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Forum Review

Extracellular Disulfide Exchange and the Regulation of Cellular Function

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ABSTRACT

An emerging concept is that disulfide bonds can act as a dynamic scaffold to present mature proteins in different conformational and functional states on the cell surface. Two examples are the conversion of the receptor, integrin $\alpha_{IIb}\beta_3$, from a low affinity to a high affinity state, and the interaction of CD4 receptor with the HIV-1 envelope glycoprotein gp120 to promote virus-cell fusion. In both of these cases there is a remodeling of the protein disulfide bonding pattern. The formation and rearrangement of disulfide bonds is modulated by a family of enzymes known as the thiol isomerases, which include protein disulfide isomerase (PDI), ERp5, ERp57, and ERp72. While these enzymes were reported originally to be restricted in location to the endoplasmic reticulum, in some cells thiol isomerases are found on the cell surface. This may indicate a wider role for these enzymes in cell function. In platelets it has been shown that reagents that react with cell surface sulfhydryl groups are capable of blocking a number of functional responses, including integrin-mediated aggregation, adhesion, and granule secretion. Furthermore, the use of function blocking antibodies to either PDI or ERp5 causes inhibition of these functional responses. This review summarizes current knowledge of the extracellular regulation of disulfide exchange and the implications of this in the regulation of cell function. *Antioxid. Redox Signal.* 8, 312–324.

INTRODUCTION

THE FORMATION of a disulfide bond between two cysteine residues in a protein is a reaction that is introduced early in biochemistry texts and classes. The role of these disulfide bonds is set commonly in a structural capacity stabilizing the formation of the mature protein. The majority of proteins secreted to the cell-surface contain at least one disulfide bond, with many proteins containing multiple bonds to confer the inter- and intramolecular covalent attachments necessary to ensure correct secondary and tertiary protein structure. These disulfide bonds are often arranged as part of distinct three-dimensional motifs, for example, the single disulfide bond forming the immunoglobulin (Ig) loop, or the multiple bonds involved in the formation of the epidermal growth factor (EGF) domains. To ensure the correct maturation of disulfide bonds in nascent polypeptides a family of enzymes located in the endoplasmic reticulum (ER), known as the thiol isomerases, catalyse their reduction, oxidation, and rearrangement (33). The thiol isomerase enzymes are requisite for protein maturation, speeding up a process that may take days to less than an hour (the time for secretion of nascent proteins). In addition, as part of this process thiol isomerase enzymes prevent the formation and accumulation of misfolded proteins, which can be involved in a number of disease states (17).

A considerable amount of data has been published recently to indicate that disulfide bond formation and rearrangement, and the roles of thiol isomerases, are more complex. It is evident that disulfide bonds can form a dynamic scaffold for protein structure that is capable of rearrangement to present a variety of physical and functional protein forms. Furthermore, it has been demonstrated that thiol isomerases are not confined to the ER, and may be involved in disulfide bond rearrangement outside the ER. This has been recognized in a number of publications that have addressed the function of

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thiol isomerase proteins (93, 100), and the role of thiol-disulfide exchange in protein and cellular function (21, 36, 66).

This review will summarize the growing evidence that demonstrates thiol isomerase activity is linked to a number of extracellular events that affect cell function, and that the thiol isomerase enzymes form an extended family that may play different roles in these processes. Particular emphasis will be placed on the role of thiol isomerase enzymes in platelet function.

Disulfide bond formation

For disulfide bond formation there are a number of thermodynamic considerations which are factors in determining whether a bond is favorable to the formation of native protein conformation. In terms of entropy (broadly speaking the number of different possible structures of a peptide chain) the added constraints of a disulfide bond serve to lower the entropy of the unfolded form and bring it closer to that of the folded protein, thereby helping protein folding. In addition there are also enthalpy factors; the potential of strain from a sterically unfavored conformation can decrease the stability of the folded form and negate the enthalpy of bond formation. Furthermore, the formation of a disulfide bond is an oxidation reaction and therefore it requires the presence of an electron acceptor, and is favored at higher pH where the thiol side chains are in the deprotonated thiolate anion form. In cellular systems the oxidized and reduced forms of glutathione (GSSG/GSH) form a redox couple and usually serve as the electron sink. Thus, the chemical environment can directly affect whether disulfide bond formation is favored, and the strain energy related to the protein conformation will affect the stability of the bond.

In the cytoplasm of the cell, where there are high concentrations of reduced glutathione and low concentrations of oxidised glutathione (ratio GSH:GSSG > 10:1) the formation of disulfide bonds is obstructed (32). Indeed cytosolic disulfide formation may be limited to transient examples of enzymes undergoing oxidation/reduction processes (12). However, in the ER the ratio of reduced to oxidized glutathione is approximately equal, therefore providing special cellular conditions to facilitate disulfide bond formation (32).

Thiol isomerase proteins

The chemical conditions in the ER are necessary, but not sufficient, for the correct folding of proteins within the time frame of protein translation and secretion. Thiol isomerase enzymes are a group of protein chaperones that facilitate the formation, reduction, and rearrangement of disulfide bonds in nascent proteins (Fig. 1). Protein disulfide isomerase (PDI) has been studied in most detail but there are also a number of other family members, that includes, ERp5, ERp57, and ERp72 (29, 35, 68).

Sequence identity between members of this family is relatively low, but they do share active-site motifs and domain structure (28, 29, 45). The proteins contain three major domain types; a thioredoxin-like catalytically active domain (commonly labelled the 'a' domain), a catalytically inactive thioredoxin fold domain (b), and a highly acidic domain, believed to be involved in peptide binding (c). These domains can be found in different orders and combinations between family members, as highlighted in Figure 2.

The catalytic site of the active domain is characterized by a conserved vicinal dithiol motif –CGHC-, which can be compared to the -CGPC- motif found in thioredoxin. For PDI the N-terminal cys residue of the first active site is stabilized partly by the histidine imidazole group and this leads to a much lower pK_a value for this cys than that of free cysteine (4.5 *cf.* 8.7) (29). This allows the thiolate anion generated to initiate nucleophilic attack on disulfide bonds, rendering the cysteine highly reactive.

Catalytic rates, binding constants, and enzyme profiles have been determined for some thiol isomerase enzymes (34, 46, 49), but not under identical conditions. Therefore, it is not possible to identify whether some enzymes are more efficient, or better suited to different conditions, than others. It is known that not all enzymes are capable of catalyzing equivalent reactions *in vitro*, and in particular it appears that rearrangement of protein disulfide bonds (isomerase activity) requires the enzyme to contain two catalytic domains (100). In addition, some members show additional functionality. This is notable for PDI, which can act as a subunit for prolyl hydroxylase and triglyceride transferase (48, 99). In these situations it is believed that the protein acts a chaperone and prevents protein aggregation rather than actively catalyzing thiol exchange reactions.

In addition to the domain architecture there is a C-terminal retention sequence, –KDEL, which through binding to –KDEL receptors ensures retention in the ER (65, 89). The KDEL motif also serves as a retrieval signal for proteins normally present in the ER that have been transported beyond this compartment (75). The mechanism of retrieval of soluble tagged proteins and some of the proteins involved in their transport (e.g., Erd2p) have been established (42, 81, 89). Although thiol isomerase enzymes are all resident normally within the ER, there is some variation between retention sequences; PDI and ERp5: –KDEL; ERp57: –QEDL; ERp72: –KEEL, which may influence their cellular sorting and localization (93).

There is little information on factors that regulate thiol isomerase activity. Although purified enzymes are functionally active *in vitro*, they are known to interact with other proteins *in vivo*. PDI and ERp57 have been shown to interact with the lectin-like chaperone calreticulin (4, 11). Furthermore, several thiol isomerases have been shown to bind calcium with low affinity and high capacity, and can be phosphorylated. PDI is substrate of sphingosine-dependent kinases (69), ERp72 can be serine phosphorylated by casein kinase II (41), and ERp57 can be tyrosine phosphorylated by Lyn kinase (18). It is not known which kinases are present within the ER and, therefore, the opportunity for regulation of thiol isomerases here is unclear. However, there will be opportunity for the regulation of thiol isomerases by protein phosphorylation for enzymes that are found beyond the ER.

Thiol isomerase enzymes beyond the ER

A number of studies have provided evidence that thiol isomerase proteins have a far broader cellular localization and interact with a wider range of proteins than believed previ-

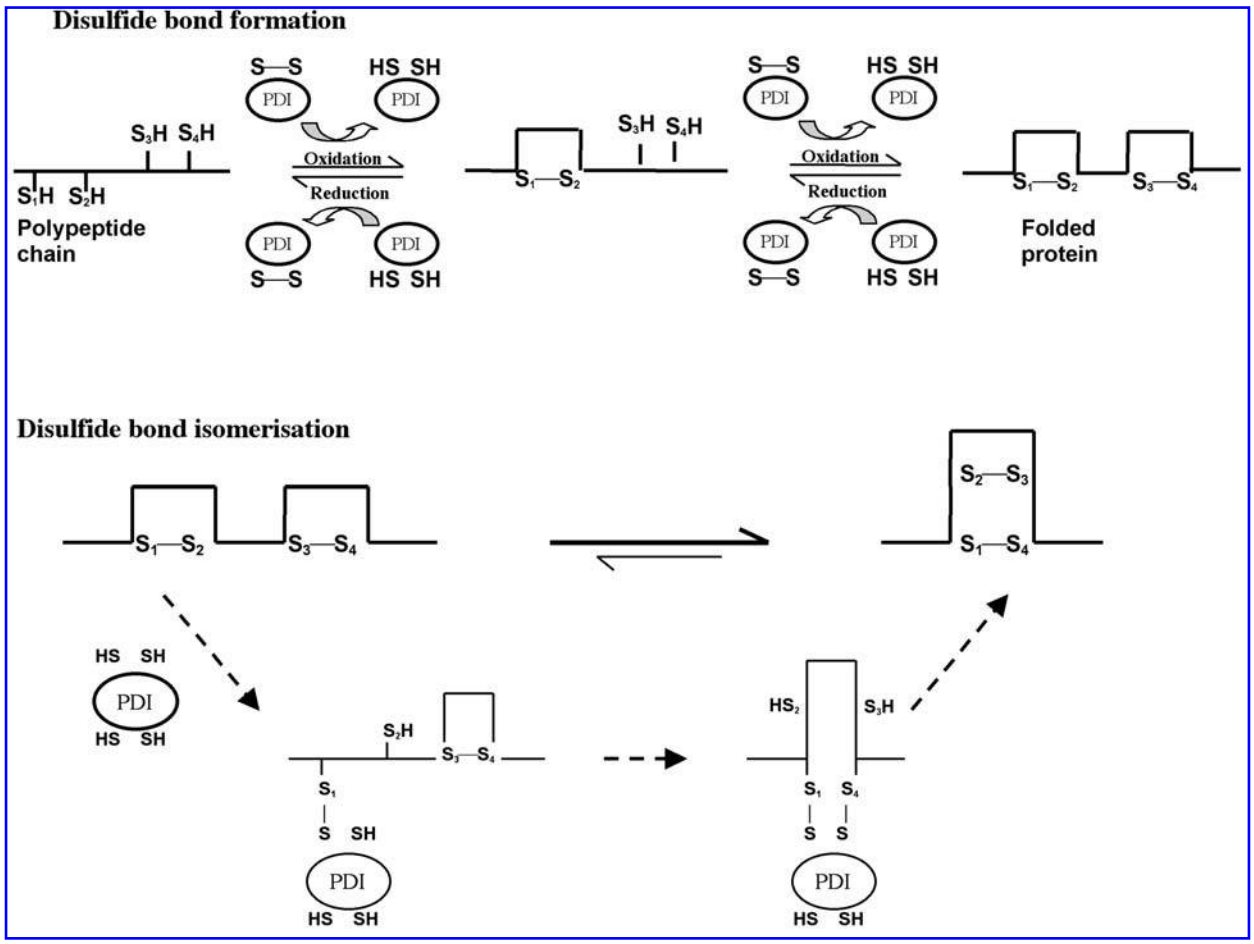

FIG. 1. Thiol isomerase catalyzed disulfide formation and isomerization. Generation of a native protein conformation from a nascent polypeptide may involve disulfide bond formation and isomerization. The disulfide-bonded active site of thiol isomerases (PDI depicted here) mediates the formation of substrate disulfide bonds by accepting electrons from the sulfhydryl groups (*oxidation*). Conversely, *reduction* of a substrate disulfide bond is achieved by donation of electrons from the sulfhydryl thiol form of thiol isomerases. Disulfide bond *isomerization* involves a change in the pattern of bonds within a protein. This can occur via an intramolecular pathway in which a mixed isomerase-substrate complex is formed (*dashed pathway*), or via successive cycles of reduction followed by reoxidation in an alternative configuration.

ously. This is likely to reflect a wider functional role for these enzymes.

Since the KDEL motif serves as both a retention and retrieval signal for maintaining proteins in the ER it would not be surprising to find that thiol isomerase enzymes are present at low levels on different cell membranes. Indeed, PDI has been identified on the surface of resting platelets (22). In addition a number of other cell types, including bovine aortic endothelial cells (38), rat hepatocytes (1, 91), human B cells (50, 87), and yeast cells (58) have been shown to secrete PDI, which associates with the cell surface. More recently proteomic analysis has been applied to the profiling of cell-surface proteins and studies have found PDI to be present of the surface of cancer (SH-SY5Y, A549) and leukaemia (Sup-B15, KG1) cell lines (40, 84), and endothelial and fibrosarcoma cell lines (19). Careful experimental practice is required to ensure correct labeling and protein separation is achieved in these technically challenging experiments. These studies found a surprisingly large pool of proteins with chaperone function on the cell surface, including PDI, and a number of heat shock proteins (HSP). Traditionally HSPs were believed to be localized to the cytoplasm, where they prevent protein misfolding. Recently it has been reported that cell surface exposure of HSPs can have functional affects, for example, mediating innate recognition of bacterial products through an interaction with the toll like receptors (92, 94, 95).

The storage of receptors in the ER and shuttling between internal organelles and the cell-surface is a common phenomenon. Recent studies have shown cell-surface expression of GluR5 kainate receptors is regulated by an endoplasmic reticulum retention signal (77) and modulated by protein phosphorylation. In addition, a number of cells, and platelets in particular, contain granules for protein storage, which can be secreted to the cell surface following cell stimulation. At the present time the exact partitioning of thiol isomerase enzymes between these different cellular locations remains to be determined.

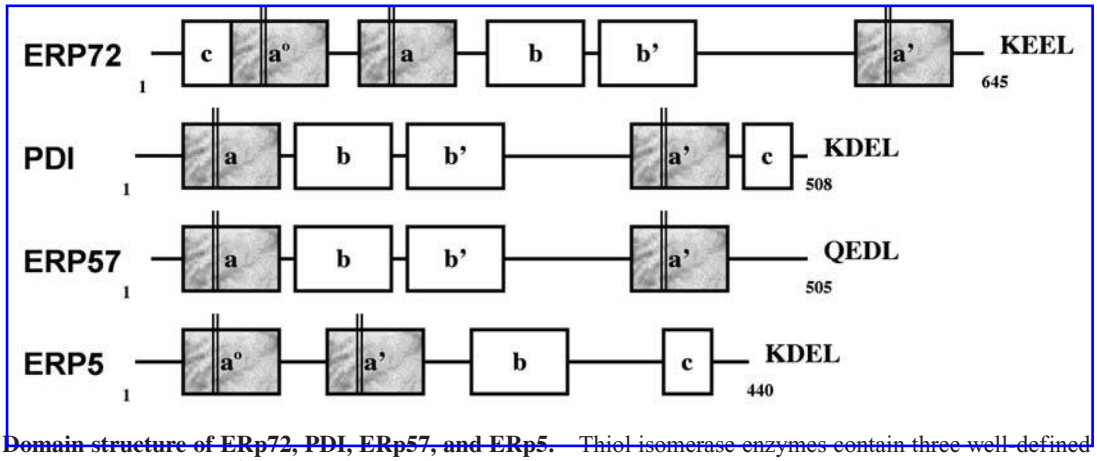

FIG. 2. Domain structure of ERp72, PDI, ERp57, and ERp5. Thiol isomerase enzymes contain three well-defined domain structures. Domains **a°**, **a**, and **a′** (*shaded boxes*) are redox-active thioredoxin domains, with *bars* indicating the positions of the active site –CGHC- sequence. Domains **b** and **b′** are not catalytically active but share a common thioredoxin fold. Domain **c** is a highly acidic region associated with peptide binding. Retention in the ER is maintained by a C-terminal signal motif –KDEL, or as given.

Strikingly the levels of thiol isomerase proteins present on the extracellular membrane of cells are highly variable and can increase significantly following agonist stimulation. In platelets this has been demonstrated for both PDI and ERp5 (8, 44). Burgess *et al.* (8) demonstrated that there was a 440% increase in the cell-surface exposure of thiol groups on the platelet surface following stimulation. We observed an increase in cell-surface exposure for both PDI and ERp5 in response to platelet stimulation. The exposure of ERp5 was shown to have distinct time-dependent and agonist-dependent profiles (Fig. 3). In response to the platelet agonist thrombin there is a biphasic profile with an initial peak at approximately 60 s, followed by a prolonged increase in exposure. For collagen, following an initial peak in exposure, cell surface ERp5 return to basal levels after 5 min. Further studies are underway to determine the basis for the different agonist induced profiles and the intracellular source of the ERp5. The kinetics of recruitment of ERp5 are similar to α-granule secretion. A number of mechanisms for cell-surface exposure of ERp5 and PDI are possible. These include a breakdown in the normal ER retrieval mechanism (89), a specific translocation signal, as observed for the kainate receptors (77), secretion from a separate enzyme pool, such as α- or dense-granules (30), an ER-Golgi independent secretory pathway (88), or membrane blebbing (39).

Identifying the interactions between thiol isomerase enzymes and cell-surface receptors and extracellular components is a key factor in defining a wider role for these enzymes. Determining the pathway for cell-surface exposure will be important as it will provide information on the scope of possible interactions. Two different pathways, which are

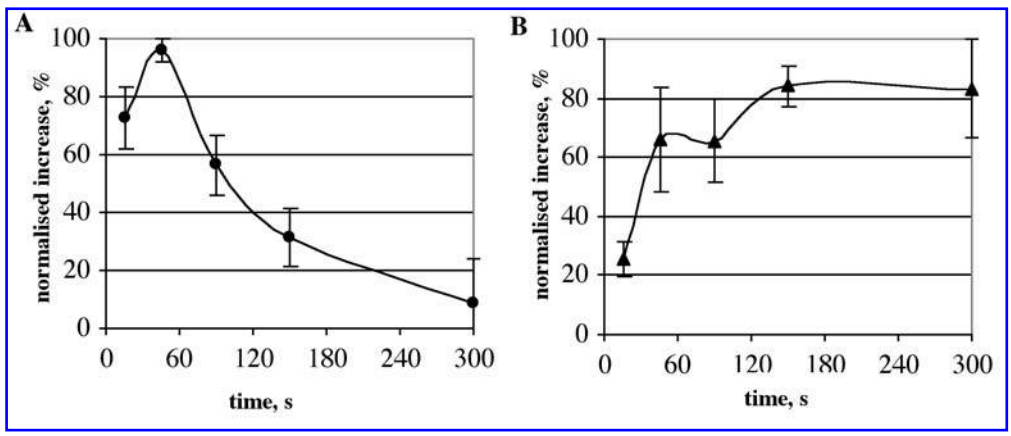

FIG. 3. ERp5 cell surface exposure increases in response to platelet stimulation. Stimulation of platelets by collagen (**A**), or thrombin (**B**) results in an increase in cell-surface exposure for ERp5 in a concentration- and time-dependent manner as determined by flow cytometry. Normalized plots for the increase in cell-surface exposure observed for ERp5 over time are given as mean ± SE ($n = 3$), where 100% represents the maximal response detected. This research was originally published in *Blood*. Jordan *et al.* A role for the thiol isomerase protein ERp5 in platelet function. *Blood* 2005; 105: 1500–1507. © The American Society of Hematology.

not necessarily mutually exclusive, can be suggested: secretion into the media and reassociation with the cell membrane, or direct placement into the cell membrane as part of a transport process. Secretion into the media would allow enzymes a greater degree of interaction with extracellular components, for example, with fibrinogen and von Willebrand factor (vWF) in the vascular system. vWF is a large disulfide-linked multimeric protein that mediates the interaction between platelets and the exposed extracellular matrix as part of an initial response in platelet thrombus formation (78, 101). The avidity of vWF for platelets is related to its multimeric structure and this is controlled in part by thrombospondin 1 (TSP-1), an enzyme with thiol reductase activity that cleaves vWF by reducing intramolecular disulfide bonds (36, 102). In turn it has been demonstrated that PDI can facilitate disulfide exchange in TSP-1 and that alternate disulfide bonded forms of TSP-1 demonstrate different reductase activity (37, 70). Although thiol isomerase activity has been determined experimentally, based upon an RNase refolding assay (10), and PDI detected (9) in the media from activated platelets, an association between PDI and vWF or TSP-1 has not been observed. Further studies are required to determine whether the thiol isomerase activity released is based on a single enzyme or whether a mixture of enzymes is present with thiol isomerase activity. In addition, the RNAse refolding assay has been used to demonstrate the presence of proteins with thiol isomerase activity on the surface of activated cells. The number of proteins contributing to this activity, and their identities, has not been determined fully.

Protein sequence studies and hydropathy plots indicate that the thiol isomerase enzymes do not contain a transmembrane sequence or lipid binding domains for attachment to membrane structures. There have been no reports detailing the presence of –KDEL receptor proteins on the cell-surface and it has been suggested that cell-surface PDI binds through electrostatic interactions (91). Two physical associations have been characterized for thiol isomerase proteins with cell surface receptors. Using a fluorescence FRET based assay, Burgess *et al.* demonstrated a physical association between PDI and the receptor glycoprotein GP1bα on platelets (8). GP1bα is a subunit of a receptor complex that binds vWF and forms an initial point of contact between platelets and the extracellular matrix. Using co-immunoprecipitation we found a physical association between ERp5 and the integrin β_3 subunit (44) following platelet activation. Integrin $\alpha_{IIb}\beta_3$ is the major receptor for fibrinogen in platelets and is critical to the late stages of platelet aggregation. The interaction was a time-dependent process demonstrating approximately maximal association following collagen stimulation after 30 s, which coincides with the maximal cell-surface exposure for ERp5 following collagen stimulation. There have been no other reports characterizing the association between thiol isomerases and cell-surface components, although given their ability to bind proteins and peptides as part of their chaperone function, further interactions are likely. The potential functional consequences of these interactions are addressed in the next section.

FUNCTIONAL EFFECTS OF CELL SURFACE THIOL ISOMERASE ACTIVITY

Platelets are involved in the primary hemostasis response, aggregating at sites of vascular damage to form a hemostatic plug and promote the cessation of bleeding. An initial tethering that occurs between platelet receptors, serum proteins (von Willebrand factor) and exposed components of the extracellular matrix (collagen), is followed by firm adhesion. The ensuing signaling events lead to a release of intracellular calcium and the secretion of dense and alpha granules that contain proaggregatory factors, such as ADP, serotonin, and fibrinogen. A three-dimensional aggregate can then form based on interactions between platelets, matrix components, and secreted proteins. Many of these interactions involve integrins, which are capable of regulating their ligand-affinity states by receptor remodeling. Thus, a series of functional markers are available to assess the activation state of these cells, some of which are known to involve disulfide bond rearrangements. Coupled with the identification of thiol isomerases on their cell surface this makes platelets a good choice for examining a wider role for thiol isomerase enzymes in regulating cellular function. The majority of studies have focused on platelets and this body of work represents the most comprehensive mechanistic picture for PDI and the only data available for ERp5. It is possible that these cells may be unique in terms of extracellular disulfide bond rearrangements, but this is not necessarily true.

A number of studies have revealed important regulatory functions for thiol isomerase enzymes following their recruitment to the cell surface. Particular attention has been focused on events that trigger $\alpha_{IIb}\beta_3$ integrin remodelling from a low to high-affinity state (21), and those that facilitate cellular entry of HIV-1 virus (66). In addition, a number of other functional affects have been observed in platelets following the inhibition of cell-surface thiol isomerase activity. These include cell adhesion, P-selectin exposure, integrin $\alpha_5\beta_1$ activation, and transnitrosation (13, 44, 51–53, 108).

Experimental approaches

In the majority of these studies, specific functions have been inferred following the inhibition of thiol isomerase activity. Three main experimental approaches for this are through the use of (i) function blocking antibodies; (ii) low-molecular-weight sulfhydryl reactive species (for example, 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), *p*-chloromercuribenzene sulfonate (pCMPS), phenylarsine oxide); and (iii) the natural antibiotic bacitracin.

While bacitracin is able to inhibit platelet activation, it is transported poorly into cells and has been shown to inhibit PDI at the platelet surface (22), and this reagent is not selective. Reagents that react with free thiol, or vicinal dithiol groups are useful in determining functional effects involving sulfhydryl groups, but are limited due to their lack of enzyme specificity. As such, the use of these compounds will inhibit all thiol isomerase enzymes present. This has proved useful in determining cellular functions in which thiol isomerase activity is involved, but does not allow identification of the indi-

vidual enzymes concerned. The use of function blocking antibodies provides an experimental approach to inhibit selectively different members of the thiol isomerase family. Indeed the observation of higher levels of inhibition for thiol reactive reagents relative to the use of inhibitory antibodies, which display enzyme specificity, would seem to indicate a role for multiple thiol isomerase enzymes in these events (52, 108).

Function blocking antibodies have been developed, for example, against ERp5 and PDI that display no cross-reactivity (44). However, neither of the antibodies used (polyclonal sheep anti-human ERp5 and monoclonal mouse anti-human PDI Ma3–019) were capable of total inhibition of enzyme activity. Therefore, even at very high antibody concentrations low levels of individual thiol isomerases remain active, thereby hindering the ability to use combinations of antibodies to probe different mechanistic pathways.

Investigations into functional effects of thiol isomerases using genetic techniques have been limited due to the essential nature of these enzymes. In yeast it has been shown that PDI is necessary for cell viability (27, 54), although some degree of redundancy has been demonstrated using complementation studies (72). In the human erythroleukemic cell line (HEL), Zai *et al.* (108) inhibited cell-surface exposure of PDI using an antisense phosphorothioate oligodeoxynucleotide against PDI mRNA. Cells remained viable in this system and a decrease in cell-surface exposure of PDI and PDI folding activity of 74% and 47%, respectively, was observed. The difference between these two values suggests that there is additional thiol isomerase activity on the cell surface that is capable of compensating for the decrease in expression of PDI. The decrease in cell-surface PDI was associated with a significant decrease in cyclic GMP generation after exposure to S-nitrosothiols (65%), with no effect of cAMP generation following exposure to prostaglandin E_1. The authors therefore concluded that cell-surface PDI may be involved in the regulation of intracellular transfer of nitric oxide. The redox control of cell surface protein thiols and disulfides by PDI was investigated by Jiang *et al.* (43) in fibrosarcoma HT1080 cell line. Densitometry of Western immunoblots allowed the visualization of 11 proteins that displayed altered disulfide bonding profiles following overexpression of PDI, and three proteins that displayed altered profiles following the underexpression of PDI. The identity of these proteins was not determined. For these experiments PDI expression in anti-sense clones was never less than ~40% of control cell expression. This indicates that genetic techniques may not be effective for the isolation of individual thiol isomerase function.

The development of inhibitors selective for different members of the thiol isomerase family would be of great practical use in determining the mechanistic pathways for the functional affects observed.

Activation of platelet integrin $\alpha_{IIb}\beta_3$

The integrins comprise a large superfamily of heterodimeric adhesion receptors that mediate the attachment of cells to one another and to the surrounding extracellular matrix (76). They are composed of two noncovalently linked transmembrane proteins, α and β subunits, each characterized by a large NH_2-terminal extracellular domain, a single transmembrane domain, and a short cytoplasmic domain. Different combinations of α and β subunits form the basis for the different ligand affinities observed for these receptors. Both α and β integrin subunits contain a large number of disulfide bonds, with α_{IIb} containing 18 and β_3 56 cysteine residues. The platelet receptor integrin $\alpha_{IIb}\beta_3$ plays a key role in the platelet aggregation process binding to fibrinogen and stabilizing the formation of a platelet plug (56, 76, 83). The receptor is constitutively present on the cell-surface of platelets in a low affinity form and there is an internal store that allows an increase in surface exposure following platelet activation (82, 96). Platelet activation by a range of agonists promotes conversion of the receptor to an active state, capable of binding to fibrinogen, via a complex series of events known as inside-out signaling (56, 83). The events involved in integrin activation and signaling can be summarized briefly as follows: (a) initial activation of the platelet by an external stimulus leads to the generation of cytoplasmic signaling; (b) this signaling results in modified interactions of the cytoplasmic tail of the integrin promoting a structural rearrangement of the extracellular domain of the receptor; (c) the remodeled receptor binds with high affinity to its ligand (fibrinogen) and receptor clustering occurs; (d) cytoplasmic (outside-in) signals are generated by the receptor in the ligand-bound clustered conformation. The mechanistic basis for inside-out signalling has been studied extensively with the final stages believed to involve binding of the cytoplasmic protein talin triggering a conformational change in the β_3 subunit that extends from the intracellular to extracellular domain of the integrin (86, 104). For $\alpha_{IIb}\beta_3$ remodeling of the integrin from a low-affinity to a high-affinity ligand-binding state involves a conformational change in which the disulfide bonding pattern of the external domain of the receptor is changed (43, 105–107) and hence this is a redox active process. Epitope mapping of monoclonal antibodies and the application of site-directed mutagenesis has been used to map regions and transformations in the β_3 subunit involved in ligand binding (85, 98, 79). These studies found that the long range Cys_{406}-Cys_{655} disulfide bond was not critical to function, but that the long range Cys_5-Cys_{435} disulfide bond and the point mutation $Cys_{560}\rightarrow$Arg directly affect ligand bind capability.

The interplay between inside-out signaling and disulfide exchange has yet to be fully elucidated. It is possible to directly effect integrin activation and fibrinogen binding in the absence of inside-out signaling using the reducing agent DTT (59–61). This process involves reduction of disulfide bonds in the integrin's cysteine-rich repeats (107). However, reduced glutathione (GSH) is not capable of activating $\alpha_{IIb}\beta_3$ even though it can increase the number of free thiols within the integrin complex (24). A combination of reduced and activated glutathione (GSH/GSSG) that matches conditions found in blood was found, however, to potentiate platelet aggregation and $\alpha_{IIb}\beta_3$ activation (25). Thus, it appears specific disulfide bonds must be reduced to form an active conformation, as indicated by site-directed mutagenesis (85, 98).

The addition of exogenous thiol isomerase enzymes to intact platelets has not been investigated to determine if they are capable of directly affecting $\alpha_{IIb}\beta_3$ activation. Function

blocking thiol isomerase antibodies to PDI and ERp5, and low molecular weight sulfhydryl reagents, prevent $\alpha_{IIb}\beta_3$ activation following platelet stimulation (and hence inside-out signaling) with a range of agonists (21, 26, 44, 62). The peptide LSARLAF is capable of directly binding $\alpha_{IIb}\beta_3$ and promoting the change from low- to high-affinity receptor state in an inside-out signaling independent manner (5, 14, 15). Activation of $\alpha_{IIb}\beta_3$ by LSARLAF was not affected by the addition of anti-PDI antibodies but was inhibited by the sulfhydryl reagent pCMBS (26), indicating that PDI may not directly activate $\alpha_{IIb}\beta_3$ and that there are other sulfhydryl(s) involved. These studies have not been performed using anti-ERp5 antibodies, but this would be useful to determine if the physical association observed between ERp5 and β_3 is capable of directly activating the integrin.

The integrin β_3 subunit has also been shown to display endogenous thiol isomerase activity (73, 97). It is not known if this activity is sufficient to promote the conformational change in the integrin in either direction. However, there must be an additional level of regulation to prevent $\alpha_{IIb}\beta_3$ from being present in a constitutively active form. Interestingly *in vitro* RNase refolding studies demonstrate that the divalent cation requirement for the thiol isomerase activity of ERp5 and PDI are opposite to those of the β_3 subunit, the former being enhanced in the presence of Ca^{2+} while the latter is favored in the presence of EDTA (44, 73).

We propose a working model for integrin $\alpha_{IIb}\beta_3$ activation as follows: (a) platelet stimulus initiates inside-out signaling; (b) this leads to the recruitment of effectors to the cytoplasmic tail of $\alpha_{IIb}\beta_3$ and an increase in the surface location of thiol isomerase enzymes; (c) conformational changes triggered from the cytoplasmic tail of $\alpha_{IIb}\beta_3$ present a critical disulfide bond in the extracellular domain to the surface, or increase the strain energy on a previously stable disulfide bond; (d) thiol isomerase enzymes reduce or rearrange the disulfide bond triggering a conformational change from an inactive to an active state (Fig. 4). The activation of $\alpha_{IIb}\beta_3$ by DTT, but not by GSH, is accounted for by the inaccessibility of the latter reagent to the critical disulfide bond or its inability to reduce a stable disulfide in the resting conformation. In keeping with the whole concept of dynamic remodeling of extracellular disulfide bonds the endogenous thiol isomerase activity of the β_3 subunit could play a role in maintaining the resting conformation of the integrin following reduction of disulfide bonds by extracellular GSH and/or by promoting the conformational rearrangement of the integrin from an active to an inactive state.

Activation of other integrins

The published studies on factors affecting integrin-ligand affinity modulation suggest that thiol isomerase activity and disulfide bond reduction or rearrangement may be a common theme.

$\alpha_2\beta_1$. Adhesion of platelets to collagen is mediated primarily through the integrin $\alpha_2\beta_1$ and it has been suggested that this is distinct from signaling events that are mediated through glycoprotein VI (GPVI) (71). Synthetic substrates have been developed that bind selectively to $\alpha_2\beta_1$ (GFOFER peptide) (47) and GPVI (collagen related peptide, -(Gly-Pro-Hyp)$_n$-, CRP) (3). This has allowed Lahav and co-workers (51–53) to examine the effect of function blocking antibodies to PDI, and sulfhydryl reagents, on collagen-mediated platelet stimulation. They found that adhesion to type I collagen was inhibited in an $\alpha_2\beta_1$ dependent manner.

$\alpha_5\beta_1$. The integrin $\alpha_5\beta_1$ acts as the receptor for fibronectin, a major constituent of extracellular matrices. The primary functional form of fibronectin is thought to be as a multimer, which is stabilized by a disulfide bond network. In a similar manner to studies with $\alpha_{IIb}\beta_3$ it has been shown that $\alpha_5\beta_1$ on leukocytes can be activated by DTT in the absence of other external stimuli (13), and that *in vitro* $\alpha_5\beta_1$ possesses endogenous thiol isomerase activity (73). In addition the ligand itself, fibronectin, has been shown to possess endogenous thiol isomerase activity (55). The functional significance of this activity and the mechanistic basis for DTT activation of this integrin remains to be determined.

$\alpha_v\beta_3$. The integrin $\alpha_v\beta_3$ acts as the receptor for vitronectin and shares a common subunit with $\alpha_{IIb}\beta_3$. It is believed that a conformational change is required for receptor activation, although it has not been demonstrated that this requires a rearrangement of the disulfide bond pattern (2, 6, 103). O'Neill *et al.* (73) have shown the receptor to possess endogenous thiol isomerase activity, and to a greater level than that observed for $\alpha_{IIb}\beta$.

Facilitation of cell entry for HIV-1 virus

It has been proposed that two of the main proteins involved in HIV-1 infection undergo redox changes (31, 64, 80). The two proteins in question are the primary HIV-1 receptor on immune cells, CD4, and the HIV-1 envelope glycoprotein gp120. The binding of HIV-1 to CD4 and a chemokine receptor (CXCR4) triggers fusion of the viral and cell membranes leading to HIV-1 entry and infection (20).

CD4 is a transmembrane glycoprotein of the Ig superfamily. The extracellular portion contains 4 Ig domains (D1–D4), three of which (D1, D2, D4) normally contain disulfide bonds. Possibly through the action of a secreted factor the disulfide bonding pattern of the CD4 receptor appears to be controlled by the activation state of the cell. The disulfide bond in the D2 domain of the protein is formed within the same β sheet structure, whereas disulfide bonds in the other domains occur across β sheets (67). Thus, this bond is under more torsional strain than the D1 and D4 disulfide bonds and this is reduced upon activation (66). Hogg identified 118 functionally distinct proteins from the Protein Data Bank with similar cross-strand disulfide bonds (36), many of which belong to mammalian cell-surface receptors and bacterial and viral proteins involved in cell fusion. gp120 is the HIV-1 envelope glycoprotein and it binds to the CD4 receptor at the D1 domain. Studies have shown that the number of free thiols in gp120 increases following interaction with $CD4^+$ cells. This step occurs following the interaction with the chemokine receptor CXCR4 and is consistent with the reduction of two disulfide bonds. It is not clear whether disulfide bond reduction in the receptor and envelope protein are linked. It has

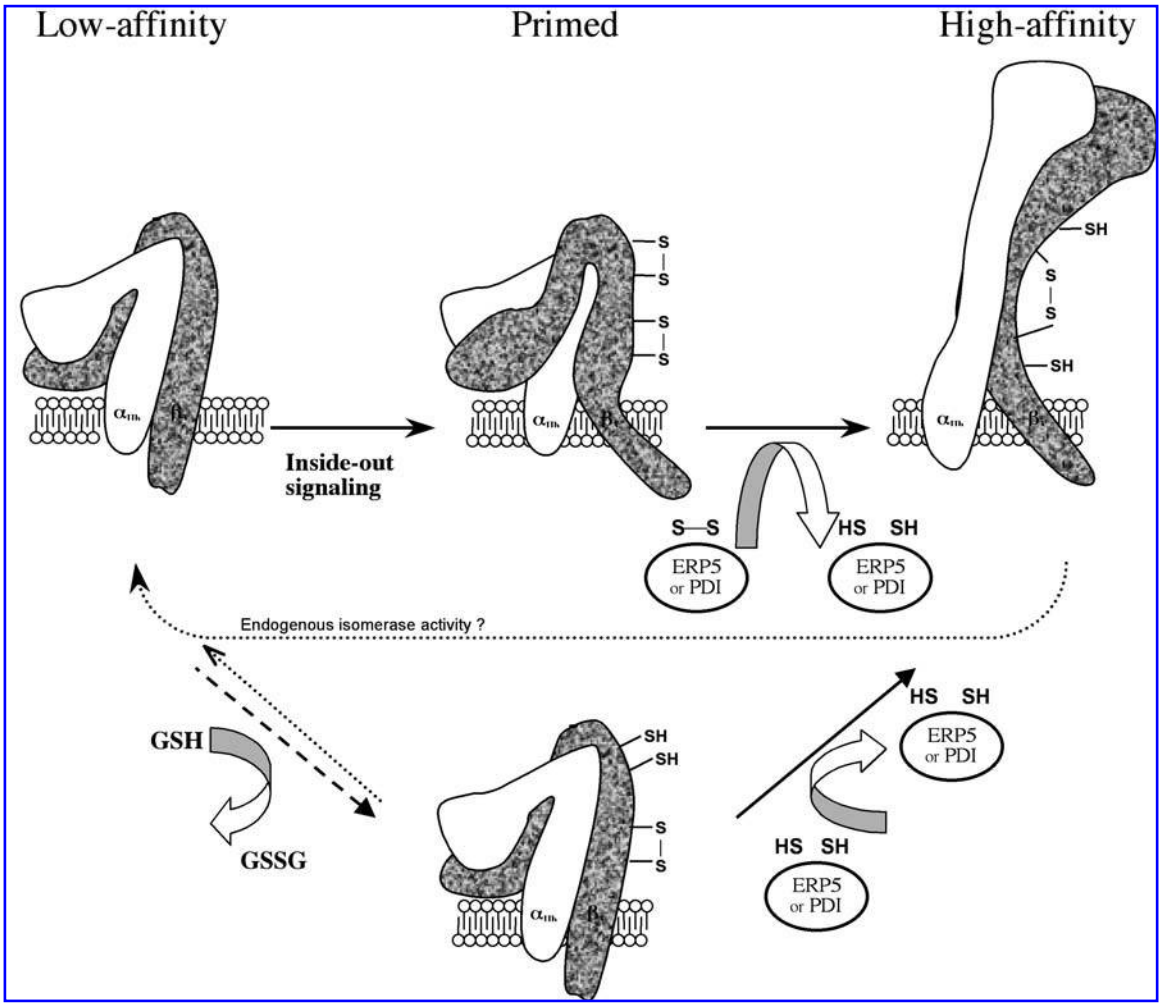

FIG. 4. Model for platelet integrin $\alpha_{IIb}\beta_3$ receptor remodeling. Transformation of platelet integrin $\alpha_{IIb}\beta_3$ from a resting conformation with low affinity for fibrinogen to a high affinity conformation involves a complex series of events, which can be blocked by sulfhydryl reactive reagents or function blocking antibodies to PDI or ERp5. In this model we propose that there are (at least) three different receptor states; low-affinity, primed (still low fibrinogen affinity), and high-affinity. Conversion from the low affinity to primed state is modulated by inside-out signaling. Reduced glutathione can also effect a transformation from a low affinity to a primed state (*dashed pathway*) although this is not necessarily the same conformation as that achieved by inside-out signaling. Conversion from the primed to the high affinity state involves a further conformational change in which there is disulfide bond reduction or isomerization. This step is sensitive to sulfhydryl reagents and may be mediated by ERp5 or PDI either directly or indirectly. The high affinity state may consist of different receptor conformations, $\alpha_{IIb}\beta_3$ contains more free thiols from platelets activated by treatment with DTT than from thrombin stimulated platelets. It is possible endogenous thiol isomerase activity of β_3 returns the receptor from high affiinity or primed states to the low affinity state (*dotted pathway*). The position of thiol groups and disulfide bonds is for illustration only.

been proposed that different factors (PDI and thioredoxin) are involved in mediating CD4 receptor and gp120 disulfide reduction, although the use of anti-PDI antibodies alone is sufficient to block viral fusion. Subsequent addition of exogenous PDI can rescue virus-cell fusion following treatment with the sulfhydryl reagent DTNB (64). This is the first example of functional rescue of a cell response following modification of cell-surface sulfhydryls and demonstrates that extracellular enzyme is capable of re-associating with the cell membrane.

The interchange of critical thiols/disulfides has also been implicated in successful viral entry for other viruses (baculovirus and vaccinia virus), in addition to HIV-1 (57, 63). This may indicate a common theme in virus entry and further developments may be expected. No studies have looked at the involvement of other thiol isomerase family members in these viral systems.

Other interactions

Selectins. The selectins are a family of adhesion receptors that are found on vascular cells and mediate cell–cell interactions. L-selectin is expressed on the majority of leukocytes (90). Studies by Bennett *et al.* (7) showed that L-selectin was

proteolytically cleaved from the cell surface following inhibition of a thiol dependent process. The greatest effect was observed using the compound phenylarsine oxide (PAO), which binds selectively to vicinal dithiol motifs, but was also seen following incubation with monoclonal anti-PDI antibodies. It was suggested that PDI was maintaining L-selectin in a compact shape that was resistant to cleavage by the constitutively active L-selectin sheddase.

P-selectin is not present constitutively on platelets, but is stored in α-granules and is presented on the cell-surface following platelet stimulation. Flow cytometry studies on P-selectin exposure on the surface of platelets is taken commonly as an indicator for α-granule secretion and platelet activation (30, 74). These experiments have been used to show that P-selectin cell-surface exposure is inhibited following treatment of platelets with inhibitory antibodies to PDI or ERp5, or following treatment with low-molecular weight sulfhydryl reagents (44, 52). This has been proposed as a consequence of functional blocking of α-granule secretion, although the mechanism for this remains unknown. It is possible that a direct interaction between a thiol isomerase enzyme and P-selectin is blocked in these studies and leads to the presentation of P-selectin in a form not recognized by the antibody used.

$P2Y_{12}$. In platelets ADP signaling occurs through two G-protein-coupled-receptors (GPCR's), $P2Y_1$, coupling through G_q, and $P2Y_{12}$, coupling through G_i. A recent report has demonstrated that $P2Y_{12}$ function can be affected by the redox state of two critical extracellular cysteine residues (16). In $P2Y_{12}$ these residues appear to be present as free thiols, whereas the corresponding residues in $P2Y_1$ form a disulfide bond. Thiol reactive reagents, and in particular the active metabolite of the $P2Y_{12}$ selective antagonist clopidogrel, react with these free cysteine residues and inhibit receptor function. The role of thiol isomerase enzymes in this process is possible, although remains unclear.

SUMMARY

The presence of multiple family members of the protein disulfide isomerase family has been recognized for some time and although enzymes share low homology overall they contain a high level of conservation around the active site motif of –CGHC–. The majority of these enzymes are capable of catalyzing the refolding of scrambled RNase *in vitro.* However, the enzyme specificity and regulation *in vivo* remains largely uncharacterized. Thiol isomerases have been found in a range of other cellular locations including the cytoplasm, nucleus, and extracellular membrane surface, in addition to their normal ER residency (93). This has lead to the proposal that they play a wider role in cell function. Indeed, a number of cell-surface events, including integrin receptor affinity modulation and virus-cell fusion, have been shown to be sensitive to agents that block thiol isomerase activity. PDI remains the best characterized isomerase, but observations of different levels of inhibition between anti-PDI antibodies and thiol isomerase reactive agents indicate that PDI may not be the only agent responsible for these functional effects. This has been supported by the observation that function blocking antibodies to another thiol isomerase enzyme, ERp5, affect platelet function. It is likely that future studies will find roles for additional thiol isomerase enzymes, or for proteins that demonstrate endogenous thiol isomerase activity.

The mechanistic basis for these observations is intriguing and would be clarified by further spatial, temporal, and physical data on thiol isomerase enzymes to identify their intracellular source, the pathway for their cell-surface exposure and means of attachment, and the primary substrates with which they interact. Furthermore, the development of selective inhibitors for thiol isomerases would allow individual mechanistic pathways to be examined to determine if common or distinct reaction configurations are involved. We propose that in platelets, thiol isomerases share a common pathway for cell-surface exposure but are then capable of interacting with different sets of receptors on the cell surface.

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