Ero1\(\alpha\) Is Expressed on Blood Platelets in Association with Protein-disulfide Isomerase and Contributes to Redox-controlled Remodeling of \(\alpha_{\text{IIb}}\beta_3\)

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Recent evidence supports a role of protein-disulfide isomerase (PDI) in redox-controlled remodeling of the exofacial domains of \(\alpha_{\text{IIb}}\beta_3\) in blood platelets. The aim of this study was to explain whether Ero1\(\alpha\) can be responsible for extracellular redox reoxidation of the PDI active site. We showed that Ero1\(\alpha\) can be found on platelets and is rapidly recruited to the cell surface in response to platelet agonists. It is physically associated with PDI and \(\alpha_{\text{IIb}}\beta_3\), as suggested by colocalization analysis in confocal microscopy and confirmed by immunoprecipitation experiments. Apart from monomeric oxidized Ero1\(\alpha\), anti-\(\alpha_{\text{IIb}}\beta_3\) immunoprecipitates showed the presence of several Ero1\(\alpha\)-positive bands that corresponded to the complexes \(\alpha_{\text{IIb}}\beta_3\)-PDI-Ero1\(\alpha\), PDI-Ero1\(\alpha\), and Ero1\(\alpha\)-Ero1\(\alpha\) dimers. It binds more efficiently to the activated \(\alpha_{\text{IIb}}\beta_3\) conformer, and its interaction is inhibited by RGD peptides. Ero1\(\alpha\) appears to be involved in the regulation of \(\alpha_{\text{IIb}}\beta_3\) receptor activity because of the following: (a) blocking the cell surface Ero1\(\alpha\) by antibodies leads to a decrease in platelet aggregation in response to agonists and a decrease in fibrinogen and PAC-1 binding, and (b) transfection of MEG01 with Ero1\(\alpha\) increases \(\alpha_{\text{IIb}}\beta_3\) receptor activity, as indicated by increased binding of fibrinogen.

The catalytic events involving the oxidation, reduction, and isomerization of disulfide bonds take place in the endoplasmic reticulum (ER).\(\ddagger\) These reactions are catalyzed by a group of ER resident proteins that belong to the protein-disulfide isomerase (PDI) family (1, 2). For proteins with few disulfides, the process can be catalyzed by oxidation of cysteine residues to form the correct native disulfide. In the case of proteins with several disulfides, an isomerization reaction is also required to correct for non-native disulfides formed following oxidation (3). In the first step, a disulfide is introduced into the substrate protein with the concomitant reduction of a disulfide within the active site in PDI (4). Then Ero1\(\alpha\) or Ero1\(\beta\) catalyzes the reoxidation of the PDI active site in a reaction involving the reduction of oxygen to liberate hydrogen peroxide (5, 6). There is a growing interest in the role of thiol-disulfide oxidoreduction of the exofacial domain protein cysteines as a means of redox regulation of their function. ER proteins, in particular PDI, were found to be associated with the surface of cellular membranes on several types of cells, including endothelial cells, hepatocytes, pancreatic cells, B cells, and cancer cells (7–11). The physiological role of cell-surface PDI has been implicated in various processes such as cellular adhesion, viral entry, and the progression of many diseases (9–12). Because it was found to be specifically exposed at the site of vessel injury, originating both from disrupted vessel wall cells and adhering platelets, it may represent an injury response signal that activates tissue factor, a major initiator protein of blood coagulation (13, 14). PDI was also identified on the platelet surface, where it appears to play an important role in platelet reactions such as adhesion, aggregation, and secretion (9). Both \(\alpha_{\text{IIb}}\beta_3\) and \(\alpha_{\beta_3}\) were reported to be substrates of PDI, and their thiol groups seem to be implicated in platelet adhesion and aggregation. Recent observations support the concept that this phenomenon may be more general in terms of integrin activation. PDI was found to directly interact with \(\alpha_{\beta_3}\) in endothelial cells, and the exofacial disulphide exchange appears to be involved in the conformational changes that follow \(\alpha_{\beta_3}\) activation (10). Similarly, a role for disulfide exchange in the conversion of \(\alpha_{\beta_3}\) and \(\alpha_{\beta_3}\) to a ligand-competent state was suggested (10, 15–18).

Because PDI alone is not sufficient to isomerize disulfide bonds and requires reoxidation, in this study we searched for any oxidoreductase that could contribute to redox that controls and/or cooperates with PDI in close vicinity to \(\alpha_{\text{IIb}}\beta_3\). We provide evidence that Ero1\(\alpha\) is bound to platelet membranes and colocalizes with \(\alpha_{\text{IIb}}\beta_3\) and PDI. Its surface expression increases upon platelet activation, and it binds more efficiently with the activated \(\alpha_{\text{IIb}}\beta_3\) conformer. Thus, Ero1\(\alpha\) can support PDI activity toward \(\alpha_{\text{IIb}}\beta_3\) providing oxidative equivalents to PDI, which in turn reduces or rearranges disulfide bonds in the cargo protein.

**EXPERIMENTAL PROCEDURES**

**Materials**—Apyrase (type VII), bovine serum albumin (fraction V), and thrombin were obtained from Sigma. CD61 and CD41 antibodies were obtained from Chemicon (Temecula, CA). Horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence detection system were
from Pierce. Monoclonal anti-PDI (MA3 019) clone RL90 and (MA3 018) clone RL77 were from Affinity Bioreagents (Golden, CO). Protein A/G-agarose was from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were of reagent grade.

Recombinant Ero1α—The gene encoding Ero1α was excised from pcDNA3.1-Ero1α-Myc (a gift from Dr. R. Sita, Universita Vita-Salute San Raffaele, Milano, Italy) by digestion with XbaI and Pmel and insertion into pRSETa (Invitrogen) for expression in Escherichia coli (BL21; Invitrogen) as a His tag fusion protein containing 12 residues (MRGSHHHHHHGS) at its N terminus. To purify the recombinant Ero1α, cells were harvested and homogenized in a French press, followed by centrifugation at 5000 × g to remove unbroken cells. The cytosol fraction in 1% Triton X-100, 5% glycerol, 50 mM Tris-HCl, pH 7.4, and protease inhibitors was then separated by chromatography on chelating Sepharose (GE Healthcare), loaded with nickel ions according to the manufacturer’s instructions. After washing with 10 mM imidazole, proteins were eluted with 500 mM imidazole in buffer containing 0.1% Triton X-100, 5% glycerol, 50 mM Tris-HCl, pH 7.4, and protease inhibitors. After dialysis against the same buffer to remove imidazole, Ero1α was bound to a 1-ml Hi-Trap Q-Sepharose column and eluted using a linear 0–1 M potassium acetate gradient. Ero1α was identified in the eluted fractions by SDS-PAGE and Coomassie Blue staining.

Anti-Ero1α Antibodies—Highly purified recombinant Ero1α was used to immunize rabbits to raise specific anti-Ero1α antisem, as described previously (19). Anti-Ero1α antibodies were purified by affinity chromatography using recombinant Ero1α immobilized on cyanogen bromide-activated Sepharose. Briefly, specific rabbit anti-Ero1α serum was diluted twice with PBS and passed through an Ero1α-Sepharose column. Nonspecifically bound proteins were removed by washing in two steps. In the first step, the column was washed with 0.01 M Tris-HCl buffer, pH 7.5, containing 150 mM NaCl and 0.1% Tween 20. In the next step, the same buffer containing 1 M NaCl was used. The excess NaCl was removed by washing with PBS, and specifically bound immunoglobins were eluted with 0.5 M acetic acid, immediately dialyzed against PBS, and stored in small volumes at −70 °C. The specificity of anti-Ero1α was determined by Western immunoblotting analysis using platelet and endothelial cell lysates. For flow cytometry studies, anti-Ero1α antibodies and nonimmune rabbit IgG were conjugated with FITC or TRITC according to standard procedure.

Platelet Preparation—All experiments using human subjects were performed in accordance with the Declaration of Helsinki. Whole blood was drawn from healthy, consenting human volunteers into tubes containing one-sixth volume of ACD (2.5 g of sodium citrate, 1.5 g of citric acid, and 2 g of glucose in 100 ml of deionized water). Blood was centrifuged at 230 × g for 20 min at room temperature to obtain platelet-rich plasma. The platelet-rich plasma was then centrifuged for 10 min at 980 × g at room temperature to pellet the platelets. Platelets were resuspended in Tyrode’s buffer (138 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 3 mM NaH₂PO₄, 5 mM glucose, 10 mM HEPES, pH 7.4, 0.2% bovine serum albumin) containing 0.1 unit/ml aprotinin (20). To 1 ml of the platelet suspension, 1 unit/ml thrombin was added to generate platelet-derived microparticles. Following a 10-min incubation period, 50 μl of EGTA (200 mM) was added, and the sample was centrifuged at 710 × g for 15 min to separate platelets from platelet microparticles. Microparticles were pelleted by centrifugation of the supernatant at 150,000 × g for 90 min at 4 °C.

Separation of Platelet Subfractions—Lysates were prepared from resting platelets using a mild detergent lysis buffer (2% Triton X-100, 10 mM EGTA, 100 mM Tris-HCl, 2 mg/ml leupeptin, 100 mM benzamidine, and 2 mM phenylmethanesulfonyl fluoride, pH 7.4). Platelet subcellular fractions were prepared from the lysates as described previously (21). The low speed insoluble fraction corresponding to the cytoskeleton-enriched fraction was obtained through centrifugation at 15,600 × g for 4 min at 4 °C, with subsequent centrifugation of the supernatant at 100,000 × g for 2.5 h to obtain the high speed insoluble fraction corresponding to the membrane-skeleton fraction. The remaining supernatant contained detergent-soluble proteins. The insoluble fractions were solubilized in buffer: 50 mmol/liter Tris, pH 7.4, containing 1 mmol/liter Na₂VO₄, 1% Triton X-100, 500 μmol/liter EDTA, 1 μmol/liter leupeptin, 1 μg/ml aprotinin and 0.1% SDS. All samples were heated to 95 °C and electrophoresed through SDS-polyacrylamide gels.

Coominprecipitation—Platelets were lysed for an hour at 4 °C using a nondenaturing lysis buffer (1% CHAPS, 25 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 2 mM NaF, 1 mM phenylmethanesulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). Cytoskeletal debris was pelleted at 10,000 × g for 10 min. Precleared lysates were incubated with specific antibodies (10 μg/ml) and protein A/G-Sepharose at 4 °C with rotation for 6 h. Captured immune complexes were washed eight times with the lysis buffer containing 0.1% CHAPS and dissolved using Laemmli sample buffer. SDS-PAGE and Western immunoblotting onto nitrocellulose membrane were performed by means of standard procedures. Primary antibodies were used at a concentration of 1 μg/ml. Horseradish peroxidase-conjugated secondary antibodies were diluted 1:8,000. Enhanced chemiluminescence images were collected on x-ray film.

Isolation of Human Platelet Ero1α—Ero1α was isolated by affinity chromatography on anti-Ero1α antibodies coupled to Sepharose 4B using either platelet releasates or solubilized platelet pellets. To determine the optimal pH for Ero1α extraction, washed platelets were suspended in 30 mM HEPES buffer containing 150 mM potassium acetate and 2.5 mM magnesium acetate at different pH values (6.5, 7.0, 7.5, 8.0, 9.0, 11.0, and 13.0). After 10 min, platelet suspensions were centrifuged at 10,000 × g for 10 min, and both supernatants and pellets were analyzed by SDS-PAGE under nonreduced conditions followed by Western immunoblotting. Platelet supernatant obtained at pH 10.0 was loaded onto the column, and after washing with PBS followed by 0.5 M NaCl, fractions containing Ero1α were eluted with 0.2 M glycine buffer, pH 2.6. Purity and specificity of eluted fractions were estimated by SDS-PAGE and Western immunoblotting using immunospecific anti-Ero1α antibodies.

Expression of Ero1α and Its Mutants—The pcDNA3.1Ero1α-myc construct containing the complete wild type coding sequence of Ero1α was gifted by Dr. Roberto Sitàia (Department of Biological and Technical Research, San Raffaele Scientific Institute, Milan, Italy) by digestion with NcoI and XhoI and insertion into pCMV-EGFP vector.
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Institute, Milan, Italy). To replace the cysteines within the conserved Ero1α CXXCXXC motif with alanines, three PCR s were performed on pcDNA3.1Ero1-α-Myc template by using the GeneTailor site-directed mutagenesis system (Invitrogen). The following pairs of oligonucleotide primers were used to obtain pcDNA3.1Ero1(C391A)-myc, pcDNA3.1Ero1(C394A)-myc, pcDNA3.1Ero1(C397A>-myc, respectively: ATATTTCTGAAT-TATTGGATGACGTTGTGTTT and ATCCATAATTCTT-GAGATTTTTATATGCGA; GATATTTGGATGATTTG-TGGTGCATTTAAATGTCG and ACCAACCACAATCAT-TATGGATGCAGTTGGTTT and ATCCATAATTCT-TGTGATTTTGGTGCATTTAAATGTCG. The resulting constructs were propagated in E coli, purified with Wizard MidiPrep (Promega), and sequenced to confirm the open reading frame. MEG01 cells were transiently transfected with wild type propogated in ACAATCCATAATTCTTG. The resulting constructs were TAAAGCACGTCTGTGGGG and TTTAAAACAACCAAC-AATTCTTGAAATATTTC; and ATTGTGTTGGTTGTTT-GTTGTCATTTAAATGTC and ACCAACACAATCCAT-TATGGATGCAGTTGGTTT and ATCCATAATTCT-

**Platelet Aggregation**—The experiments were carried out in a dual channel aggregometer (Labor, Ahrensburg, Germany). Washed platelet suspensions contained 2–4 × 10^8/ml platelets to which fibrogen (200 μg/ml) was added. The examined samples, after mixing, were preincubated at 37 °C for 10 min, without stirring, and placed into the instrument. Immunochemically purified anti-Ero1α or control IgG was preincubated with the platelets for 10 min, although a 1-min preincubation proved almost as effective. Platelet activation was initiated by adding ADP (1–10 μM) to platelets under constant stirring conditions at 37 °C and was observed for 12 min. The results were recorded with a dual channel plotter. At the end of each experiment, platelet responses were restested without inhibitors to ensure continued responses.

**Two-dimensional Polyacrylamide Gel Electrophoresis**—Captured immune complexes obtained by immunoprecipitation of platelet proteins with anti-Ero1α antibody were dissolved in lysis buffer (9 m urea, 4% CHAPS, 1% DTT, 2% Pharmalyte, and Complete inhibitors (Roche Applied Science)). A buffer volume approximately equal to the packed cell volume was used. To improve resolution and recovery of proteins in two-dimen-

**Flow Cytometry**—Platelets, resting or activated with thrombin (0.5 unit), ADP (5 μM), and collagen (5 μg/ml), were incubated for 10 min with anti-Ero1α antibodies labeled with rho-

**Mass Spectrometry, Data Base Search, and Data Processing**—Proteins in each gel slice were subjected to reduction with 10 mm dithiothreitol, alkylation with 50 mm iodoacetamide, and trypptic digestion with modified trypsin (10 μg/ml, Promega) at 37 °C for 14 h. After in-gel digestion, the product peptides were extracted stepwise with three portions of 60 μl 0.1% TFA in 2% acetonitrile and loaded on an RP-18 pre-column (LC Packings). Peptides were eluted to a nano-HPLC RP18 column (75 μm × 15 cm capillary; LC Packings) by acetonitrile gradient in the presence of formic acid and directly applied into an electro-

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FIGURE 1. Ero1α is localized on the surface membrane of blood platelets. A shows the purity of recombinant Ero1α separated by SDS-PAGE (lane a) and the specificity of immunologically purified anti-Ero1α antibodies, shown by Western immunoblotting of blood platelets (lane b) and endothelial cells (lane c). B shows Ero1α associated with platelet pellets and supernatants (Sup.). Platelet lysates were preincubated for 10 min at pH ranging from 7.0 to 10.0, centrifuged for 10 min at 10,000 × g, and analyzed by Western blotting using anti-Ero1α and anti-actin antibodies. Further subcellular fractionation of platelets showed that Ero1α is mostly attached to the membranes (C). Cytoskel., cytoskeleton; Supern., supernatant; Plt., platelet; Membr., membrane. The identity of platelet membranes was proved by the high content of αIIbβ3. The location of Ero1α on the external platelet surface of resting platelets was evaluated with anti-Ero1α labeled with TRITC using confocal microscopy (D) and by FACS analysis (E). All figures show representative data obtained during several 3–5 experiments. Intrinsic fluorescence of platelets is shown by black tracing. For experimental details see “Experimental Procedures.”

Recent studies showed that substantial amounts of PDI, a major Ero1α-binding protein, are present on the surface of blood platelets (9). Therefore, in this study we assessed whether Ero1α could be found in association with PDI on platelet membranes. For this purpose, we expressed recombinant Ero1α in E. coli as a His tag fusion protein, purified on a nickel-chelating column, and refolded by sequential dialysis to remove the denaturant. The final product was soluble in aqueous buffers and was homogeneous as assessed by SDS-PAGE (Fig. 1A). We then raised polyclonal antisera against recombinant Ero1α in rabbits and anti-Ero1α antibodies immunologically purified from serum by immunoaffinity chromatography on immobilized recombinant Ero1α. The concentration of anti-Ero1α antibodies in the original antisera was 600–1094 μg/ml. Anti-Ero1α antibodies were monospecific by Western immunoblotting, giving a single band corresponding to the molecular weight of Ero1α in human platelet lysates and extracts of endothelial cells (Fig. 1A, lanes b and c).

Localization of Ero1α in Platelets—Because human Ero proteins do not contain transmembrane domains, first we assessed the location of Ero1α in platelet cytosol and platelet pellets corresponding to the membrane-enriched fraction. For this purpose, platelet lysates were preincubated for 10 min at different pH values (6.5, 7.0, 7.5, 8.0, 9.0, 11.0, and 13.0) and centrifuged for 10 min at 10,000 × g, then aliquots of supernatants and insoluble pellet fractions were analyzed by Western immunoblotting. Fig. 1B shows that at pH 7.0, a majority of Ero1α is accumulated in pellets, reflecting its interaction with integral membrane proteins. With the increase of pH up to 10.0, there was a dramatic decrease in the amount of Ero1α bound to pellets associated with its release to supernatant. A complete extraction of Ero1α from pellets was accomplished at pH 13.0 (data not shown). Subcellular fractionation of platelets demonstrated that Ero1α is in fact not present in platelet cytosol but is associated with the cytoskeleton, obtained after centrifugation of platelet lysate at 15,600 × g for 4 min at 4 °C, as well as in the high speed insoluble fraction. As evidenced by the high content of αIIbβ3, it corresponds to the membrane-skeleton fraction isolated after subsequent centrifugation of the supernatant for 2.5 h at 100,000 × g (Fig. 1C). The membrane-bound Ero1α appears to be exposed on the platelet surface as further demonstrated by confocal microscopy and flow cytometry. For this purpose, resting platelets were fixed in 3% paraformaldehyde, incubated with anti-Ero1α antibodies conjugated with rhodamine, and subjected to confocal microscopy analysis. Fig. 1D shows that fluorescence derived from Ero1α is localized to the periphery or rim of the platelets. This staining is specific because nonimmune rabbit IgG used in the place of anti-Ero1α antibodies did not produce any reaction (data not shown). The surface exposure of Ero1α was next confirmed by flow cytometry (Fig. 1E). There was significant binding of anti-Ero1α antibodies to platelets indicating significant expression of Ero1α on the resting platelets. In this experiment, anti-PDI and nonimmune rabbit IgGs were used as positive and negative controls, respectively. Furthermore, Ero1α was also present in microparticles generated by activation of platelets with thrombin. In contrast to nonimmune IgG, platelet-derived microparticles strongly stained with anti-Ero1α antibodies (Table 1).
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TABLE 1

Ero1α in platelet-derived microparticles generated by thrombin

| Buffer       | Control IgG | Anti-Ero1α |
|--------------|-------------|------------|
| Mean fluorescence |             |            |
| 13.71 ± 3.45 | 11.02 ± 5.70 | 296.32 ± 36.68 |

Platelet Proteins Interacting with Ero1α—To search for proteins that interact with Ero1α in blood platelets, platelet lysates were immunoprecipitated with anti-Ero1α antibodies, separated by SDS-PAGE under nonreducing conditions, and immunoblotted with anti-Ero1α antibodies. Fig. 2A shows the presence of three bands with molecular masses of ~48, 60–75, and 250–300 kDa that consistently reacted with anti-Ero1α antibodies. Reduction of these complexes resulted in the presence of a double band with molecular masses of 52 and 48 kDa, respectively, which correspond to the reduced and oxidized forms of Ero1α. Small amounts of the oxidized Ero1α were also present when separation was performed under nonreducing conditions. Both bands were identified with confidence by peptide sequencing and peptide mass fingerprinting using an electrospray mass spectrometer. To identify proteins that coimmunoprecipitate with Ero1α, samples of immunoprecipitates obtained with anti-Ero1α antibodies were separated by two-dimensional gel electrophoresis and stained with silver. The first dimension was run using pH that ranged from 3.0 to 10.0, and 12.5% gels were used for the second dimension (Fig. 2B). As a control, proteins obtained during immunoprecipitation using control nonimmune IgG were separated in parallel (data not shown). They showed the presence of IgG but no platelet proteins. Following silver staining, protein spots were excised and subjected to microsequencing using an electrospray mass spectrometer. Criteria for positive protein identification were set as follows: at least three matching peptide sequences, the molecular weight of an identified protein should match estimated values, and a top score given by software not lower than 70. These data show that Ero1α alone or in complex with PDI can interact with a number of platelet proteins that are associated with the platelet cytoskeleton and with integrin αIIbβ3. Among them there were vinculin, filamin, vimentin, and fibrinogen (Fig. 2C).

Ero1α Associates with PDI and αIIbβ3 Integrin in Platelets—We next turned to immunoprecipitation experiments to detect association of Ero1α with PDI or αIIbβ3 in blood platelets. To identify the Ero1α-PDI complex, the blood platelet lysates were incubated with immunochemically purified antibodies either to platelet Ero1α or monoclonal antibody to PDI, respectively. The immunoprecipitates were then isolated using a 50% slurry of protein A/G-agarose (Santa Cruz Biotechnology) overnight at 4 °C. Specifically bound proteins were solubilized in 40 μl of Laemml sample buffer and separated by SDS-PAGE under nonreducing (Fig. 3A) or reducing (Fig. 3B) conditions using a 10% separating gel. Both proteins were identified by Western immunoblotting of immunoprecipitates using specific antibodies (Fig. 3). Thus, the presence of Ero1α in high molecular mass complexes with PDI showing molecular masses of 250–300, 150, and 100 kDa was evidenced (Fig. 3A) by the following: (a) blotting with anti-Ero1α the immunoprecipitate pulled down with anti-PDI antibodies, and (b) blotting with anti-PDI antibodies the immunoprecipitate pulled down with anti Ero1α antibodies. Similar experiments performed to search for the Ero1α-αIIbβ3 complex demonstrated that both proteins can be detected in immunoprecipitates produced either with anti-Ero1α or anti-αIIbβ3 antibodies, respectively (Fig. 3, C and D). Interestingly, in addition to being covalently bound to αIIbβ3 detectable in a broad band of 250–300 kDa, Ero1α appears to be also noncovalently associated with the integrin in a complex that upon SDS treatment dissociates releasing its oxidized 48-kDa form (Fig. 3C, 2nd lane). Finally, confocal microscopy was used to search for the presence of Ero1α on the surface of platelets. For this purpose, human platelets were stained with immunochemically purified antibodies to platelet Ero1α conjugated with rhodamine. The same cells were also incubated with monoclonal antibodies to PDI, αIIbβ3, or β3 conjugated with fluorescein (FITC). Fig. 4, A–C, shows that Ero1α, PDI, and β3 are present on the surface of unstimulated blood platelets, and as shown in the overlay images, both PDI and αIIbβ3 colocalized well with Ero1α. Incubation with nonimmune IgG did not show any staining (data not shown). To evaluate Ero1α and PDI on the surface, the relative brightness of platelets stained with anti-Ero1α and anti-PDI antibodies was taken by a laser-scanning confocal microscope and presented as a percentage of the average brightness of platelets stained with anti-αIIbβ3. Fig. 4D shows several optical sections of platelets stained with anti-Ero1α or anti-PDI. Plots of the relative brightness composition of the image shown in Fig. 4E illustrate an average.
Optical sections of platelets stained with anti-Ero1 antibody binding and flow cytometry were performed in buffers containing 1 mM Ca²⁺ as reflected by the mean fluorescence intensity. Both approaches suggest that Ero1α and anti-PDI antibodies used at concentrations of ≥1.0 and 1.2 μg/ml, respectively. Fig. 4F shows the mean fluorescence intensity of nonactivated platelets stained with anti-Ero1α and anti-PDI antibodies used at concentration of 1 μg/ml. Data obtained by both approaches suggest that Ero1α and PDI are in similar concentrations close to the membrane.

To further confirm colocalization of Ero1α, PDI, and αIIbβ3 on the cellular surface, we next used the megakaryocyte cell line MEG01 in confocal microscopy analysis. MEG01 stained with anti-Ero1α and anti-PDI showed high accumulation and colocalization close to the membranes (Fig. 5A). Although staining with anti-αIIbβ3 was much more intensive than that produced by anti-Ero1α, colocalization of both proteins was clearly visible (Fig. 5B).

Ero1α Is Implicated in the Regulation of Platelet Activation—In the next experiments, expression of Ero1α on blood platelets activated with ADP, collagen, or thrombin was evaluated by flow cytometry. Fig. 6A shows that activation of blood platelets with all agonists resulted in significantly increased binding of anti-Ero1α to their surface indicating enhanced association of Ero1α with platelet membranes. Table 1 shows the mean fluorescence of platelets activated with ADP, collagen or thrombin when tested by FACS after binding of anti-Ero1α or nonimmune IgG. Expression of Ero1α significantly increased upon activation with all three agonists when compared with control unstimulated platelets (p < 0.001). To determine whether αIIbβ3 is involved in this interaction, platelets were activated in the presence of 1 mM RGD or RGE peptides then extracted with a nondenaturing lysis buffer containing 1% CHAPS, and immunoprecipitated with antibodies specific to the β3 integrin subunit. The immunoprecipitate was isolated using protein A/G-Sepharose and after extensive washing was solubilized in Laemmli sample buffer. Fig. 6B shows the separation of anti-β3 immunoprecipitates by SDS-PAGE followed by transfer onto nitrocellulose and immunostaining with anti-Ero1α antibodies. Control blood platelets showed the presence of monomers and dimers of Ero1α. In addition, its complex with PDI and αIIbβ3 with molecular masses of 100 and 250–300 kDa, respectively, could be seen. Activation of platelets with collagen in the presence of RGE resulted in significantly higher binding of Ero1α to...
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FIGURE 5. Colocalization of Ero1α, PDI, and αιιβ₃₃ analyzed by confocal microscopy of megakaryocyte cell line MEG01. Representative thin section confocal fluorescence micrographs of MEG01 were obtained during three different experiments. Cells were stained with antibody to Ero1α conjugated with FITC and counterstained with antibodies conjugated with TRITC specific to PDI (A) or integrin αιιβ₃₃ (B). In these experiments, we used the affinity-purified rabbit polyclonal antibodies to platelet Ero1α or PDI and monoclonal antibodies to αιιβ₃₃ (CD41α, BD Biosciences). The average fluorescence derived from Ero1α and PDI or αιιβ₃₃ when merged, revealed extensive colocalization of these proteins.

FIGURE 6. Stimulation of blood platelets with ADP, collagen, or thrombin increases expression of Ero1α on their surface. Blood platelets were stimulated with ADP (10 μM), collagen (1 μg/ml), or thrombin (0.5 units) for 10 min and then analyzed by flow cytometry to evaluate binding of anti-Ero1α antibodies conjugated with FITC (A). Binding of anti-Ero1α and anti-PDI antibodies to resting platelets is shown as controls. B shows immunoprecipitates obtained with monospecific antibody to β₃, integrin subunit using lysates of resting platelets and collagen-activated platelets in the presence of RGD or RGE peptides (1 mM). The immunoprecipitates were isolated with protein A/G-Sepharose, washed, and solubilized in Laemmli sample buffer. After separation by SDS-PAGE under nonreducing conditions and electrophoretic transfer onto nitrocellulose, proteins were immunostained with anti-Ero1α antibodies, followed by second antibodies conjugated with horseradish peroxidase. The presented data are representative of at least three different experiments.

αιιβ₃₃ as demonstrated by its increased level found in the anti-β₃ immunoprecipitate, both covalently (broad band 250–300 kDa) and noncovalently bound (bands of 48, 70, and 100 kDa). RGD markedly reduced the amount of Ero1α bound to αιιβ₃₃, well documented by decreased intensity of all bands positively stained with anti Ero1α antibodies. To express these data in a quantitative manner, gels with separated immunoprecipitates, obtained during several experiments, were scanned densitometrically, and changes in amounts of Ero1α as well as of its complexes were expressed as the percentage of that found in the control immunoprecipitate (Table 3). This supports the concept that binding of Ero1α to the activated form of αιιβ₃₃ is enhanced. To see whether increased interaction of Ero1α with αιιβ₃₃ affects its receptor activity, we next checked whether anti-Ero1α antibodies interfere with fibrinogen binding to platelets and platelet aggregation. Immunochemically purified anti-Ero1α antibodies inhibited ADP aggregation of washed platelets in a concentration-dependent manner (Fig. 7A). Control IgG had no effect. In the next experiment, resting platelets or platelets activated with collagen were incubated with FITC-labeled fibrinogen and analyzed by flow cytometry. As shown in Fig. 7B, blocking of Ero1α with specific antibodies reduced binding of fibrinogen to activated platelets. This inhibitory effect was consistently observed in three separate experiments, and inhibition of binding produced by anti-Ero1α antibodies relative to normal IgG amounted to 56 ± 12% (p < 0.001). Preincubation of platelets with IgG isolated from nonimmune serum had no effect on levels of FITC-fibrinogen binding. The effect of anti-Ero1α on the activation of integrin αιιβ₃₃ was further evaluated by measuring binding of PAC-1 antibody to resting or stimulated platelets. For this purpose, platelets were activated with 5 μM ADP or 5 μg/ml collagen, as described under “Experimental Procedures.” Fig. 7C shows that anti-

### TABLE 2

| Agonist | Control IgG | Anti-Ero1α | p values |
|---------|-------------|------------|----------|
| Control | 12 ± 5      | 25 ± 12    |          |
| ADP     | 13 ± 3      | 61 ± 10    | p < 0.001|
| Collagen| 15 ± 3      | 142 ± 15   | p < 0.001|
| Thrombin| 10 ± 4      | 328 ± 25   | p < 0.001|

### TABLE 3

| Band Composition | Control platelets + Collagen + RGE | Collagen + RGD | p values |
|------------------|-----------------------------------|----------------|----------|
| 48 Ero1α         | 100 ± 52                          | 103 ± 22       | p < 0.05 |
| 70 Ero1α/Ero1α   | 100 ± 45                          | 43 ± 11        | p < 0.001|
| 100 Ero1α/PDI    | 100 ± 58                          | 33 ± 20        | p < 0.001|
| 250 Ero1α/PDI/αιιβ₃₃ | 100 ± 32                         | 50 ± 15        | p < 0.001|

### Figure 7

- **A**: Flow cytometry histograms showing binding of FITC-labeled fibrinogen to platelets activated with ADP, collagen, or thrombin. The histograms show a significant decrease in fibrinogen binding in the presence of anti-Ero1α compared to control IgG.
- **B**: Western blots showing increased expression of Ero1α in platelets activated with ADP, collagen, or thrombin. The bands corresponding to Ero1α increase in intensity in the presence of the agonists.
- **C**: Immunohistochemical staining of αιιβ₃₃ in platelets activated with ADP, collagen, or thrombin, showing enhanced expression of αιιβ₃₃ in activated platelets.
Ero1α antibodies, similar to anti-PDI antibodies, significantly inhibited binding of PAC-1 to integrin αIIbβ3 stimulated by either ADP or collagen.

Finally, to further indicate a role of Ero1α in αIIbβ3 receptor activity, we used the megakaryocyte cell line MEG01 transfected with wild type Ero1α and its inactive mutants and tested their interaction with fibrinogen. For this purpose, we replaced the cysteines within the conserved Ero1α CXXCXC motif with alanines and produced the following constructs: pcDNA3.1Ero1C391A-myc, pcDNA3.1Ero1C394A-myc, and pcDNA3.1Ero1C397A-myc. Table 4 shows that in contrast to pcDNA3.1Ero1-myc, pcDNA3.1Ero1C397A-myc resulted in significantly increased binding of fibrinogen, and this effect was associated with increased exposure of thiol groups as evaluated by binding of 3-N-maleimidopropionyl biocytin. This effect was abolished by RGDS.

**DISCUSSION**

Activation of αIIbβ3 is a dynamic multistep process that involves disulfide bond exchange-independent and -dependent mechanisms (24–26). The redox-controlled remodeling of αIIbβ3 results in forming two distinct conformers, active and nonactive, which differ in arrangement of disulfide bonds. Interestingly, this process takes place in the exofacial domains of αIIbβ3. There are two possible mechanisms by which isomerization of such bonds can occur extracellularly. By using either (a) inherent enzymatic PDI activity of αIIbβ3, it has been shown that the β3 integrin subunit possesses active site motifs CGXC and expresses endogenous thiol isomerase activity, but it is not known if this activity is sufficient to promote the conformational change in either direction (14), or (b) isomerization of selected disulfide bonds in αIIbβ3 is catalyzed by PDI, although to date, the only physical association on the platelet surface that has been shown for PDI is with glycoprotein Ibα (27). However, it is noteworthy that we described such direct interaction of PDI with αIIbβ3 integrins in endothelial cells (22), which recently was confirmed by in vivo studies using β3-null mice and multichannel fluorescence intravital microscopy (28). It was shown that accumulation of PDI observed near the site of vessel injury was almost abolished in β3-null mice compared with wild type mice. Obviously, in the second mechanism for PDI to be reoxidized would require cooperation with Ero1α or another ER enzyme (5).

Our experiments show for the first time that Ero1α can be found on the membrane of resting blood platelets and forms complexes both with PDI and αIIbβ3 that are localized extracellularly. Prior to this study, it was known that PDI was present on the external membrane of activated and resting platelets, and it could be secreted from activated platelets (27). Blocking PDI with inhibitory antibodies abolished a number of platelet responses, including aggregation, adhesion, fibrinogen binding, and integrin activation (10, 16, 29). PDI is generally believed to be the primary acceptor of disulfide bonds from Ero1α, and both proteins can be found as a covalent complex linked by an inter-chain disulfide bond when cell lysates are separated by SDS-PAGE under nonreducing conditions (30–34). Unexpectedly, our proteomic analysis showed that there are numerous proteins that can be bound directly or indirectly to Ero1α and that coimmunoprecipitate with anti-Ero1α antibodies. Among them were all components of focal adhesion contacts in the activated platelets, including vinculin, vimentin, actin, and...
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fibrinogen, as well as αIibβ3 and PDI. Therefore, to prove that Ero1α interacts with PDI and αIibβ3, we performed coimmunoprecipitation experiments using specific antibodies to Ero1α, PDI, and αIibβ3. They showed that Ero1α can be found in several complexes with PDI and αIibβ3, both covalently and noncovalently linked. The latter dissociated in the presence of SDS. All three types of antibodies immunoprecipitated high molecular mass complexes, which migrated as a broad band corresponding to 250–300 kDa. It contained Ero1α, PDI, and αIibβ3. Interestingly, the immunoprecipitate obtained with anti-αIibβ3 antibodies also contained, in addition to covalently bound Ero1α, the monomeric oxidized and dimeric forms of Ero1α as well as a covalent complex between Ero1α and PDI linked by an inter-chain disulfide. This complex had a molecular mass of 110 kDa. The Ero1α-dependent PDI oxidation involves several steps as follows: docking of reduced PDI; formation of PDI-Ero1α-mixed disulfides; detachment of oxidized PDI; and recharging of Ero1α. Hence, the presence of such heterogeneous complexes of Ero1α found in the coimmunoprecipitates is consistent with the mechanism by which it may cooperate with PDI during isomerization of disulfide bonds in αIibβ3. All these species containing Ero1α increased on the platelet surface upon platelet activation, suggesting that active Ero1α readily becomes available for redox regulation of αIibβ3. However, given the inhibitory role of RDG peptides for the association of Ero1α with integrin αIibβ3 (Fig. 6B and Table 3) and the presence of fibrinogen in the protein complex immunoprecipitated by anti-Ero1α antibodies (Fig. 2), an alternative interpretation of the data might be that Ero1α actually binds to the integrin. Indeed, it is likely that both RDG peptides and Ero1α compete for fibrinogen binding through occupancy of the RDG binding sequence.

Recently, additional thiol isomerase enzymes, called endoplasmic reticulum proteins 5, 29, 44, 57, and 72 (ERP5, ERP29, ERp44, ERp57, and ERp72) and transmembrane thiol isomerase 3 (TMX3), were identified in platelets and megakaryocytes (35, 36). ERP5 was found to behave similarly to PDI because of its levels on the platelet surface but did not increase following activation. Extracellular PDI was implicated in the regulation of coagulation by the modulation of tissue factor activity (28). ERp57 was also identified within platelet-derived microparticle fractions, suggesting that ERp57 may also be involved in the regulation of coagulation as well as platelet function. Whether Ero1α cooperates with ERp5 and other thiol isomerase enzymes is not known.

To sum up, transition of the integrin αIibβ3 from an inactive to a ligand-binding state involves rearrangement of the disulfide bonding pattern. We have demonstrated a coassociation of Ero1α with PDI and αIibβ3 in activated platelets. We suggest that when associated with PDI, Ero1α, by providing oxidative equivalents, is able to assist in the conformational change of the integrin from an inactive to an active state. The mechanism through which this is regulated is uncertain. Formation of disulfide bonds within the endoplasmic reticulum requires the combined activities of Ero1α and PDI. As Ero1α produces hydrogen peroxide during oxidation, to ensure sustained oxidation in the endoplasmic reticulum it requires the inclusion of glutathione. How this process occurs extracellularly is unknown. The interaction with and regulation by other molecules, such as the interaction of ERp5, may also play a role. Because it is possible to inhibit integrin ligation by inhibitory antibodies to Ero1α, it appears that Ero1α can play a role upstream of both in the activation process.

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