Integrin $\alpha_{M}\beta_2$ Orchestrates and Accelerates Plasminogen Activation and Fibrinolysis by Neutrophils*

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Plasmin, the pivotal thromolytic enzyme, is generated on the surface of many cell types, where urokinase receptor (uPAR)-bound urokinase (uPA) activates cell-bound plasminogen (Plg). It has been reported that neutrophils mediate endogenous thrombolysis involving a uPA-dependent mechanism, and we previously demonstrated that both uPAR and integrin $\alpha_{M}\beta_2$ recognize uPA to control cell migration and adhesion. In the present study, we report that the $\alpha_{M}\beta_2$ regulates neutrophil-dependent fibrinolysis. Phorbol 12-myristate 13-acetate (PMA)-stimulated neutrophils, both uPA and Plg were co-immunoprecipitated with purified, soluble uPA (sc-uPA)/Plg mixture, decreased the $K_{d}$ 50-fold. The binding of sc-uPA to Plg ($K_{d} = 40 \text{ nM}$) and Plg ($K_{d} = 1 \text{ mM}$) in a dose-dependent and saturable manner. In Plg activation assays, addition of purified $\alpha_{M}\beta_2$, but not a control protein, to a single chain uPA (sc-uPA)/Plg mixture, decreased the $K_{d}$ from 2 to 0.1 $\mu\text{M}$, thereby augmenting the overall reaction efficiency by 50-fold. The binding of sc-uPA to $\alpha_{M}\beta_2$ was critical for the $\alpha_{M}\beta_2$-mediated enhancement of plasmin (Plm) generation, because this effect was lost when WT-sc-uPA was replaced with a kringle-less mutant ($\Delta\text{K-sc-uPA}$), which does not bind to $\alpha_{M}\beta_2$. Plm inactivation by $\alpha_{2}$-antiplasmin was significantly delayed when Plm was preincubated with purified, soluble $\alpha_{M}\beta_2$. Thus, assembly of Plg and uPA on integrin $\alpha_{M}\beta_2$ regulates Plm activity and, thereby, plays a crucial role in neutrophil-mediated thrombolysis.

Critical to the biological functions of neutrophils (PMNs) is their capacity to rapidly mobilize exuberant proteolytic and adhesive responses. Components of the plasminogen (Plg) system and members of the integrin family contribute to the proteolytic and adhesive potentials of the PMN (1–4). Among the proteolytic responsibilities of PMNs is their contribution to the degradation of fibrin-rich blood clots (5, 6). Recent data have emphasized the importance of urokinase (uPA), released from intracellular stores, to activate Plg to plasmin (Plm) and, thereby, contribute to the fibrinolytic activity of these cells (5, 7). PMNs also express the uPA receptor, uPAR, on their surface (7). Most recent studies of uPAR have emphasized its participation in cell migration, but its contribution to Plg activation has been difficult to demonstrate not only in vitro but also in vivo using deficient mice (8–10). We have recently shown that the leukocyte integrin $\alpha_{M}\beta_2$ can function as a uPA receptor on PMN (11). This recognition influences PMN adhesion and migration (11), but the role of $\alpha_{M}\beta_2$ interaction with uPA in Plg fibrinolysis has not been investigated. Plg can also bind to PMNs via its lysine binding sites (LBS) associated with its kringle domains (12), and numerous Plg-binding proteins, some present in PMN, have been identified (13–16). Thus, PMNs exhibit multiple interactions with components of the Plg system, but it is unclear if and how these interactions affect the primary function of Plg, its role in fibrinolysis.

The adhesive and migratory responses of PMNs are critical to their participation in the inflammatory response, and numerous in vitro and in vivo studies have demonstrated that $\alpha_{M}\beta_2$ is an important mediator of these activities (4, 17). Fibrinogen and ICAM-1 are among the ligands of this integrin that have been implicated in endothelial transmigration of PMNs (17, 18). These and a myriad of other ligands for $\alpha_{M}\beta_2$ interact with its I domain, an inserted domain of ~200 amino acids in the $\alpha_M$ subunit (19). As noted above, uPA is a ligand for $\alpha_{M}\beta_2$ and can support both adhesion and migration of $\alpha_{M}\beta_2$-bearing cells, including PMNs (11). uPAR also interacts within a 17-amino acid sequence, M25, located within W4 repeat of the $\beta$-propeller (20) of the $\alpha_M$ subunit, a region lying outside of the $\alpha_M$ domain, and a physical complex between uPAR and $\alpha_{M}\beta_2$ has been demonstrated on the PMN surface (21, 22). The interaction between uPAR and $\alpha_{M}\beta_2$ augments $\alpha_{M}\beta_2$-mediated functions such as adhesion to fibrinogen, ICAM-1, and other ligands of this integrin (23, 24), but uPAR contribution to Plg activation remains uncertain.

In this study, we identify a previously unrecognized function of $\alpha_{M}\beta_2$, a direct and primary role of this integrin in Plg activation. This activity is demonstrated with purified $\alpha_{M}\beta_2$ and components of the Plg system as well as with intact PMNs and is shown to regulate the participation of PMNs in fibrinolysis. This function is particularly relevant in view of the preferential accumulation of these cells within thrombi (6) and the prominent role of Plg in the proteolytic and migratory responses of PMNs.
Human platelet-poor plasma was prepared from citrated blood by centrifugation at 37°C for up to 5 h. For experiments in which PMNs were activated, KIM 185 or Mn2+ was added to the platelet-poor plasma. Clotting was initiated by addition of 0.5 NIH units/ml thrombin, and the reaction was monitored as the changes of absorbance at 405 nm for up to 5 h. In experiments where α2-antiplasmin (1 µM) or α1-proteinase inhibitor (100 nM) was added, the PMNs were incubated for 15 min at 37°C in the presence of 1 mM MnCl2, with or without 20 µl of the clotting mixture. The resin was washed twice with ice-cold lysis buffer, the wells were dried and 50 µl of a 1:10 dilution of PtI antibody was added for 1 h at 37°C. The resin was washed again, and 50 µl of a 1:10 dilution of goat anti-mouse IgG was added. After a 1 h incubation, the bound PtI was quantitated by measuring fluorescence at λmax = 480 and λmax = 530 nm. In the inhibition studies, NIF (100 nM) or the KD of uPA (50 nM) was preincubated with the integrin and added to the sensitive uPA/Plg mixture. When the effect of α2-antiplasmin on the inactivation of PtI by α2-antiplasmin was measured, PtI (200 nM) was preincubated in the presence or absence of α2-antiplasmin (600 nM) for 10 min at 37°C followed by addition of S-2251 (0.5 mM). Hydrolysis of the chromogenic substrate was measured continuously, and at 2 min the α2-antiplasmin (1 µM) was added.

**125I-HMW-tc-uPA** was preincubated with the α2-antiplasmin or I domains (0–100 nM) prior to the addition to the α2-antiplasmin-coated wells. To measure binding, varying concentrations of 125I-Glu-Plg (0–14 µM) were added to the α2-antiplasmin or BSA-coated plates in the presence of 1 mM MnCl2, with or without 50 µl unlabeled Plg. After 2 h incubation at 37°C, the wells were washed, and bound 125I-Glu-Plg was measured as described for uPA binding. Data were determined as triplicate measurements at each experimental point.

Disopropyl fluorophosphate-treated Plm was labeled with Alexafluor-488 according to the manufacturer's protocol (Molecular Probes, Eugene, OR), incubated in the presence of increasing concentrations of α2-antiplasmin (0–12 µM) of purified α2-antiplasmin, and collected on the PMN surface. In the kinetic study rates of Plm generation were plotted against Glu-Plg complexes (25, 26). The recombinant uPA domains: GFD (residues 4–18) were from Sigma Chemical Co. (St. Louis, MO). Neutrophils Preparation—Granulocytes were isolated from human peripheral blood of healthy volunteers drawn into sterile citrate-dextrose (1/10 vol. 145 mM sodium citrate, pH 4.6, and 2% dextrose). Isolation was performed by means of density gradient centrifugation on Lymphoprep (Amersham Biosciences, Uppsala, Sweden), followed by dextran sedimentation of erythrocytes and hypotonic lysis of residual erythrocytes. Cytometric Analysis—PMN purified by dextran sedimentation was measured in a system using purified reagents or in plasma. In the former assay, resting or PMN-stimulated PMNs were added at a final concentration of 1.5 × 106 cells/well to microtiter plates containing 2.9 mM dextrose (1/7 vol. 145 mM sodium citrate, pH 4.6, and 2% dextrose). The mAb to annexin II (clone Z014), which blocks its interaction with Plg (29), was from Zymed Laboratories Inc. (San Francisco, CA). The mAb to annexin II (clone Z014), which blocks its interaction with Plg (29), was from Zymed Laboratories Inc. (San Francisco, CA). All other reagents were from Sigma Chemical Co. (St. Louis, MO).

**Neutrophils Preparation—Granulocytes** were isolated from human peripheral blood of healthy volunteers drawn into sterile citrate-dextrose (1/10 vol. 145 mM sodium citrate, pH 4.6, and 2% dextrose). Isolation was performed by means of density gradient centrifugation on Lymphoprep (Amersham Biosciences, Uppsala, Sweden), followed by dextran sedimentation of erythrocytes and hypotonic lysis of residual erythrocytes.
Data Analysis—The data are expressed as means ± S.E. To determine the significance of differences between two groups, Student’s t test was performed using the Sigma-Plot software program (SPSS Inc.); p < 0.05 was considered significant.

RESULTS

Integrin αMβ2 Promotes PMN-mediated Fibrinolysis—In view of the known accumulation of PMNs in thrombi, recent data implicating uPA in the capacity of these cells to spontaneously lyse blood clots (1, 6, 35), and our own results showing that integrin αMβ2 (11) contributes to the adhesive and migratory responses to uPA, we sought to determine whether the integrin might influence PMN-mediated fibrin clot lysis. Isolated human blood PMNs were incorporated into fibrin clots, which were prepared in microtiter wells. B, in the inhibition experiments, cells were preincubated with the following reagents: 6-aminohexanoic acid (5 mM), Trasylol (100 units/ml), amiloride (300 μM), NIF (100 nM), or F(ab)2 fragments of either function blocking antibodies to uPA, αMβ2 (44A), β2 (TS1/18) or control non-immune mouse IgG (50 μg/ml) and then were added to the wells, which also contained these reagents at the same concentrations. C, PMNs stimulated with 20 nM PMA, 0.5 mM Mn2+, or 20 μg/ml KIM 185 were preincubated with sc-uPA (50 nM) in the presence or absence of the αMβ2 function blocking mAbs (as above), washed, and incorporated into fibrin clots. Clot lysis was monitored at 405 nm at 37 °C for up to 5 h. D, PMN were pretreated with the reagents as specified in B and added at 5 × 10⁷/well to a total volume of 150 μl of fresh human platelet-poor plasma. Thrombin (2 NIH units/ml) was added, the plates were incubated at 37 °C, and the absorbance at 600 nm was monitored for up to 20 h. The data are expressed as percent fibrinolysis calculated from the relative clot turbidity after 20 h in the absence of PMNs. The data are the means ± S.E. of quadruplicates measurements and are representative of three experiments.

Fig. 1. PMN-dependent lysis of fibrin clots is influenced by αMβ2 and uPA. A, resting or PMA-stimulated PMNs were incorporated into fibrin clots, which were prepared in microtiter wells. B, in the inhibition experiments, cells were preincubated with the following reagents: 6-aminohexanoic acid (5 mM), Trasylol (100 units/ml), amiloride (300 μM), NIF (100 nM), or F(ab)2 fragments of either function blocking antibodies to uPA, αMβ2 (44A), β2 (TS1/18) or control non-immune mouse IgG (50 μg/ml) and then were added to the wells, which also contained these reagents at the same concentrations. C, PMNs stimulated with 20 nM PMA, 0.5 mM Mn2+, or 20 μg/ml KIM 185 were preincubated with sc-uPA (50 nM) in the presence or absence of the αMβ2 function blocking mAbs (as above), washed, and incorporated into fibrin clots. Clot lysis was monitored at 405 nm at 37 °C for up to 5 h. D, PMN were pretreated with the reagents as specified in B and added at 5 × 10⁷/well to a total volume of 150 μl of fresh human platelet-poor plasma. Thrombin (2 NIH units/ml) was added, the plates were incubated at 37 °C, and the absorbance at 600 nm was monitored for up to 20 h. The data are expressed as percent fibrinolysis calculated from the relative clot turbidity after 20 h in the absence of PMNs. The data are the means ± S.E. of quadruplicates measurements and are representative of three experiments.
PMA-stimulated PMNs. Together, these results verify the prominent role of uPA-mediated Plg activation in the fibrinolytic response of the cells.

The involvement of integrin αMβ2 in fibrinolysis was evaluated using a blocking approach. When neutrophil inhibitory factor (NIF), a high affinity ligand antagonist of αMβ2 (36, 37) was added, it was as effective as the uPA and Plg inhibitors in suppressing clot lysis (Fig. 1B). Similarly, F(ab)2 fragments of two different mAbs to αMβ2, one to the αM and the other to the β2 subunit, were effective in inhibiting PMN-dependent fibrinolysis (Fig. 1B), whereas non-immune mouse F(ab)2 fragments had no effect. These data implicate not only Plg and uPA but also indicate that αMβ2 plays a major role in PMN-mediated lysis of fibrin clots. PMN-dependent fibrinolysis was not completely blocked by the anti-integrin reagents. The participation of plasminogen receptors (14, 38), plasm-in-dependent fibrin degradation by neutrophil proteases (39) or activation of Plg that dissociates from cell surface by cell-bound or released uPA may all contribute to integrin-independent fibrinolysis. We directly assessed the role of the two prominent Plg receptors on PMNs, annexin II (13) and α-endole (14), using blocking mAbs and found that these reagents suppressed fibrinolysis by 15 and 25%, respectively (Fig. 1B), compared with the 50% inhibition by the integrin αMβ2-blocking mAbs.

Because PMA not only induces integrin activation but also stimulates sc-uPA secretion, we investigated whether αMβ2 activation in itself is sufficient to affect PMN-mediated fibrinolysis. When PMNs were incubated with known activators of αMβ2, 0.5 mM MnCl$_2$ (40), or the β2-activating mAb, KIM 185 (27), the cells were not capable of supporting fibrin degradation (not shown); i.e. they behaved the same as non-stimulated PMN (see Fig. 1A). These reagents did induce αMβ2 activation as verified with mAb CBRM 1/5, which reacts with activated αMβ2 and does not induce sc-uPA and uPAR expression on PMN surface as assessed by FACS (not shown). However, when the cells were preincubated with exogenous sc-uPA, washed, and incorporated into fibrin clots, both MnCl$_2$ and KIM 185 induced PMN-mediated fibrinolysis (60 and 45%, respectively) as compared with unstimulated cells (Fig. 1C). The fibrinolysis induced by these reagents was dramatically reduced by αMβ2-blocking mAbs (44A, TS1/18), confirming the crucial role of αMβ2 in the response. In control samples, KIM 185 did not induce fibrinolysis in the absence of cells.

We sought to determine if the role of αMβ2 in fibrinolysis could be demonstrated in a more physiological milieu, i.e. in plasma (Fig. 1D). Isolated PMNs lysed plasma clots within a 20-h period of time, which is a time consistent with such plasma clot lysis assays (41). Similar to the results shown in Fig. 1B, clot lysis in plasma was mediated by urokinase and plasmin, because 50–60% inhibition was observed upon addition of 6-AHA, Trasylol, or a function-blocking mAb to uPA. F(ab)$_2$ fragments of mAbs to αMβ2 and NIF reduced the total clot lysis by 30%, which accounts for approximately 60–70% of uPA-mediated fibrinolysis (Fig. 1D). Thus, the involvement of αMβ2 integrin in PMN-mediated fibrinolysis was observable even in the presence of physiological concentrations of α2-antiplasmin, the major plasma inhibitor of plasmin, and other regulators of fibrinolysis.

Recognition of uPA and Plg by Purified αMβ2—To define the mechanism by which αMβ2 influences fibrinolysis, we examined the capacity of the purified receptor to interact with uPA and Plg. αMβ2 was isolated by affinity chromatography on a mAb (LM2/4, 6.11) (34) from HEK 293 cells expressing the recombining receptor. The inset in Fig. 2A shows that the αMβ2 used was intact and free of detectable contaminants by SDS-PAGE. Also, in an enzyme-linked immunosorbent assay with two different mAbs (clones 3936 and 62022.11), we excluded contamination of αMβ2 preparation with uPAR. The binding functions of purified receptor were evaluated by immobilizing it onto microtiter plates coated with purified αMβ2 or BSA, in the absence or presence of 2 mM MnCl$_2$. The inset characterizes the αMβ2 used to coat the wells by SDS-PAGE. The gel is 10% acrylamide run under reducing conditions. The bound HMW-tc-uPA was quantitated by counting the bound radioactivity in a γ-counter. The data are expressed as means ± S.E. of quadruplets of four representative experiments. B, radiolabeled HMW-tc-uPA was incubated with increasing concentrations (0–100 nM) of purified, recombinant αM or αMβ2 domains and added to αMβ2- or BSA-coated wells. After 4 h, the plates were processed as described. The background binding to BSA, which did not exceed 20% of total binding, has been subtracted. The data are expressed as means ± S.E. of quadruplets of four representative experiments.

![Fig. 2. Interaction of uPA with purified αMβ2](image-url)
immobilized \( \alpha_{M}\beta_2 \) molecule bound a single HMW-tc-uPA molecule. The growth factor domain (GFD) and kringle domain (KD) reside in the N-terminal aspects of single-chain (HMW-sc)-uPA, and thiszymogen form is converted to HMW-tc-uPA by cleavage of a single peptide bond. The capacity of different amounts of various derivatives of uPA to inhibit the binding of 125I-HMW-tc-uPA to \( \alpha_{M}\beta_2 \) was tested. IC\(_{50}\) values, estimated from the inhibition curves, are summarized in Table I. The IC\(_{50}\) values for both HMW-sc-uPA and HMW-tc-uPA (−100 nm) are similar and consistent with the estimated \( K_d \) (40 nm) of HMW-te-uPA for \( \alpha_{M}\beta_2 \), providing validation of these data. The KD was a potent inhibitor with an IC\(_{50}\) of 54 nm, whereas, the GFD, which interacts with uPAR (44), showed no propensity to interact with \( \alpha_{M}\beta_2 \).

When immobilized \( \alpha_{M}\beta_2 \) was preincubated with mAbs (clones 44a and 904) to the \( \alpha_M \) domain, a region of ∼200 amino acids within the \( \alpha_M \) subunit that is involved in the binding of multiple ligands to the integrin (19), 125I-HMW-te-uPA binding was inhibited by 60–80%, whereas normal mouse IgG had no effect (not shown). These results are consistent with the effects of these same mAbs on PMN-dependent fibrin clot lysis (Fig. 1C). Recombinant \( \alpha_I \) domain, but not \( \alpha_M \) domain, inhibited 125I-HMW-te-uPA binding to \( \alpha_{M}\beta_2 \) in a dose-dependent manner (Fig. 2B). Together, these results suggest that interaction of uPA with \( \alpha_{M}\beta_2 \) depends upon engagement of the \( K_d \) of the ligand with the \( \alpha_M \) domain of the receptor. This profile derived with the purified receptor is entirely consistent with results previously obtained with \( \alpha_{M}\beta_2 \)-expressing cells (11).

Having established that u-PA can interact with purified \( \alpha_{M}\beta_2 \), we sought to determine whether Plg itself also could bind to the isolated receptor. 125I-Plg did bind to immobilized \( \alpha_{M}\beta_2 \) in a concentration-dependent and saturable manner (Fig. 3A). Under the same conditions, the binding of the highest concentration of 125I-Plg (14 μM) to BSA-coated wells did not exceed 20% of its binding to the \( \alpha_{M}\beta_2 \)-coated wells, and its binding to the receptor was abrogated (90% inhibition) by 50 μM (a 3.6-fold molar excess) unlabeled Plg. The data in Fig. 3A were analyzed with a Scatchard plot. The linearity of the plot indicated a single class of binding sites and a \( K_d \) of 1 μM, i.e. lower than the physiological concentration of Plg in plasma (2 μM). When the binding of Alexa 488-labeled Plg to \( \alpha_{M}\beta_2 \) reached equilibrium the labeled ligand was replaced with 50 μM unlabeled Plg to assess the reversibility of Plg binding to the integrin. In these kinetic dissociation experiments Plg fell off \( \alpha_{M}\beta_2 \) within 10 min at a dissociation rate constant \( k_{off} = 0.28 \) min\(^{-1}\) and a halftime of 2.5 min (not shown). Thus, Plg binding to the integrin is reversible, although dissociation seems to be rather slow for a low affinity interaction. Additionally, this binding was inhibited in a dose-dependent manner by 6-AHA (85% inhibition by 10 mm) (Fig 3B), implicating the LBS of Plg, which are known to mediate the interaction of Plg with cell surfaces (14, 45), in \( \alpha_{M}\beta_2 \) recognition. Like u-PA, Plg binding to \( \alpha_{M}\beta_2 \) appeared to involve the \( \alpha_M \) domain. The interaction was inhibited by mAbs to the \( \alpha_M \) domain and by the purified \( \alpha_M \) domain, but not by the \( \alpha_I \) domain or mAbs to other regions of the \( \alpha_M \) subunit (Fig. 3C). Although \( \alpha_M \) domain is the major uPAP/Plg binding site, these interactions were also reduced by anti-\( \beta_2 \) mAb indicating that both subunits contribute to recognition, a pattern that is observed with certain other \( \alpha_{M}\beta_2 \) ligands, such as fibrinogen (46).
Integrin αMβ2 Regulation of Plasminogen Activation

**Fig. 4.** αMβ2 simultaneously interacts with u-PA and Plg. A. Biotinylated soluble αMβ2 integrin was immobilized on Streptavidin-agarose and nonspecific binding sites were blocked with biotin. Immobilized αMβ2 integrin on the beads was mixed with combinations of Plg (20 μg/μl) or sc-αMβ2 (10 μg/μl) as indicated. The reactions were preincubated with unlabeled DIP-uPA (B), or radiolabeled 125I-DIP-uPA was preincubated with unlabeled Plg (C). Subsequently, the mixtures were added to and incubated for 2 h with αMβ2 immobilized on 96-well plates. In control samples, the immobilized integrin was preincubated with unlabeled DIP-uPA or Plg, followed by addition radiolabeled uPA or Plg. After washing, the bound radioactivity was counted in a γ-counter. The data are expressed as means ± S.E. of quadruplicates of two representative experiments.

**Simultaneous Interaction of uPA and Plg with αMβ2—Biotinylated αMβ2, captured onto streptavidin-conjugated agarose, was incubated with Plg and/or sc-uPA.** Following washing, bound proteins were eluted from the beads by boiling in SDS-PAGE sample buffer. After SDS-PAGE, nitrocellulose transfers were probed with anti-Plg, anti-uPA, or anti-αM mAbs (Fig. 4A). Blots developed with anti-αM mAbs documented the capture of αMβ2 onto the agarose, and neither Plg nor sc-uPA affected the amount of the integrin bound. Although immobilized αMβ2 was capable of binding both Plg and sc-uPA, unarmored beads failed to bind either ligand. To confirm that both ligands could bind simultaneously to the same receptor, the experiment was repeated using radiolabeled Plg or sc-uPA. With 125I-Plg, increasing amounts of uPA (DPP-inactivated) did not interfere with Plg binding to immobilized αMβ2 (Fig. 4B); and, conversely, with 125I-sc-uPA, increasing amounts of Plg failed to affect the amount of sc-uPA bound (Fig. 4C). The binding of both ligands was reduced by anti-αM and anti-β2 mAbs, indicating specificity. All of the results observed in the direct binding studies shown in Figs. 2–4 were confirmed by FACS analysis using Alexa-488-labeled uPA and Plg (not shown); i.e. PMMA-stimulated PMNs bound the two ligands, and the interactions were suppressed by αMβ2 blocking reagents.

**uPA Binding to αMβ2 Accelerates Plg Activation—**The inhibitory effect of αMβ2 function blocking antibodies on PMN-dependent fibrinolysis and on direct binding of uPA and Plg to purified αMβ2 raised the possibility that these interactions might influence Plg activation. To test this hypothesis, we measured Plm generation in the presence of soluble, purified integrin, and sc-uPA, the inactive form of uPA secreted by PMNs (7, 35). Sc-uPA was incubated in the presence or absence of soluble αMβ2, followed by addition of increasing concentrations of Plg (0–10 μM) and Plg-specific chromogenic substrate (S-2251). Substrate hydrolysis was subsequently monitored at 405 nm. The kinetics of Plg activation by sc-uPA in the presence of αMβ2 followed a Michaelis-type mechanism as evidenced by the linearity of double-reciprocal plots of the data (Fig. 5A, inset). These data were derived at the optimal αMβ2 concentration of 50 nM. As the concentrations of αMβ2 added to sc-uPA (23 nM) and Plg (300 nM) were increased from 0–200 nM, the rates of Plm generation also increased at integral concentrations below 50 nM and then no further increase in Plm generation was observed. In control samples, direct activation of either Plg or sc-uPA by αMβ2 was not detected (not shown). These observations indicate that the potentiation of Plg activation is specifically mediated by αMβ2, rather than by a protease activity contaminating the integrin preparations. In control experiments, tc-uPA activated Plg as expected, but further enhancement of plasmin formation by soluble αMβ2 was not observed.

When compared with Plg activation in the absence of the integrin (Fig. 5A), αMβ2 enhanced the overall efficiency (kcat/Km) of the reaction (4.07 versus 0.067 μM−1 s−1) (Table II) by ~60-fold. The individual kinetic constants derived from these data (Table II) indicate that this increased efficiency was primarily due to a 20-fold reduction in the Km for Plg activation from 2 to 0.1 μM. These data can be most readily interpreted in terms of formation of a ternary complex between Plg, sc-uPA, and the integrin resulting in an increase in the apparent affinity of Plg for uPA, which leads to a reduced Km for Plg activation. αMβ2 increased the catalytic constant kcat only by 3-fold, indicating a less marked effect of the integrin on the dissociation of tc-uPA-plasminogen(ogen) complex.

The kringle domain of uPA is critical for its recognition by αMβ2 but not for its activation of Plg. We utilized this distinction to assess the importance of the uPA-αMβ2 interaction in the enhancement of Plg activation, employing the following recombinant sc-uPAs: wild type (WT-sc-uPA), sc-uPA lacking the KD (ΔK-sc-uPA), and sc-uPA with deleted GFD (ΔGFD-sc-uPA). In the absence of the integrin, the sc-uPA forms used showed low intrinsic Plg activator activities, which were assayed a value of 1 (Fig. 5B). Integrin αMβ2, but not human serum albumin, increased velocity of Plm generation by 17- to 19-fold when WT-sc-uPA or ΔGFD-sc-uPA were added. This effect was not observed with ΔK-sc-uPA, which is unable to bind αMβ2. Moreover, consistent with the direct involvement of the interaction of sc-uPA with αMβ2 in the potentiation of Plg activation, preincubation of the integrin with NIF or recombi-
Experimental Procedures

Detected spectrophotometrically as described under "Experimental Procedures" and are plotted against Plg concentration in a double-reciprocal manner. The linearity of the plot in the presence of αMβ2 is demonstrated in the inset, where the data are replotted on a different scale. The data are the mean of quadruplicates from two separate experiments and gave Km = 2 μM and Vmax = 2.8 nm s⁻¹ in the absence of αMβ2 and Km = 0.1 μM and Vmax = 9.22 nm s⁻¹ in the presence of αMβ2. B, WT-sc-uPA and mutant forms of sc-uPA (23 nm) were incubated in the absence or presence of soluble αMβ2 (50 nm) or control human serum albumin (20 μg/ml), and then Glu-Plg (300 nm) and the Plm-specific chromogenic substrate, S-2251 (0.5 nm) were added. Plm activity of Glu-Plg incubated alone or with αMβ2 was undetectable. The low intrinsic activities of utilized forms of sc-uPA in the absence of integrin were assigned a value 1. C, soluble αMβ2 was either untreated or incubated with NIF (100 nm) or the kringle domain of uPA (50 nm), followed by addition of sc-uPA/Plg mixture. To assess the effect of NIF and KD on the uPA intrinsic activity WT-sc-uPA was treated with these reagents as described above, then Plg was added. Plg activation was detected spectrophotometrically as described under "Experimental Procedures." The data are expressed as means ± S.E. of quadruplicates of two representative experiments.

Determine whether αMβ2, Plg, and uPA could co-associate on the leukocyte surface. Plg was added to resting or PMA-stimulated PMNs, followed by lysis of the cells and immunoprecipitation with a non-function blocking mAb (OKM1) to the αM subunit. Immunoprecipitates were analyzed on Western blots with anti-Plg immunoprecipitated with the integrin subunit. Because αMβ2-mediated assembly of the Plg system on PMNs—To determine whether αMβ2, Plg, and uPA could co-associate on the leukocyte surface, Plg was added to resting or PMA-stimulated PMNs, followed by lysis of the cells and immunoprecipitation with a non-function blocking mAb (OKM1) to the αM subunit. Immunoprecipitates were analyzed on Western blots with antibodies to Plg, uPAR, uPA, or αM (Fig. 7A). Non-immune mouse IgG showed no protein bands in the αM, Plg, uPAR, or uPA regions of the gels, and OKM1 did not react with Plg, uPA, and uPAR (not shown), establishing the specificity of the analytical system. This anti-αM mAb immunoprecipitated similar amounts of the subunit from both resting and stimulated PMNs as indicated in the blot probed with a second anti-αM mAb (ICO-GH1). A faint band of uPAR was detected in αM immunoprecipitates from unstimulated cells incubated in the absence of Plg in agreement with previous data (22, 23) demonstrating direct interaction between αMβ2 and uPAR. When Plg was added, uPAR and uPA immunoprecipitated with the integrin subunit. Because both αMβ2 and uPAR are translocated from intracellular granules to the cell surface in PMA-stimulated PMNs (7, 50) and uPA interacts with both receptors (11), consistently ~20-fold more uPA was present in complexes from stimulated as compared with resting PMNs. Notably, upon stimulation with PMA, substantial amounts of Plg co-precipitated with the αM subunit from the

Table II

Comparison of the apparent kinetic parameters for Glu-Plg activation by uPA in the presence and absence of purified αMβ2

| Kinetic parameters | Absence of αMβ2 | Presence of αMβ2 |
|-------------------|----------------|-----------------|
| Km (μM)           | 2              | 0.1             |
| kcat (s⁻¹)        | 0.135          | 0.405           |
| kcat/Km (μM⁻¹ s⁻¹)| 0.087          | 4.07            |

Figure 5. Urokinase binding to integrin αMβ2 enhances Plg activation. A, WT-sc-uPA (23 nm) was incubated with varying concentrations of Glu-Plg (0–10 μM) in the absence or presence of soluble αMβ2 (50 nm). Initial rates of Plg generation were determined as described under "Experimental Procedures" and are plotted against Plg concentration in a double-reciprocal manner. The linearity of the plot in the presence of αMβ2 is demonstrated in the inset, where the data are replotted on a different scale. The data are the mean of quadruplicates from two separate experiments and gave Km = 2 μM and Vmax = 2.8 nm s⁻¹ in the absence of αMβ2 and, Km = 0.1 μM and Vmax = 9.22 nm s⁻¹ in the presence of αMβ2. B, WT-sc-uPA and mutant forms of sc-uPA (23 nm) were incubated in the absence or presence of soluble αMβ2 (50 nm) or control human serum albumin (20 μg/ml), and then Glu-Plg (300 nm) and the Plm-specific chromogenic substrate, S-2251 (0.5 nm) were added. Plm activity of Glu-Plg incubated alone or with αMβ2 was undetectable. The low intrinsic activities of utilized forms of sc-uPA in the absence of integrin were assigned a value 1. C, soluble αMβ2 was either untreated or incubated with NIF (100 nm) or the kringle domain of uPA (50 nm), followed by addition of sc-uPA/Plg mixture. To assess the effect of NIF and KD on the uPA intrinsic activity WT-sc-uPA was treated with these reagents as described above, then Plg was added. Plg activation was detected spectrophotometrically as described under "Experimental Procedures." The data are expressed as means ± S.E. of quadruplicates of two representative experiments.

Indicate that binding of uPA via its KD to αMβ2 is essential for stimulation of Plg activation.

αMβ2 Delays Plm Inactivation by α2-Antiplasmin—The involvement of the LBS in Plg binding to αMβ2 implies that Plm will also bind to the receptor and further suggests that receptor binding might influence its inactivation by its primary physiological inhibitor, α2-antiplasmin, which interacts initially with the LBS and then with the active site of Plm (8, 47, 48). This prediction was tested. First, the capability of αMβ2 and its I domain to compete with α2-antiplasmin for Plm binding was examined. As shown in Fig. 6A, both αMβ2 and αM I domains, but not αM I domain, suppressed Plm binding to immobilized α2-antiplasmin. These results are consistent with the interaction of Plg with the αM I domain and suggest that Plm also binds to this domain of αMβ2. Next, we tested the capacity of Plm bound to αMβ2 to be inhibited by α2-antiplasmin (Fig. 6B).Plm was preincubated with or without soluble αMβ2 followed by addition of the Plm chromogenic substrate S-2251. Plm activity was monitored by the changes of absorbance at 405 nm (Fig. 6B). The activities of free and αMβ2-complexed Plm were similar, indicating that integrin binding did not influence Plm activity. At 2 min, α2-antiplasmin was added. In the absence of αMβ2, rapid and complete inhibition of Plm activity by the inhibitor was achieved. In contrast, αMβ2 suppressed the inhibition of Plm activity by α2-antiplasmin. At the physiological concentration of α2-antiplasmin (∼70 μg/ml), the delay in inactivation by the integrin was ~7 min. The slow but time-dependent decrease in Plm activity in the presence of the integrin may reflect rapid neutralization as Plm dissociates from Plm bound to the integrin. The protective effect of αMβ2 on Plm inactivation by α2-antiplasmin is consistent with that observed upon its binding to cell surfaces (8) as well as other candidate Plg receptors (14, 49).

αMβ2-Mediated Assembly of the Plg System on PMNs—To determine whether αMβ2, Plg, and uPA could co-associate on the leukocyte surface, Plg was added to resting or PMA-stimulated PMNs, followed by lysis of the cells and immunoprecipitation with a non-function blocking mAb (OKM1) to the αM subunit. Immunoprecipitates were analyzed on Western blots with antibodies to Plg, uPAR, uPA, or αM (Fig. 7A). Non-immune mouse IgG showed no protein bands in the αM, Plg, uPAR, or uPA regions of the gels, and OKM1 did not react with Plg, uPA, and uPAR (not shown), establishing the specificity of the analytical system. This anti-αM mAb immunoprecipitated similar amounts of the subunit from both resting and stimulated PMNs as indicated in the blot probed with a second anti-αM mAb (ICO-GH1). A faint band of uPAR was detected in αM immunoprecipitates from unstimulated cells incubated in the absence of Plg in agreement with previous data (22, 23) demonstrating direct interaction between αMβ2 and uPAR. When Plg was added, uPAR and uPA immunoprecipitated with the integrin subunit. Because both αMβ2 and uPAR are translocated from intracellular granules to the cell surface in PMA-stimulated PMNs (7, 50) and uPA interacts with both receptors (11), consistently ~20-fold more uPA was present in complexes from stimulated as compared with resting PMNs. Notably, upon stimulation with PMA, substantial amounts of Plg co-precipitated with the αM subunit from the
In the present study, we have shown that integrin αMβ2 participates in Plg activation on the surface of human PMNs and, thereby, influences the fibrinolytic response of these cells. Specifically, we have provided evidence that 1) stimulated PMNs have the ability to lyse fibrin clots in an integrin-dependent manner and 2) the mechanism underlying this involvement depends upon the direct and simultaneous binding of uPA and Plg to the integrin. These interactions were demonstrable with purified αMβ2 and on the surface of PMNs. The concurrent binding of sc-uPA and Plg to αMβ2 enhanced Plm formation by ~60-fold, primarily due to 20-fold decrease in Km, uPA binding to αMβ2 via its kringle domain is crucial for this effect; a mutant form of sc-uPA lacking the kringle, 4K-sc-uPA, failed to enhance Plg activation. Demonstrating the importance of Plg binding to αMβ2 in this effect, 6-AHA, which interferes with the LBS activities of Plg but does not inhibit uPA binding, suppressed the integrin-dependent enhancement of Plg activation. The capacity of uPA, Plg, and αMβ2 to interact on intact PMNs was detected by co-immunoprecipitation, and each of these interactions was necessary for αMβ2 to support enhanced Plg activation on the PMN surface. An additional important consequence of the binding of Plg/Plm to αMβ2 is a substantial delay in the inhibition of Plm activity by α2-antiplasmin. It has been estimated that blockade of the LBS of Plm prolongs its half-life with α2-antiplasmin by ~50-fold (48). Consequently, the overall effect of αMβ2 on Plm activity may be ~3000-fold.

Thus, the mechanism underlying the capacity of stimulated PMNs to induce fibrinolysis depends upon the formation of a complex between Plg, sc-uPA, and αMβ2, and the functional consequences of these interactions on Plm activity.

Purified and immobilized αMβ2 bound uPA. This interaction is mediated primarily by the αM domain based upon inhibition by: (a) recombinant αM domain but not αI domain; (b) NIF, a high affinity αM domain ligand (11); and (c) mAbs, which bind to the domain (not shown). Optimal recognition of uPA may also involve the β2 subunit, based upon mAb inhibition, and appears to require activation of αMβ2, as evidenced by the enhanced interaction observed in the presence of Mn2+. Mn2+ activates αMβ2 by inducing an “open” conformation of the αM domain (40). In addition, purified αMβ2 bound Plg, and Plg co-immunoprecipitates with αMβ2 from the lysates of PMNs, indicating that this interaction can occur on the cell surface. It has been shown that cell binding of intact Glu-Plg facilitates its conversion to Lys-Plg, which lacks 80–90 amino acids from the N terminus and is more readily activated to Plm (51). Whether this mechanism contributes to the increase in Plg activation induced by αMβ2 remains to be tested. The C-terminal lysine analog, 6-AHA, inhibited Plg binding to the integrin, implicating the LBS of Plg in the interaction (12, 52). Thus, αMβ2 resembles other Plg/Plm receptors, including integrins αIbβ2 (15) and αIIbβ3 (53), as well as many non-integrin Plg receptors, including annexin II (13) and α-enolase (14), with regard to involvement of the LBS in recognition. Although αMβ2 apparently does not contain a C-terminal lysine, certain internal basic amino acids can mimic these and interact with LBS (54). We cannot exclude limited proteolysis of the αMβ2 preparations that might generate a C-terminal lysine; however, the αMβ2 preparations seemed to be intact by SDS-PAGE, and the involvement of the internally located αM domain would argue
against this possibility. The role of the LBS in αMβ2 recognition of Plg implicates kringle 1 and/or 5 in the interaction (55). Because the kringle of uPA lacks LBS activity (56), it is reasonable that both uPA and Plg could bind simultaneously to αMβ2 via their kringle as we have found and yet not compete with one another.

Although other Plg binding molecules are expressed on PMN, notably annexin II (13) or α-enolase (14), αMβ2 appears to account for ~40% of total Plg binding capacity of PMA-stimulated PMNs under the conditions of our analyses, whereas 15 and 20% inhibition was achieved with anti-α-enolase or anti-annexin II mAbs. Additionally, uPA-mediated Plg activation on PMN surface was reduced by 50% by laser or anti-annexin II mAbs. Additionally, uPA-mediated Plg activation in the absence of uPAR is not without precedence. Nevertheless, on the surface of PMN, we were able to implicate uPAR in Plg activation with uPAR mAbs. This involvement is consistent with our previous data implicating uPAR in αMβ2-dependent cell migration (11) as well as the results of other studies (23, 24). Potentially, uPAR may serve as an enhancer of αMβ2 functions by presenting uPA to the integrin, or by influencing the activation of the integrin, which is necessary for uPA binding. Either of these effects of uPAR would enhance the apparent affinity of αMβ2 for uPA, which is relatively low (K_d ~ 40 nM) compared with that uPAR (K_d ~ 1 nM). Consequently, uPAR, uPA, αMβ2, and Plg may form a multicontact, tetramolecular complex on the PMN surface, which profoundly influences the thrombolytic functions.

Plg interacted with αMβ2 as a soluble ligand, and this interaction was not inhibited by Fg. We have also observed that Fg does not inhibit PMN adhesion to Plg (not shown). These observations suggest that αMβ2 can recognize Plg even in the face of a vast excess of another ligand of the integrin. We have previously shown that Fg also is a poor inhibitor of uPA binding to αMβ2, reducing the interaction by only ~40% at physiological concentrations of Fg (11). Our observation that αMβ2 can mediate plasmin-dependent fibrinolysis (Fig. 1B) adds credence to the biological significance of the recognition of fibrinolytic component occurrence by the integrin. Previously, Simon et al. (61) implicated αMβ2 in plasmin-independent fibrin clearance involving internalization by monocytes. Our data suggest a second role for the integrin in fibrinolysis, a Plg-dependent func-
tion. The capacity of αMβ2 to bind and activate Plg may also be particularly relevant to migration and invasion of PMNs and other leukocytes during inflammatory responses. This mechanism may contribute to the blunting of inflammatory responses observed in mice deficient in αMβ2 (4) and components of the Plg system (2, 3).

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REFERENCES

1. Plow, E. F., Herren, T., Redlitz, A., Miles, L. A., and Hoover-Plow, J. L. (1995) Thromb. Diath. Haemorrh. 73, 35–39
2. Ploplis, V. A., French, E. L., Carmeliet, P., Collen, D., and Plow, E. F. (1998) J. Biol. Chem. 273, 24519–24527
3. Gyetko, M. R., Libre, E. A., Fuller, J. A., Chen, G.-H., and Toews, G. B. (1999) J. Biol. Chem. 274, 23243–23254
4. Mizgerd, J. P., Kubo, H., Kutkoski, G. J., Bhagwan, S. D., Scharffetter-Kochanek, K., Beaudet, A. L., and de Boer, R. H. (1997) J. Exp. Med. 186, 1337–1344
5. Moir, E., Booth, N. A., Bennett, B., and Ribe, K., Al-Mehdi, A. B., Mazaa, A., Cines, D. B., and Higazi, A. A. (2000) FASEB J. 14, 1411–1422
6. Nagar, T., Haj-Yehia, A., Akkawa, S., Kuo, A., Bdeir, K., Mazaa, A., Cines, D. B., and Higazi, A. A. (2000) J. Biol. Chem. 277, 40499–40504
7. Robinson, M. K., Andrew, D., Rosen, H., Brown, D., Oettle, S., and Stephens, P. E. F. (1992) J. Immunol. 148, 1080–1085
8. Miles, L. A., and Plow, E. F. (1987) Thromb. Haemost. 58, 936–942
9. Facone, D. J., Borth, W., Khan, K. M. F., and Hajjar, A. A. (2001) Blood 97, 777–784
10. Wu, S. C., Castellino, F. J., and Wong, S. L. (2003) J. Biol. Chem. 278, 18199–18206
11. Hajjar, L., Friedenburg, J., and Nesheim, M. (1990) J. Biol. Chem. 265, 16945–16954
12. Miles, L. A., and Plow, E. F. (1985) J. Biol. Chem. 260, 4303–4311
13. Wei, Y. Luksheev, M., Simon, D. I., Bodary, S. C., Rosenberg, S., Davie, M. Y., and Chapman, H. A. (1990) Science 253, 1551–1555
14. Miller, L. J., Wiebe, J., and Springer, T. A. (1987) J. Immunol. 138, 2381–2383
15. Heipke, J. M., and Ososkow, L. (1986) J. Exp. Med. 164, 844–846
16. Zhang, L., and Plow, E. F. (1997) J. Biol. Chem. 272, 17558–17564
17. Rieu, P., Ueda, T., Haruta, I., Sharma, C. P., and Arnaout, M. A. (1994) J. Cell Biol. 127, 2081–2091
18. Palone, D. J., Borth, W., Mathew, J., Guevara, C., and Hajjar, K. A. (1995) FASEB J. 9, 2388a
19. Plow, E. F., Gramaz, M., and Havemann, K. (1988) J. Lab. Clin. Med. 102, 858–869
20. Li, R. P., Griffith, D. L., Scott, D., and Arnaout, M. A. (1988) J. Cell Biol. 134, 1523–1534
21. Taylor, F. R., and Lockhart, M. S. (1986) Thromb. Res. 37, 639–649
22. Smith, J. W., Portwicz, R. S., and Mathis, D. (1994) J. Biol. Chem. 269, 960–967
23. Muchowski, P. J., Zhang, L., Chang, E. R., Soule, H. E., Plow, E. F., and Move, M. (1994) J. Biol. Chem. 269, 26419–26423
24. Appellia, E., Robinson, K. A., Ullrich, S. J., Stoppeil, M. P., Corti, A., Cassani, G., and Bital, F. (1987) J. Biol. Chem. 262, 4437–4440
25. Plow, E. F., Freaney, D. E., and Plow, E. F. (1993) J. Clin. Invest. 92, 1467–1476
26. Morris, J. P., and Castellino, F. J. (1988) Biochim. Biophys. Acta 705, 264–270
27. Plow, E. F., Freaney, D. E., Plescia, J., and Miles, L. A. (1986) J. Cell Biol. 103, 2441–2452
28. Kassam, G., Le B. H., Chai, K.-S., Kang, H.-M., Fitzpatrick, S.-L., Lou, P., and Warsman, D. M. (1998) Biochemistry 37, 16958–16966
29. Sengelov, H., Kjeldsen, L., Diamond, M. S., Springer, T. A., and Porrenga, N. (1993) J. Clin. Invest. 92, 1467–1476
30. Miles, L. A., Castellino, F. J., and Gong, Y. (2003) Trends Cardiovasc. Med. 13, 21–30
31. Longstaff, C., Merton, E., Miles, L. A., Ruf, W., and Tukada, Y. (2002) J. Biol. Chem. 277, 33564–33570
32. Nissenweiz, W., Schenkel, W. J. G., Yonekawa, O., Tesser, G. I., and Voskuilen, M. (1990) J. Exp. Med. 181, 2381–2383
33. Bohuslav, J., Horejsi, V., Hansmann, C., Stock, J., Weide, U. H., Majdic, O., Bartke, J., Knapp, W., and Stockinger, H. (1995) J. Exp. Med. 181, 1331–1340
34. Simon, D. I., Rao, N. K., Xu, H., Wei, Y., Majdic, O., Ronne, E., Kobzik, L., and Chapman, H. A. (1996) Blood 88, 3185–3194
35. Sitrin, R. G., Todd, F. F., III, Petty, H. R., Brock, T. G., Shollenger, S. B., Albrecht, E., and Gyetko, M. R. (1996) J. Clin. Invest. 97, 1942–1951
36. Haj-Yehia, A., Nazzar, T., Sachais, B., Kuo, A., Bdeir, K., Al-Mehdi, A. B., Mazaa, A., Cines, D. B., and Higazi, A. A. (2000) FASEB J. 14, 1411–1422
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