Arg-Gly-Asp–Dependent Occupancy of GPIIb/IIIa by Applaggin: Evidence for Internalization and Cycling of a Platelet Integrin

By June D. Wencel-Drake, Andrew L. Frelinger III, Michael G. Dieter, and Stephen C.-T. Lam

Using indirect immunofluorescence microscopy we examined the distribution and cycling of GPIIb/IIIa after binding to applaggin, a high-affinity Arg-Gly-Asp (RGD)–containing ligand. Resting, unfixed platelets were incubated with applaggin for 30 minutes at 37°C, and bound applaggin was detected by an affinity-purified rabbit anti-applaggin antibody. Examination of intact cells showed a rim pattern for applaggin, consistent with its binding to the platelet surface. Staining of Triton X-100–permeabilized cells showed an intracellular pool of applaggin. Competition of applaggin binding by either AP-2, an anti-GPIIb/IIIa monoclonal antibody (MoAb) that blocks fibrinogen binding, or the synthetic peptide RGDW eliminated both surface and intracellular staining, indicating that applaggin is binding to GPIIb/IIIa in an RGD-dependent manner. Inhibition of platelet activation by PGE, and theophylline had no effect on the observed staining patterns, indicating that cellular activation is not required for surface binding and subsequent internalization. To evaluate whether occupancy of functional binding sites on GPIIb/IIIa is required for internalization, we used mAb15, an anti-GPIIIa antibody that neither blocks fibrinogen binding nor induces the expression of ligand-induced binding sites on GPIIb/IIIa. In these studies mAb15 was internalized in a manner analogous to both AP-2 and applaggin, showing that occupancy of the RGD binding site is not required to initiate receptor internalization. To estimate the size of the newly internalized pool of applaggin, 125I-applaggin–binding studies were performed. Displacement of bound 125I-applaggin by excess unlabeled applaggin or EDTA showed that at least 17% of bound applaggin was nondisplaceable when binding was performed under conditions permitting membrane flow and internalization. These data indicate that GPIIb/IIIa is internalized in unstimulated platelets independent of cellular activation or occupancy of the functional binding site(s) of GPIIb/IIIa by RGD-containing ligands. Thus, internalization of GPIIb/IIIa may represent a mechanism by which the surface expression of this adhesion receptor is regulated.

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Blood Platelets participate in hemostasis by adhering to damaged vessel walls and by coadhering to each other. Both events are mediated in part by the recognition of adhesive proteins by their receptors on the platelet surface. It is well established that the major platelet membrane glycoprotein (GP)IIb/IIIa, a member of the integrin superfamily of adhesion receptors, binds several adhesive ligands including fibrinogen, fibronectin, vitronectin, and von Willebrand factor. All of these adhesive proteins contain Arg-Gly-Asp (RGD) sequences that serve as recognition sites for binding to GPIIb/IIIa. In human platelets GPIIb/IIIa has been localized to the plasma membrane, vacuolar and open canicular membrane systems, as well as the α granule membrane, a platelet secretory organelle. Additionally, α granules contain several adhesive ligands for GPIIb/IIIa, including fibrinogen. It has been shown in megakaryocytes that fibrinogen is incorporated into α granules from plasma. In this regard, a proposed mechanism for transport of plasma proteins into intracellular organelles is that of endocytosis. In previous studies, we identified a mobile pool of GPIIb/IIIa in human platelets that is apparently internalized via the formation of endocytic vesicles. Moreover, these vesicles represent a functional intracellular pool that can be transferred to the surface following thrombin stimulation. These immunolocalization experiments were performed by incubating resting unfixed platelets with antibodies directed against GPIIb/IIIa. The use of Fab fragments in these studies ruled out Fc-mediated events or internalization resulting from cross-linking by a bivalent ligand. However, because the antibodies used in these studies block binding of the physiologic ligand fibrinogen, we could not distinguish between internalization mediated by antibody binding or by occupancy of the functional binding site of GPIIb/IIIa. To distinguish between these possibilities, we wanted to examine the distribution and cycling of GPIIb/IIIa bound to an RGD-containing ligand. Recently, several high-affinity RGD-containing peptides have been isolated from snake venoms. In particular, applaggin, isolated from Agkistrodon piscivorus piscivorus, binds saturably to platelets, resulting in inhibition of platelet functions. Unlike the binding of fibrinogen, which requires platelet activation, applaggin binds both to resting and to stimulated platelets. The binding of applaggin to platelets has been shown to be inhibited by an anti-GPIIb/IIIa monoclonal antibody (MoAb), indicating that GPIIb/IIIa is the major receptor for applaggin on the platelet surface. Therefore, in the present study we used applaggin as a probe to address mechanistic issues pertaining to the internalization and cycling of GPIIb/IIIa in human platelets.

MATERIALS AND METHODS

Chemicals/reagents. Bovine serum albumin (BSA; once recrystallized, essentially globulin free) and poly-L-lysine were purchased from Sigma Chemical Company, St Louis, MO. Paraformaldehyde was obtained from Polysciences, Warrington, PA. Sepharose 2B.
Sephadex G-100 superfine, and cyanogen bromide-activated Sepharose 4B were obtained from Pharmacia, Inc, Piscataway, NJ. Protein-A agarose was obtained from Pierce Inc, Rockford, IL. Triton X-100 was purchased from J.T. Baker Chemicals, Phillipsburg, NJ. RGDW was obtained from Peninsula Laboratories, Inc, Belmont, CA. All other chemicals were reagent grade.

**Applaggin preparation and purification.** Applaggin was isolated from Agkistrodon piscivorus piscivorus venom as previously described.14 Amino acid analysis showed that 84% of the mass was peptide, with a composition consistent with that previously reported for applaggin.15 Purity determined by high performance liquid chromatography analysis was greater than 90%. Positive ion mass spectrometry was performed by M-Scan (West Chester, PA) on applaggin, dissolved in dilute acetic acid then diluted with 90% methanol, using a Bio-Q instrument with a quadrupole mass analyzer (VG Biotech, Manchester, UK). Myoglobin was used to calibrate the instrument. Applaggin (10 μL) was injected into the instrument source and elution was performed using a 1:1 vol/vol methanol:water solution at a flow rate of 5 μL/min. A strong positive ion electrospray-MS spectrum was observed with several series of multiply charged signals as shown in the insert in Fig 1. When deconvoluted, these data show a major component of molecular mass 7,666 D and additional minor components of 7,537 and 7,764 D (Fig 1). Because the calculated molecular weight of applaggin based on its amino acid sequence is 7,666 D, there was no evidence of dimerization in our applaggin preparation.

For binding studies, applaggin was radioiodinated to a specific activity ranging between 150 to 300 mCi/μmol by a modified chloramine-T procedure. Briefly, purified applaggin (25 μg/mL) was incubated with 1 mCi Na125I (Amersham, Corp, Arlington Heights, IL) and 40 μg chloramine-T (Sigma) for 5 minutes at room temperature. The reaction was halted by addition of sodium metabisulfite. Free iodine was removed by gel filtration on a PD-10 column (Pharmacia, Inc). The fluorescein-to-protein ratio was calculated to be 7.7 by the optical density (OD) values at 280 nm and 495 nm.

**Platelet preparation.** Platelet-rich plasma (PRP) was prepared from acid citrate dextrose-anticoagulated whole blood obtained from healthy, aspirin-free volunteers as described.19 Human platelets were isolated by centrifugation and gel filtration on Sepharose 2B.20 For binding studies, PRP was prepared from 0.38% sodium citrate-anticoagulated whole blood and platelets were isolated by gel filtration of PRP on Sepharose 2B.

**Immunofluorescence.** Immunofluorescent staining of platelets was performed as previously described.11 Briefly, resting platelets were incubated with 0.1 to 2.0 μmol/L applaggin or applaggin plus competitors or inhibitors for 30 minutes at 22°C or 37°C. Cells were subsequently fixed with 1% paraformaldehyde on ice for 1 hour. Unreacted aldehyde was blocked with NH4Cl-Tris-buffered saline, pH 7.4, and the cells were permitted to settle on polylysine-coated glass coverslips. In order to visualize newly internalized applaggin-tagged membrane glycoproteins, fixed cells were treated with 0.1% Triton X-100 for 3 minutes to render them permeable. Permeable or intact cells were rinsed with Tris-buffered saline containing 0.1% BSA. Following incubation with affinity-purified rabbit anti-applaggin IgG for

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**Fig 1.** Positive ion electrospray mass spectroscopy of applaggin. The insert displays a typical ion spectra observed for applaggin analyzed as described in Materials and Methods. This data was deconvoluted to generate the mass profile shown above.
20 minutes, cells were sequentially stained with a biotinylated goat (Fab); anti-rabbit IgG (Accurate Chemical and Scientific Corporation, Westbury, NY) and rhodamine-avidin (Vector Laboratories Inc, Burlingame, CA). Staining specificity was routinely assessed by using irrelevant ascites as the primary antibody. In double-label experiments the same cells were counterstained for 20 minutes with fluoresceinated goat (Fab); anti-human fibrinogen (Organon-Tecknika Corporation, Cappel Research Products Division, Durham, NC).

In internalization studies using MoAbs, resting platelets were incubated with an appropriate dilution of primary antibody or ascites control. Samples incubated for 30 minutes at 37°C were fixed and processed as described above. Permeable or intact cells were sequentially stained with a biotinylated goat (Fab); anti-mouse IgG (Organon-Tecknika Corporation) followed by rhodamine-avidin and were mounted on a droplet of FITC-Guard (Testog Inc, Chicago, IL). Platelets were viewed with a Jenaval phase/fluorescence microscope equipped with an HBO 50-watt mercury lamp, IVFl epifluorescence condenser with BP 450-560, LP590 barrier filters (Jenoptik Jena GmbH, Germany). Platelets were photographed with Tri-X panchromatic film (Eastman Kodak, Rochester, NY).

**Flow cytometry.** Citrated PRP (approximately 3.0 × 10^7 cells/5 μL) was added to 50 μL Tyrode’s buffer containing either applaggin (2 μmol/L), mAb15 Fab (125 μg/mL), ascites control (120 μg/mL), or buffer control and incubated for 15 minutes at 37°C. In some cases, incubation with mAb15 was followed by an additional 15-minute incubation with applaggin. After addition of FITC-anti-LIBS1 (58 μg/mL), samples were incubated in the dark at room temperature for 5 minutes, diluted 1:10 in Tyrode’s buffer, and analyzed immediately by flow cytometry.

Samples were analyzed in a Coulter Epics 753 flow cytometer (Coulter Electronics, Inc, Hialeah, FL) formatted for single-color analysis. The fluorescence channel was set at logarithmic gain and was calibrated using 1-μm uniform latex particles (Seradyn Inc, Indianapolis, IN) by gating on forward angle light scatter. Values acquired reflect the mean fluorescence intensity for 10,000 events.

**Applaggin binding.** Binding of 125I-applaggin to platelets was performed as previously described. Briefly, gel-filtered platelets (1 × 10^9/mL) were incubated with 125I-applaggin for 60 minutes at 4°C or 37°C in Tyrode’s buffer. Platelet-bound applaggin was quantitated by centrifuging the platelets through 20% sucrose and determining radioactivity associated with the platelet pellet. Nonspecific binding was determined by competition of 125I-applaggin with a 30-fold molar excess of unlabeled applaggin. In some experiments, EDTA (12 mmol/L) was also used to inhibit applaggin binding. In displacement studies, 125I-applaggin, bound for 60 minutes as described above, was displaced by addition of either excess unlabeled applaggin or EDTA, followed by a 30 minute incubation at 37°C. After correction for nonspecific binding, percent displacement was calculated as 1 minus the ratio of nondisplaceable to total binding.

**RESULTS**

**Specificity of applaggin binding and internalization in resting, unfixed platelets.** Resting, unfixed platelets were incubated with 2 μmol/L applaggin for 30 minutes at 37°C. Following fixation and staining with an affinity-purified rabbit anti-applaggin IgG, intact cells demonstrated a rim-staining pattern consistent with applaggin binding to its receptor(s) on the platelet surface (Fig 2a and b). Permeabilization showed extensive intracellular staining for applaggin similar to the vacuolar pattern previously observed using anti-GPIIb/IIIa antibodies. Inasmuch as the cells were incubated with applaggin before fixation, washing and subsequent permeabilization, intracellular labeling patterns only represent newly internalized bound applaggin.

![Image](https://www.bloodjournal.org/)

It has previously been reported that an anti-GPIIb/IIIa antibody (LJ-CP3) inhibits applaggin binding to intact platelets. We therefore examined whether AP-2, a complex specific anti-GPIIb/IIIa antibody that blocks fibrinogen binding would inhibit the observed binding and internalization of applaggin. In competition experiments platelets were incubated with a mixture of 0.1 μmol/L applaggin and either 0.13 μmol/L AP-2 (C and D) or 1 mmol/L RGDW (E and F) (original magnification ×1,500).

![Figure 2. Internalization of GPIIb/IIIa in resting, unfixed platelets.](https://www.bloodjournal.org/)

To demonstrate the specificity of applaggin binding to GPIIb/IIIa, additional competition experiments using RGDW were performed. Incubation of resting platelets with a mixture of 1 mmol/L RGDW and 0.1 μmol/L applaggin resulted in essentially complete inhibition of applaggin binding and subsequent internalization (Fig 2e and f). These data confirm that applaggin binding to GPIIb/IIIa occurs via an RGD-dependent mechanism.

**Requirements of functional binding site occupancy for internalization of GPIIb/IIIa.** To evaluate whether receptor internalization is triggered by occupancy of functional binding
site(s), we next investigated the ability of mAb15, an anti-GPIIb/IIIa antibody that does not block fibrinogen binding,23 to become internalized. As can be seen in Fig 3, when platelets were incubated with mAb15 (0.17 μmol/L) for 30 minutes at 37°C, intact cells showed the typical rim pattern consistent with surface distribution. Permeable cells showed an intracellular labeling pattern for mAb15 identical to those obtained from cells incubated with either applaggin (Fig 1) or AP-2.11 Identical results were obtained with both whole IgG and Fab fragments of mAb15.

It has been shown that occupancy of GPIIb/IIIa by RGD peptides induces conformational changes in the receptor resulting in the expression of neoantigenic sites on GPIIb/IIIa termed ligand-induced binding sites (LIBS).26 Therefore, we examined whether a ligand-occupied conformer of the receptor was required for internalization. To accomplish this we made use of anti-LIBSI, an antibody that recognizes a specific ligand-occupied conformer of GPIIb/IIIa.21 As expected, applaggin (2 μmol/L) induced expression of LIBSI on GPIIb/IIIa, as measured by binding of FITC-labeled, anti-LIBSI antibody (Table 1). In contrast, preincubation with mAb15 failed to induce the expression of LIBSI on GPIIb/IIIa. However, sequential incubation with mAb15 followed by applaggin gave results comparable to applaggin alone, suggesting that although mAb15 does not induce LIBSI expression, it does not prevent induction. Taken collectively, the observation that mAb15 was internalized in a manner analogous to applaggin yet failed to induce the expression of LIBSI epitope suggests that neither occupancy of the RGD binding site nor LIBSI induction is required for internalization.

Cellular activation requirements for internalization of applaggin-tagged GPIIb/IIIa. To investigate the cellular activation requirement for internalization of applaggin-tagged GPIIb/IIIa, platelets were incubated with agents that increase cAMP levels; elevation of which is commonly associated with inhibition of platelet functions.27 Pretreatment of platelets with 2.5 μmol/L PGE1 and 1.1 mmol/L theophylline completely inhibited platelet aggregation in response to 1 U/mL thrombin (data not shown). As shown in Fig 4, pretreatment of platelets with these agents had no apparent effect on binding and subsequent internalization of applaggin as measured by immunofluorescence (Fig 4a and b) and (c and d). In control samples, cells incubated without applaggin showed minimal labeling, confirming the specificity of our anti-applaggin antibody (Fig 4e and f). These studies suggest that platelet activation is not required for binding and subsequent internalization of applaggin.

Effect of temperature on applaggin internalization. Preliminary studies with AP-2 showed that internalization of GPIIb/IIIa requires active metabolic function.11 To extend this observation to the present studies, resting platelets were incubated with applaggin for 30 minutes at various temperatures. As shown in Fig 5, incubation with applaggin at 37°C resulted in a typical rim-staining pattern in intact cells (Fig 5a) as well as an intracellular vacuolar pattern in permeabilized cells (Fig 5b). In contrast, when applaggin binding was performed at 22°C, only surface labeling was observed in both intact and permeabilized cells (Fig 5c and d). Identical results were obtained in parallel studies performed at 15°C and 4°C (data not shown). These observations suggest that lowering the incubation temperature prevents internalization of applaggin-tagged GPIIb/IIIa.

**Table 1. FACS Analysis of Anti-LIBSI Binding Induced by Applaggin and mAb15**

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<th>Addition(s)</th>
<th>Mean Fluorescence Intensity</th>
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<td>Control (buffer)</td>
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<td>Applaggin (2 μmol/L)</td>
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<tr>
<td>mAb15 Fab (2.5 μmol/L)</td>
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<tr>
<td>mAb15 Fab (2.5 μmol/L) + Applaggin (2 μmol/L)</td>
<td>139</td>
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Values reflect mean fluorescence intensity per 10,000 events.

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**125I-applaggin binding: Competition and displacement studies.** To quantitate the size of the observed intracellular pool of applaggin-tagged GPIIb/IIIa, we performed binding studies with 125I-applaggin (Table 2). Resting platelets were incubated with 0.6 μmol/L 125I-applaggin for 60 minutes at
either 37°C or 4°C to achieve steady-state binding. Nonspecific binding, ranging between 2% to 4% of total binding was determined by simultaneous addition of a 30-fold molar excess of unlabeled applaggin or 12 mmol/L EDTA. To estimate the amount of applaggin being internalized, 125I-applaggin on the cell surface was displaced by the addition of either unlabeled applaggin or EDTA at time (t) = 60 minutes. When binding was performed at 4°C in the absence of internalization, essentially complete displacement was achieved (98.4% with unlabeled applaggin and 99.9% with EDTA). However, when cells were incubated with 125I-applaggin at 37°C thereby permitting internalization, only 82.5% and 83% were displaced by the additions of unlabeled applaggin and EDTA, respectively. These results show that approximately 17% of platelet-bound applaggin was not displaceable under conditions permitting membrane flow and internalization. When platelets were incubated for shorter periods of time (15 or 30 minutes) before addition of excess unlabeled applaggin, the nondisplaceable pool was smaller (5% and 10%, respectively).

**DISCUSSION**

The major findings of the present study are: (1) applaggin bound to platelets is internalized in a time- and temperature-dependent manner; (2) binding and internalization are mediated by GPIIb/IIIa and occur via an RGD-dependent mechanism; (3) platelet activation is not required for either surface binding or subsequent internalization of applaggin; (4) neither occupancy of the functional binding site of GPIIb/IIIa nor occupancy-dependent conformational changes of GPIIb/IIIa is required for the observed trafficking of the receptor; and (5) dissociation of 125I-applaggin bound to platelets at 4°C or 37°C indicates that at least 17% of bound applaggin is internalized.

Using an anti-GPIIb/IIIa MoAb, AP-2, we previously reported the existence of a mobile pool of GPIIb/IIIa, which is actively internalized and can be reexpressed on the platelet surface following thrombin stimulation. However, because AP-2 blocks fibrinogen binding, these studies did not distinguish between events mediated by either antibody binding or occupancy of the functional binding site(s) of GPIIb/IIIa. The advantage of using applaggin in the present study is that this ligand interacts directly with the RGD binding site of GPIIb/IIIa. By indirect immunofluorescence microscopy, we observed that applaggin is internalized in a manner analogous to AP-2. However, similar internalization patterns were also observed with mAb15, an anti-GPIIIa antibody (Fig 3), and with Tab, an anti-GPIIb antibody (data not shown). Because mAb15 and Tab do not inhibit fibrinogen binding, these results suggest that occupancy of the fibrinogen binding site is not required for receptor internalization. Additionally, using an anti-LIBS1 antibody we found that mAb15 did not induce the expression of the LIBS1 epitope, suggesting that ligand-induced conformational changes in the receptor are also not required for internalization. Furthermore, applaggin binding to intact platelets has been shown to be independent of cellular activation. The present study confirms and extends this observation by showing that internalization of applaggin-tagged GPIIb/IIIa was not inhibited when platelets were incubated with agents that elevate cellular cAMP levels. Although these data suggest that neither cellular activation nor occupancy of the RGD binding site of GPIIb/IIIa is required for receptor internalization, they do not address whether unoccupied GPIIb/IIIa undergoes continuous cycling or turnover in unstimulated platelets. In this regard, an alternative method for studying cycling integrins has been reported by Br€otscher who used a thiol sensitive reagent, 125I-labeled N'-hydroxyphenyl propionyl N[sulphosuccinimidyl di(thiopropionyl) glycyl] cystine, to chemically modify the receptors. Following internalization, removal of surface label with glutathione and quantitative immunoprecipitation, it was reported that the αβ1, α5β1, and Mac-1 integrins all participate in the endocytotic cycle. Although these results suggest that cycling of certain integrins occurs in the absence of ligand or antibody binding, any modification of cell surface proteins may lead to physical or biochemical alterations in the receptors thereby influencing the observed rates of internalization. Thus, a detailed study of rates of internalization of GPIIb/IIIa using a variety of probes is necessary to distinguish between initiation, augmentation, or retardation of receptor cycling in platelets. Although the binding of applaggin to platelets was inhibited by RGD peptides and anti-GPIIb/IIIa antibodies, it should be noted that other integrins (α6β1 and α5β1) have been identified on platelets that bind RGD-containing ligands and may interact with applaggin. In the present study we observed that RGDW completely blocked surface binding and inter-
INTERNALIZATION OF HUMAN PLATELET GPIIb/IIIa

Fig 5. Effect of temperature on internalization of applaggin-tagged GPIIb/IIIa. Resting platelets were incubated with 2 μmol/L applaggin for 30 minutes at 37°C (a and b) or 22°C (c and d) and were processed and stained as in Fig 2. Permeabilization was verified by double labeling cells with FITC conjugated goat anti-human fibrogen (not shown) (original magnification X1,500).

Previously, Chao et al reported that applaggin exists as a disulfide-linked dimer, as suggested by its migration on reduced and nonreduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), thereby raising the possibility that our observed internalization of applaggin-tagged GPIIb/IIIa is mediated by cross-linking of the receptor by a bivalent ligand. However, analysis of purified applaggin by mass spectroscopy showed the presence of applaggin monomers with no evidence of dimerization. This discrepancy may be caused by differential amounts of SDS bound to nonreduced and reduced applaggin molecules, creating a charge-to-mass ratio that does not behave normally in SDS-PAGE. Nonetheless, the absence of applaggin dimers in our preparation, as evidenced by mass spectroscopy data, excluded crosslinking as a potential mechanism mediating the internalization of GPIIb/IIIa. This conclusion is further supported by our observation that Fab fragments of various GPIIb/IIIa antibodies also report internalization of the receptor.

It is well established that membrane flow is a temperature-dependent process. Similar to studies in which GPIIb/IIIa trafficking was followed with MoAbs, internalization of applaggin-tagged GPIIb/IIIa also showed temperature dependence as evidenced by a lack of intracellular staining at 22°C (Fig 5), 15°C, or 4°C (data not shown). Therefore, we made use of the observed temperature dependence of internalization to quantitate the relative amounts of applaggin present in surface and intracellular pools. Our results indicate that approximately 17% of bound applaggin was not displaceable when binding was performed at 37°C; however, surface-bound applaggin (4°C binding) was completely displaced with both unlabeled applaggin and EDTA. These data coupled with immunofluorescence studies suggest that the nondisplaceable applaggin is present in an intracellular pool. It should be noted that 125I-applaggin was displaced at 37°C,
conditions that may permit recycling of applaggin-tagged GP IIb/IIIa to the cell surface. Therefore, the size of the intracellular pool being 17% of total bound applaggin may represent the lower limit of the amount of internalized ligand at 60 minutes.

Although internalization of soluble and particulate ligands by platelets has long been a subject of interest, there remains considerable controversy regarding the mechanism(s) by which this is accomplished. In this regard, evidence has been provided supporting platelet-mediated endocytosis. However, conflicting results were reported in pulse chase studies using gold-labeled anti-platelet antibodies and cationized ferritin. If internalization occurs via lateral diffusion into the OCS, labeling of intracellular vesicles/OCS with both gold and ferritin would be expected. On the contrary, it was reported that the two ligands appeared in different intracellular compartments, suggesting that internalization occurs as the result of new invaginations or endocytosis of the plasma membrane. In this regard, studies by our laboratory using an anti-GP IIb/IIIa MoAb reported that GP IIb/IIIa is internalized via the formation of endocytic vesicles. Because incorporation of plasma fibrinogen into α granules has been suggested to occur by endocytosis, these cycling vesicles may function as transport vehicles for plasma proteins. More recently, it has been reported that antibody-tagged GP IIb/IIIa was distributed to α granule membranes of unstimulated platelets. Thus, it is interesting to speculate that ligand- and/or antibody-bound GP IIb/IIIa is internalized via endocytic vesicles that may cycle to and fuse with secretory granules or may cycle back to the surface.

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REFERENCES

23. Wencel-Drake JD, Plow EF, Zimmerman TS, Painter RG, Ginsberg MH: Immunofluorescent localization of adhesive glyco-
29. Bretscher MS: Circulating integrins: αβ3, αβ4, and Mac-1, but not αβ1, αβ1, or LFA-1. EMBO J 11:405, 1992
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