Aggregation of Mammalian Cells Expressing the Platelet Glycoprotein (GP) Ib-IX Complex and the Requirement for Tyrosine Sulfation of GP Ibα

By Jing-fei Dong, William Hyun, and José A. López

The glycoprotein (GP) Ib-IX complex mediates platelet aggregation in response to high shear forces by binding von Willebrand factor (vWF) in the plasma. We investigated the possibility that the complex could mediate a similar phenomenon if expressed in nonhematopoietic cells. When agitated on a tabletop shaker, CHO and L cells expressing the complex formed large aggregates in the presence of vWF and the modulator ristocetin. When the rate of agitation was increased, aggregation occurred without added ristocetin and appeared to require only the application of a physical force. The aggregation was homophilic and temperature-dependent and required a functional ligand-binding subunit of the GP Ib-IX complex, GP Ibα. Posttranslational tyrosine sulfation of GP Ibα was required for aggregate formation and stability. Thus, aggregation of cells expressing the GP Ib-IX complex is a unique example of a ligand-receptor interaction induced by mechanical forces and demonstrates an important biological role for sulfation of tyrosine residues.

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THE ADHESION OF circulating platelets to the blood vessel subendothelium and to one another involves molecular interactions that must be precisely regulated to achieve hemostasis while averting thrombosis. Each adhesive event requires specific receptors on the platelet surface and ligands on the subendothelial matrix or in the plasma. The platelet receptor primarily responsible for adhesion to the subendothelium is the glycoprotein (GP) Ib-IX complex, which recognizes von Willebrand factor (vWF) affixed to the matrix at regions of blood vessel injury. Adhesion, through this ligand-receptor interaction increases as fluid shear forces increase, an ingenious mechanism that has evolved for overcoming the natural tendency of flow to limit platelet adhesion in regions of intact vascular endothelium.

If fluid shear forces reach very high levels—as those found in regions of arterial stenosis—plasma vWF binds GP Ib-IX on the platelet surface, an interaction that leads to platelet activation and aggregation. Shear apparently induces a change in the affinity of vWF for GP Ib-IX, because under static flow conditions soluble vWF does not bind to the receptor unless an appropriate modulator, such as ristocetin or botrocetin, is present. These modulators apparently mimic the change in conformation of the receptor and/or ligand that is induced by shear forces.

The specific structural requirements of the receptor for the shear-induced interaction with vWF are not known. However, in many respects the requirements parallel those for the ristocetin-induced binding of vWF. The region of the complex containing the vWF-binding site resides on GP Ibα, one of the three homologous polypeptides that make up the GP Ib-IX complex (the other two are GP Ibβ and GP IX). An acidic region within the globular N-terminus of GP Ibα has been implicated as important for binding vWF. We recently showed that this region contains three tyrosine residues that are posttranslationally sulfated and that this modification is necessary for optimal ristocetin-induced vWF binding by the complex.

The events that follow vWF binding to the GP Ib-IX complex and culminate in the formation of a platelet aggregate are also poorly understood. On the one hand, metabolically dead formaldehyde-fixed platelets can be agglutinated through an interaction between GP Ib-IX and vWF in the presence of ristocetin. This phenomenon is likely the consequence of direct physical cross-linking of the platelets by the binding of multimeric vWF to GP Ib-IX molecules on adjacent platelets. On the other hand, in live platelets the shear stress-induced binding of vWF to GP Ib-IX induces a calcium influx, which results in activation of the platelet integrin, GP Ib-IIIa. Glycoprotein Ib-IIIa then mediates platelet aggregation by binding another site on vWF. The platelet aggregation induced by vWF binding to GP Ib-IX is, therefore, similar to that induced by a number of soluble platelet agonists, including adenosine diphosphate, which itself is released by platelets after vWF binds the GP Ib-IX complex.

Thus, the study of shear-induced aggregation is complicated by the presence of a number of agonists released by the platelets themselves and by the presence of other cell adhesion molecules on the platelet surface. A model system for studying the functional role of the receptor apart from other platelet proteins is, therefore, desirable. Molecular cloning and cell transfection techniques have made it possible to establish stable mammalian cell lines that express the complete complex or combinations of its components, but neither the aggregation nor the aggregation of such cells has been reported. In the current report, we present evidence that the GP Ib-IX complex mediates vWF-dependent cell aggregation in the presence of ristocetin and, in the absence of ristocetin, in the presence of mechanical forces by a process that may mimic shear-induced platelet aggregation. We also demonstrate that sulfation of tyrosine residues in the ligand-binding subunit, GP Ibα, is a major structural determinant of the function of the complex in this model system of vWF-dependent cell aggregation.

MATERIALS AND METHODS

vWF: Purified human vWF was provided by Dr. Zaveri Ruggeri of the Scripps Research Institute, La Jolla, CA.

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Cell lines. We have previously reported several transfected cell lines that stably express high levels of the entire GP Ib–IX complex or different combinations of its three subunits.\textsuperscript{4,2} The cell lines used in this study were: L2H cells (mouse L cells expressing the entire complex), CHO αβIX cells (CHO cells that stably express the entire complex), and CHO βIX cells (which express GP Ibβ and GP IX). Untransfected parental L and CHO cells were used as controls. L cells were grown in a 1:l mixture of Dulbecco’s modified Eagle’s medium and F12 medium (DMEM/F12, Gibco, Grand Island, NY), and CHO cells were grown in a minimum essential media (α-MEM, Gibco). Both media were supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, UT). To maintain high expression of the recombinant proteins of CHO αβIX cells, or L2H cells were procured using Serumfree cells, 400 μg/mL G4 I8 and 100 pmol/L thymidine (HAT, Gibco) for L2H cells.

Suspension cell culture. Monolayers of CHO αβIX, CHO βIX, or L2H cells were procured using 0.53 mmol/L EDTA to separate single-cell suspension in the same medium used for monolayer culture. One hundred milliliters of this cell suspension was transferred into a 250 mL spinner flask to an initial concentration of 50 × 10⁶ cells/ml. With continuous stirring, the suspension cultures could be maintained at 37°C with 5% CO₂ for at least 1 week without spontaneous cell clumping or adhesion of the cells to the culture vessel.

Inhibition of tyrosine sulfation of GP Ibα. To inhibit sulfation of GP Ibα, L2H and CHO αβIX cells were cultured in standard medium to 80% confluence and then switched to sulfate-free medium with 2% of the normal concentrations of methionine and cysteine and supplemented with 10% dialyzed FBS. Sodium chlorate (Aldrich, Milwaukee, WI) and guaicol (Sigma Chemical Co, St Louis, MO) were added to final concentrations of 5 mmol/L and 0.2 mmol/L, respectively. Cells were maintained in this condition for 24 hours and then processed for the cell aggregation assay. Control cells were grown in complete medium.

Trypsinization of cells. To evaluate the effect on cell aggregation of trypsin treatment of surface glycoproteins, an aliquot of cells grown in suspension was pelleted at 150 g, washed with phosphate-buffered saline (PBS), and resuspended in a concentration of 1 × 10⁸ cells/mL in complete culture medium without serum. Trypsin from bovine pancreas (Sigma) was added to a final concentration of 50 μg/mL, and the cells were incubated at 37°C for 30 minutes. Trypsin activity was then stopped by the addition of an equal volume of 10% heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, UT). The cells to be aggregated were pelleted by centrifugation at 150 g, washed with PBS, and then resuspended in aggregation buffer (PBS containing 1 mmol/L CaCl₂, 1% bovine serum albumin [BSA], and 6 mg/mL D-glucose) for further assays. Cell viability was evaluated after trypsin treatment by the trypan blue dye exclusion test and was not significantly decreased compared with the viability of cells that had not been subjected to enzymatic digestion.

Paragominaldehyde fixation of cells. Ten-milliliter aliquots of CHO αβIX or L2H cells were mixed with equal volumes of 4% paraformaldehyde and incubated at 4°C for 30 minutes with stirring. Fixed cells were then washed and resuspended in aggregation buffer to a final concentration of 1 × 10⁶ cells/mL for further assay.

Cell aggregation. The cells to be aggregated were pelleted by centrifugation at 100 g, washed with PBS, and then resuspended in the aggregation buffer to a final concentration of 1 × 10⁶ cells/mL, unless otherwise specified. Cell viability determined by trypan blue exclusion was typically found to be 95% to 99%. Aliquots of 500 μL of the cell suspension were loaded onto a 24-well culture dish on a rotary shaker, and cell aggregation was then initiated by adding 5 μg purified bovine vWF and 1.4 mg/mL ristocetin (Sigma), with constant shaking at six cycles/second. To induce cell aggregation in the absence of ristocetin, the cells were shaken at a frequency of 10 cycles/second in the presence of vWF. The extent of cell aggregation was determined semiquantitatively under a phase-contrast microscope by estimating the number of cells per aggregate and quantitatively by flow cytometry as the percentage of cells in aggregates.

To determine the role of vWF in aggregation, cells were preincubated with the GP Ibα monoclonal antibody AN51 (5 μg/10⁶ cells) at 37°C for 30 minutes with constant shaking before adding inducer.

Flow cytometry. To evaluate the extent of cell aggregation by flow cytometry, aliquots of the cell suspension were removed at different times after shaking was initiated and analyzed for changes in forward and orthogonal light scatter using a FACS 440 flow cytometer (Becton Dickinson, San Jose, CA) equipped with CICERO electronics (Cytomation, Fort Collins, CO) and modified optics and fluids for large-event analysis. The cytometer argon-ion laser (Spectra-Physics, Mountain View, CA) was tuned to 488 nm and set at 500 mW. To widen the laser beam at the point of cell interrogation so that large aggregates could be faithfully detected, a special lens configuration—consisting of a spherical lens mounted adjacent to a cylindrical lens—was used to focus the laser beam as a 200-μm-wide ellipse. The fluids were modified to accommodate a 240-μm nozzle holder with larger sample intake tubing of 220 μm internal diameter.

The forward-scatter diode gain and the orthogonal-scatter photomultiplier tube voltage were calibrated using a single-cell suspension such that the single-cell population appeared in the lower quarter of the dynamic range. Any event detected above the single-cell threshold was considered to represent a cell aggregate.

vWF binding. Flow cytometry was also used to determine the effect of fixation on vWF binding. First, cells expressing the GP Ibα complex were collected by centrifugation. Half of the cells were then fixed in 4% paraformaldehyde followed by extensive washing with PBS. Aliquots of 1 × 10⁶ fixed and unfixed cells were incubated in 1 mL PBS buffer containing 1% BSA for 20 minutes at room temperature to block nonspecific binding. Five micrograms of vWF and 1.4 mg of ristocetin were then added to the cell suspension and incubated for 30 minutes at room temperature. Unbound vWF was removed by washing the cells with PBS; the cells were also dispersed vigorously to dissociate any aggregates that may have formed. Cells were then incubated with 1 μg of rabbit anti-vWF antibody (DAKO, Carpinteria, CA) for 1 hour at room temperature, washed with PBS, and incubated for 30 minutes with a fluorescein isothiocyanate (FITC)–conjugated goat antirabbit IgG (Zymed, South San Francisco, CA). The cells were then washed with PBS and dispersed to ensure a single cell population, and their surface labeling with the anti-vWF antibody was analyzed by flow cytometry. As an internal control, the same cells were incubated with 1% BSA without the primary antibody and analyzed similarly.

Measurement of calcium influx. Cell suspensions were incubated at 37°C in the dark with 10 μmol/L of fura2-AM ester (Molecular Probes, Eugene, OR) for 20 minutes followed by an additional 20-minute incubation after the dye was diluted 10-fold. The calcium influx ([Ca²⁺]₁) was determined using a Hitachi F-2000 fluorescence spectrophotometer (Naperville, IL) that has dual-wavelength excitation capacity. Before the assay, cells were washed with fresh PBS and resuspended in aggregation buffer to a final concentration of 1 × 10⁶ cells/mL. One milliliter of cell suspension was loaded onto a 1.8-mL cuvette containing a stir bar. Purified vWF plus ristocetin were added into the cell suspension 10 seconds after the measurement started and [Ca²⁺]₁ was monitored for 5 minutes. The calcium influx was determined using the ratio of absorbances at 510 nm after exciting the dye at 340 nm and 380 nm, using a kd for fura2 of 224 mmol/L.\textsuperscript{21,24} Determining the nature of cell aggregation. CHO αβIX cells
were first stained with the fluorescent lipophilic dye, Dil (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, Molecular Probes). Pelleted CHO αβIX or L2H cells were briefly resuspended at 5 × 10^6 cells/mL in α-MEM containing 1% BSA. The fluorescent dye was then added to a final concentration of 10 μg/mL, and the cells were incubated at 37°C in the dark for 30 minutes with stirring. After they were washed to remove free dye, the labeled CHO αβIX cells were mixed with an equal number of unstained CHO βIX or CHO cells and then evaluated using the aggregation assay. The cell aggregates were viewed under a phase-contrast microscope with a rhodamine filter.

RESULTS

A note about nomenclature: throughout this manuscript we refer to cell aggregation. Our meaning of aggregation is the formation of cell aggregates and implies nothing about the mechanism by which these aggregates form. Unlike the distinction made when describing platelet clumping, aggregation as we use it for cells does not imply an active process or the involvement of a cell-surface integrin.

Ristocetin-induced aggregation of cells expressing the GP Ib-IX complex. To determine if the GP Ib-IX complex can mediate the aggregation of nonhematopoietic cells, we agitated cells expressing the complex on a tabletop shaker in the presence of vWF and ristocetin. When shaken at moderate frequencies (four to six cycles/second), CHO αβIX and L2H cells (CHO and L cells that express the complete GP Ib-IX complex) formed multicellular clusters within 1 to 5 minutes after the addition of vWF and ristocetin (Fig 1). The percentage of cells in these aggregates increased steadily as the time of shaking increased and plateaued after approximately 15 to 25 minutes at room temperature (Fig 2). After this time, the aggregates contained as many as several hundred cells (Fig 1B), and they remained stable without disaggregating for at least 2 hours after shaking ceased. Cell aggregation required the presence of both ristocetin and vWF; omission of either component completely prevented aggregation (Table 1). In contrast to cells expressing the full GP Ib-IX complex, neither parental CHO nor L cells nor CHO βIX cells aggregated under identical conditions (Fig 1A and Table 1).

The requirement of GP Ibα for this phenomenon was demonstrated by the experiments in which CHO βIX cells failed to aggregate when agitated in the presence of vWF and ristocetin, thus suggesting that the requirements for aggregation of GP Ib-IX–expressing cells may be similar or identical to those for vWF-mediated platelet aggregation. To test this possibility further, we measured aggregation of CHO αβIX and L2H cells after preincubating them with the anti-GP Ibα monoclonal antibody AN51, which blocks vWF binding to platelets.23 This treatment completely inhibited aggregation, even after the cells had been agitated for 2 hours (Tables 1 and 2).

We further investigated the requirement for GP Ibα by digesting CHO αβIX and L2H cells with 50 μg/mL trypsin for 30 minutes, a treatment known to proteolytically remove the 45-kD globular ligand-binding region of GP Ibα.24 Less than 10% of the trypsin-treated cells aggregated, even after prolonged shaking (Table 1). Trypsin treatment had no effect on cell viability, as judged by the trypan blue exclusion test.

Ristocetin-independent cell aggregation. Because the interaction between GP Ib-IX and vWF in platelets can be induced by shear-stress alone in the absence of a modulator such as ristocetin, we speculated that with high-frequency shaking we might observe ristocetin-independent aggregation of cells expressing the complex. In contrast to their failure to aggregate in the absence of ristocetin when shaken at a frequency of six cycles/second (Table 1), both CHO αβIX and L2H cells aggregated in the presence of vWF alone when the shaking frequency was increased to 10 cycles/second. Parental cells and CHO βIX cells failed to aggregate under these conditions (Table 2). Like the aggregation induced by ristocetin, cell aggregation induced by higher frequency shaking was completely blocked by AN51 (Table 2). The onset of cell aggregation at higher shaking frequencies was slower than at lower frequencies with ristocetin (Fig 2B), although the morphologies of the aggregates formed under these two conditions were indistinguishable.

Tyrosine sulfation and cell aggregation. In a previous study, we showed that inhibition of tyrosine sulfation does not affect the synthesis or reduce cell-surface expression of the GP Ib-IX complex, but does significantly reduce its abil-
The state of GP Ibα sulfation also influenced aggregate stability after shaking ceased. Unlike sulfate-replete cells, which remained in aggregates for up to 2 hours after shaking was discontinued, the sulfate-depleted cells disaggregated within 5 minutes of discontinuing shaking (Fig 3).

An even more striking effect of inhibiting sulfation was observed in aggregation at higher shaking frequencies without ristocetin. Under these conditions, cells depleted of sulfate failed to aggregate altogether, even at the higher cell concentration (Table 3).

Aggregation of cells expressing the GP Ib-IX complex is homophilic. We next examined whether cell aggregation involved only cells that expressed the full complex or could involve nonexpressing cells in a heterophilic manner. CHO αβIX cells were first labeled with a fluorescent dye and then mixed with an equal number of unlabeled CHO βIX or CHO cells. The cell suspension was then agitated at both moderate and high frequencies. The resultant cell aggregates contained only CHO αβIX cells, which formed sharply edged intensely fluorescent regions on a background of unlabeled single CHO βIX cells (Fig 4).

Aggregation of fixed cells. vWF can mediate platelet aggregate formation by at least two mechanisms: the first, essentially a cross-linking of the cells by the multivalent vWF molecule, can occur in formalin-fixed platelets; the second requires signal transduction and integrin activation. To determine whether a component of the aggregation of GP Ib-IX complex-expressing cells requires signal transduction and cell activation, we analyzed the aggregation of paraformaldehyde-fixed CHO αβIX and L2H cells under the same conditions used for live cells. Fixed cells were still able to aggregate, although the extent of aggregation was about 30% to 40% less than that of live cells (43% ± 19% vs 26% ± 12%, \( \chi^2 \) test, \( P < .001 \)). Aggregation of fixed cells also required the interaction of GP Ibo with vWF, as it was completely blocked by AN51.

Because fixation may have decreased aggregation by reducing vWF binding, we studied directly its effect on binding using a flow cytometry assay. Cell-bound vWF was detected by an anti-vWF antibody and a FITC-conjugated secondary antibody. Fixation did not decrease vWF binding (Fig 5).

Cell aggregation and calcium influx. To evaluate further the possible involvement of a signal transduction mechanism in cell aggregation, we measured changes in intracellular calcium in cells during aggregation induced by ristocetin and vWF. Cells preloaded with fura2-AM ester were placed onto a 1.8-mL cuvette with a stir bar, and aggregation was induced by adding vWF and ristocetin to the cell suspension. Intracellular calcium concentration was monitored for 5 minutes. Although the cells clearly aggregated during this time, no significant increases in calcium concentration were detected.

Effect of temperature on cell aggregation. Another clue to the possible requirement of signal transduction in cell aggregation would be a demonstration of temperature dependence. Therefore, we performed the aggregation assay at three temperatures: 4°C, 25°C, and 37°C. At both moderate

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**Fig 2.** Aggregation of CHO αβIX cells as measured using flow cytometry. (A) A typical histogram of cell aggregation after adding vWF and ristocetin. The cell suspension was agitated at 6 cycles/s on a tabletop shaker. The cell population at time zero reflects the size distribution of single cells. As time of shaking increased, larger particles representing aggregates increased, and the percentage of total cells that remained as single cells decreased. Gate 1, single-cell population; gate 2, aggregates. (B) Time course of cell aggregation as determined by flow cytometry. At each time point, flow cytometry was performed and the percentage of cells in aggregates was determined by subtracting the number of cells in gate 1 at the specific time from the number in gate 1 at time zero.
and high shaking frequencies, the extent of aggregation of live cells was clearly temperature-dependent (Fig 6). In contrast, aggregation of fixed cells was temperature-independent. Temperature had a greater effect on the ristocetin-independent aggregation induced at higher shaking frequencies than it did on ristocetin-dependent aggregation; after prolonged shaking (>30 minutes), the percentages of cells in the aggregates induced by higher shaking frequencies compared with those induced by ristocetin were 61.9%, 66.9%, and 48.1% at 37°C, 25°C, and 4°C, respectively (Table 1).

In living cells, temperature also affected the lag time to initiation of aggregation. At 37°C, the lag times were as short as 20 seconds and 60 seconds at the moderate and higher shaking frequencies, respectively, whereas at 4°C the lag times were approximately 5 minutes at both shaking frequencies. Temperature did not affect the lag time to aggregation of fixed cells, which was about 5 minutes at each temperature.

**DISCUSSION**

In the current report, we demonstrate that mammalian cells expressing the platelet GP Ib-IX complex can be induced to aggregate in the presence of vWF and ristocetin. More important, our studies show for the first time that the interaction between cell-surface GP Ib-IX and vWF, and subsequent cell aggregation, can be induced in a nonplatelet system in the absence of modulators by merely increasing the cation does not affect the synthesis or cell-surface expression of the complex, but it does contribute to its vWF-binding capacity in the presence of ristocetin. The experiments in the present study demonstrate that tyrosine sulfation is also very important for the aggregation of GP Ib-IX-expressing cells, particularly in the absence of ristocetin. These results corroborate the hypothesis that the interaction between GP Ib-IX and vWF has a major electrostatic component. Evidence from these experiments and others indicates that the aggregation is absolutely dependent for its initiation on an interaction between GP Ib-IX and vWF; the actual aggregation requires that platelet GP IIb-IIIa be activated and bind von Willebrand factor.

The aggregation of cells expressing the GP Ib-IX complex, like shear-induced platelet aggregation, also appears to be mediated by an interaction between the GP Ib-IX complex and vWF. Several lines of evidence support this conclusion: (1) aggregation was homophilic, involving only cells that express the full complex on their surfaces; (2) aggregation was inhibited by a GP Ibα antibody and by proteolytic removal of the GP Ibα ligand-binding domain from the cell surface; and (3) parental CHO and L cells and CHO βIX cells were unable to aggregate under the conditions that induce aggregation of cells expressing the entire GP Ib-IX complex. These characteristics of cell aggregation reflect intrinsic features of the GP Ib-IX complex, as transfected cells derived from different parental cell lines (CHO and L cells) behaved identically in these experiments.

Another key finding of this study is the demonstration that tyrosine sulfation plays an important role in cell aggregation mediated by the interaction of GP Ibα and vWF. We recently found that three tyrosine residues in an acidic region of GP Ibα are modified by posttranslational sulfation. This modification does not affect the synthesis or cell-surface expression of the complex, but it does contribute to its vWF-binding capacity in the presence of ristocetin. The experiments in the present study demonstrate that tyrosine sulfation is also very important for the aggregation of GP Ib-IX-expressing cells, particularly in the absence of ristocetin.

### Table 1. Cell Aggregation Under Moderate Shaking Frequency

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Controls</th>
<th>37°C</th>
<th>Room Temperature</th>
<th>4°C</th>
<th>vWF Only</th>
<th>Ristocetin Only</th>
<th>Pretreated With AN51</th>
<th>Pretreated With Trypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO α/IX cells</td>
<td>0</td>
<td>64.8 ± 12.1</td>
<td>41.9 ± 17.1</td>
<td>28.8 ± 9.2*</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6.9 ± 1.7</td>
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<tr>
<td>L2H cells</td>
<td>0</td>
<td>58.4 ± 15.3</td>
<td>36.6 ± 19.2</td>
<td>24.0 ± 8.6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5.4 ± 2.1</td>
</tr>
<tr>
<td>CHO β/IX cells</td>
<td>0</td>
<td>3.2 ± 2.9</td>
<td>1.7 ± 0.9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CHO cells</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The numbers indicate the percentage of cells in aggregates as determined by cytometry (Fig 2A) 30 minutes after shaking was begun and are the means derived from four individual experiments. Shaking frequencies were 6 cycles/s.

χ² tests: * P < .01; † P < .05.

### Table 2. Cell Aggregation Under Higher Shaking Frequency (10 cycles/s)

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Controls</th>
<th>37°C</th>
<th>Room Temperature</th>
<th>4°C</th>
<th>vWF Only</th>
<th>Ristocetin Only</th>
<th>Pretreated With AN51</th>
<th>Pretreated With Trypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO α/IX cells</td>
<td>0</td>
<td>39.5 ± 9.5</td>
<td>28.0 ± 14.1</td>
<td>12.9 ±</td>
<td>2.2*</td>
<td>0</td>
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<tr>
<td>L2H cells</td>
<td>0</td>
<td>41.2 ± 10.6</td>
<td>26.6 ± 10.7</td>
<td>6.1*</td>
<td>0</td>
<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>CHO β/IX cells</td>
<td>0</td>
<td>2.9 ± 2.5</td>
<td>3.4 ± 2.9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CHO cells</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The values are the means of three independent experiments.

* χ² test, P < .005.
Table 3. Role of Tyrosine Sulfation in Cell Aggregation

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Moderate Shaking Frequencies (vWF and ristocetin)</th>
<th>Higher Shaking Frequencies (with vWF only)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sulfate-Replete</td>
<td>Sulfate-Depleted</td>
</tr>
<tr>
<td>CHO αβIX cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High cell density</td>
<td>69.8 ± 9.6</td>
<td>67.4 ± 12.9</td>
</tr>
<tr>
<td>Low cell density</td>
<td>49.8 ± 11.2</td>
<td>0</td>
</tr>
<tr>
<td>L2H cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High cell density</td>
<td>65.1 ± 17.5</td>
<td>57.9 ± 13.5</td>
</tr>
<tr>
<td>Low cell density</td>
<td>56.4 ± 21.1</td>
<td>0</td>
</tr>
<tr>
<td>L2H cells: 5 min after shaking ceased</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High cell density</td>
<td>71.5 ± 14.9</td>
<td>0</td>
</tr>
<tr>
<td>Low cell density</td>
<td>42.1 ± 6.7</td>
<td>0</td>
</tr>
</tbody>
</table>

dence has been presented that a region of high negative charge between residues 268 and 297 plays an important role in botrocetin-induced vWF binding to GP Iba; a direct consequence of tyrosine sulfation is to add more negative charges to this region. These electronegative regions may bind directly to cationic regions of vWF; alternatively, the added negative charges may in some way stabilize a conformational change of GP Iba induced by a physical force that allows optimal exposure of the ligand-binding site (much as γ-carboxylation of glutamate residues in the vitamin K-dependent blood clotting factors adds negative charges that, in the presence of calcium ions, allow the region containing these modified amino acids to assume a conformation suitable for binding to phospholipid). The second possibility is consistent with the observation that the botrocetin-induced aggregates of cells that have undergone sulfate starvation completely disaggregate within 5 minutes after shaking is discontinued, suggesting that sulfate groups aid in maintaining the conformational change that is induced by the combination of botrocetin and shaking. In the absence of the physical force contributed by shaking, botrocetin alone is insufficient to maintain that conformation. Another less likely explanation for the observed effects of sulfate starva-
tion on cell aggregation is that this manipulation has altered one or more other molecules that are also vital for aggregation to proceed.

Shaking also likely contributes to aggregation by bringing the cells together. Thus, aggregation is probably a function of the frequency of cell collision—which is determined by the velocity, size, concentration, and rate of agitation of the cells—and by the efficiency of these collisions, which is affected by the shear force to which the cells are exposed and by the viscosity of the liquid milieu. In addition, increasing the shaking frequency from six cycles/s to 10 cycles/s also changes the nature of fluid flow within the wells in which the experiments are performed, converting a predominately laminar flow pattern to a turbulent pattern, a condition favoring cell collisions. The data in the current study thus raise the possibility that shear stress may not be the only physical force (or even the predominant force) that induces the interaction between vWF and GP Ib-IX and the subsequent aggregation of platelets; the process may also involve other phenomena that occur concomitantly with shear stress. For example, higher shear stresses may increase the velocity of platelet-platelet collisions, and thereby the kinetic energy that is imparted to each cell on collision. Kinetic energy may

Fig 3. Effect of tyrosine sulfation on the duration of cell aggregation. Ristocetin-induced cell aggregates remained intact for more than 2 hours in sulfate-replete CHO αβIX cells (A), whereas the aggregates of sulfate-depleted CHO αβIX cells fell apart within 5 minutes after shaking stopped (B) (bar = 100 μm).
AGGREGATION OF GP Ib-IX-EXPRESSING CELLS

Fig 4. Homophilic cell aggregation. Fluorescent dye-labeled CHO αβIX cells were mixed with an equal number of unlabeled CHO βIX cells and subjected to the aggregation assay. Aggregates formed only between CHO αβIX cells; unlabeled CHO βIX cells remained unaggregated (bar = 50 μm).

thus be another important determinant of aggregation under conditions of high shear, which might explain why we are able to induce aggregation of cells expressing the complex under shear stresses that, although difficult to quantitate, are likely to be below the threshold for platelet aggregation. The cells are much larger than platelets; collisions between cells therefore release much more kinetic energy than do collisions between platelets at a given velocity. The role of kinetic energy remains to be tested under conditions of precisely controlled collision frequency and velocity.

Once shear stresses have induced the interaction between vWF and the GP Ib-IX complex in platelets, the platelets become activated and aggregation is facilitated by binding of GP Ib-IIIα to another site on vWF. Cell aggregation observed in our experiments may also involve more than one mechanism. On the one hand, it resembles passive agglutination and may be caused by a physical coupling between GP Ib-IX complexes on adjacent cells bridged by vWF. This type of mechanism is almost certainly involved in the aggregation of paraformaldehyde-fixed cells, which is temperature-independent. On the other hand, in living cells the temperature dependence of the aggregation suggests that an active process is involved. When

Fig 5. Effect of paraformaldehyde fixation on vWF binding of cells expressing the GP Ib-IX complex. Paraformaldehyde-fixed cells and control cells were incubated with vWF for 30 minutes, washed, and bound vWF was detected with an anti-vWF antibody followed by an FITC-conjugated secondary antibody. Fixation did not decrease vWF binding.

Fig 6. Effect of temperature on cell aggregation. The extent of aggregation of live CHO αβIX cells at either 6 cycles/s with ristocetin or 10 cycles/s without ristocetin was significantly higher when shaking was conducted at 37°C compared with shaking at 4°C (t-test P < .001 for 6 cycles/s and P < .05 for 10 cycles/s), whereas there was no significant difference in the aggregation of fixed CHO αβIX cells at the different temperatures.
cell aggregation was induced at 4°C, the lag time—ie, the time between addition of activator and initiation of cell aggregation—was approximately 15 times longer than at 37°C (300 seconds at 4°C v 20 seconds at 37°C). Furthermore, the extent of cell aggregation decreased as temperature decreased, the greatest reduction being at 4°C. We have no evidence that a GP Ib-IIIa-like integrin capable of binding vWF is expressed on either L or CHO cells.

The temperature-dependence of aggregation can be explained in at least two ways. First, the interaction of vWF with the GP Ib-IX complex on the cell surface may be facilitated by diffusion of the complex within the plane of the plasma membrane, a thermodynamic process that would be expected to increase with higher temperatures. This mechanism is compatible with the notion of a multivalent interaction between vWF (which is multimeric) and GP Ib-IX complexes on the cell surface. Second, an enzymatic process could be involved that facilitates aggregation by activating another receptor on the cell surface. The fact that cell aggregation at higher shaking frequencies was more sensitive to changes in temperature may suggest an active process involving intracellular signaling; ristocetin-induced cell aggregation, on the other hand, may involve two pathways: one similar to that involved at higher shaking frequencies, another by agglutination.

Arguing against a pathway involving signal transduction is the lack of a detectable increase in intracellular calcium, a process that in platelets is usually associated with activation. Two possible explanations could account for the failure to observe a calcium influx. First, cell aggregation could indeed be a calcium-independent phenomenon, a possibility for which there is biological precedent. Three Drosophila proteins that are members of the leucine-rich motif family to which the polypeptides of the GP Ib-IX complex also belong—Toll, chaoptin, and connectin—all mediate cell aggregation in a calcium-independent manner similar to what we found with GP Ib-IX--vWF-mediated cell aggregation. Second, the reactive calcium flux in these cells may have been too small to be detected by the fluorescence spectrophotometer used, which measures the intracellular calcium concentrations in cell populations rather than in individual cells. Compared with the studies performed on platelets, in our studies both the density of receptors expressed on the cell surface and the number of cells in a defined volume are likely to be smaller, so that calcium fluxes, even if they occur, may be below the limits of detection.

In summary, we have found that the GP Ib-IX complex expressed in nonhematopoietic cells is capable of mediating cell aggregation induced solely by mechanical forces. The mechanical forces may include kinetic energy generated by cell-cell collisions, suggesting a different interpretation of the forces required for pathological shear stress-induced platelet aggregation. In addition, these studies have identified tyrosine sulfation of the receptor as a vital structural determinant of the receptor’s ability to interact with its ligand and to mediate cell aggregation.

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Aggregation of mammalian cells expressing the platelet glycoprotein (GP) Ib-IX complex and the requirement for tyrosine sulfation of GP Ib alpha

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