We have analyzed tyrosine phosphorylation associated with retraction of the fibrin clot by washed platelets in purified fibrinogen. Retraction was dependent on integrin αIIbβ3, based on absence of retraction of αIIbβ3-deficient thrombasthenic platelets. However, only a subset of αIIbβ3-blocking antibodies or peptides were able to inhibit retraction, suggesting a differential engagement of αIIbβ3 in fibrin clot retraction versus aggregation. Immunoblotting demonstrated a phosphorylated protein pattern comparable with aggregation at early time points. However, as opposed to aggregation, tyrosine phosphorylation decreased rapidly in parallel to retraction (up to 60 min). Dephosphorylation was αIIbβ3-dependent, since it was blocked by αIIbβ3-specific inhibitors and was absent in thrombasthenic platelets. Inhibition of platelet clot retraction by phenylarsine oxide and peroxovanadate, suggested a role for tyrosine phosphatases. Cytochalasin D and E (5 μM) blocked fibrin clot retraction and tyrosine dephosphorylation, suggesting regulation by actin cytoskeleton assembly. Tyrosine phosphatase activities were found associated with clot retraction using the "in-gel" tyrosine phosphatase assay; however, none were αIIbβ3-dependent. An 85-kDa protein and to a lesser degree "Src" showed the closest dose-dependent correlation between inhibition of tyrosine dephosphorylation and inhibition of retraction. We thus postulate that αIIbβ3 engagement in fibrin clot retraction drives, in an actin cytoskeleton-dependent manner, the interaction of tyrosine phosphatases and of the tyrosine-phosphorylated substrates 85-kDa protein and Src, the dephosphorylation of which regulates the force generation and/or transmission required for full contraction of the fibrin matrix.

Matrix retraction (or contraction) is a cellular event subsequent to cell matrix adhesion that is biologically significant, since it is involved in such relevant phenomena as morphogenesis during embryogenesis, wound healing, or the final steps of hemostasis (1, 2). Cellular matrix retraction corresponds macroscopically to the reduction of the matrix volume, due to the activity of cells that actively reorganize the extracellular matrix by shortening and thickening matrix fibers (3). Matrix contraction is exhibited by numerous cell types, including smooth muscle cells, fibroblasts, monocytes, endothelial cells, or platelets. Although the underlying mechanisms involve integrin engagement, cytoskeletal reorganization, and force generation by molecular motors (1, 2), the exact molecular requirements for each of these steps as well as their coordination are still poorly understood.

Platelets provide a particularly attractive model with which to address the issue of matrix retraction, since 1) platelets are easy to isolate and to analyze, 2) they develop a contractile activity in fibrin clot that is easy to assess, and 3) retraction activity is linked to engagement of a well characterized integrin, αIIbβ3 (also termed glycoprotein IIb-IIIa, or gpIIb-IIIa). αIIbβ3 is the most abundant integrin at the platelet surface (4) and acts as a fibrinogen receptor in platelet aggregation. Both structure-function relationships and the signaling pathways triggered by αIIbβ3 engagement have been defined in detail for aggregation and adhesion (5). In contrast, engagement of αIIbβ3 in fibrin clot retraction has been the subject of only a limited number of studies and remains poorly understood. The first evidence for a role of αIIbβ3 in retraction has been deduced from studies of a human hereditary hemorrhagic condition termed Glanzmann thrombasthenia, which is characterized by a quantitative or a qualitative defect in αIIbβ3 (6). In this recessive disorder, bleeding tendency is associated with the inability of the patient’s platelets to aggregate and to retract a fibrin clot. More direct evidence for a role of αIIbβ3 in fibrin clot retraction was gained from studies using αIIbβ3-specific monoclonal antibodies and αIIbβ3 antagonist peptides (7–9). In addition, a differential engagement of αIIbβ3 in fibrin clot retraction versus aggregation has been suggested (10, 11). Altogether, these studies have demonstrated that αIIbβ3 is an important component of fibrin clot retraction, but the presence of plasma in most studies has precluded more refined biochemical characterization as well as the study of retraction-associated platelet signaling.

Signaling associated with platelet aggregation, with adhesion to immobilized adhesive proteins (fibrinogen, fibrin, von Willebrand factor), or with spreading has been extensively studied (for a review, see Ref. 5). In these experimental settings, signaling as mediated by αIIbβ3 was shown to involve tyrosine kinases, including Syk, Fak, and Src, as well as tyrosine phosphatases, such as PTP1B (5, 12, 13). In contrast, signaling associated with clot retraction by platelets has been the subject of only a few reports. These include the observation that tyrosine kinase inhibitors inhibit fibrin clot retraction (14), a study of calpain engagement in clot relaxation, in which partial inhibition of retraction was restored by the calpain inhibitor calpeptin (15), and a study on Rho-A, an actin cytoskeleton regulator of the Rho family of small G-proteins, for which no involvement in clot retraction could be demonstrated (16). Finally, a report demonstrated that knock-in mice expressing the conservative mutations Tyr → Phe747 and Phe759 of the β3 cytoplasmic tail exhibited platelets with altered clot retraction ability (17), suggesting a role for β3 cytoplasmic tail tyrosine phosphorylation in clot retraction. However signaling events associated with clot retraction by...
tion were not examined. To date, no thorough analysis of signal- and particularly protein tyrosine phosphorylation during clot retraction has been reported.

In the present paper, we analyze retraction of a fibrin clot by washed platelets in a plasma-free system. We confirm that like in platelet-rich plasma, α1β3 is central to retraction, based on experiments using α1β3-specific antagonists or α1β3-deficient thrombasthenic platelets. Next, we demonstrate that clot retraction is associated with a strong and short initial wave of tyrosine phosphorylation, followed by a sustained α1β3-dependent tyrosine dephosphorylation of several polypeptides. Dephosphorylation parallels retraction, is specifically blocked by α1β3 blockers, and is absent in thrombasthenic platelets. Dephosphorylation is specific for retraction, since it is not observed with thrombin alone. In turn, tyrosine phosphatase inhibitors block clot retraction, thus suggesting a functional role for tyrosine phosphatases in retraction. Tyrosine dephosphorylation was inhibited by actin polymerization inhibitors. Tyrosine phosphatase activities were found associated with clot retraction using the “in-gel” tyrosine phosphatase assay, but none were α1β3-dependent. Thus, α1β3 engagement in retraction does not induce specific sets of tyrosine phosphatases but rather may drive the specific interaction of preexisting tyrosine phosphatases with tyrosine-phosphorylated substrates. Among the latter, an 85-kDa protein exhibited the highest sensitivity to phenylarsine oxide (PAO). We conclude that α1β3 engagement in fibrin clot retraction drives, in an actin cytoskeleton-dependent manner, the interaction of tyrosine phosphatases and of specific substrates and particularly an 85-kDa protein, the dephosphorylation of which may be involved in force generation and/or force transmission required for full contraction of the fibrin matrix.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human fibrinogen was purchased from Stago (Courbevoie, France) or Kordia (Leiden, The Netherlands). Sodium orthovanadate (NaVO₄), H₂O₂, PAO, genistein, erstatin A, RGDS, RGES, the γ-400–411 dodecapeptide, cytochalasin D and E, and the monoclonal anti-actin IgG were from Sigma-Aldrich (Meylan, France). α1β3-specific monoclonal antibodies were kindly provided by Dr B. S. Coller for α1β3-deficient thrombasthenic platelets. Next, we demonstrate that clot retraction is associated with a strong and short initial wave of tyrosine phosphorylation, followed by a sustained α1β3-dependent tyrosine dephosphorylation of several polypeptides. Dephosphorylation paralleled retraction, is specifically blocked by α1β3 blockers, and is absent in thrombasthenic platelets. Dephosphorylation is specific for retraction, since it is not observed with thrombin alone. In turn, tyrosine phosphatase inhibitors block clot retraction, thus suggesting a functional role for tyrosine phosphatases in retraction. Tyrosine dephosphorylation was inhibited by actin polymerization inhibitors. Tyrosine phosphatase activities were found associated with clot retraction using the “in-gel” tyrosine phosphatase assay, but none were α1β3-dependent. Thus, α1β3 engagement in retraction does not induce specific sets of tyrosine phosphatases but rather may drive the specific interaction of preexisting tyrosine phosphatases with tyrosine-phosphorylated substrates. Among the latter, an 85-kDa protein exhibited the highest sensitivity to phenylarsine oxide (PAO). We conclude that α1β3 engagement in fibrin clot retraction drives, in an actin cytoskeleton-dependent manner, the interaction of tyrosine phosphatases and of specific substrates and particularly an 85-kDa protein, the dephosphorylation of which may be involved in force generation and/or force transmission required for full contraction of the fibrin matrix.

**RESULTS**

**Plasma-free Clot Retraction Assay Is Dependent upon α1β3 Integrin Engagement**—We have developed a plasma-free retraction assay using purified fibrinogen and washed platelets. Photographs of a typical control clot retraction are shown in Fig. 1A (upper panel) from 0 to 60 min. The extent of retraction was assessed from quantitation of clot surface area (see “Experimental Procedures”) and was expressed as percentage of total clot surface versus time, as illustrated in Fig. 1A, lower panel, control curve. In preliminary experiments (not shown), we have tested varying conditions including thrombin concentrations from 0.1 to 4 units/ml and Ca²⁺ concentrations from 1 to 10 mM, at the physiological fibrinogen concentration of 2 mg/ml. No difference was found in retraction kinetics (nor in protein tyrosine phosphorylation patterns). We therefore considered our conditions non-limiting and chose arbitrarily to perform experiments at concentrations of 1 mM Ca²⁺ and 1 unit/ml thrombin.

Previous studies have demonstrated that α1β3 integrin involvement in retraction of fibrin clot in plasma (7–10). To verify the engagement of α1β3 in our plasma-free conditions, we used various α1β3-specific inhibitors (Fig. 1). Among several α1β3-blocking monoclonal antibodies (Fig. 1A, lower panel), the β3-specific monoclonal antibody 7E3 inhibited retraction efficiently (80%), confirming previous observations (7). In addition, combi-
nations of monoclonals, ineffective when used alone, such as AP3 (β₃-specific) and Tab (α₃b₃-specific), or either one in combination with 10E5 (α₁b₃-specific) also blocked retraction efficiently. Surprisingly, the aggregation-blocking 10E5 (as well as AP2, not shown) was unable to block retraction when used alone. This suggests a differential engagement of α₁b₃ with fibrin compared with fibrinogen and/or in retraction compared with aggregation.

In Fig. 1A, a comparative analysis was conducted with the α₁b₃ antagonists RGDS and γ₄₀₀–₄₁₁ dodecapeptide and the highly specific peptide Integrilin®. Integrilin® inhibited retraction efficiently (82 ± 11% inhibition at 25 μM), confirming the specific engagement of α₁b₃ in fibrin clot retraction. Of note, neither RGDS nor the γ₄₀₀–₄₁₁ dodecapeptide had any effect; this result together with the higher concentration of Integrilin® required for full inhibition of retraction (25 μM) versus aggregation (2 μM; Ref. 23 and data not shown) suggests higher affinity/avidity of fibrin for α₁b₃ compared with fibrinogen, possibly due to the polymeric state of the former.

In Fig. 1C, α₁b₃ engagement in fibrin clot in our assay conditions was confirmed by the low extent of retraction of platelets from a type I thrombasthenic patient exhibiting no detectable α₁b₃ by Western blotting (see Refs. 26 and 28; data not shown). This result is comparable with that obtained with the monoclonal antibody mix Tab + AP3.

Altogether, our data 1) confirm the involvement of α₁b₃ integrin in fibrin clot retraction in plasma-free conditions, and thereby validate our assay and 2) suggest differential engagement of α₁b₃ in retraction versus aggregation.

**Fibrin Clot Retraction Kinetics Correlates with Protein Tyrosine Dephosphorylation** — We then asked whether differential engagement of α₁b₃ corresponded to differential α₁b₃ signaling; we thus assessed tyrosine phosphorylation associated with clot retraction, since it is a prominent signaling pathway triggered by α₁b₃ (5). Fig. 2 shows phosphotyrosine protein patterns during retraction, as assessed by Western blotting and the corresponding band intensity quantitation. In Fig. 2A, a time course of phosphotyrosine protein phosphorylation during fibrin retraction with normal platelets showed an initial pattern comparable with platelet aggregation (26), including a 125/130- and a 100/105-kDa band (the latter slightly altered in migration by a 100-kDa nonphosphorylated protein identified as the cross-linked γγ fibrinogen/fibrin chain dimer; data not shown), a faint 85-kDa band, cortactin as a 77–80-kDa doublet, a 64-kDa band, Src as a 60-kDa band, and a 48-kDa band. Cortactin and Src have been identified previously by immunoprecipitation (28). This pattern evolved with time, exhibiting a short initial wave of phosphorylation culminating at 5 min, followed by a slower wave of dephosphorylation (see “Experimental Procedures”). Values are plotted as a function of time. Control platelets are shown by open diamonds (continuous line). Monoclonal antibodies used were 10E5 (anti-α₁b₃, open diamonds, dashed line), Tab (anti-α₁b₃, open circles, dashed line), AP3 (anti-β₃, open squares, dotted line), 7E3 (anti-β₃, closed squares, dashed line), or mixes: 10E5 + AP3 (closed triangles, continuous line), 10E5 + Tab (closed triangles, dotted line), 7E3 + Tab (closed squares, dotted line), or Tab + AP3 (closed circles, continuous line). Data correspond to means ± S.E. from 5–9 experiments. B, inhibition of retraction by α₁b₃-specific peptide antagonists. Platelets were preincubated with antagonists at 37 °C for 10 min prior to the addition of fibrinogen and clot initiation by thrombin. Antagonists were RGDS (striped bar), the γ₄₀₀–₄₁₁ dodecapeptide from fibrinogen (horizontal striped bar), and Integrilin® (closed bars). Values are maximal retraction obtained at 60 min and are expressed as means ± S.E. from three experiments for RGDS and γ₄₀₀–₄₁₁ dodecapeptide and nine experiments for Integrilin®. C, defective clot retraction in plasma-free conditions with α₁b₃-deficient thrombasthenic platelets. Platelets from thrombasthenic patient CB (open circles) were washed and subjected to clot retraction in buffer, comparatively with control platelets (closed diamonds) or control platelets added with Tab + AP5 mix (20 μg/ml each) (closed triangles). Values are mean ± S.E. from one experiment in triplicate.

**Fig. 1.** Fibrin clot retraction in plasma-free conditions is mediated by integrin α₁b₃ engagement. A, kinetics of fibrin clot retraction in buffer and washed platelets and its inhibition by α₁b₃-specific monoclonal antibodies. Platelets were washed and resuspended at 3 x 10⁶/ml in buffer (see “Experimental Procedures”) in the presence of 2 mg/ml human fibrinogen, and fibrin clot was initiated by 1.0 unit/ml bovine thrombin at 37 °C. Upper panel, time frames of a retracting clot at the indicated time points. Lower panel, clot retraction kinetics curves in the absence or the presence of anti-α₁b₃ monoclonal antibodies. Clot surface areas were assessed by digital processing and plotted as percentage of maximal retraction (i.e. volume of platelet suspension) (see “Experimental Procedures”). Values are plotted as a function of time. Control platelets are shown by open diamonds (continuous line). Monoclonal antibodies used were 10E5 (anti-α₁b₃, open diamonds, dashed line), Tab (anti-α₁b₃, open circles, dashed line), AP3 (anti-β₃, open squares, dotted line), 7E3 (anti-β₃, closed squares, dashed line), or mixes: 10E5 + AP3 (closed triangles, continuous line), 10E5 + Tab (closed triangles, dotted line), 7E3 + Tab (closed squares, dotted line), or Tab + AP3 (closed circles, continuous line). Data correspond to means ± S.E. from 5–9 experiments. B, inhibition of retraction by α₁b₃-specific peptide antagonists. Platelets were preincubated with antagonists at 37 °C for 10 min prior to the addition of fibrinogen and clot initiation by thrombin. Antagonists were RGDS (striped bar), the γ₄₀₀–₄₁₁ dodecapeptide from fibrinogen (horizontal striped bar), and Integrilin® (closed bars). Values are maximal retraction obtained at 60 min and are expressed as means ± S.E. from three experiments for RGDS and γ₄₀₀–₄₁₁ dodecapeptide and nine experiments for Integrilin®. C, defective clot retraction in plasma-free conditions with α₁b₃-deficient thrombasthenic platelets. Platelets from thrombasthenic patient CB (open circles) were washed and subjected to clot retraction in buffer, comparatively with control platelets (closed diamonds) or control platelets added with Tab + AP5 mix (20 μg/ml each) (closed triangles). Values are mean ± S.E. from one experiment in triplicate.
phorylation, which paralleled most of the retraction extent (Fig. 2B). This phenomenon was not due to protein degradation, as ascertained by even Ponceau S patterns (not shown) and replotting of the blot with anti-actin (bottom panel, actin). Dephosphorylation was not due to thrombin stimulation per se, since platelets stimulated with thrombin in the absence of added fibrinogen yielded a tyrosine phosphorylation pattern that remained steady with time, as opposed to platelets in conditions of retraction (Fig. 2C). Thus, the pattern of tyrosine phosphorylation, characterized by a short initial wave of tyrosine phosphorylation followed by a slower dephosphorylation pattern, is specific to fibrin clot retraction.

Tyrosine Dephosphorylation Is Dependent on αIIbβ3 Integrin Engagement—Fig. 3 shows that when retraction was inhibited by the retraction-blocking association of anti-αIIbβ3 (Tab, AP-3) monoclonal antibodies (middle panel), initial tyrosine phosphorylation occurred in all conditions but was not followed by dephosphorylation, as opposed to control platelets (left panel). The same absence of dephosphorylation was noted when retraction was blocked by the synthetic inhibitor Integrilin® (not shown) or when thrombasthenic platelets lacking αIIbβ3 were used (right panel). The correlation in kinetics between retraction and protein tyrosine dephosphorylation and the absence of dephosphorylation in retraction-blocking conditions both argue strongly in favor of a protein tyrosine dephosphorylation activity specifically triggered by αIIbβ3 engagement in retraction. Interestingly, the initial wave of tyrosine phosphorylation appears αIIbβ3-independent, since it is preserved in αIIbβ3-blocking conditions or in thrombasthenic platelets.

Tyrosine Phosphatase Inhibitors Block Clot Retraction—To test whether tyrosine phosphatases played any role in retraction, we performed clot retraction in the presence of various concentrations of PAO or of peroxovanadate (H₂O₂ + NaVO₄), two distinct inhibitors of tyrosine phosphatases (29, 30). Fig. 4, A and B, demonstrates that both inhibitors totally inhibited clot retraction, with IC₅₀ of 0.4 μM for PAO and 4–800 μM for peroxovanadate, while enhanced tyrosine phosphorylation was demonstrated by Western blotting for the major phosphorylated substrates p48, Src, p64, cortactin, p85, 100/105, and 125/130 (not shown). Altogether, our results suggest that tyrosine phosphatase activity is directly or indirectly involved in clot retraction.

**Cytochalasin D, an Inhibitor of Actin Polymerization, Inhibits Clot Retraction and αIIbβ3-dependent Protein Tyrosine Dephosphorylation—**Cytochalasins are known to inhibit clot retraction (3). To check if inhibition of actin cytoskeleton affected αIIbβ3-dependent tyrosine dephosphorylation, we preincubated platelets with cytochalasin D, a well studied inhibitor of actin polymerization. We obtained a dose-dependent inhibition of clot retraction (Fig. 5A), with full inhibition at 2–5 μM. The dose-dependent inhibition of retraction was comparable between cytochalasin D and E (not shown). Analysis of protein tyrosine phosphorylation by Western blotting showed that inhibition of actin polymerization completely inhibited protein tyrosine dephosphorylation (Fig. 5B). These results thus suggest that αIIbβ3 regulates protein tyrosine dephosphorylation via actin cytoskeleton assembly.

**Platelet Tyrosine Phosphatase Patterns during Clot Retraction by in-gel Analysis—**One interpretation of our results is that dephosphorylation during retraction is due to induction by αIIbβ3 of a de novo tyrosine phosphatase activity. Alternatively, αIIbβ3 may regulate tyrosine phosphatase/tyrosine-phosphorylated substrate interaction. To distinguish between these possibilities, we looked for correlations between retraction blockade by Integrilin® or cytochalasin D and variations in the SDS-PAGE pattern of tyrosine phosphatase activities of platelets, obtained by the
in-gel method (see “Experimental Procedures”). Fig. 6 shows a negative autoradiograph after in-gel phosphatase activity analysis of platelets after retraction for 5 and 60 min, compared with retraction inhibited by the α_{IIb}β_{3}-blocker Integrilin® or by cytochalasin D. Resting platelets (lane 1) exhibit two major bands at 130 and 32 kDa, as well as several minor bands at 100, 85, 49, and 22 kDa. After 5-min retraction, the 130-kDa and the 32-kDa bands were strongly diminished, while a new 26-kDa band appeared, the other bands remaining unchanged (lanes 2 and 3).

After 60-min retraction, all bands diminished except for the unchanged 26-kDa band. Thus, retraction correlated with a specific pattern of tyrosine phosphatases, which evolved with time. However, the same pattern was obtained when clot retraction was inhibited by the α_{IIb}β_{3}-blocker Integrilin® (lanes 4 and 5) or by cytochalasin D (lanes 6 and 7). This result thus suggests that tyrosine phosphatases induced in conditions of fibrin clot retraction are independent from α_{IIb}β_{3} engagement or from actin polymerization. Although we cannot rule out a possible “technical” artifact, this result suggests that dephosphorylation during retraction is not due to the de novo induction of a new tyrosine phosphatase by α_{IIb}β_{3}. It thus follows that α_{IIb}β_{3}-dependent dephosphorylation is due to the regulation by α_{IIb}β_{3}-dependent interaction of tyrosine phosphatases and of tyrosine-phosphorylated platelet proteins in the course of clot retraction in the presence of 5 μM cytochalasin D was assessed by Western blotting. Left panel, control platelets; right panel, platelets treated with cytochalasin D. Shown is a representative autoradiogram from three experiments. Bands are identified on the right.

<FIG. 3. Immunodetection of tyrosine-phosphorylated platelet proteins by Western blotting during clot retraction in the presence of α_{IIb}β_{3}-specific monoclonal antibodies or of α_{IIb}β_{3}-defective thrombasthenic platelets. Platelets were subjected to retraction for different times and immediately solubilized. Proteins were then separated by SDS-PAGE and electroblotted, and tyrosine-phosphorylated proteins were detected by a phosphotyrosine-specific monoclonal antibody, luminol-based chemiluminescence, and autoluminogram. Left panel, control platelets; medium panel, platelets in the presence of a mix of anti-α_{IIb} and anti-β_{3} monoclonal antibodies (20 μg/ml of Tab and AP3, respectively); right panel, platelets from a thrombasthenic patient (no α_{IIb}β_{3} integrin detectable by Western blot). Shown are representative autoluminograms from four separate experiments for each. Percentage of retraction values (% retr.) are indicated at the top of each blot at corresponding times. Bands are identified between Control and anti-α_{IIb}β_{3} panels.

<FIG. 4. Effect of the tyrosine phosphatase inhibitors PAO and peroxovanadate on platelet clot retraction. Various concentrations of the tyrosine inhibitors PAO (A, control (closed diamonds); 0.25 μM (closed squares); 0.30 μM (closed triangles); 0.50 μM (open squares); 0.75 μM (open triangles); 1 μM (open circles)) or peroxovanadate (1 mM H_{2}O_{2} + NaVO_{4} (B, control (closed diamonds); 0.1 μM (closed squares); 0.4 μM (closed triangles); 1 μM (closed circles); 2 μM (open circles); 4 μM (open diamonds)) were preincubated for 10 min at room temperature with platelets prior to thrombin induction of clot retraction. Quantitation was as in Fig. 1. Data represent the means ± S.E. of nine experiments for PAO and eight experiments for peroxovanadate.

<FIG. 5. Effect of cytochalasin D on clot retraction and on tyrosine phosphorylation of platelet proteins. A, control platelets (closed diamonds) or platelets preincubated with cytochalasin D at various concentrations (0.5 μM (closed squares); 1 μM (closed circles); 5 μM (open circles)) were subjected to clot retraction, and data were expressed as percentage of complete retraction. Data are the means ± S.E. of four experiments. B, the kinetics of tyrosine phosphorylation of platelet proteins in the course of clot retraction in the presence of 5 μM cytochalasin D was assessed by Western blotting. Left panel, control platelets; right panel, cytochalasin D-treated platelets. Shown is a representative autoradiogram from three experiments. Bands are identified on the right.
phosphorylated substrates, possibly through actin cytoskeleton assembly.

Inhibition of Clot Retraction by Integrilin®, PAO, and Cytochalasin D Correlate with Dose-dependent Patterns of Protein Tyrosine Dephosphorylation—We thus reasoned that if relevant to retraction, tyrosine phosphatase substrates should exhibit dose-dependent inhibition of dephosphorylation with preferably all retraction blockers. The ratios of phosphorylation levels at 60 min versus 5 min of clot retraction, in the absence or the presence of the inhibitors Integrilin®, PAO, and cytochalasin D were calculated for each substrate determined by Western blotting and compared. A ratio of 1 or more indicates absence of dephosphorylation or rephosphorylation, respectively. In Fig. 7A, 60:5 ratios were plotted as a function of Integrilin® concentration. The 100–105-kDa, 85-kDa, cortactin, Src, and 48-kDa substrates reached a ratio of 1 (i.e. without dephosphorylation from 5 until 60 min) at 10 and 25 μM, efficient inhibitory concentrations (see retraction dose-response curve, upper right inset). 85-kDa substrate exhibited the largest difference in ratio between 0 and 25 μM, indicating the highest sensitivity to Integrilin®. Quantitation of dephosphorylation in the presence of PAO (Fig. 7B) showed high and dose-dependent ratios for 85-kDa, 48-kDa, and 64-kDa substrates and Src. Of note, 85-kDa ratios were dose-dependent throughout the whole range of PAO concentrations tested (0.3–0.6 μM), best fitting the retraction dose-response curve (upper right inset). In addition, 85-kDa protein demonstrated the largest difference between ratios at 0.6 μM (r = 2.0) and 0 μM (r = 0.2), indicating again the highest sensitivity to PAO. Thus 85-kDa phosphorylation appears particularly sensitive to both an αIβIIIβ3 block by Integrilin® and to tyrosine phosphatase inhibition and correlates dose-dependently with clot retraction inhibition. Fig. 7C analyzes the phosphorylation ratio of platelet proteins during retraction in the presence of the actin polymerization inhibitor, cytochalasin D. Several substrates showed a high 60:5 ratio, particularly 64-kDa protein, Src, and 85-kDa protein, suggesting that regulation of their phosphorylation state is highly dependent on tyrosine phosphatase activities dependent on actin cytoskeleton assembly. Altogether, these results suggest that the 85-kDa protein, as a substrate for tyrosine phosphatase activity, and its αIβIIIβ3 and actin polymerization-dependent dephosphorylation are functionally related to retraction.

DISCUSSION

In the present study, we have addressed the questions of adhesive and signaling determinants of retraction of the fibrin clot by platelets. To this end, we have developed an assay with washed platelets and pure fibrinogen to analyze platelet proteins...
by Western blotting. Since most previous studies on platelet retraction were carried out in plasma (8, 9), we sought to validate our newly designed plasma-free assay. Because of the minimal composition of our system compared with plasma, we wished to check the characteristics of $\alpha_{IIb}\beta_3$ integrin engagement. The efficient blocking of clot retraction by the $\beta_3$-specific monoclonal antibody 7E3 and the $\alpha_{IIb}\beta_3$-specific antagonist peptide Integrilin® confirmed the engagement of $\alpha_{IIb}\beta_3$. The inability of several $\alpha_{IIb}\beta_3$-specific aggregation-blocking agents to alter retraction, including RGDS and the $\gamma$-400–411 dodecapeptide, as well as the monoclonals 10E5 and AP2 (not shown), is suggestive of a differential engagement of $\alpha_{IIb}\beta_3$ in retraction versus aggregation. This is consistent with the work by Cohen et al. (7) who first showed that RGDS and 10E5 did not inhibit (but instead increased) isometric clot tension. Our results are also consistent with the work of Rooney et al. (10), who showed that recombinant fibrinogen in which the $\gamma$408–411 region was deleted did support platelet retraction and not aggregation. The molecular mechanism underlying differential engagement of $\alpha_{IIb}\beta_3$ with fibrin in retraction versus fibrinogen in aggregation remains to be elucidated, although differences in avidity of polymeric(fibrin) versus monomeric (fibrinogen) ligands for $\alpha_{IIb}\beta_3$ may be at play.

Protein tyrosine phosphorylation is one of the major “outside-in” signaling events triggered by clustering of fibrinogen and has been thoroughly studied in platelet aggregation and spreading (5). We found that retraction was characterized by a brief initial phase of protein tyrosine phosphorylation followed by a dephosphorylation wave, affecting the same substrates previously found in aggregation or platelet adhesion: 125/130- and 100/105-kDa doublets, cortactin, 64-kDa protein, and the Src tyrosine kinase (Refs. 5 and 26 and Fig. 2). Tyrosine dephosphorylation paralleled retraction kinetics during most of its extent and was not due to thrombin activation of platelets. In contrast, the initial tyrosine phosphorylation was clearly $\alpha_{IIb}\beta_3$-independent, since neither its intensity nor its pattern were affected when $\alpha_{IIb}\beta_3$ was blocked by Integrilin® or monoclonal antibodies or when $\alpha_{IIb}\beta_3$-defective thrombasthenic platelets were used (Fig. 3). A corollary to this observation is that tyrosine phosphorylation in platelet fibrin clot retraction is under the control of an $\alpha_{IIb}\beta_3$-independent system, which remains to be determined. In conclusion, as opposed to aggregation, where $\alpha_{IIb}\beta_3$ engagement clearly controls successive waves of tyrosine phosphorylation and dephosphorylation (5, 31, 32), our data are consistent with $\alpha_{IIb}\beta_3$ engagement in the fibrin clot controlling essentially activation of tyrosine dephosphorylation.

Two hypotheses, not necessarily mutually exclusive, could account for $\alpha_{IIb}\beta_3$-dependent tyrosine dephosphorylation: 1) $\alpha_{IIb}\beta_3$ specifically activates one or several tyrosine phosphatase activity(ies), or 2) $\alpha_{IIb}\beta_3$ mediates the specific interaction of tyrosine phosphatases with their tyrosine-phosphorylated substrates. To distinguish between these two possibilities, we used the in-gel technique developed by Burridge and Nelson (27), the only technique among several tested that preserved tyrosine phosphatase activity after the denaturation conditions required to solubilize the fibrin clot (i.e. proteins boiled in SDS for 20 min, in the presence of a dithiulfide reducing agent). Using that elegant method, we found a pattern of tyrosine phosphatase activities specifically induced during retraction. However, we found that this pattern was $\alpha_{IIb}\beta_3$-independent, since it was unaffected during retraction inhibition by Integrilin® or cytochalasin D. In addition, it was similar to that described previously by Pasquet et al. (33) in platelets activated by thrombin and collagen and neither subjected to aggregation or retraction and is thus probably not specific to retraction. Thus, within the limits of this technique, we cannot conclude as to the induction of a specific tyrosine phosphatase activity by $\alpha_{IIb}\beta_3$ engagement in clot retraction. Altogether, our results are in favor of the idea that $\alpha_{IIb}\beta_3$-dependent dephosphorylation during clot retraction is the consequence of an $\alpha_{IIb}\beta_3$-driven specific tyrosine phosphatases/tyrosine-phosphorylated substrates interaction rather than activation of tyrosine phosphatases.

An important finding in the present work is that tyrosine phosphatase inhibitors, PAO and peroxovanadate, which act through different mechanisms, inhibited retraction. This clearly suggests a functional role for tyrosine phosphatases and thus for dephosphorylation in retraction. We have also found that the strong inhibitory effect of cytochalasins on retraction correlated with inhibition of protein tyrosine dephosphorylation (Fig. 5). Altogether, our results are thus consistent with a model in which $\alpha_{IIb}\beta_3$ drives actin cytoskeleton assembly, where the interaction of tyrosine phosphatases with phosphoprotein substrates may take place. Dephosphorylation of these substrates may then be involved directly or indirectly in force generation and/or force transmission, leading to reorganization of the fibrin matrix and clot retraction. Ezumi et al. (32) have also reported tyrosine dephosphorylation in the course of thrombin-induced platelet aggregation. This dephosphorylation was thought to be the consequence of the translation of the tyrosine phosphatases PTP1B and PTP1C (also known as SHP1) to the actin cytoskeleton in an $\alpha_{IIb}\beta_3$-dependent manner (32). From these studies, it was difficult to conclude as to the exact significance of dephosphorylation in aggregation, in particular since no attempt at inhibiting phosphatase activity was made and also because it only correlated with the late phase of aggregation. We propose that tyrosine dephosphorylation in aggregation corresponds to reinforcement of aggregates, due to either force generation and/or reorganization of the actin cytoskeleton allowing force transmission and leading to the irreversible phase of aggregation. Thus secondary aggregation may be equivalent to retraction. This postulate is the subject of current investigations.

Tyrosine dephosphorylation has been shown to correlate with platelet microparticle release (33). Interestingly, microparticles were elicited by the synergistic activation of platelets by collagen and thrombin, (or the A23187 Ca$^{2+}$ ionophore), thrombin alone being ineffective. However, only a subpopulation of stimulated platelets bound annexin V, which was used to probe for phosphatidylinositol surface exposure, a marker of microparticle release. This platelet subpopulation exhibited extensive protein tyrosine dephosphorylation. These results led the authors to conclude that there is a strong correlation between microparticle release and protein tyrosine dephosphorylation. Other investigators have demonstrated that platelet microparticle release involves cortical actin membrane skeleton reorganization, possibly controlling membrane phospholipid redistribution (34, 35). It is therefore tempting to speculate that tyrosine dephosphorylation has a common role in both platelet microparticle release and fibrin retraction, possibly via actin cytoskeleton reorganization.

We have sought to determine whether pathways other than tyrosine phosphorylation and known to be dependent upon $\alpha_{IIb}\beta_3$ engagement were involved in fibrin clot retraction. The signaling and $\alpha_{IIb}\beta_3$-dependent protease $\mu$-calpain may positively regulate aggregation (36). Interestingly, one of its targets is PTP1B (37), thus making $\mu$-calpain an attractive candidate for activating $\alpha_{IIb}\beta_3$-dependent tyrosine phosphatase activity in clot retraction. However, calpeptin (as well as other calpain inhibitors) was without effect on retraction (data not shown). This strongly suggested that calpains are not predominant positive regulators of fibrin clot retraction, at least in our conditions. This is consistent with the work of Schoenwaelder et al. (15), who clearly demonstrated that calpains regulate clot...
retraction negatively, since the calpain inhibitor calpeptin suppressed partial inhibition of retraction as induced by platelet stirring. We were also unable to demonstrate any role in clot retraction for the phosphatidyl 3-kinase or mitogen-activated protein kinases, based on the absence of inhibitory effect of the phosphatidyl 3-kinase inhibitors wortmannin and Ly-294002 and the mitogen-activated protein kinase/extracellular signal-regulated kinase inhibitor PD98059 (data not shown).

Among the four tyrosine phosphatases described in platelets (PTP1B, SHP1, SHP2, and HePTP; Refs. 12, 13, 31, 32, and 37), PTP1B appears as the most likely candidate, because of its strict αmβ3-dependent and complete cytoskeletal translocation upon platelet thrombin aggregation (32). However, we have not been able to test this hypothesis, because the denaturing conditions required for disrupting the fibrin clot were not compatible with isolation of intact cytoskeleton. Nonetheless, information concerning tyrosine-phosphorylated fibrin clot were not compatible with isolation of intact cytoskeleton linkages, as the denaturing conditions required for disrupting the fibrin matrix induces a strengthening of cytoskeleton linkages, as a consequence of a cytoskeletal rearrangement dependent on tyrosine dephosphorylation of p85. An alternative hypothesis, not necessarily exclusive from the first, is that tyrosine dephosphorylation may act positively on the molecular motor(s) in retraction.

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REFERENCES

1. Grinnell, F. (1999) Curr. Top. Pathol. 93, 61–73
2. Cheresh, D. A., Leng, J. & Klemke, R. L. (1999) J. Cell Biol. 146, 107–116
3. Cohen, I., Gerrard, J. M. & White, J. G. (1982) J. Cell Biol. 93, 775–787
4. Phillips, D. R., Charon, I. F., Parise, L. V. & Fitzgerald, L. A. (1988) Blood 71, 831–843
5. Shattil, S. J., Kashiwagi, H. & Pampori, N. (1998) Blood 91, 2645–2657
6. Norden, A. T. (1999) Thromb. Haemost. 82, 468–480
7. Cohen, I., Burk, D. L. & White, J. G. (1989) Blood 73, 1880–1887
8. Carr, M. E., Jr., Carr, S. L., Hanig, R. R. & Braaten, J. (1995) Thromb. Haemost. 73, 499–505
9. Jennings, L. K., White, M. M. & Mandrell, T. D. (1995) Thromb. Haemost. 74, 1511–1556
10. Rooney, M. M., Parise, L. V. & Lord, S. T. (1996) J. Biol. Chem. 271, 8553–8555
11. Hantgan, R. R. & Mousa, S. A. (1998) Thromb. Res. 90, 271–279
12. Jackson, S. P., Schoenwaelder, S. M., Yuan, Y., Salem, H. H. & Cooray, P. (1996) Thromb. Haemost. 76, 640–650
13. Levy-Toledano, S., Gallet, C., Nadal, F., Bryckaert, M., Maclouf, J. & Rosa, J.-P. (1997) Thromb. Haemost. 78, 226–233
14. Schoenwaelder, S. M., Jackson, S. P., Yuan, Y., Teadale, M. S., Salem, H. H., Mitchell, C. A. (1994) J. Biol. Chem. 269, 32479–32487
15. Schoenwaelder, S. M., Kulkani, S., Salem, H. H., Inajah-Ohmi, S., Yamao, Harigaya, W., Saijo, T. C. & Jackson, S. P. (1997) J. Biol. Chem. 272, 24876–24884
16. Leng, L., Kashiwagi, H., Ren, X. D. & Shattil, S. J. (1998) Blood 91, 4206–4215
17. Law, D. A., DeGuzman, F. R., Heiser, P., Ministri-Madrid, K., Killeen, N. & Phillips, D. R. (1999) Nature 401, 868–871
18. Collier, B. S., Peerschke, E. I., Scudeller, L. E. & Sullivan, C. A. (1995) J. Clin. Invest. 71, 253–338
19. Collier, B. S. (1985) J. Clin. Invest. 76, 101–108
20. Pidard, D., Montgomery, R. R., Bennett, J. S. & Kunicki, T. J. (1985) J. Biol. Chem. 258, 12562–12566
21. Newman, P. J., Allen, R. W., Kahn, R. A. & Kunicki, T. J. (1985) Blood 65, 527–532
22. McEver, R. P., Baenziger, N. L. & Majerus, P. W. (1980) J. Clin. Invest. 66, 1311–1318
23. Scarbrough, R. M., Naughton, M. A., Teng, W., Rose, J. W., Phillips, D. R., Manizzi, L., Arfsten, A., Campbell, A. M. & Char, I. P. (1995) J. Biol. Chem. 262, 1066–1073
24. Carteaux, J. P., Steiner, B. & Roux, S. (1993) Thromb. Haemost. 70, 817–821
25. Rosenthal, W. C., Kirchhofer, P., Hedwary, P., Eckehof, A. & Weiss, T.
26. Penninger, G., Baumgartner, H. R., Jennings, L. K. & Steiner, B. (1992) Blood 80, 2539–2547
27. Rosa, J.-P., Artçanuthurry, V., Grelas, F., Maclouf, J., Caen, J. P. & Levy-Toledano, S. (1997) Blood 88, 4385–4392
28. Burridge, K. & Nelson, A. (1995) Anat. Biochem. 232, 56–64
29. Djaflar, I., Caen, J. P. & Rosa, J.-P. (1993) Hum. Mol. Genet. 2, 2183–2185
30. Garcia-Morales, P., Minami, Y., Klausner, R. D. & Samelson, L. E. (1990) Proc. Natl. Acad. Sci. USA 87, 9255–9259
31. Pumiglia, K. M., Lai, L. F., Huang, C. K., Burroughs, S. & Feinstein, M. B. (1992) Biochem. J. 286, 441–449
32. Takayama, H., Ezumi, Y., Ichihara, T. & Okuma, M. (1995) Biochem. Biophys. Res. Commun. 194, 472–477
33. Ezumi, Y., Takayama, H. & Okuma, M. (1995) J. Biol. Chem. 270, 11927–11934
34. Pasquet, J.-M., Dachary-Prigent, J. & Norden, A. T. (1996) Biochem. J. 333, 591–599
35. Yamao, Harigaya, W., Saijo, T. C. & Jackson, S. P. (1997) Biochem. J. 324, 232–239
36. Fox, J. E., Austin, C. D., Boyles, J. K. & Steffen, P. K. (1990) J. Cell Biol. 111, 483–493
37. Fox, J. E., Taylor, R. G., Taffarel, M., Boyles, J. K. & Goll, D. E. (1993) J. Cell Biol. 120, 1501–1507
38. Frangioni, J. V., Oda, A., Smith, M. E., Neel, B. J. (1992) EMBO J. 11, 4843–4856
39. Li, R. Y., Gaits, F., Ragab, A., Ragab-Thomas, J. M. & Chab, H. (1994) FEBS Lett. 343, 89–93
40. Yu, D. H., Qu, C. K., Henegariu, O., Lu, X. & Feng, G. S. (1998) J. Biol. Chem. 273, 21125–21131
41. Choquet, D., Felsenfeld, D. P. & Sheetz, M. P. (1997) Cell 88, 39–48
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