Redox Control of Exofacial Protein Thiols/Disulfides by Protein Disulfide Isomerase*

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Protein disulfide isomerase (PDI) catalyzes thiol-disulfide interchanges that can result in formation, reduction, or rearrangement of protein disulfide bonds. It is generally considered that PDI is important for proper folding and disulfide bonding of nascent proteins in the endoplasmic reticulum and is secreted by cells and associates with the cell surface. We examined the consequence of over- or underexpression of PDI in HT1080 fibrosarcoma cells for the redox state of cell-surface protein thiols/disulfides. Overexpression of PDI resulted in 3.6–4.2-fold enhanced secretion of PDI and 1.5–1.7-fold increase in surface-bound PDI. Antisense-mediated underexpression of PDI caused 38–53% decreased secretion and 10–33% decrease in surface-bound PDI. Using 5,5′-dithio-bis(2-nitrobenzoic acid) to measure surface protein thiols, a 41–50% increase in surface thiols was observed in PDI-overexpressing cells, whereas a 29–33% decrease was observed in underexpressing cells. Surface thiol content was strongly correlated with cellular (r = 0.998) and secreted (r = 0.969) PDI levels. The pattern of exofacial protein thiols was examined by labeling with the membrane-impermeable thiol reactive compound, 3-(N-maleimidylpropionyl)biocytin. Fourteen identifiable proteins on HT1080 cells were labeled with 3-(N-maleimidylpropionyl)biocytin. The intensity of labeling of 11 proteins was increased with overexpression of PDI, whereas the intensity of labeling of 3 of the 11 proteins was clearly decreased with underexpression of PDI. These findings indicated that secreted PDI was controlling the redox state of existing exofacial protein thiols or reactive disulfide bonds.

Protein disulfide isomerase (PDI) catalyzes thiol-disulfide interchanges that can result in formation, reduction, or rearrangement of protein disulfide bonds. It is generally considered that PDI is important for proper folding and disulfide bonding of nascent proteins in the endoplasmic reticulum (1–4). PDI contains a C-terminal KDEL anchor (9) that mediates interaction of PDI with the KDEL receptor on membranes of the Golgi and the intermediate compartment. The PDI-KDEL receptor complex is recycled back to the endoplasmic reticulum (10). Despite this retrieval mechanism, PDI is exported from cells and binds to the cell surface. Secreted PDI retains the KDEL anchor (11, 12). Cultured rat hepatocytes (12) and pancreatic cells (13) secrete PDI that associates with the cell surface, and murine fibroblasts secrete PDI in response to treatment with calcium ionophore (14). PDI is also on the surface of B cells (15, 16) and platelets (17, 18).

Cell-surface PDI has been implicated in regulation of the disulfide-linked diphtheria toxin heterodimer (19, 20), cell-surface events which trigger entry of the human immunodeficiency virus into lymphoid cells (21), and shedding of the human thyrotropin receptor ectodomain (22). PDI has also been implicated as a cell-surface recognition/adhesion molecule during neuronal differentiation of the retina (23) and in redox control of exofacial protein thiols/disulfides of lymphocytes (16, 24).

In this study we examined the consequence of over- or underexpression of PDI for the redox state of cell-surface protein thiols/disulfides. Human fibrosarcoma cells (HT1080) were stably transfected with a PDI expression vector or with a PDI antisense construct. Overexpression of PDI resulted in enhanced secretion of PDI but not two other KDEL-containing proteins and enhanced cell-surface association of PDI. Similarly, antisense-mediated underexpression of PDI caused decreased secretion and cell-surface localization of PDI. By using two different membrane-impermeable thiol-specific reagents, we showed that increased or decreased secretion of PDI correlated with increased or decreased protein thiols on the cell surface. At least 14 proteins on the surface of control and PDI-transfected cells contained redox-active thiols/disulfides that were regulated by the level of secreted PDI. These results demonstrated that secreted PDI was controlling the redox state of certain cell-surface protein thiols/disulfides.

EXPERIMENTAL PROCEDURES

HT1080 Cell Culture—HT1080 cells from ATCC (Rockville, MD) were maintained in DMEM containing 10% fetal bovine serum (FBS), 2 mM glutamine, 10 units/ml penicillin G, and 10 μg/ml streptomycin sulfate. All media components were from Life Technologies, Inc.

Generation of HT1080 Cells Overexpressing or Underexpressing PDI—A 1.7-kilobase pair PDI cDNA was isolated by reverse transcriptase-polymerase chain reaction from total RNA extracted from primary human foreskin fibroblasts and cloned into the vector, pGEM-T (Promega, Madison, WI). The PDI primers were ATTGATGGATCCAT-GCTGCGCCGCGCTCTGCT (PDI sequence position 76–96) and GCGAGGTAGTGCTTCTCCTGTGGTGGT (PDI sequence position 176–196). The PDI sequence was confirmed by automatic sequencing (ABI-377 Automatic Sequencer, Applied Biosystems) and was the same as the reported sequence (25).

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The PDI cDNA was derived as a BamHI fragment and inserted into the mammalian expression vector pCDNA3 in either the sense or antisense direction (Invitrogen, San Diego, CA). HT1080 cells were transfected with either 5 μg of un-manipulated vector or vector containing the sense or antisense PDI cDNA using calcium phosphate (Life Technologies, Inc., Gaithersburg, MD) as described by Vandeputte et al. (26). For secreted GSH levels, HT1080 cells at 80% confluence were washed twice with PBS and incubated in DMEM without FBS for 6 h. Cells (5 x 10^6) were lysed in TRIZOL buffer (Life Technologies, Inc.) and total RNA extracted as described by the manufacturer. Total RNA (20 μg) was blotted onto nylon Hybond transfer membrane (Amersham Australia, Sydney, Australia) and probed with a riboprobe complementary to PDI mRNA. The riboprobe was transcribed from the pCDNA3-PDI vector using SP6 polymerase. The ribopropes were visualized using digoxigenin detection (Boehringer Mannheim, Sydney, Australia).

Measurement of Total Cellular and Secreted Glutathione and Cellular Protein-bound Glutathione and Protein Thioli Levels—Reduced glutathione (GSH) and oxidized glutathione (GSSG) levels were determined as described by Vandepuye et al. (26). For secreted GSH levels, HT1080 cells at 80% confluence were washed twice with PBS and incubated in DMEM without FBS for 6 h (0.7 x 10^6 cells per ml of medium). Conditioned media were centrifuged at 3000 x g for 10 min to remove cell debris. For cellular GSH levels, HT1080 cells (1.5 x 10^6) were detached from culture flasks using 5 mM EDTA in PBS, washed twice with PBS, resuspended in 0.3 ml of PBS, and snap-frozen in liquid nitrogen. Cellular protein was precipitated with 5-sulfosalicylic acid according to Vandepuye et al. (26), and the GSH and GSSG content of the supernatant was determined. To determine protein-bound GSH, the pellets from the acid precipitation were resuspended in 1% sodium borohydride, incubated for 10 min at 4 °C, and centrifuged at 10,000 x g for 1 h at 25 °C. The resulting supernatant was neutralized with 100 mM potassium phosphate, pH 7.4 buffer, and the GSH content determined. Total thiol content of HT1080 cell lysates was determined using 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB, Sigma) (27) after denaturating the proteins with 5% SDS. Cellular protein thioli content was calculated by subtracting the cellular GSH content from the total thiol content.

ELISA for PDI—Affinity-purified anti-PDI polyclonal antibodies (100 μl of 5 mg/ml in 15 mM Na_2CO_3, 35 mM NaHCO_3, 0.02% azide, pH 9.6 buffer) were adsorbed to Nunc PolySorp 96-well plates overnight at 4 °C in a humid environment. Wells were washed once with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS/Tween), non-specific binding sites blocked by adding 200 μl of 2% bovine serum albumin in PBS and incubating for 90 min at 37 °C, and then washed two times with PBS/Tween. HT1080 cells (5 x 10^5) were detached from culture flasks using 5 mM EDTA in PBS, washed twice with PBS, resuspended in 1 ml of ice-cold 50 mM Tris/HCl, pH 8, buffer containing 0.5 mM NaCl, 1% Triton X-100, 10 μg leupeptin, 2 mM phenylmethylsulfonyl fluoride (Sigma), 5 mM EDTA, and 10 μg aprotinin (Bayer Australia Ltd., Sydney, Australia), and sonicated on ice. Purified placenta PDI and HT1080 cell lysates were diluted in PBS/Tween and 100-μl aliquots added to antibody-coated wells and incubated for 30 min at room temperature with orbital shaking. Wells were washed three times with PBS/Tween and 100 μl of 5 μg/ml of the murine anti-PDI monoclonal antibody, M10, added and incubated for 30 min at room temperature with orbital shaking. The M10 antibody was a gift from Prof. Johan Stenflo, Lund University, Sweden. Wells were washed three times with PBS/Tween, and rabbit anti-mouse IgG horseradish peroxidase-agarose conjugated antibody was added at 1 in 500 dilution in 100 μl of PBS/Tween and incubated for 30 min at room temperature with orbital shaking. Wells were washed three times with PBS/Tween and the color developed with 100 μl of 0.03% H_2O_2, 1 ml, 22-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) in 50 mM citrate, pH 4.5 buffer, for 20 min at room temperature with orbital shaking. Absorbsances were read at 405 nm using a Molecular Devices Thermostak Kinetic Microplate Reader ( Molecular Devices). Results were corrected for control wells not coated with polyclonal antibody. The ELISA is linear up to a PDI content of 200 ng/ml.

Detection of Total and Reduced Cell-surface PDI Protein—HT1080 cell-surface PDI was estimated by labeling with either sulfo-succinimidobiotin (SSB) (Pierce) (28), or 3-(N-maleimidylpropionyl)boc-tycin (MPB) (Molecular Probes) (28). Both SSB and MPB are membrane-impermeable. SSB labeled the primary amines in PDI, and MPB labeled the reactive site sulphydryls. HT1080 control, HT1080s, or HT1080as cells (5 x 10^6) were detached from culture flasks using 5 mM EDTA in PBS, washed twice, resuspended in 1 ml of PBS containing 100 μM of either SSB or MPB, and incubated for 30 min at room temperature. Unreacted SSB was quenched with 200 μM glycine (Sigma) for 10 min at room temperature. Unreacted MPB was quenched with 200 μM GSH (Sigma) for 10 min at room temperature, and remaining sulphydryl groups were quenched with 400 μM iodoacetamide (Sigma) for 10 min at room temperature. The cells were washed three times with 1 ml of PBS, sonicated in lysis buffer as described above, and incubated with 100 μl of a 50% slurry of streptavidin-agarose (Sigma) for 60 min at 4 °C with rotary mixing. Bound proteins were washed five times with 50 mM Tris/HCl, pH 8, buffer containing 0.15 mM NaCl and 0.05% Triton X-100, resolved on 10% SDS-PAGE, transferred to PVDF membrane, and the SSB- or MPB-labeled PDI detected by Western blot.

Detection of Secreted PDI Protein—Secretion of PDI by HT1080 control, HT1080s, or HT1080as cells was assessed by washing 80% confluent cultures twice and incubating in DMEM without FBS for 6 h. Conditioned media were centrifuged at 3000 x g for 10 min to remove cell debris, and media from 3 x 10^6 cells were resolved on 10% SDS-PAGE, transferred to PVDF membrane, and detected by Western blot.

Quantitation of HT1080 Cell-surface Protein Thiols—Protein thiols on control HT1080, HT1080s, or HT1080as cells were quantitated using either DTNB (27) or MPB (see above). Cells at 80–90% confluence were detached from culture flasks using 5 mM EDTA in phosphate-buffered saline (PBS) and washed twice with PBS. For DTNB labeling, 1.5 x 10^6 cells were resuspended in 1 ml of PBS, DTNB added to a final concentration of 200 μM, and the mixture incubated for 30 min at room temperature with gentle rolling. Cells were pelleted at 300 x g for 5
min, and 2-nitro-5-thiobenzoic acid content of the supernatant was measured from absorbance at 412 nm using an extinction coefficient of 14,150 M−1 cm−1 (27). For MPB labeling, 5 × 105 cells were resuspended in 1 ml of PBS, MPB added to a final concentration of 100 μg/ml MPB, and the mixture incubated for 30 min at room temperature with gentle rolling. Unreacted MPB was quenched with GSH and iodoacetamide as described above. Labeled cells were sonicated in 1 ml of lysis buffer as described above. Lysate corresponding to 2.5 μg of protein (approximately 1 × 10^6 cells) was resolved on SDS-PAGE, transferred to PVDF membrane, and MPB-labeled proteins detected by blotting with streptavidin peroxidase. On some occasions, cells at 80–90% confluence were washed with PBS and incubated with either 50 μg/ml preimmune rabbit IgG or affinity purified anti-PDI rabbit polyclonal antibodies in serum-free DMEM for 48 h prior to labeling with MPB. Rabbit polyclonal antibodies were developed against purified human placenta PDI in New Zealand white rabbits and affinity purified on a PDI-Affigel 15 matrix (Bio-Rad). PDI was purified from human placenta as described previously (30) with modifications (31).

Electrophoresis and Blotting—Samples were resolved on either 10 or 5–15% SDS-PAGE under non-reducing conditions (32) and transferred to PVDF membrane. Proteins were detected by Western blot using affinity purified anti-human PDI rabbit polyclonal antibodies (used at 3 μg/ml), a anti-human Grp78/Grp94 murine monoclonal antibody (used at 5 μg/ml) from Stressgen, British Columbia, Canada, or an anti-human thioredoxin murine monoclonal antibody (used at 5 μg/ml) from Dr. Frank Clarke, Griffith’s University, Brisbane, Australia. Swine anti-rabbit horseradish peroxidase-conjugated antibodies and rabbit anti-mouse horseradish peroxidase-conjugated antibodies (Dako Corporation, Carpinteria, CA) were used at 1:2000 and 1:1000 dilution, respectively. MPB-labeled proteins were blotted with streptavidin peroxidase. On some occasions, cells at 80–90% confluence were washed with PBS and incubated with either 50 μg/ml preimmune rabbit IgG or affinity purified anti-PDI rabbit polyclonal antibodies in serum-free DMEM for 48 h prior to labeling with MPB. Rabbit polyclonal antibodies were developed against purified human placenta PDI in New Zealand white rabbits and affinity purified on a PDI-Affigel 15 matrix (Bio-Rad). PDI was purified from human placenta as described previously (30) with modifications (31).

RESULTS

Characterization of PDI Messenger Levels and Protein in Control Versus PDI Sense and Antisense Transfected Cells—HT1080 cells were transfected with either the unmanipulated pCDNA3 mammalian expression vector (HT1080) or the vector containing PDI cDNA inserted in either the sense (HT1080s) or antisense (HT1080as) direction. Stable transfectants were selected with G418 and cloned. One control and two sense and two antisense clones were selected for investigation. These clones have been called HT1080 control, HT1080s1 and 2 and HT1080as1 and 2, respectively. PDI mRNA in the control, sense, and antisense clones is shown in Fig. 1A. HT1080s1 and HT1080s2 clones contained 2.9- and 3.0-fold more PDI mRNA than control cells, respectively, whereas PDI mRNA in HT1080as1 and HT1080as2 clones was 42 and 40% that in control cells, respectively.

PDI protein in cell lysates of HT1080 control, HT1080s, or HT1080as clones is shown in Figs. 1B. Consistent with the mRNA levels, HT1080s1 and HT1080s2 clones contained 1.9- and 2.1-fold more PDI protein per mg of cell protein than control cells, respectively, whereas PDI protein in HT1080as1 and HT1080as2 clones was 53 and 37% that in control cells, respectively.

There was no difference in the amount of urokinase plasminogen activator receptor or β1 integrin protein in whole cell lysates, or plasminogen activator inhibitor-1 in the extracellular matrix, of control versus HT1080s2 or HT1080as2 cells (not shown). It was suggested that PDI overexpression did not have a general effect on protein synthesis.

Comparison of Cell Growth and Morphology in Control Versus PDI Sense and Antisense Transfected Cells—Control and PDI sense and antisense transfected cells were plated at 3000 cells per well in 6-well plates, and cell number was determined each day for 4 days. The values and error bars represent the mean and S.E. or triplicate determinations. B, phase contrast micrographs of HT1080 control (a), HT1080s2 (b), and HT1080as2 (c) cells at high density. Cells were plated at 3000 cells per well in 6-well plates, and micrographs were taken after 5 days growth.

FIG. 2. Comparison of cell growth and morphology in control versus PDI sense and antisense transfected cells. A, HT1080 control and PDI sense (HT1080s2) and antisense (HT1080as2) transfected cells were plated at 3000 cells per well in 6-well plates, and cell number was determined each day for 4 days. The values and error bars represent the mean and S.E. of triplicate determinations. B, phase contrast micrographs of HT1080 control (a), HT1080s2 (b), and HT1080as2 (c) cells at high density. Cells were plated at 3000 cells per well in 6-well plates, and micrographs were taken after 5 days growth.
control Versus PDI Sense and Antisense Transfected Cells—The intracellular GSH and protein-bound GSH content of control and PDI sense and antisense transfected cells was very similar (Fig. 3). The intracellular GSSG content was less than 1% of the GSH content and was also similar in all cells (not shown). In contrast, the sense and antisense transfected cells secreted an average 1.9- and 4.1-fold more GSH, respectively, than control cells. The total protein thiol content of transfected cells was slightly higher than control cells, whereas it was slightly lower than control in antisense transfected cells.

Characterization of PDI Secretion Levels in Control Versus PDI Sense and Antisense Transfected Cells—PDI was secreted by the control and transfected cells (Fig. 4A). The extent of PDI secretion paralleled the PDI protein levels in cell lysates (see Fig. 1B). Purified placenta PDI is shown as control and mitochondria PDI sense and antisense transfected cells was determined as described by Vandeputte et al. (26). Protein-bound GSH (PSSG) was determined using sodium borohydride. Total PDI content of HT1080 cell lysates determined in SDS was determined using DTNB. Cellular protein thiol content (PSH) was calculated by subtracting the cellular GSH content from the total thiol content.

Thioredoxin is a 12-kDa redox active protein that can be secreted by cultured cells (34). Interestingly, PDI sense transfected cells contained ~40% less thioredoxin than control antisense transfected cells (Fig. 4C). The reason for this is unknown but may reflect coordinated expression of PDI and thioredoxin. For instance, overexpression of thioredoxin reduced expression of glutaredoxin in Escherichia coli and vice versa (35). Thioredoxin was not secreted by control or transfected HT1080 cells within the limits of detection of the Western blot (not shown). Comparison of secreted PDI relative to cellular PDI content with the cellular content of thioredoxin (from Figs. 1B and 4, A and C) indicated that if thioredoxin was secreted then the level of secretion was <10% of the level of PDI secretion.

Characterization of Cell-surface PDI Levels in Control Versus PDI Sense and Antisense Transfected Cells—PDI associates with the cell surface after secretion (12, 13). To examine cell-surface associated PDI, HT1080 control and sense and antisense cells were labeled with either the membrane-impermeable amine-reactive reagent, SSB, or the membrane-impermeable thiol-reactive reagent, MPB. PDI contains two active site sulfhydryl groups in the common sequence WCG-PCK which have a redox potential of ~110 mV (3) and can be labeled with MPB (36). Therefore, SSB-labeled PDI is a measure of total cell-surface PDI, whereas MPB-labeled PDI is a measure of reduced cell-surface PDI.

HT1080 cell-surface SSB- or MPB-labeled proteins were collected on streptavidin-agarose, separated on 12% SDS-PAGE, transferred to PVDF membrane, and blotted with PDI polyclonal antibodies (SSB, Fig. 5A; MPB, Fig. 5B). The results...
represent labeling of $5 \times 10^5$ HT1080 cells. SSB- or MPB-labeled placenta PDI is shown as control and migrated with the expected mass of $\approx 57$ kDa. The amount of SSB- or MPB-labeled cell-surface PDI paralleled the PDI protein levels in cell lysates (see Fig. 1B) and secreted PDI protein (see Fig. 4A). HT1080s1 and HT1080s2 clones contained 1.5- and 1.7-fold more total PDI on their surface than control cells, respectively, whereas total PDI levels on HT1080as1 and HT1080as2 clones were 90 and 67% that on control cells, respectively (Fig. 5A). A similar ratio was observed for reduced surface PDI levels (Fig. 5B), although the absolute levels of reduced PDI were approximately one-third of the total PDI levels. This result implied
that surface PDI was a mixture of reduced and oxidized forms, although the level of reduced PDI may have be underestimated if the efficiency of labeling by MPB was not optimal.

By having demonstrated that over- or underexpression of PDI in HT1080 cells correlated with increase or decrease, respectively, in secretion and cell-surface levels of PDI, we were in the position to test whether PDI controlled the redox state of cell-surface protein thiols/disulfides. We used two different thiol-reactive reagents to examine this question, DTNB and MPB. Both of these reagents are membrane-impermeable and thiol-specific at neutral pH and have been used previously to assess cell-surface thiol status (27, 29).

Quantitation of Cell-surface Protein Thiol Levels in Control Versus PDI Sense and Antisense Transfected Cells—HT1080 control and PDI sense (HT1080s) and antisense (HT1080as) transfected cells were reacted with the membrane-impermeable thiol-specific reagent, MPB. The MPB-labeled proteins were resolved on 10% SDS-PAGE, transferred to PVDF membrane, blotted with streptavidin-peroxidase, and detected using chemiluminescence. Lanes 1–5 are MPB-labeled cell-surface proteins from 2.5 μg of cell lysate (1 × 10⁶ cells). The positions of M₀ markers are shown at left. Fourteen identifiable proteins were labeled with MPB. These proteins have been indicated by arrows. A, correlation between surface PDI level and labeling by MPB. Surface PDI level was taken from Fig. 5A. Total MPB label was estimated by the total densitometry units of each lane in A. The solid line represents the linear regression fit to the data (r = 0.969). B, densitometric analysis of the MPB-labeled proteins from HT1080 control and HT1080s2 and HT1080as2 cells shown in A. The individual protein peaks have been identified by numbers and correspond to the arrows in A. Three proteins (1–3) were only labeled on sense transfected cells. The intensity of labeling of 11 proteins (4–14) was increased with overexpression of PDI, whereas the intensity of labeling of 3 of the 11 proteins (5–7) was clearly decreased with underexpression of PDI. C, densitometric analysis of the MPB-labeled proteins from HT1080 control and HT1080s2 cells shown in A. The individual protein peaks have been identified by numbers and correspond to the arrows in A. Three proteins (1–3) were only labeled on sense transfected cells. The intensity of labeling of 11 proteins (4–14) was increased with overexpression of PDI, whereas the intensity of labeling of 3 of the 11 proteins (5–7) was clearly decreased with underexpression of PDI. D, effect of anti-PDI antibodies on labeling of control and PDI sense transfected cells with MPB. HT1080 control and HT1080s2 cells were incubated with either preimmune rabbit IgG or affinity purified anti-PDI rabbit polyclonal antibodies (50 μg per ml) for 48 h in serum-free medium. The cells were labeled with MPB and processed as described in A. Total MPB label was estimated by the total densitometry units of each lane.
HT1080 cells, control, sense, and antisense transfected cells were labeled with MPB. The MPB-labeled proteins were resolved on SDS-PAGE, transferred to PVDF membrane, blotted with streptavidin-peroxidase, and detected using chemiluminescence. The results represent MPB-labeled cell-surface proteins in 2.5 μg of cell lysates (approximately 1 × 10⁶ cells). The relative intensity and pattern of MPB-labeled cell-surface proteins of control, sense, and antisense cells is shown in Fig. 8A.

The surface of HT1080s1 and HT1080s2 cells incorporated 2.9- and 3.0-fold more MPB than control cells, respectively, whereas MPB label on the surface of HT1080as1 and HT1080as2 cells was 71 and 49% that on control cells, respectively. Surface PDI level was strongly correlated with extent of incorporation of MPB label (r = 0.969) (Fig. 8B).

Densitometric analysis of the MPB-labeled proteins from HT1080 control and HT1080s2 and HT1080as2 cells is shown in Fig. 8C. Fourteen identifiable proteins were labeled with MPB. Three proteins (1–3) were only labeled on sense transfected cells. The intensity of labeling of 11 proteins (4–14) was increased with overexpression of PDI, whereas the intensity of labeling of 3 of the 11 proteins (5–7) was clearly decreased with underexpression of PDI. Therefore, at least 14 proteins on the surface of control and PDI transfected cells contained redox active thiols/disulfides that were regulated by the level of secreted PDI.

To confirm that secreted PDI was controlling the redox state of cell-surface protein thiols/disulfides, HT1080 control and HT1080as2 cells were incubated with either preimmune rabbit IgG or affinity purified anti-PDI rabbit polyclonal antibodies incorporated 3.4-fold less MPB label than cells incubated with preimmune IgG. Similarly, HT1080s2 cells incubated with anti-PDI antibodies incorporated 4.1-fold less MPB label than cells incubated with preimmune IgG. Similarly, HT1080as2 cells incubated with anti-PDI antibodies incorporated 3.4-fold less MPB label than cells incubated with control IgG.

**DISCUSSION**

Increase or decrease in cellular PDI levels equated with increased or decreased secretion and cell-surface localization. The C-terminal KDEL sequence in PDI is thought to trap PDI in the Golgi and intermediate compartment and recycle it back to the endoplasmic reticulum (11, 12). However, PDI is transported from the cell despite the C-terminal KDEL anchor. Theories as to how PDI escapes recycling and is secreted have been proposed (11, 12) and include saturation of the KDEL receptor, a defect in the retention system, and escape from a salvage compartment. These ideas imply unregulated leakage of PDI. However, specific overexpression of PDI in Chinese hamster ovary cells caused enhanced secretion of PDI but not other resident endoplasmic reticulum proteins containing the KDEL sequence (33), and we have shown herein that overexpression of PDI in HT1080 cells caused enhanced secretion of PDI but not the KDEL-containing proteins, Grp78 and Grp94. Also, appendage of the KDEL sequence to the C termini of two secretory proteins retarded transport from the endoplasmic reticulum but did not cause permanent retention (37). These observations argue against saturation of the KDEL receptor as the cause of PDI secretion. These findings and the diversity of cell types that express PDI on their surface, including platelets, suggest that PDI secretion and cell-surface localization is a specific event.

Antisense-mediated underexpression of PDI in HT1080 cells caused the cells to accumulate in cell islands, and complete confluency was never reached. In contrast, the morphology of HT1080 cells overexpressing PDI was not obviously different from control cells. PDI expression in all the antisense clones examined was never less than ~40% of control cell expression. It may be that further decrease in PDI expression is not compatible with cell survival. This notion is supported by the observation that PDI is essential for yeast viability (38). The intracellular GSH, GSGG, and protein-bound GSH content of control and PDI sense and antisense transfected cells was very similar. In contrast, the sense and antisense transfected cells secreted an average 1.9- and 4.1-fold more GSH, respectively, than control cells. The total protein thiol content of sense transfected cells was slightly higher than control cells, whereas it was slightly lower than control in antisense transfected cells. The difference in PDI levels in the sense versus antisense transfected cells would have probably contributed to the small differences in cellular protein thiol.

The ability of PDI to manipulate disulfide bonds in proteins resides in two very reactive dithiols/disulfides that share the common sequence WCGHCK (3). These dithiols/disulfides catalyze thiol-disulfide interchanges that can lead to the net formation, the net rearrangement, or the net reduction of protein disulfide bonds depending on the nature of the protein substrate, the redox conditions, and the presence of other thiols and disulfides. Increase or decrease in surface protein thiol groups strongly correlated with increase or decrease in total cellular (r = 0.998) or cell surface (r = 0.969) PDI. A 41–51% increase in surface thiols was observed in PDI-overexpressing cells using DTNB, whereas a 2.9–3.0-fold increase was observed using MPB. The difference in quantitation between the two thiol-reactive reagents probably related to the accessibility of the reagents to protein thiols. DTNB is a bulky aromatic compound that would have only reacted with exposed protein thiols, whereas the maleimide moiety of MPB is attached through a propionyl spacer arm to biotin which makes the maleimide more accessible to partly buried protein thiols. Secreted PDI was responsible for the redox control of surface protein thiols/disulfides as anti-PDI antibodies reduced labeling of HT1080 surface thiols by MPB to less than control in both control and sense transfected cells.

Fourteen identifiable proteins on HT1080 cells were labeled with MPB. Three of the proteins (1–3, Mₓ of ~200, ~120, and ~80) were only labeled on sense transfected cells. The intensity of labeling of the remaining 11 proteins (4–13, Mₓ between 70 and 25) was increased with overexpression of PDI, whereas the intensity of labeling of 3 of the 11 proteins (5–7, Mₓ of ~65, ~58, and ~55) was clearly decreased with underexpression of PDI. This result implied that certain protein disulfide bonds were more susceptible to reduction by PDI than others. That is, high levels of PDI were required to reduce disulfide bond(s) in proteins 1–3, whereas a proportion of proteins 4–14 contained reduced disulfide bond(s) on control cells, but this proportion increased with increasing PDI. Similarly, decrease in secreted PDI reduced the proportion of proteins 5–7 that contained reduced disulfide bond(s). The difference in susceptibility of different protein disulfide bonds to reduction by PDI was probably a consequence of the relative stability of the disulfide bond...
and/or accessibility of the disulfide bond to PDI.

These findings implied that the cell-surface environment favors net disulfide bond reduction by PDI in certain proteins, which is in accordance with reported functions of secreted PDI. Cell-surface PDI has been implicated in reduction of the disulfide-linked diphteria toxin homodimer (20, 21) and reduction and shedding of the human thyrotropin receptor ectodomain (22), and PDI can reduce disulfide bonds in thrombospondin (31) and plasmin (36). It is possible that dithiols/disulfides in other cell-surface proteins were also influenced by secreted PDI but were not detected because of their paucity or refractiveness to labeling by DTNB and/or MPB. PDI may have also catalyzed isomerization of disulfide bonds in certain HT1080 cell-surface proteins; however, because isomerization does not change sulphydryl content, these proteins would not have been resolved by DTNB or MPB labeling. It was noteworthy that at least a third of the HT1080 surface PDI was labeled with MPB. This finding implied that one or both of the active site dithiols/disulfides of a substantial fraction of the surface PDI was in the reduced dithiol form and suggested that a mechanism may exist to maintain PDI in a reduced state on the cell surface. The concentration of GSH in the medium of $1 \times 10^6$ HT1080 cells after 6 h of incubation was $\sim 1 \mu M$, which would not be sufficient to reduce the active site dithiols/disulfides of oxidized PDI. One possibility is the plasma membrane NADH-oxidoreductase system (40) which has been implicated in reduction of extracellular protein disulfide bonds.

A model for the effects of secreted PDI on exofacial protein thiol(s)/disulfide(s) is shown in Fig. 9. The figure is a cartoon of a transmembrane protein but could also be a glycosylphosphatidylinositol-linked protein or a protein non-covalently bound at the cell surface. The protein exists in one of two configurations, a form in which an exofacial reactive dithiol is oxidized to form a disulfide bond and the other in which the disulfide bond is reduced. The exofacial thiol(s) on the reduced form can be labeled with MPB. The two forms exist in equilibrium which is influenced by the level of secreted PDI. The results of MPB labeling support the equilibrium depicted in the cartoon.

It is apparent that the function of some intracellular proteins is controlled by the redox state of their cysteine residues (41). We suggest that the function of certain extracellular proteins is similarly controlled by the redox state of their cysteine/cysteine residues. Our findings indicate that the cell can manipulate the redox state of extracellular protein thiols/disulfides through secretion of PDI. A scenario in which cell-surface redox potential is controlled by PDI suggests that the rate of PDI secretion will vary with cellular activity. In support of this hypothesis, heat shock has been shown to induce secretion of PDI in primary rat hepatocytes (12), whereas calcium ionophore enhanced secretion of PDI in NIH 3T3 cells (14) and Chinese hamster ovary cells (33).

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Redox Control of Exofacial Protein Thiols/Disulfides by Protein Disulfide Isomerase
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