2, were added to the coated wells and incubated for 90 min at 22 °C. Non-adherent cells were removed by washings before microscopic observation and CHO cell enumeration was done as indicated above. Platelets were washed several times with flow cytometric buffer as above. For blocking assays, the cells were pretreated with specific peptides and their corresponding random counterpart (125 μM final concentration) or different mAbs (5 μg for 5 × 10^6 platelets or 1.0 × 10^7 CHO cells/100 μl) for 30 min at 22 °C prior addition to protein-coated wells.

RESULTS

RBCs Interact with Activated Platelets—To analyze molecular events occurring during RBC-platelet interaction, in vitro cell adhesion assays were developed using RBCs from donors of
common and rare phenotypes immobilized to plastic surface via anti-GPA binding and platelets from normal healthy donors, pretreated or not with the synthetic GRGDSP peptide in the presence of inhibitors of platelet activation, thus resulting in specific $\alpha_{\text{IIb}}\beta_3$ integrin activation and the acquisition of high affinity $\text{Fc}$-binding state without addition of a cellular agonist (37). Accordingly, in addition to bind $\text{Fg}$, GRGDSP-treated platelets reacted strongly with the mAb PAC-1, which binds to the activated $\alpha_{\text{IIb}}\beta_3$ complex (40), but no reactivity with the mAb AK-4 (41), which binds to P-selectin normally contained in intracellular $\alpha$-granules (not shown). As shown in Fig. 1, GRGDSP-activated platelets adhered more efficiently than unactivated platelets to immobilized ICAM-4-positive RBCs from control donors. The 100% relative binding was equivalent to $220 \pm 100$ GRGDSP-activated platelets adhered to $1.0 \times 10^9$ immobilized RBCs. When unactivated platelets were used as control, a 69% reduced adhesion was noted that corresponded to a mean background of $31 \pm 12 \%$. As preliminary assays showed that similar results were obtained with fresh and unfrozen RBCs (not shown), the following studies were performed with thawed RBCs since rare RBC variants lacking different membrane proteins were available from our frozen collection.

Activated platelets bind to coated RBCs lacking the blood group proteins Lu (CD239, laminin receptor of 78–85 kDa), JMH (CD108, 80 kDa), and DO (ADP-ribosyltransferase 4 of 47–67 kDa) but expressing normal levels of ICAM-4, as efficiently as would normal ICAM-4-positive RBCs. Interestingly, when ICAM-4 negative (LW null) RBCs lacking the ICAM-4/LW glycoprotein (42 kDa) from three unrelated donors were coated to plastic wells, a 40% decrease binding of GRGDSP-activated platelets was observed after deduction of the mean background corresponding to the unactivated platelet adhesion to all types of RBCs ($p < 0.001$ versus unactivated platelets and $p < 0.05$ versus controls).

In order to confirm that ICAM-4 plays a role in RBC-platelet interactions, RBC adhesion on adherent platelets stimulated by thrombin, a more physiologically relevant platelet activator than the RGDS peptide, was also analyzed although in this assay platelets are more activated with $\alpha$-granule release than GRGDSP-activated platelets (see above). Although ICAM-4-positive RBCs did not bind to unstimulated adherent platelets in the presence of PGE1 and aprotase (not shown), they bind strongly to thrombin-stimulated platelets (Fig. 2). This binding was efficiently decreased to 50 ± 9% and 11 ± 1% by mAb BS56 and soluble ICAM-4-Fc protein, respectively, whereas the mAb AICD58 reacting with the erythroid membrane CD58 protein and the soluble ICAM-2-Fc protein had only a minor inhibitory effect (88 ± 4 and 85 ± 7%, respectively). Similarly, mAbs anti-β3 (LO1-15C9), anti-β1 (BAM9917) and anti-MER2 (1D12 or 2F7) directed against various RBC surface membrane proteins did not exhibit any effect (not shown). Unfortunately, the nonspecific adhesion of frozen RBCs in this assay made impossible the comparative analysis between the ICAM-4-positive and -negative RBCs. Altogether, these data suggests that ICAM-4 might take a significant part (about 50%) in the adhesion of RBCs to activated platelets. As the GRGDSP peptide is a trigger of a high affinity state of $\alpha_{\text{IIb}}\beta_3$ integrin, which mediates Fg binding and platelet aggregation (37), our data suggested that ICAM-4 might interact with $\alpha_{\text{IIb}}\beta_3$ integrin but also with other adhesive molecules.

**RBC-Platelet Interaction Is Mediated via ICAM-4** —To obtain further evidence that ICAM-4 might interact with a high affinity state of $\alpha_{\text{IIb}}\beta_3$ integrin, type-I glanzmann’s thrombocytopenia platelets from two unrelated patients who both exhibit a 6-bp deletion in exon 7 of the $\beta_3$ gene (42), were used for cell adhesion assays to coated ICAM-4-Fc protein. Fig. 3A shows that unactivated platelets from normal control donors did not bind to immobilized ICAM-4-Fc, as expected from above data, whereas the same platelets activated by the GRGDSP peptide bound readily to coated ICAM-4-Fc, but not to immobilized ICAM-1. The 100% relative binding of GRGDSP-activated platelets to ICAM-4-Fc was equivalent to $12.5 \pm 3.0\%$ of the total added platelets. Conversely, platelets from the thrombocytopenic patients type 1 with a severe defect of $\alpha_{\text{IIb}}\beta_3$ integrin surface expression, either unactivated (not shown) or GRGDSP-activated, failed to bind to coated ICAM-4-Fc (Fig. 3A).

In order to determine the specificity of these interactions, the effect of different mAbs and synthetic peptides on the platelet adhesion to immobilized ICAM-4-Fc protein was investigated (Fig. 3B). Adhesion of activated platelets from normal control donors was efficiently blocked (approximately, 70 and 60%, respectively) by P2 and AP2 mAbs specific for the $\alpha_{\text{IIb}}\beta_3$-i integrin in the presence of the $\beta_3$-chain and the complex-specific epitope of the $\alpha_{\text{IIb}}\beta_3$ integrin, respectively. SZ21 and SZ22 mAbs that recognize the $\beta_3$- and $\alpha_{\text{IIb}}\beta_3$-chains alone, respectively, and the BS56 mAb specific for ICAM-4, partially but significantly inhibited the interaction between ICAM-4 and activated platelets, whereas the SZ22 mAb directed against the GPIb platelet glycoprotein and the control mouse IgG had no significant effect (Fig. 3B). In addition, mAbs FA6 and 3E12 directed against CD36 and CD47, respectively, did not inhibit the platelet-ICAM-4 interaction. Blocking experiments by synthetic peptides revealed that the RGD peptide that binds to $\alpha_{\text{IIb}}\beta_3$ integrin and inhibits $\text{Fg}$ binding, strongly reduced by 75% the adhesion of activated platelets to ICAM-4, whereas the RGE peptide had no effect.

**RBC-Platelet Interaction Is Mediated via ICAM-4/$\alpha_{\text{IIb}}\beta_3$ Integrin** —To provide further evidence that ICAM-4 may interact with the $\alpha_{\text{IIb}}\beta_3$ integrin, stable CHO transfectants expressing recombinant human $\alpha_{\text{IIb}}\beta_3$ were generated and used in cell adhesion assays (Fig. 4). Several $\alpha_{\text{IIb}}\beta_3$-CHO transfectants
32% of the total added DTT-activated /H9251 cells, either or not treated with DTT, did not bind at all. About

tibody (binds to RBCs, unrelated mouse IgG an-

clude mAb anti-CD58 (AICD58), which

bound to adherent platelets. Controls in-

top right of each field of view. 100% cor-

responds to the total number of RBCs bound to adherent platelets. Controls include mAb anti-CD58 (AICD58), which

binds to RBCs, unrelated mouse IgG an-
tibody (ctrl IgG) and ICAM-2-Fc protein.

were obtained, and one clone expressing a high level of αIIIβ3 integrin (αIII, 18,600 molecules/cell and β3, 67,000 molecules/ cell, as estimated by flow cytometric analysis with specific mAbs) was chosen for further studies. The αIIIβ3 integrin of these cells was activated by DTT treatment and the adhesion of DTT-activated and unactivated αIIIβ3-CHO transfectants to immobilized ICAM-4-Fc was examined (Fig. 4). In a preliminary experiment we found that these cells also reacted with PCA-1 mAb that recognized the activated αIIIβ3 integrin complex (not shown). DTT-activated αIIIβ3-CHO transfectants dose-dep

ependently bind to coated ICAM-4-Fc protein, whereas un-

-treated αIIIβ3-CHO transfectants as well as parental CHO cells, either or not treated with DTT, did not bind at all. About

32% of the total added DTT-activated αIIIβ3-CHO transfectants adhered to coated ICAM-4-Fc, but there was no binding to immobilized ICAM-1-Fc protein used as control (Fig. 4B). Identical results were obtained when the αIIIβ3-CHO transfectants were activated by the GRGDSP-peptide instead of DTT (not shown). The binding of DTT-activated αIIIβ3-CHO transfectants to immobilized ICAM-4-Fc could be blocked by ~50% by mAbs specific for ICAM-4 (BS56) or for the complex-specific epitope of the αIIIβ3 integrin (AP2), but not with mAbs to the β3-chain (SZ21) and αIII-chain (SZ22 and P2) of αIIIβ3 complex (AP2), CD47 (3E12), and CD36 (FA6.152), or pretreated with 125 μM (fi-
nal concentration) of RGE and RGD peptides. The results are expressed as the relative percentage of activated platelets bound to coated ICAM-Fc proteins. 100% value is calculated from the total number of activated platelets bound in the absence of peptides or mAbs. Negative con-
trols include mAb SZ2 specific for platelet gpIb, unrelated mouse IgG antibody (ctrl IgG), ICAM-1-Fc and wells without coated protein. The mean ± S.E. from three experiments is shown. By Student’s t test analysis: **, p < 0.01, and *, p < 0.05.

Putative Domains on ICAM-4 That Interact with the αIIIβ3 Integrin—As an attempt to localize the αIIIβ3 integrin binding site on ICAM-4, domain deletion mutants lacking either extracellular Ig-like domain D1 or domain D2 were produced and used in cell adhesion assays to chimeric Fc proteins. Fig. 5A showed that the binding of DTT-activated αIIIβ3-CHO transfectants via the αIIIβ3 integrin required the presence of both

**FIG. 2. Adhesion of RBCs to adher-
ent platelets.** Adhesion of ICAM-4-positi-
vie RBCs to adherent platelets stimu-
lated by thrombin (0.5 unit/well). RBCs and stimulated adherent platelets were pretreated or not with saturating concentra-
tion of mAbs to ICAM-4 (BS56) and ICAM-4-Fc protein, respectively. The per-
centage of bound RBCs is indicated on the top right of each field of view. 100% cor-
responds to the total number of RBCs bound to adherent platelets. Controls include mAb anti-CD58 (AICD58), which

binds to RBCs, unrelated mouse IgG an-
tibody (ctrl IgG) and ICAM-2-Fc protein.

**FIG. 3. Adhesion of platelets to im-
obilized ICAM-4-Fc protein.** A, mi-
crophotographs showing the comparative adhesion of GRGDSP-activated platelets from normal or type-I glanzmann’s thrombasthenia (GT type-I) patients to ICAM-Fc proteins coated to flat-bottom 96-well microtiter plates (1 μg/well). ICAM-1-Fc protein and unactivated nor-
mal platelets were used as negative con-
trols. B, adhesion of normal GRGDSP-ac-
tivated platelets to coated ICAM-Fc proteins (1 μg/well) and pretreated or not with saturating concentrations of mAbs specific for ICAM-4 (BS56), β3-chain (SZ21), αIII-chain (SZ22 and P2), αIIIβ3 complex (AP2), CD47 (3E12), and CD36 (FA6.152), or pretreated with 125 μM (fi-
nal concentration) of RGE and RGD peptides. The results are expressed as the relative percentage of activated platelets bound to coated ICAM-Fc proteins. 100% value is calculated from the total number of activated platelets bound in the absence of peptides or mAbs. Negative con-
trols include mAb SZ2 specific for platelet gpIb, unrelated mouse IgG antibody (ctrl IgG), ICAM-1-Fc and wells without coated protein. The mean ± S.E. from three experiments is shown. By Student’s t test analysis: **, p < 0.01, and *, p < 0.05.
domains D1 and D2, since a 50% decrease binding was observed in the absence of either domain D1 or D2. Similar effects with less amplitude were observed using GRGDSP-activated platelets (see Fig. 5B).

Further blocking experiments with synthetic peptides were performed. Adhesion of activated-platelet was efficiently inhibited to 14 and 58% by the FBI, Fg γ-chain residues 400–411) peptide and the ICAM-4 peptide Gly–Val (residues 65–74), respectively, two peptides exhibiting a QXXDV motif involved in the fibrinogen/αIIIβ3 integrin interaction (Fig. 5B). When DTT-activated αIIIβ3 CHO transfectants were used, a 78% decrease in binding was observed in the presence of the ICAM-4-derived peptide Gly–Val whereas the peptide FBI failed to inhibit (Fig. 5A). The lack of inhibition by the peptide FBI, might result from some changes of αIIIβ3 integrin when expressed in CHO transfectants, as suspected for the reactivity of mAb P2 (see above). Neither the random peptides of FBI and Gly-65–Val-74 nor a control ICAM-4 peptide (Cys–Arg, residues 180–192) had any inhibitory effect. Altogether, these results demonstrate that the αIIIβ3 binding site on ICAM-4 encompassed domains D1 and D2 and that it seems to reside at the tip of the E strand of domain D1, which is in contact with the loop C′-E of domain D2.

**DISCUSSION**

In this report in vitro cell adhesion assays have been developed to evaluate the capacity of red cell ICAM-4 to interact with platelets and to identify the molecular basis of the interaction. The αIIIβ3 integrin (platelet fibrinogen receptor GPIb-IIIa) was identified as the receptor for RBC ICAM-4. However, we found that the αIIIβ3 integrin had to be in its high affinity state to bind ICAM-4, as the interaction occurred only after synthetic GRGDS peptide activation, but not with untreated resting platelets. This was based on the following evidence: (i) activated platelets adhered less efficiently to immobilized ICAM-4-negative (LW-mul) than to ICAM-4-positive RBCs, (ii) monoclonal antibodies specific for a complex-specific epitope of the αIIIβ3 integrin or to the β3-chain alone and specific for
ICAM-4 to a lesser extent, inhibited platelet adhesion, (iii) activated platelets from two unrelated type-I glanzmann's thrombasthenia patients that are deficient for the αIIbβ3 integrin (and vitronectin receptor αvβ3) did not bind to coated ICAM-4-Fc protein, and (iv) DTT-activated αIIbβ3-CHO transfectants strongly adhere to coated ICAM-4-Fc protein but not to coated ICAM-1-Fc, and this was inhibitable by specific antibodies. It should be mentioned that αIIbβ3 integrin activation occurred in the absence of any signaling or secretion (37, 39) and that antibodies specific for GPIb, the von Willebrand receptor of platelets, for CD36 (platelet GPIV) and CD47 (IAP, integrin-associated protein), two multifunctional membrane proteins acting as thrombospondin receptors (43, 44), did not inhibit the platelet adhesion to immobilized ICAM-4-Fc protein. As thombasthenic platelets that expressed normal levels of other platelet receptors like the α2β1 integrin (collagen receptor), and the fibronectin and laminin receptors (GPIa-IIa and GPIc-Ⅲa, respectively), did not adhere to ICAM-4-Fc protein, it is assumed that these proteins do not play a significant role in RBC-platelet interaction under the experimental conditions used.

Further analysis with platelets, αIIbβ3-CHO transfectants and ICAM-4-Fc mutant proteins have shown that the two Ig-like domains of ICAM-4 are required for αIIbβ3 integrin interaction, since domain deletion mutants lacking either the first (D1) or second (D2) Ig-domain exhibited significant reduced binding (see Fig. 5, A and B). A similar effect has been observed when ICAM-4 mutant proteins interact with the leukocyte αIIbβ3 (Mac-1) integrin, whereas interaction with the αIIbβ3 (LFA-1) integrin requires predominantly the first Ig domain D1 (26). The binding of adhesive proteins to αIIbβ3 integrin is predominantly mediated by the RGD peptide motif present on the respective adhesive ligands (45), but this peptide, which is absent from ICAM-4, blocks the ICAM-4/αIIbβ3 interaction. Therefore, binding inhibition by antibodies specific for GPIb, the von Willebrand receptor of platelets, for CD36 (platelet GPIV) and CD47 (IAP, integrin-associated protein), two multifunctional membrane proteins acting as thrombospondin receptors (43, 44), did not inhibit the platelet adhesion to immobilized ICAM-4-Fc protein. As thrombasthenic platelets that expressed normal levels of other platelet receptors like the α2β1 integrin (collagen receptor), and the fibronectin and laminin receptors (GPIa-IIa and GPIc-Ⅲa, respectively), did not adhere to ICAM-4-Fc protein, it is assumed that these proteins do not play a significant role in RBC-platelet interaction under the experimental conditions used.

Our studies therefore indicate that adhesion of normal RBCs to activated platelets occurs through a specific ligand/receptor interaction. Whether or not signaling events across the platelet and/or RBC membranes are triggered by the interaction of the αIIbβ3 integrin receptor with its RBC ICAM-4 ligand is currently unknown.
chain (CD41) of the α_{IIb}β_{3} integrin (62). Although no effect of monoclonal antibodies to GPIb and CD36 was found in static conditions of assay, our results indicate that the interaction of thrombin-activated platelets with intact RBCs (Fig. 2) and of GRGDSP-activated platelets with immobilized ICAM-4 (Fig. 3B) could be inhibited by soluble ICAM-4 (by 89%) and to at least 50% by the monoclonal antibody P2 recognizing the α_{IIb}-chain in the presence of the β_{3}-chain, or by the monoclonal antibody AP-2 specific for a complex-specific epitope of the α_{IIb}β_{3} integrin, respectively. Monoclonal antibodies S22 and S221 to the α_{IIb}-chain and β_{3}-chain alone, respectively, were weak inhibitors in the latter condition (Fig. 3B). Consistent with the above results, our studies have shown that ICAM-4 interaction with β integrins is calcium-dependent (26). Obvi-
ously, distinct experimental conditions (platelet activation, flow conditions) and monoclonal antibodies used may explain the reported differences.

In conclusion, although passive entrapment of RBCs during coagulation or thrombosis is commonly accepted, these data provide independent evidence indicating that a physiological interaction between RBCs and activated platelets (and neutrophils) mediated by specific receptor/ligand interactions can occur in a variety of biological processes, notably during normal hemostatic conditions ( clot formation), pathological occlusion conditions (deep vein thrombosis, sickle cell disease) and possibly inflammation, particularly under low blood flow conditions, close to static, which may facilitate RBC adhesion events.

Although ICAM-4 may play a significant role, clearly other receptor/ligand interactions are likely to occur which deserves further analysis.

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