A role for the thiol isomerase protein ERP5 in platelet function

Running title: Thiol isomerase ERP5 and platelet function


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Abstract

Formation and rearrangement of disulphide bonds during the correct folding of nascent proteins is modulated by a family of enzymes known as thiol isomerases, which include protein disulphide isomerase (PDI), ERP5 and ERP57. Recent evidence supports an alternative role for this family of proteins on the surface of cells, where they are involved in receptor remodelling and recognition. In platelets, blocking PDI with inhibitory antibodies inhibits a number of platelet activation pathways including aggregation, secretion, and fibrinogen binding.

Analysis of human platelet membrane fractions identified the presence of the thiol isomerase protein ERP5. Further study showed that ERP5 is mainly resident on platelet intracellular membranes, although it is rapidly recruited to the cell surface in response to a range of platelet agonists. Blocking cell-surface ERP5 using inhibitory antibodies leads to a decrease in platelet aggregation in response to agonists, and a decrease in fibrinogen binding and P-selectin exposure. It is possible that this is based upon the disruption of integrin function as we observed that ERP5 becomes physically associated with the integrin $\beta_3$ subunit during platelet stimulation. These results provide new insights into the involvement of thiol isomerases and regulation of platelet activation.
Introduction

In classical terms reduction / oxidation systems within a cell have been represented very simply. The cytoplasmic environment is hypoxic, and reducing in nature, whereas the extracellular environment is normoxic, and oxidising. The generation of a disulfide bond from two cysteine residues is an oxidation reaction. To correctly generate these bonds inside the cell there are, therefore, a group of enzymes known as the thiol isomerases. These are capable of the formation, reduction, and rearrangement of the disulfide bonding patterns of proteins, often as part of folding of nascent proteins. The thiol isomerase enzymes are anchored to the endoplasmic reticulum via KDEL-receptor proteins1-3. Recent studies have suggested additional functionality for thiol isomerase enzymes – on the surface of cells, where they participate in receptor activation and remodelling, and substrate processing4-6.

Protein disulphide isomerase (PDI) is the best characterised thiol isomerase to demonstrate this dual functionality. A number of cell types including bovine aortic endothelial cells7, rat hepatocytes8,9, and human B cells5,10, have been shown to secrete PDI, which associates with the cell surface. Cell-surface PDI has been implicated in the reduction of the disulphide linked diptheria toxin heterodimer11 and events triggering entry of the HIV virus into lymphoid cells6,12. Based upon a series of investigations, initially by Detweiller and co-workers, a role for PDI in platelet physiology is now established4,13-16. Early studies demonstrated PDI was present on the external membrane of activated and resting platelets and proteins with thiol isomerase activity were secreted from activated platelets. Indeed, cell-surface exposure of free thiol groups, and those from PDI in particular, are elevated following platelet activation17. Further studies demonstrated inhibition of PDI with inhibitory antibodies can block a number of platelet responses including aggregation, adhesion, fibrinogen binding, and integrin activation16,18-20. Reagents that block cell-surface thiol groups such as para-chloromercuriphenyl sulfonate, dithiobisnitrobenzoic acid, and bacitracin have also been shown to inhibit these functions19,21. This inhibition has often been to a greater degree than that
observed for anti-PDI antibodies indicating that there may be additional proteins involved in this process. The mechanistic basis for these observations has not been determined, although it has been proposed that they are based upon interaction with integrins, in particular integrins $\alpha_2\beta_1$ and $\alpha_{III}\beta_3$. Studies have shown that the different affinity states for the ecto-domain of $\alpha_{III}\beta_3$ have different conformations and evidence indicates switching between states is a redox active process with a different arrangement of disulphide bonds in the two conformations. It has been shown that $\alpha_{III}\beta_3$ and $\alpha_V\beta_3$ possess endogenous thiol isomerase activity, but it is not known if this activity is sufficient to promote the conformational change in either direction. However, there must be an additional level of regulation to prevent the receptor being presented in a constitutively active form. It is possible that this could involve PDI, although, to date, the only physical association on the platelet surface that has been shown for PDI is with glycoprotein Ib$\alpha$ and not $\alpha_{III}\beta_3$.

In this study we report the isolation of an additional thiol isomerase enzyme from human platelet membranes, which was identified as ERP5. Following platelet activation levels of ERP5 on the platelet surface were rapidly elevated. Antibodies that block the thiol isomerase activity of ERP5 were found to inhibit platelet function. Notably, ERP5 was found to become associated with the integrin $\beta_3$ subunit on platelet stimulation.
Materials and Methods

Materials

Platelet agonists: collagen (Horm, type I from equine tendon, Nycomed, Munich, Germany), convulxin, and thrombin (Sigma, Poole, UK). Convulxin was purified from snake venom and a generous gift from Drs. M. Leduc and C. Bon (Institut Pasteur, Paris, France). Two venom preparations were used, with different potencies. Horseradish peroxidase- conjugated secondary antibodies and the enhanced chemiluminescence detection system were from Amersham Bioscience; RNase (Roche, Lewes, UK); bovine serum albumin (First Link, Birmingham, UK); anti-β3 clone AP3 was kindly provided by Prof. P. Newman (Blood Research Institute of Southeastern Wisconsin, USA); monoclonal anti-PDI, MA3-019 (Affinity Bioreagents, Golden, CO); PE-conjugated anti-CD62p, P-Selectin, (BD Biosciences, Oxford, UK). IV.3 hybridoma cell line was from the ATCC (HB-217). F(ab) fragments of the IV.3 antibody were generated using Immunopure kit (Pierce, Tattenhall, UK). All other reagents were purchased from Sigma. Protein concentrations were determined by Bradford assay (BioRad, Hemel Hempstead, UK).

ERP5 purification

Platelet membranes prepared from ~5 units of blood (provided by Dr P Smethurst, University of Cambridge, UK) were solubilised in buffer containing 1% (v/v) Triton X-100. Affinity chromatography was performed using 50µg convulxin coupled to 200µl Sepharose 4B. Eluted fractions were subjected to SDS-PAGE, western blotted onto PVDF membrane and stained. Bands of approximately 50kDa were subjected to protein sequencing by Edman degradation.

Antibody generation

Full-length ERP5 gene (cDNA clone provided by Prof. M. Kikuchi, Ritsumeikan University, Japan\cite{26,27}) was cloned into the pGEX4T2 expression vector, to generate a ERP5-GST fusion protein. Recombinant protein was located in inclusion bodies from E. coli and isolated via urea solubilization and refolding to >98% purity, as estimated by SDS-PAGE. Polyclonal antibodies were raised
against the fusion protein and purified using protein G-sepharose. Specificity was determined by immunoblotting platelet lysates and comparing with anti-PDI, and anti-CaBP1 (anti-CaBP1 antibody to the rat homologue of ERP5 was a gift from Dr. D. Ferrari, Max Planck Institute, Germany), and with antibody neutralised using recombinant ERP5. Polyclonal anti-PDI antibodies were raised in rabbits using purified recombinant PDI (human PDI expression vector (pLWRP62) was provided by Dr L.W. Ruddock, Biocenter Oulu, Finland).

**Preparation of highly purified platelet plasma (PM) and intracellular membranes (IM)**

Platelet PM and IM were prepared as described in detail previously. Briefly, platelets were separated from human blood and treated with neuraminidase (type X, 0.05 U/ml) for 20 min at 37°C. After washing, platelets were disrupted by sonication and the platelet homogenate centrifuged at 42,000 x g for 90 min on a linear (1-3.5 M) sorbitol density gradient to obtain a mixed membrane (MM) fraction (free of granular contamination). MM were separated into PM and IM by free-flow electrophoresis using an Octopus apparatus (Dr. Weber Gmbh, Germany) running at 750V, 100mA. Two discrete peaks comprising PM and IM (more electronegative) were obtained. Tops of peaks were pooled, centrifuged (100,000 x g, 60 min) and resuspended in 0.4 M sorbitol, 5% glycerol and 10 mM triethanolamine pH 7.2.

**Preparation and stimulation of washed platelets**

Human platelets from drug-free volunteers were prepared freshly by differential centrifugation as described previously and resuspended in modified Tyrode’s/Hepes buffer (134mM NaCl, 0.34mM Na₂HPO₄, 2.9mM KCl, 12mM NaHCO₃, 20mM Hepes, 5mM glucose, 1mM MgCl₂, pH 7.3).

Stimulation of platelets with collagen, convulxin, and thrombin was performed in an optical aggregometer (Chrono-log) at 37°C with continuous stirring. For platelet aggregation and flow cytometry studies platelets were stimulated at 4x10⁸ cells/ml, for immunoprecipitation studies platelets were stimulated at 1x10⁹ cells/ml. Where necessary non-aggregating conditions were
maintained by addition of EGTA (1mM), and secondary stimulation by released thromboxane A2 or secreted ADP prevented by inclusion of indomethacin (10µM) and apyrase (2U/ml), respectively.

**Co-immunoprecipitation**

Standard procedures for immunoprecipitation were followed\(^3\). Platelets were lysed with ice-cold NP-40 buffer (300mM NaCl, 20mM Tris, 10mM EDTA, 2% NP-40, 1mM phenylmethylsulfonyl fluoride, 2mM Na\(_3\)VO\(_4\), 10µg/ml leupeptin, 10µg/ml aprotinin, and 1µg/ml pepstatin A, pH 7.3). Pre-cleared lysates were incubated with specific antibodies (1µg) and protein A (or G) sepharose at 4°C with rotation for 90 minutes. Following SDS-PAGE and Western blotting onto PVDF immunoblotting was performed using standard procedures\(^2\). Primary antibodies were used at a concentration of 1µg/ml. Horseradish peroxidase conjugated secondary antibodies were diluted 1:10,000. Blots were stripped and reprobed to verify equivalent levels of protein loading. ECL images were collected on X-ray film.

**Flow cytometry**

Platelets were stimulated with agonist in the presence of EGTA (1mM), indomethacin (10µM), and apyrase (2U/ml). Stimulation was terminated by addition of modified Tyrode’s/Hepes buffer containing 1% (w/v) bovine serum albumin, 1mM EGTA, 200µM sodium azide. Primary antibody was added at appropriate dilutions and incubated for 1hr on ice. Secondary antibody (fluorescein isothiocyanate-conjugated IgG) was used at 1:2000 dilution and incubated for 1hr on ice in the dark. Data were collected and analysed using a Becton Dickinson FACScan flow cytometer and CELLQuest software.

**Fibrinogen binding**

FITC-labelled human fibrinogen binding was measured as for flow cytometry with the omission of EGTA from all buffers. Where required, samples were incubated with α-ERP5 antibodies, or control pre-immune antibodies, prior to platelet stimulation. All samples were pre-incubated with saturating concentrations of a F(ab) fragment of monoclonal antibody IV.3 to prevent signalling through the
FcγRIIA receptor. Saturating concentrations were determined as the amount of IV.3 F(ab) fragment, pre-incubated with platelets, required to completely inhibit platelet aggregation caused by subsequent incubation with whole IV.3 and cross-linking using anti-mouse IgG2a F(ab’)2 fragment31.

RNAse activity assay

Thiol isomerase activity was assessed by the ability to renature reduced and denatured RNAse (rdRNAse). The assay was performed as outlined by Pigiet et al 25,32. Reactivated RNAse was assayed by the degradation of cyclic 2’-3’cytidine monophosphate (cCytP), measured by increase in absorbance at 284nm in refolding buffer (50mM NaPO₄, pH 7.2, 50mM NaCl, 1.5mM GSH, 500μM GSSG, 400μM CaCl₂, 400µM MgCl₂). Controls of rdRNAse only, cCytP substrate only (blank), protein and cCytP substrate but no rdRNAse, were run with each set of experiments. The activity was expressed relative to native RNAse, or as a percentage of inhibition of activity for antibody blocking experiments.

Calcium mobilisation assay

Mobilisation of calcium from intracellular stores was measured using a fast-filter ratiometric technique based on fluorescence of the calcium binding compound Fura-231,33. Platelets (~5x10⁹ cells/ml) were loaded with Fura-2-am (5µM) and resuspended at 4x10⁸ cells/ml in tyrodes buffer31,33. Prior to stimulation EGTA (1mM) was added to each sample. Data was collected on a Perkin Elmer LS50B fluorimeter and analysed using FLWinLab software as described previously31,33.

Data Analysis

Data was analysed using SPSS software to calculate two-tailed paired t-test at a 95% confidence value.
Results

**ERP5 is present in human platelets – associated with intracellular and plasma membranes**

An unknown protein was purified from human platelet membrane fractions using a convulxin affinity column. Convulxin, isolated from the venom of the rattlesnake, possess a high affinity for the platelet glycoproteins GP VI and GP Ib\(^{34,35}\). N-terminal sequence data was obtained for the first fifteen residues of the protein (LYSSSDDVIELTPSN). A BLAST search revealed this to be identical to the sequence of ERP5 following cleavage of a predicted signal sequence.

ERP5 was cloned by Hayano *et al* from a placental cDNA library while screening for proteins related to protein disulphide isomerase, PDI\(^{26}\). The gene sequence encodes a 48kDa protein containing two active thioredoxin domains (containing CGHC motifs) that share 47% aa sequence identity with human PDI, a C-terminal peptide binding domain, and a KDEL sequence for retention in the endoplasmic reticulum\(^{26}\). Sequence alignment studies by Ferrari and Soling suggest that ERP5 and PDI share a similar domain structure\(^{2,36}\).

Given its expected restriction to the ER, the presence of ERP5 in membrane fractions was confirmed by immunoblot analysis (not shown). The membrane association was examined more closely using intracellular (IM) and plasma membranes (PM) from resting platelets isolated by free-flow electrophoresis, Figure 1A. ERP5 was present on both intracellular and plasma membranes. The IM fraction contains membranes of intracellular origin such as endoplasmic reticulum, but excludes granule membranes and surface-connected membranes. The higher loading of PM indicate that the predominant location of ERP5 in resting platelets is the intracellular membranes. The purity of platelet membrane fractions was assessed by complete separation of the two peaks following FFE, absence of SERCA 2b in PM and absence of GP1b in IM (not shown). Full characterization of these fractions has been extensively reported previously\(^ {28,37}\). ERP5 was detected using polyclonal antibodies raised to a GST-fusion protein containing full-length human ERP5. A protein was detected on Western blots (Figure 1B) of the same mobility as that recognised by antibodies to
CaBP1, the rat homologue of ERP5 (not shown)\textsuperscript{36}. These antibodies showed no cross-reactivity with human recombinant and platelet PDI (Figure 1B).

Flow cytometry was employed to confirm cell surface expression of ERP5 and investigate whether this was a static or dynamic process. Washed platelets were stimulated with agonists convulxin, collagen or thrombin and the concentration- and time-dependent patterns of cell-surface exposure for ERP5 studied (Figure 2). Low levels of cell-surface ERP5 were found to be substantially and rapidly increased following stimulation with each agonist in a concentration-dependent manner. To investigate the trends observed in time-dependence profiles, data were normalised to the greatest response within an individual experiment, and averaged to overcome donor variability (Figure 2.iii). All three agonists demonstrate biphasic profiles, where there is an initial rapid increase in cell-surface exposure, which peaks at approximately 45s, with substantial increases seen as rapidly as 15s. For convulxin and thrombin this is followed by a lag phase where exposure levels are maintained or dip slightly between 60s and 120s before beginning to rise again over the period of 150-300s. These data suggest there are secondary effectors or mechanisms present for promoting a second wave of cell-surface expression of ERP5 in response to these agonists. These experiments were performed in the presence of EGTA, apyrase, and indomethacin which block the second wave of platelet aggregation responses based on fibrinogen, ADP, and thromboxane A2. Microaggregates based upon $\alpha_{ib}\beta_3$ interactions have been reported to form in the presence of EGTA\textsuperscript{38}, this may account for a second wave of signalling. A different profile was observed for the time-dependent increase in cell-surface exposure of ERP5 with the agonist collagen. Again there was a rapid increase which peaked at approximately 45s but subsequently these levels decreased to approximately baseline values by 300s.

**ERP5 protein has thiol isomerase activity**

Based upon refolding of reduced denatured RNAse, previous studies have demonstrated thiol isomerase activity for bovine liver ERP5, CaBP1, and human PDI\textsuperscript{36}. To verify that human ERP5 is a functionally active thiol isomerase we analysed the recombinant fusion protein in this assay system.
The protein was found to possess thiol isomerase activity, with activity approximately 70% of that measured for molar equivalents of the PDI recombinant fusion protein. Human ERP5 immunoprecipitated from platelet samples using a non-function-blocking antibody also demonstrated thiol isomerase activity (not shown). It was found that under the assay conditions employed thiol isomerase activity of both ERP5GST and PDIIHis was dependent on divalent cations and inhibited in the presence of EDTA (not shown). This is opposite to the observed thiol isomerase activity profile for the integrin sub-unit β3, which has been shown to display enhanced activity in the presence of EDTA25. Such differential cation-dependence for thiol isomerase activity may be important in the cellular regulation of the activity of these proteins and may imply distinct functions.

The effect on enzymatic activity of ERP5 by antibodies to ERP5 was investigated. Antibodies raised in sheep against recombinant ERP5 were found to inhibit enzyme activity, where pre-immune IgG, and monoclonal antibodies against human PDI, displayed no effect on activity (Figure 3B). In addition anti-ERP5 antibodies showed no inhibitory effect on the thiol isomerase activity of recombinant human PDI (not shown). It was not possible to block completely the thiol isomerase activity of ERP5GST, even at very high antibody concentrations, consistent with studies performed on PDI with function-blocking antibodies.

Platelet aggregation is inhibited by inhibition of ERP5 activity

Activity-blocking anti-ERP5 antibodies were used to investigate the potential involvement of ERP5 in the regulation of platelet function. Platelets were stimulated with collagen or convulxin following incubation with anti-ERP5 antibodies or control IgG purified from pre-immune serum from the animal used to raise the antibodies. Prior to addition of inhibitory antibodies platelets were incubated with saturating concentrations of F(ab) fragment of the monoclonal antibody IV.3 to prevent signalling through the FcγRIIa receptor31. The traces shown in Figure 4 demonstrate anti-ERP5 antibodies inhibit the aggregation response induced by low concentrations of convulxin and collagen. Platelet aggregation was reduced substantially by 6μg/ml anti-ERP5 in response to
2.5µg/ml collagen. The aggregation profile was reversible with platelets showing signs of disaggregating after 120s. Addition of higher concentrations of anti-ERP5 antibodies further decreased the level of aggregation observed. For convulxin, aggregation was reduced substantially following incubation with 12µg/ml of antibody and it was possible to completely inhibit aggregation at higher antibody concentrations. For both collagen and convulxin, pre-incubation with antibodies did not inhibit shape change. At higher concentrations of collagen and convulxin it was possible to overcome inhibitory effects of anti-ERP5 antibodies. The additive effect of anti-ERP5 and anti-PDI antibodies on platelet aggregation was investigated (figure 4.ii). Sub-maximal antibody concentrations (with regard to inhibitory activity) were used in these experiments because at high concentrations aggregation is completely inhibited. Total antibody concentrations were controlled for using pre-immune IgG in samples containing only a single inhibitory antibody. The results indicate that there is a modest additive effect on the inhibition of platelet aggregation when anti-ERP5 and anti-PDI antibodies are used in combination. This does not appear to be synergistic as there is no enhancement of the effect when the two antibodies are combined.

Agonist-stimulated mobilisation of calcium from intracellular stores was measured to determine whether anti-ERP5 antibodies affected platelet activation signalling itself, and thereby reduced platelet activation. Mobilisation of calcium from intracellular stores was not affected by incubation of platelets with anti-ERP5 antibodies (Figure 4C). Platelet activation signalling per se is therefore unaffected by anti-ERP5 treatment, but partial inhibition of ERP5 affects downstream functional responses that lead to aggregation

**ERP5 is implicated in the regulation of fibrinogen binding**

In view of the inhibition of aggregation observed following blockade of ERP5, and previous studies that have reported anti-PDI-mediated inhibition of fibrinogen binding4,19, we investigated the ability of platelets to bind fibrinogen in the presence of inhibitory anti-ERP5 antibodies. Flow
cytometry was used to measure the binding of FITC-labelled fibrinogen to collagen- and convulxin-stimulated platelets (Figure 5). A greater shift in fluorescence is observed by flow cytometry at higher agonist concentrations. Therefore, to increase the dynamic range for these experiments higher agonist concentrations were used relative to the aggregation studies. Stimulation of platelets resulted in an increase in the level of binding of FITC-fibrinogen, consistent with an increase in affinity of integrin $\alpha_{\text{IIb}}\beta_3$\textsuperscript{39,40}. Incubation of platelets with anti-ERP5 antibodies, or monoclonal anti-PDI antibodies, resulted in a significant decrease in platelet binding to fibrinogen for both agonists. In response to 100ng/ml convulxin, platelet binding of fibrinogen reduced by 70% and 91% for anti-ERP5 (P<0.005) and anti-PDI (P<0.005) antibodies respectively. When 10$\mu$g/ml collagen was used the reduction in fibrinogen binding was more modest, at 25% and 29% for anti-ERP5 (P<0.05) and anti-PDI (P<0.05) respectively. This is consistent with the more modest inhibitory effect of anti-ERP5 on collagen stimulated platelet aggregation. Pre-incubation of platelets with IgG from pre-immune sera had no effect on levels of FITC-fibrinogen binding. The effect of a combination of anti-ERP5 and anti-PDI antibodies on fibrinogen binding was studied (figure 5.ii.). For both convulxin and collagen stimulated platelets an additive effect was observed for the reduction in platelet binding of fibrinogen, at sub-maximal concentrations of inhibitory antibodies. For either single antibody with respect to a combination of antibodies (anti-ERP5 plus anti-PDI) the difference in fibrinogen binding is significant (P<0.05), but does not appear to be synergistic. At higher concentrations of inhibitory antibodies this effect is reduced and a less-than-additive effect is observed (data not shown).

\textit{\textalpha-\text{granule secretion is inhibited by anti-ERP5}}

P-selectin exposure, a marker of $\alpha$-granule secretion \textsuperscript{41,42}, was inhibited in response to the agonists collagen and convulxin by blocking ERP5 or PDI with specific function-blocking antibodies (Figure 6). In response to 100ng/ml convulxin, there was a decrease in surface exposure for P-Selectin of 73% and 94% in the presence of anti-ERP5 (P<0.01) and anti-PDI (P<0.001) antibodies,
while in response to 10µg/ml collagen a decrease of 39% (P<0.02) and 46% (P<0.005) was measured. The effect of a combination of anti-ERP5 and anti-PDI antibodies on P-Selectin exposure was also studied (figure 6.ii.). For both convulxin- and collagen-stimulated platelets an additive effect was observed for the reduction in the cell surface expression of P-selectin, at sub-maximal concentrations of inhibitory antibodies. In a similar manner to results for fibrinogen binding a combination of antibodies was significantly different (P<0.05) from the use of a single antibody. This held for all examples apart from anti-P5 following stimulation with convulxin. Again at higher concentrations of inhibitory antibodies a less-than-additive effect is observed (not shown).

The data obtained in the thiol isomerase assay (figure 3 and 25) demonstrate that inhibitory antibodies are incapable of blocking completely enzyme activity. Therefore, even in the presence of high concentrations of inhibitory antibodies there will still be low levels of thiol isomerase activity on resting and activated platelets. Thus, it is hard to determine the relative contributions of ERP5 and PDI proteins to fibrinogen binding and P-Selectin exposure assays used here. However, these data implicate strongly cell-surface ERP5 and PDI in the regulation of platelet thrombus formation.

**ERP5 associates with integrin β3 in stimulated platelets**

To investigate whether there was a direct association between ERP5 and the fibrinogen receptor integrin αIIbβ3, co-immunoprecipitation studies were performed. The integrin β3 sub-unit was found to associate with ERP5 in platelets activated by agonists convulxin and thrombin (Figure 7). This association was observed using complimentary experimental techniques (*i.e.* for immunoprecipitation using either anti-ERP5 or anti-β3 antibodies). The degree of association was agonist concentration and time dependent, increasing with increasing concentrations of agonist, and peaking at approximately 30s post-stimulation. The data shown in Figure 2.iii, for the cell-surface exposure of ERP5, demonstrate there is a peak at approximately 45s for the exposure of ERP5 in response to both of the agonists convulxin and thrombin.
Similar experiments were performed to examine the potential interaction of PDI with $\beta_3$, but no such interaction was observed. Interactions between ERP5 and PDI were also not observed.
Discussion

Recent studies have developed the concept of redox controlled receptor remodelling as part of the activation process in platelets. It has been proposed that these reactions are based upon thiol isomerase activity, the ability to generate, reduce, or rearrange disulphide bonds in proteins\textsuperscript{23-25}. Resting platelets display low levels of thiol isomerase activity on the cell surface, and these levels are enhanced dramatically when platelets are stimulated by agonists\textsuperscript{17}. The functional importance of this activity is demonstrated by the fact that blocking thiol isomerases, inhibits a number of key events in the platelet activation process including adhesion, aggregation, fibrinogen binding and P-Selectin exposure\textsuperscript{4,19,20}. The only thiol isomerase enzyme characterised in platelets previously is PDI. We report the presence of an additional thiol isomerase enzyme, ERP5, on the surface of platelets. The contribution to the cell-surface thiol isomerase activity by other enzymes, such as ERP5 could be the basis for the observation that chemical modification reagents consistently inhibit platelet activation markers to a greater extent than specific antibodies that inhibit PDI.

In theory a small number of thiol isomerases could activate a large number of receptors as they do not have to form long-term stable complexes. The balance in this scenario will be time, because fewer proteins will take longer to activate all receptors. Indeed, limiting surface expression could be seen as another form of setting the gain, or threshold, for platelet activation by modulating the response time for complete activation. This characteristic of extended periods of shape change and slower onset of aggregation is observed when platelets are incubated with low levels of inhibitory antibodies.

Shuttling of receptors between internal organelles and the cell-surface is a common phenomenon and recent studies have shown cell-surface expression of GluR5 kainate receptors is regulated by an endoplasmic reticulum retention signal\textsuperscript{43}. Both ERP5 and PDI are recruited to the cell surface, as shown in figure 2 and Burgess \textit{et al}\textsuperscript{17} respectively, although the intracellular source, whether \(\alpha\)-granules or IMs, are presently unclear. The kinetics of recruitment of ERP5 are similar to
α-granule secretion. In response to convulxin and thrombin there is a biphasic profile with an initial peak at approximately 60s followed by prolonged increase in exposure. For collagen, following an initial peak in exposure, cell surface levels of ERP5 return to basal after 5 mins. It may be that stimulation with collagen is unable to mobilise a second wave of cell-surface exposure for ERP5, but given that collagen and Cvx are both able to stimulate platelet activation via GPVI, this would be surprising.

Until recently it would have been easy to attribute the differences observed in the time-dependent exposure profiles to the fact that these agonists stimulate platelets through different signalling pathways. Thrombin through G-protein-coupled-receptor pathways via PAR1 and PAR4, collagen through the integrin α2β1, and convulxin through the receptor GPVI. However, recent reports have suggested that this distinction is less clear; convulxin has been shown to bind GP1b, and it has been proposed that the central receptor responsible for collagen signalling is GPVI, with integrin α2β1 being responsible primarily for adhesion. Thus, one may expect thrombin to be distinct and not collagen. Previous reports have indicated a link between thiol isomerase activity of PDI and integrin activation. It is possible, therefore, that the different profiles seen are based upon a separate pathway following activation via integrins as opposed to other stimuli. In platelets Wang et al have observed internalisation of soluble collagen via the integrin α2β1 over a period of 30 minutes. It is possible that ERP5 may internalise with α2β1, although under the conditions presented in this study, the kinetics of disappearance of ERP5 from the platelet surface are faster. Another possible mechanism for the loss from the platelet surface, is that ERP5 is released from collagen-stimulated platelets. Indeed platelets stimulated with thrombin have been shown to release 10% of total PDI.

The observation that small, thiol-reactive reagents modulate platelet function suggests the enzymatic activity of the proteins underlie their function on the cell-surface. Little is known of the mechanism by which this occurs. Essex et al have proposed PDI acts downstream of the primary activation process, but prior to activation of the integrin receptor α4β3. They observed anti-PDI
inhibitory antibodies were able to block conversion of integrin $\alpha_{\text{Iib}}\beta_3$ to the activated state recognised by PAC-1 antibody, but not block activation via a peptide (LSARLAF) that has been shown to bind $\alpha_{\text{Iib}}\beta_3$ and directly stimulate aggregation and secretion. We believe this is a good starting point, which can be developed further to incorporate the presence of additional thiol isomerase proteins on the platelet cell-surface, such as ERP5. A question introduced by the discovery of ERP5 is whether this and PDI act through a common mechanism. When a combination of inhibitory antibodies to ERP5 and PDI are used at sub-maximal concentrations (figures 4, 5, and 6) an additive effect on platelet function was detected. If a common pathway was involved the effects of competitive inhibition may promote a less-than-additive effect. At higher concentrations of inhibitory antibody a less-than-additive effect is observed for the fibrinogen binding and P-Selectin exposure assays. It is possible this indicates that both ERP5 and PDI operate through a common mechanistic pathway, although they may be acting at different points. Future work to establish the respective substrate preferences of ERP5 and PDI will be required to shed more light on this issue.

The ability of ERP5 to regulate the binding of fibrinogen, cell-surface exposure of P-Selectin, and co-association of $\beta_3$ integrin (Figures 5, 6 and 7), highlight the potential inter-relationships between different thiol isomerase enzymes and receptor activation on the platelet cell-surface. Following pre-incubation of platelets with function-blocking antibodies to either ERP5 or PDI the binding of fibrinogen and cell-surface exposure of P-Selectin was found to be inhibited significantly in platelets. Differences were observed in agonist and inhibitory antibody responses with greater inhibition observed for convulxin rather than collagen, and for blocking PDI rather than ERP5. The different levels of inhibition observed may be based upon different potencies of the agonists used, different activation pathways and the respective amounts of ERP5 and PDI on the platelet surface at the time of assay. Lahav et al have reported an approximate 55% decrease in fibrinogen binding and approximate 30% decrease in P-Selectin exposure for collagen stimulated platelets pre-incubated with the monoclonal anti-PDI antibody RL-90$^{19}$. These results are consistent with the data we have
obtained showing a 29% decrease in fibrinogen binding and 47% decrease in P-Selectin exposure in response to blocking with the monoclonal anti-PDI antibody Ma3-019. There is no statistical significance (P>0.25) between the reductions in fibrinogen binding and P-Selectin exposure in this study. This suggests that the differences observed in the relative levels of inhibition for the two sets of data are based on experimental protocol. The greater inhibition observed in this study for PDI relative to ERP5 may be due to different affinities and function-blocking properties of the antibodies used. Interestingly, the extent of inhibition of fibrinogen binding and P-Selectin exposure is closer for anti-PDI and anti-ERP5 blocking in response to collagen (P>0.2) than in response to convulxin (P<0.05), suggesting that PDI is more important than ERP5 upon convulxin stimulation. This may be due to different signalling pathways affected by these agonists. Notably, convulxin is capable of stimulating GPVI and GP1b, and Burgess et al have demonstrated that there is a physical association between PDI and GP1b17.

Remodelling of the integrin $\alpha_{IIb}\beta_3$ from an inactive to a ligand-binding state involves a conformational change in which the disulphide bonding pattern of the receptor is changed$^{39,40}$. The $\beta_3$ sub-unit of the integrin possesses inherent thiol isomerase activity$^{25}$, although it is unknown whether this activity is sufficient to promote the conformational change in either direction. We have demonstrated a co-association of ERP5 with the $\beta_3$ sub-unit of integrin $\alpha_{IIb}\beta_3$ in activated platelets. We suggest that, when associated with $\beta_3$, ERP5 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, with increased cell-surface exposure and $\beta_3$ association likely to be involved. The interaction with, and regulation by, other molecules, such as the interaction of PDI, or calreticulin$^{51}$, may also play a role. However, it is possible to inhibit integrin ligation and still trigger expression of P-Selectin (or vice-versa). Therefore, ERP5 may play a role upstream of both in the activation process.
Acknowledgements

The authors wish to thank Gwenda Graham (University of Reading) and Dr Sheila Hassock (King’s College) for technical assistance.
References


Figure Legends

Figure 1A: Localisation of ERP5 to intracellular and plasma membranes of human platelets

Human platelet intracellular (IM - 40µg) and plasma (PM - 80µg) membranes from resting platelets prepared by high-voltage free flow electrophoresis were separated by SDS-PAGE and immunoblotted. ERP5 protein was detected using specific polyclonal antibodies.

B. Specificity of anti-ERP5 antibodies:

Recombinant human PDI (4µg) and human platelet lysates (4µg and 20µg) were Western blotted and probed for ERP5 and PDI using sheep polyclonal anti-ERP5 and mouse monoclonal anti-PDI respectively.

Figure 2: ERP5 cell surface exposure increases in response to platelet stimulation

Stimulation of platelets by convulxin (A), collagen (B), or thrombin (C) results in an increase in cell-surface expression for ERP5 in a concentration- and time-dependent manner.

i. Concentration-dependence; Ai. Basal ■, Cvx 10ng —, Cvx 40ng ⋯; Bi. Basal ■, Coll 25µg —, Coll 100µg ⋯; Ci. Basal ■, Thr 0.2U —, Thr 1.0U ⋯, all for 90s stimulation.

ii. Time dependence; Aii. Basal ■, t=45s —, t=300s ⋯, Cvx 40ng; Bii. Basal ■, t=45s —, t=300s ⋯, Coll 25µg; Cii. Basal ■, t=45s —, t=300s ⋯, Thr 1.0U.

iii. Normalised plots for the increase in cell-surface exposure observed for ERP5 over time are given for convulxin (Aiii), collagen (Biii), and thrombin (Ciii); mean ± SE (n=3), where 100% represents the maximal response detected.
**Figure 3: Thiol isomerase activity of human ERP5**

Thiol isomerase activity was assessed as the ability to refold denatured, scrambled RNase and enhance the degradation of cyclic 2’,3’-cytidine monophosphate, as followed by UV/vis spectroscopy.

**A.** Activity was observed for a recombinant ERP5 fusion protein (21 µg/ml) and a recombinant PDI fusion protein (11 µg/ml) relative to samples containing only cyclic 2’,3’-cytidine monophosphate (blank), or only fusion protein.

**B.** Anti-ERP5 polyclonal antibodies (24 µg/ml) raised in sheep were able to partially inhibit the thiol isomerase activity of a recombinant ERP5 fusion protein (30 µg/ml). Pre-immune IgG (24 µg/ml) and monoclonal anti-PDI antibodies (28 µg/ml) were found to possess no such inhibitory activity.

Data are mean ± SE from 3 different determinations, * P < 0.05

**Figure 4: An inhibitory antibody for ERP5 inhibits platelet aggregation**

Platelets (4x10^8 cells/ml) were incubated with anti-ERP5 IgG or control IgG at the concentrations given for 2.5 minutes prior to addition of agonist.

**Ai. Concentration effect of inhibitory antibodies:** Collagen, 2.5 µg/ml, incubated with: Pre-immune IgG (36 µg/ml); anti-ERP5 antibody (6 µg/ml); anti-ERP5 antibody (36 µg/ml).

**Aii. Additive effect for inhibitory antibodies for ERP5 and PDI:** Collagen, 4.0 µg/ml, incubated with: Pre-immune IgG (36 µg/ml); {anti-ERP5 (12 µg/ml) + pre-immune IgG (19 µg/ml)}; {anti-PDI (6 µg/ml) + pre-immune IgG (19 µg/ml)}; and {anti-ERP5 (12 µg/ml) + anti-PDI (6 µg/ml)}.

**Bi. Concentration effect of inhibitory antibodies:** Convulxin, 30 ng/ml, incubated with: Pre-immune IgG (24 µg/ml); anti-ERP5 antibody (12 µg/ml); anti-ERP5 antibody (24 µg/ml).
Bii. Additive effect for inhibitory antibodies for ERP5 and PDI: Convulxin, 150ng/ml, incubated with: Pre-immune IgG (36µg/ml); {anti-ERP5 (12µg/ml) + pre-immune IgG (19µg/ml)}; {anti-PDI (6µg/ml) + pre-immune IgG (19µg/ml)}; and {anti-ERP5 (12µg/ml) + anti-PDI (6µg/ml)}.

Prior to addition of antibodies platelets were pre-incubated with a saturating concentration of a F(ab) fragment of the IV.3 protein to prevent signalling through the FcγRIIa receptor. Control IgG was purified from the pre-immune serum of the animal used to raise antibodies in. Traces shown are representative from that observed for at least three different donors.

C. Mobilization of calcium from intracellular stores: Ca²⁺ release was measured by Fura-2 fluorescence in platelets stimulated with convulxin (300ng/ml, ■) and collagen (4µg/ml, □). Prior to stimulation platelet were incubated with anti-ERP5 antibodies, control antibodies (24µg/ml) from pre-immune sera.

Data are presented as mean ± S.E for three separate experiments.

Figure 5: Binding of fibrinogen is inhibited in platelets following blocking of cell-surface ERP5

Binding of FITC labelled fibrinogen was measured using flow cytometry on platelets stimulated with the agonists convulxin or collagen. Prior to stimulation platelets were incubated with anti-ERP5, anti-PDI, antibodies or control antibodies from pre-immune sera.

A. Histogram for fluorescence of FITC-fibrinogen labelled platelets in response to the agonist convulxin (100ng/ml); control IgG (12µg/ml) ■, anti-ERP5 antibody (12µg/ml) —, anti-PDI antibody (33µg/ml) ⋯

i. Residual binding of FITC-fibrinogen following incubation of platelets with pre-immune IgG (12µg/ml), anti-ERP5 antibody (12µg/ml), anti-PDI antibody (33µg/ml). Agonists, B convulxin 100ng/ml ■; C collagen 10µg/ml.

ii. Additive effect for inhibitory antibodies on residual binding of FITC-fibrinogen following incubation of platelets with; pre-immune IgG (36µg/ml); {anti-ERP5 (12µg/ml) + pre-immune IgG
Figure 6: P-Selectin expression is inhibited in platelets following blocking of cell-surface ERP5
Binding of PE conjugated anti-CD62p was measured using flow cytometry on platelets stimulated
with the agonists convulxin or collagen. Prior to stimulation platelets were incubated with anti-
ERP5, anti-PDI, antibodies or control antibodies from pre-immune sera.

A. Histogram for fluorescence of PE anti-CD62p labelled platelets in response to the agonist
convulxin (100ng/ml); control IgG (12µg/ml) ■, anti-ERP5 antibody (12µg/ml) —, anti-PDI
antibody (33µg/ml) ■

i. Residual binding of PE anti-CD62p following incubation of platelets with pre-immune IgG
(12µg/ml), anti-ERP5 antibody (12µg/ml), anti-PDI antibody (33µg/ml). Agonists, B convulxin
100ng/ml ■; C collagen 10µg/ml .

ii. Additive effect for inhibitory antibodies on residual binding of PE anti-CD62p following
incubation of platelets with; pre-immune IgG (36µg/ml); {anti-ERP5 (12µg/ml) + pre-immune IgG
(19µg/ml)}; {anti-PDI (6µg/ml) + pre-immune IgG (19µg/ml)}; and {anti-ERP5 (12µg/ml) + anti-
PDI (6µg/ml)}. Agonists, B convulxin 300ng/ml ■; C collagen 4µg/ml .

Data are presented as mean ± S.E for four separate experiments, * P < 0.05, ** P < 0.005.

Figure 7: Stimulation dependent association of ERP5 with integrin β3
Platelets (1x10^9 cells/ml) were stimulated in the presence of EGTA, apyrase and indomethacin at
varying concentrations of convulxin (ng/ml) or thrombin (U/ml) for 90s (A), or at fixed
concentrations of agonist (Cvx 100ng, Thr 1.0U) for increasing duration (B). Following sample lysis
proteins were precipitated and separated with specific antibodies and protein A sepharose.
Immunoblotting was used to show interacting proteins. Blots were stripped and re-probed to verify equivalent levels of target antigen in each sample lane.
Figure 1

A.

<table>
<thead>
<tr>
<th>kDa</th>
<th>IM (40μg)</th>
<th>PM (80μg)</th>
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B. i. anti-P5  
Platelet
PDI  Lysate

ii. anti-PDI  
Platelet
PDI  Lysate

55 kDa
Figure 3

A.

% Activity

0 20 40 60 80 100

RNAse  PGST + RdRNAse  PDI + RdRNAse  Blank  PGST only  PDI only

B.

% maximal activity

0 20 40 60 80 100

control IgG  anti-PDI  anti-P5
Figure 4

A. Collagen

B. Convulxin

C. Normalized % maximum calcium mobilisation

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<th>Condition</th>
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<td>Control IgG</td>
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<td>anti-P5</td>
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Figure 5

A.

Bi. Convulxin

Ci. Collagen

ii. **

Control IgG anti-IP5 anti-PDI

% residual binding

0 20 40 60 80 100

0 20 40 60 80 100

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Figure 6

A.

B. Convulxin

Bi.

C. Collagen

Ci.
Figure 7

IP: $\beta_3$

Blot: ERP5

Blot: $\beta_3$

IP: ERP5

Blot: ERP5

Blot: $\beta_3$
A role for the thiol isomerase protein ERP5 in platelet function

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